Pol. J. Food Nutr. Sci., 2022, Vol. 72, No. 2, pp. 119–128
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/147913
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Control of Mould Spoilage on Apples Using Yeasts as Biological Control Agents

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Key words: mould spoilage, fungicides, postharvest control, biocontrol agents (BCAs), apples, yeast

Considerable quantities of fruit are lost during pre- and post-harvest stages due to mould spoilage. The aim of this study was to evaluate the antagonistic effect of selected yeasts against spoilage mould *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae*. One hundred and four yeast isolates were screened for antagonistic activity against *B. cinerea*, *P. expansum* and *A. alstroemeriae* using radial inhibition, dual and mouth-to-mouth plate assays. Sixty-seven out of 104 yeasts showed growth inhibition activity against *P. expansum*, while 36 yeasts inhibited *B. cinerea*, 47 yeasts inhibited *A. alstroemeriae,* but only 22 yeasts displayed inhibition activity against all three moulds. *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Zygoascus hellenicus* Y89 had the highest inhibition activity against all three moulds, when mode of inhibition was due to direct contact. Volatile organic compounds produced by *Pichia kluyveri* Y64, *C. pyralidae* Y63 and *M. guilliermondii* Y88 demonstrated the highest growth inhibition against all three moulds. These yeasts were also evaluated against all three moulds on apples. *P. kluyveri* Y64 displayed 100%, 57% and 26% growth inhibition against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively, on apples and performed slightly better than a commercial fungicide against *B. cinerea* and *P. expansum*. While *M. guillermondii* Y88 showed 100%, 60% and 18% inhibition on apples against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively. *P. kluyveri* Y64 and *M. guilliermondii* Y88 demonstrated potential as biofungicides and warrant further investigation.

INTRODUCTION

Fruits are commercially and nutritionally important commodities and play an important role in human health by supplying vitamins and minerals [Al-Hindi *et al*., 2011]. Globally, apple (*Malus domestica*) production increased from 75 to more than 85 million tonnes per annum from 2014 to 2020 [FAOstat, 2020]. South Africa is a relatively small apple grower in terms of global hectares and produces approximately 1.3 million tonnes each year, with a total value of more than 8 billion rands [Du Plessis, 2017]. Ninety-two percent of this income is generated by fresh fruit sales [Du Plessis, 2017]. Agricultural products are subject to mould spoilage before, during and after harvest, as well as during transportation and processing [Romanazzi *et al*., 2016; 2017]. Fruit losses due to spoilage mould pose several challenges to the agrifood industry [Parveen *et al*., 2016]. Each year, 25% of the total fruits produced is lost in industrialised countries and more than 50% in developing countries [Droby, 2005; Nunes, 2012]. During the pre- and post-harvest stages, considerable amounts of fruits including apples are lost due to mould diseases caused by *Botrytis cinerea* (grey mould), *Penicillium expansum* (blue mould), *Alternaria* spp. (necrotic leaf blotch), *Venturia inaequalis* (apple scab), *Cladosporium* spp. (Sooty spot) and *Colletotrichum gloeosporioides* (bitter rot) [Sharma *et al*., 2009].

Spoilage moulds need to be controlled to maintain the quality and abundance of fruit produced around the world [Mercier & Lindow, 2001]. Currently, fruit producers and exporters are using costly spraying programs incorporating synthetic chemicals, which are labour intensive and require the application of various classes of fungicides sprayed up to 20 times during the growing season. These practices, even at the lowest dose, can negatively affect the health of consumers,

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Submitted: 15 December 2021
Accepted: 31 March 2022
Published on-line: 30 May 2022

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the environment, and the taste and aroma of the food being preserved [Benito *et al*., 2009; Contarino *et al*., 2019; Oliveira *et al*., 2014]. Some mould can become resistant to fungicides as farmers use the chemicals regularly [Fernández-Ortuño *et al*., 2008].

The desire to minimise chemical residues and to offset rising prices of new synthetic chemicals is fostering the search for alternatives to synthetic chemical fungicides [Quaglia *et al*., 2011; Robiglio *et al*., 2011]. The recent trend is shifting towards safer and environmentally friendly alternatives for the control of post-harvest decay [Sharma *et al*., 2009]. Biological control using yeasts demonstrated great potential as an alternative to chemical fungicides [Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019a] and is more environmentally friendly and cost effective [Bonaterra et al., 2012]. In the last years, a considerable amount of microbial-based commercial products and patents have been developed worldwide in order to exploit the microbial strategies to counteract the growth of spoilage and/or pathogenic microorganisms in pre and post-harvest [De Simone *et al*., 2021].

Yeasts can be used as an alternative to synthetic chemicals because of their ability to compete for nutrients and space, their ability to grow faster than most fungal pathogens and the production of inhibitory growth compounds [Liu *et al*., 2013]. Yeasts, such as *Meyerozyma guilliermondii*, *Candida pyralidae* and *Hanseniaspora* species, have the ability to secrete extracellular metabolites, such as volatile organic compounds (VOCs), acetic acid, hydrogen sulphide and cell wall-degrading enzymes, which have antimicrobial properties against many fruit spoilage moulds [Al-Maawali *et al*., 2021; Cordero-Bueso *et al*., 2017; Grevesse *et al*., 2003; Han *et al*., 2021; Hua *et al*., 2014; Mewa-Ngongang *et al*., 2019b; Ruiz-Moyano *et al*., 2020; Zhou *et al*., 2018]. The aim of this study was to screen yeasts for growth inhibition activity against *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae* under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Culturing conditions and inoculum preparation

One hundred and four yeast isolates were obtained for evaluation from the ARC Infruitec-Nietvoorbij (the Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa), the Instituto Superior de Agronomia (Lisbon, Portugal), the Centraal Bureau voor Schimmelcultures (Utrecht, Netherlands), the Gulbenkian Institute of Science (Oeiras, Portugal) and the Council for Scientific and Industrial Research (Pretoria, South Africa) (Table S1 in the Supplementary Materials). Yeast selection was based on previous research and the fact that the yeasts were isolated from different fruits and environments. The yeasts were cultured on yeast malt agar (YMA) (1% glucose, 0.3% malt extract, 0.5% peptone, 2% bacteriological agar) for 2 days at 28°C. A wire loopful of each pure yeast colony on the plates was transferred into test tubes containing 5 mL of sterilised yeast malt broth (YMB) (Sigma-Aldrich, Saint Louis, USA) and incubated at 28°C for 2 days. Thereafter 1 mL of the culture was transferred to a sterile 2 mL microtube and centrifuged at 20,400×*g* for 5 min. The supernatant was discarded, and the pellet resuspended in 100 μL of sterile distilled water. Yeast cells were counted using a Neubauer haemocytometer (Sigma-Aldrich, Darmstadt, Germany) and a microscope (Euromex, Arnhem, Netherlands), at 400× magnification, in order to prepare the yeast inoculum (1×10^8 cells/mL).

For the apple bioassay, grape pomace extract was obtained by pressing "Chenin Blanc" grape pomace from the ARC Infruitec-Nietvoorbij research farm (Stellenbosch) at 200 kPa. The resultant grape pomace extract was frozen in a 25 L polypropylene bucket at −20°C. Prior to use, the grape pomace extract was thawed and diluted with sterile distilled water to a sugar concentration of 100 g/L. Yeast strains Y63 (*C. pyralidae*), Y88 (*M. guilliermondii*) and Y64 (*P. kluyveri*) from the ARC Infruitec-Nietvoorbij culture collection, were grown in 5 mL of YMB for 2 days at 28°C and then transferred to Erlenmeyer flasks containing 50 mL of sterile grape pomace extract broth (GPB), incubated at 28°C and agitated at 150 rpm, using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 2 days. The yeast cultures were then transferred to 500 mL of GPB and grown at 28°C for 24 h under agitation. The yeast inoculum of 1×10^8 cells/mL was used, as mentioned previously.

The fruit spoilage moulds, *B. cinerea* FFD 003–15, *P. expansum* C370V59 and *A. alstroemeriae* C370V51, were obtained from the fungal genebank and Post-harvest Pathology laboratory at ARC Infruitec-Nietvoorbij and cultured for 7 to 14 days at 25°C on potato dextrose agar (PDA, Merck, Johannesburg, South Africa). Spores were harvested by gently scraping them from the surface of the agar and rinsing with sterile distilled water to attain a volume of 50 mL of the spore suspension in a sterile 250 mL Schott bottle. Prior to the dilution with sterile distilled water, a haemocytometer and a microscope (400× magnification) were used to count the spores in the initial spore solution, as previously described. The inoculum was prepared by diluting the spore suspension to 1×10^5 spores/mL.

Radial growth inhibition assay

The radial growth inhibition assay was applied as described by Núñez *et al*. [2015], with some modifications. In brief, a 5 mm mycelial disk, obtained from a 7-day old mould culture, was placed at the centre of a fresh YMA plate using a sterile cork borer. Subsequently, 15 μL of the yeast cells suspension (1×10^8 cells/mL) was spotted 25 mm away from the mycelial disk. Four different yeast isolates were spotted per plate and incubated at 25°C for 7 days. All yeast treatments had three replicates. The control plates only contained the 5 mm diameter mycelial disk of the respective mould. Results were recorded as (-) no activity, (+) mild activity, (++) medium activity, (+++) strong activity. Positive (+) growth inhibition results were observed by the presence of C-shaped growth around the yeast colonies, as shown in Figure 1. Yeasts with strong inhibition activity (+++) had the biggest C-shaped inhibtion zone and the distance between the yeast colony and the mould growth exceeded 10 mm. Yeasts with medium inhibtion activity (++) had a smaller C-shaped zone and the distance between the yeast colony and the mould growth was between 6 and 9 mm. For yeasts with mild

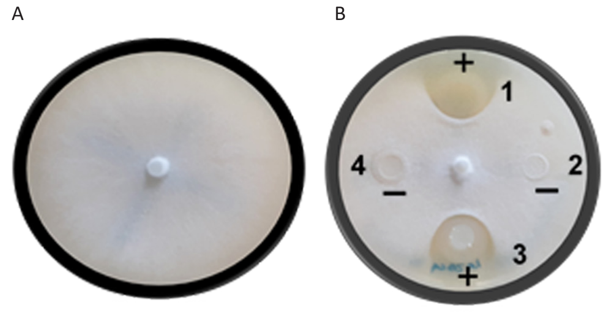


FIGURE 1. An example of the radial growth inhibition assay results. *Botrytis cinerea* growth (A) and antagonistic effect of selected yeast isolates *Candida pyralidae* Y63 (1), *Pichia kluyveri* Y64 (2), *Meyerozyma guilliermondii* Y88 (3) and *Debaryomyces hansenii* Y8 (4) against *B. cinerea* (B) on yeast malt agar. The positive sign (+) represents growth inhibition activity and the negative sign (-) represents no inhibition. This is a representative example of three replicates.

activity (+), the distance between the yeast and mould colony was less than 6 mm and no activity (-) meant that no mould growth inhibition was observed.

Diffusible metabolites assay

The dual assay described by Chen *et al*. [2018] was used to evaluate 23 yeasts (Table 1). Only those yeasts that showed growth inhibition activity against all three mould species during the radial growth inhibition assay were evaluated further. The yeast Y64 (*Pichia kluyveri*) was used in a previous study by Mewa-Ngongang *et al.* [2019a,b] and was included as the reference strain. Similar to the radial growth inhibition assay, a 5 mm mycelial disk was placed at the edge of the YMA plate. Subsequently, 20 μL of the yeast suspension (1×10^8 cells/mL) was spotted 40 mm away from the mycelial disk (Figure S1 in the Supplementary Materials) and incubated at 25°C for 9 days. The negative control (C) plates contained only the 5 mm diameter mycelial disk of the respective mould. All treatments had three replicates. The percentage inhibition was calculated as fungal radial growth inhibition (FRGI) using the following mathematical expression:

$$FRGI = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (1)$$

with D_0 representing the horizontal growth average of the fungal colony on the negative control plates and D_t representing the horizontal growth average of the fungal colony on the yeast treated plates (Figure S1 in the Supplementary Materials), as described by Núñez *et al.* [2015].

Volatile organic compound (VOCs) assay

To assess the effect of VOCs produced by the 23 yeasts used in relation to their growth inhibition potential against fruit spoilage organisms, the mouth-to-mouth assay described by Medina-Córdova *et al*. [2016] was used. Two YMA plates facing each other were sealed with laboratory film, per experimental repeat. The bottom plate was spread with 100 μL of the yeast suspension (1×10^8 cell/mL), while the top plate contained a 5 mm mould mycelial disk placed at the centre. The negative control treatment (C) only contained the 5 mm diameter mycelial disk in the centre of the plate, while no yeast was spread on the second plate. The plates were incubated at 25°C for 7 days. All treatments had three replicates. The VOC inhibition activity (VOCIA) was calculated using the following mathematical expression [Núñez *et al*., 2015]:

$$VOCIA = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (2)$$

with D_0 representing the average diameter of the fungal colony on the negative control plates and D_t representing the diameter of the fungal colony on the yeast treated plates, as shown in Figure S2 in the Supplementary Materials.

TABLE 1. Yeasts selected for the dual and mouth-to-mouth assays on yeast malt agar.

Yeast code	Species
Y6	*Aureobasidium melanogenum*
Y11	*Debaryomyces hansenii*
Y17	*Hanseniaspora occidentalis*
Y24	*Meyerozyma guilliermondii*
Y35	*Rhodotorula dairenensis*
Y39	*Meyerozyma guilliermondii*
Y63	*Candida pyralidae*
Y64	*Pichia kluyveri**
Y65	*Meyerozyma guilliermondii*
Y74	*Torulaspora delbrueckii*
Y75	*Saccharomyces cerevisiae*
Y83	*Brettanomyces lambicus*
Y84	*Debaryomyces hansenii*
Y88	*Meyerozyma guilliermondii*
Y89	*Zygoascus hellenicus*
Y91	*Zygosaccharomyces rouxii*
Y92	*Zygosaccharomyces rouxii*
Y93	*Zygosaccharomyces microellipsoides*
Y95	*Zygosaccharomyces florentinus*
Y96	*Zygosaccharomyces fermentati*
Y97	*Zygosaccharomyces bisporus*
Y102	*Candida magnolia*
Y103	*Saccharomyces cerevisiae*

*Used as reference yeast.

Post-harvest application using apple bioassay

The post-harvest biocontrol efficacy assay was performed on the apple cultivar "Panorama Goldens" and sixteen treatments were applied (Table 2). Each treatment had five replicates. Each replicate consisted of a rectangular fruit-packaging box containing five apples. Ethanol (70%, *v/v*) was sprayed on the apples to eradicate any microorganisms on the surface and allowed to dry completely before wound infliction.

TABLE 2. Treatments applied on apples during postharvest biocontrol trials*.

Treatment	Treatment description
Treatment 1	Sterile distilled water (Control)
Treatment 2	*Botrytis cinerea*
Treatment 3	*Penicillium expansum*
Treatment 4	*Alternaria alstroemeriae*
Treatment 5	*B. cinerea* and *Candida pyralidae* Y63
Treatment 6	*P. expansum* and *C. pyralidae* Y63
Treatment 7	*A. alstroemeriae* and *C. pyralidae* Y63
Treatment 8	*B. cinerea* and *Meyerozyma guilliermondii* Y88
Treatment 9	*P. expansum* and *M. guilliermondii* Y88
Treatment 10	*A. alstroemeriae* and *M. guilliermondii* Y88
Treatment 11	*B. cinerea* and *Pichia kluyveri* Y64
Treatment 12	*P. expansum* and *P. kluyveri* Y64
Treatment 13	*A. alstroemeriae* and *P. kluyveri* Y64
Treatment 14	*B. cinerea* and Captan
Treatment 15	*P. expansum* and Captan
Treatment 16	*A. alstroemeriae* and Captan

*Apples were incubated in rectangular fruit packaging boxes and five boxes (replicates) were used per treatment, with each box containing five apples.

TABLE 3. Species identity and growth inhibition activity* of the yeasts screened against selected mould on yeast malt agar.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y1	*Rhodotorula dairenensis*	***	+	–	++
Y2	*Hanseniaspora uvarum*	***	+	–	–
Y3	*Hanseniaspora uvarum*	91	–	+++	–
Y5	*Saccharomyces uvarum*	***	+	–	+
Y6	*Aureobasidium melanogenum*	99	++	+++	++
Y7	*Aureobasidium melanogenum*	***	–	++	+++
Y8	*Debaryomyces hansenii*	***	+	–	–
Y10	*Saccharomyces uvarum*	***	+	–	+
Y11	*Debaryomyces hansenii*	***	+	++	++
Y12	*Rhodotorula dairenensis*	***	++	–	++
Y13	*Hanseniaspora opuntiae*	99	–	++	–
Y14	*Saccharomyces uvarum*	97	+	–	+++
Y15	*Hanseniaspora uvarum*	***	–	–	+
Y16	*Hanseniaspora uvarum*	***	+	–	–
Y17	*Hanseniaspora occidentalis*	89	++	+++	+++
Y18	*Debaryomyces hansenii*	98	–	+	+
Y19	*Hanseniaspora uvarum*	98	+	–	+
Y20	*Hanseniaspora uvarum*	99	+++	–	–
Y21	*Debaryomyces hansenii*	***	+++	–	–
Y24	*Meyerozyma guilliermondii*	***	++	++	+++
Y25	*Hanseniaspora uvarum*	93	+++	++	–
Y26	*Hanseniaspora uvarum*	***	+	–	–
Y27	*Hanseniaspora uvarum*	***	–	–	+
Y30	*Candida oleophila*	***	+	–	+
Y31	*Candida oleophila*	92	+	+	–
Y32	*Candida oleophila*	***	+	–	–
Y34	*Candida oleophila*	99	+	–	–
Y35	*Rhodotorula dairenensis*	99	++	+++	++
Y36	*Candida oleophila*	***	++	–	–
Y37	*Candida oleophila*	99	++	–	–
Y38	*Hanseniaspora uvarum*	99	+	–	+

Apples were uniformly wounded (approximately 5 mm diameter and 3 mm deep), with a sterile cork borer. After 15 min, 15 μL of sterile purified water was inoculated into the wound of the blank treatment, while the other treatments received 15 μL of the respective mould spore suspension (1×10^5 cells/mL) and then 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, *N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide, common name Captan (800 g/kg; Universal Crop Protection (Pty) Ltd, Kempton Park, South Africa) at a concentration of 0.5 g/L, was introduced into the wound. The negative control treatments were only infected with *B. cinerea*, *P. expansum* or *A. alstroemeriae* and not treated with yeast or the commercial fungicide. Treated apples were incubated at ±20°C for 7–20 days at a relative humidity of 80%. Growth inhibition results were characterised by the absence of mould development. Lesion diameters were measured, and percentage growth inhibition was calculated and analysed statistically to determine the effectiveness of the treatments.

Yeast identification

Twenty-four isolates that showed growth inhibition activity were identified during this study (Table 3). Yeast DNA was extracted using the method described by Lõoke *et al.* [2011]. Yeast identification to species level was performed by amplification of the 5.8S-internal transcriber spacer (ITS) ribosomal region, using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [Mitchell *et al.*, 1994]. PCR reaction mixture (50 μL)

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y39	*Meyerozyma guilliermondii*	90	+	+++	+++
Y43	*Hanseniaspora guilliermondii*	99	+	–	–
Y45	*Zygosaccharomyces bailii*	***	+	–	–
Y47	*Hanseniaspora opuntiae*	99	++	–	–
Y50	*Candida stellimalicola*	97	+	–	+++
Y51	*Pichia kudriavzevii*	100	++	–	–
Y53	*Rhodotorula dairenensis*	***	–	–	+++
Y54	*Hanseniaspora guilliermondii*	***	+	–	–
Y55	*Pichia kudriavzerii*	94	+	–	–
Y56	*Pichia fermentans*	***	–	–	+
Y57	*Hanseniaspora valbyensis*	***	–	+	+
Y58	*Saccharomyces cariocanus*	***	+	–	–
Y61	*Dekkera anomala*	***	+	–	+
Y62	*Dekkera anomala*	***	–	+	–
Y63	*Candida pyralidae*	***	+++	+++	+++
Y65	*Meyerozyma guilliermondii*	98	+	+++	++
Y67	*Brettanomyces lambicus*	***	+	+	–
Y69	*Zygosaccharomyces bailii*	***	–	–	++
Y70	*Lanchancea thermotolerans*	***	–	–	+
Y71	*Torulaspora delbrueckii*	***	+	–	–
Y72	*Metschnikowia pulcherrima*	***	–	+	–
Y73	*Lanchancea thermotolerans*	***	+	–	–
Y74	*Torulaspora delbrueckii*	93	++	+++	++
Y75	*Saccharomyces cerevisiae*	***	+	+	++
Y76	*Zygosaccharomyces bailii*	***	+	–	–
Y78	*Meyerozyma guilliermondii*	98	+	–	–
Y79	*Pichia kluyveri*	***	+	–	–
Y80	*Zygoascus hellenicus*	***	+++	–	–
Y81	*Meyerozyma guilliermondii*	***	+	–	+
Y82	*Meyerozyma guilliermondii*	***	+	–	+
Y83	*Brettanomyces lambicus*	***	+	+++	+++

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y84	*Debaryomyces hansenii*	***	++	+++	++
Y85	*Pichia kluyveri*	98	+	–	+
Y87	*Meyerozyma guilliermondii*	***	+	–	–
Y88	*Meyerozyma guilliermondii*	***	+++	+++	+++
Y89	*Zygoascus helenicus*	***	+++	+++	+++
Y90	*Zygosaccharomyces bailii*	***	+	–	++
Y91	*Zygosaccharomyces rouxii*	***	+	+++	++
Y92	*Zygosaccharomyces rouxii*	***	++	+	++
Y93	*Zygosaccharomyces microellipsoides*	***	+	+++	++
Y94	*Zygosaccharomyces cidri*	***	+	–	–
Y95	*Zygosaccharomyces florentinus*	***	+	+++	++
Y96	*Zygosaccharomyces fermentati*	***	+	++	++
Y97	*Zygosaccharomyces bisporus*	***	+	+	++
Y98	*Zygosaccharomyces bisporus*	***	++	+	–
Y99	*Brettanomyces bruxellensis*	***	–	+	–
Y100	*Brettanomyces bruxellensis*	***	–	+	–
Y101	*Brettanomyces lambicus*	***	–	+	+
Y102	*Candida magnoliae*	***	+	+	++
Y103	*Saccharomyces cerevisiae*	95	+	++	++
Y104	*Saccharomyces cerevisiae*	***	+	–	+
Y105	*Meyerozyma guilliermondii*	***	+	–	–

*(-) no growth inhibition, (+) mild activity: inhibition zone less than 6 mm, (++) medium activity: inhibition zone 6 to 9 mm, (+++) strong activity: inhibition zone exceeded 10 mm.
**Percentage similarity compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool.
***Not identified during this study, previously identified yeasts.

contained 5 μL of SuperTherm Taq buffer, 0.2 μL of SuperTherm Taq polymerase (Separation Scientific SA (Pty) Ltd, Johannesburg, South Africa), 1.5 μL of 25 mM $MgCl_2$, 1 μL of 2.5 mM deoxynucleotide (dNTP) solution, 3 μL of each primer (2.5 mM), 0.5 μL of bovine serum albumin (BSA), 5 μL of template DNA (100 ng/μL) and 30.8 μL of sterile dH_2O. The PCR conditions used were: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were submitted to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for Sanger sequencing. The sequenced fragments were then compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nih.gov/BLAST).

Statistical analyses

Growth inhibition data were subjected to the analysis of variance (ANOVA) using XLSTAT software (Version 18.07.39157, Addinsoft, New York, NY, USA) and the general linear model (GLM) procedure of SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). Fisher's least significant difference (LSD) values were calculated at the 5% probability level (p=0.05) to facilitate comparison between treatment means.

RESULTS AND DISCUSSION

Radial growth inhibition assay and yeast identification

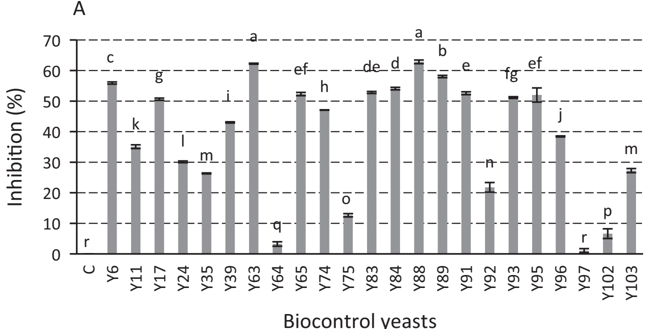
Out of the 104 yeasts tested, 83 showed growth inhibition activity against the selected mould species. Sixty-seven yeasts showed growth inhibition activity against *P. expansum*, 47 against *A. alstroemeriae,* 36 against *B. cinerea*, and 22 yeasts showed inhibition activity against all three moulds (Table 3). Most of the yeasts that showed growth inhibition activity against at least one mould species belonged to the genus *Hanseniaspora*, with *Hanseniaspora uvarum* being the predominant species. However, of the 22 yeasts that showed activity against all three mould species, most of the isolates belonged to the genus *Zygosaccharomyces* (Table 1). Only 24 of the 83 isolates that showed growth inhibition activity were identified during this study (Table 3). The identities of the other 59 isolates that showed growth inhibition activity were already known.

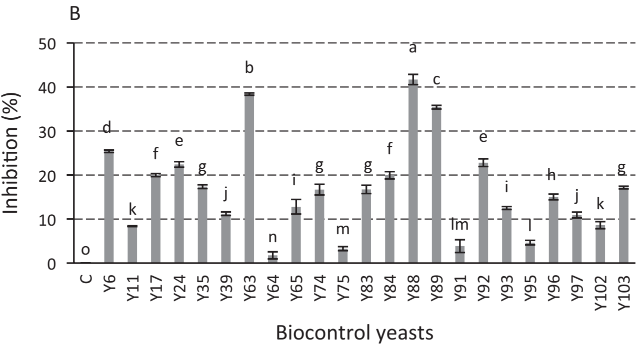
Yeasts can inhibit the growth of mould in different ways, such as the ability to grow faster than the spoilage mould by rapidly colonising surfaces, competition for nutrients or by production of growth inhibition compounds [Banjara *et al*., 2016; Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019b].

Diffusible metabolites assay

The 22 selected yeast strains and the reference strain (Y64) showed varying levels of antagonistic effects against *B. cinerea, P. expansum* and *A. alstroemeriae* (Figure 2). In general, the selected yeasts showed the highest inhibition activity against *B. cinerea* (39% mean inhibition) and lower activity against *A. alstroemeriae* (31% mean inhibition) and *P. expansum* (17% mean inhibition).

Y88 (*Meyerozyma guilliermondii*), Y63 (*Candida pyralidae*) and Y89 (*Zygoascus hellenicus*) exhibited the highest growth inhibition activity against *B. cinerea*, with 63%, 62% and 58%, respectively (Figure 2a). Y88, Y63 and Y89 showed significantly higher inhibition activity than the other 20 yeast treatments. Yeast Y64 (*Pichia kluyveri*), which was selected as the reference yeast, showed low inhibition activity (3%) against *B. cinerea*. Yeasts Y88*,* Y63 and Y89 also exhibited the highest growth inhibition activity against *P. expansum*, with 42%, 38% and 35%, respectively, and were significantly better than the other yeast treatments (Figure 2b). The reference yeast strain (Y64) showed 2% inhibition against *P. expansum*. The same three yeasts (Y88, Y63 and Y89) also exhibited the highest inhibition activity against *A. alstroemeriae*, with 41%, 37% and 35%, respectively (Figure 2c). The reference yeast (Y64) showed 7% inhibition activity against *A. alstroemeriae*. *Meyerozyma guilliermondii* strain Y88 had the highest inhibition activity against all three





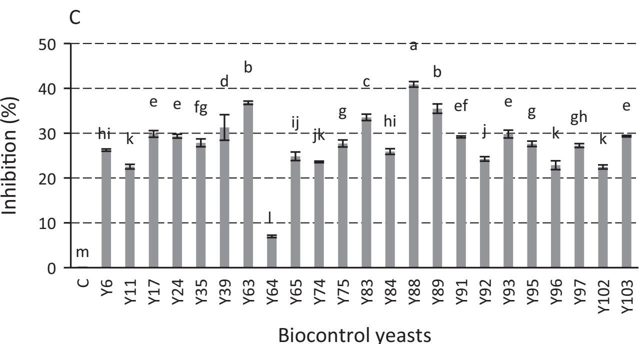


FIGURE 2. Growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the diffusible metabolites assay results. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences (p<0.05) between treatments. The plates of negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

mould species. This is the first report of growth inhibition activity of the *M. guilliermondii* species against *A. alstroemeriae*. Al-Rahbi *et al*. [2021] and Al-Maawali *et al*. [2021] reported that *M. guilliermondii* had an antagonistic effect against *Alternaria alternata* under *in vitro* conditions. In turn, Wang *et al*. [2018] reported that *M. guilliermondii* exerted antagonistic effects against two strains of *B. cinerea*, while inhibition of *P. expansum* growth by *M. guilliermondii* was reported by Han *et al.* [2021].

Candida pyralidae Y63 was the second-best performing yeast against all three moulds. This is in agreement with the findings of Mewa-Ngongang *et al*. [2019b], who reported the antagonistic effects of *C. pyralidae* against the germination of *B. cinerea* spores under *in vitro* conditions. This is the first report of the growth inhibition properties of *C. pyralidae* against *P. expansum* and *A. alstroemeriae,* and of the growth inhibition properties of *Z. hellenicus* against *B. cinerea, P. expansum* and *A. alstroemeriae.* Nally *et al.* [2012] and Mewa-Ngongang *et al*. [2019b] also reported that different yeast species showed growth inhibition activity at different levels against fruit spoilage mould, which is in agreement with the findings of the current study.

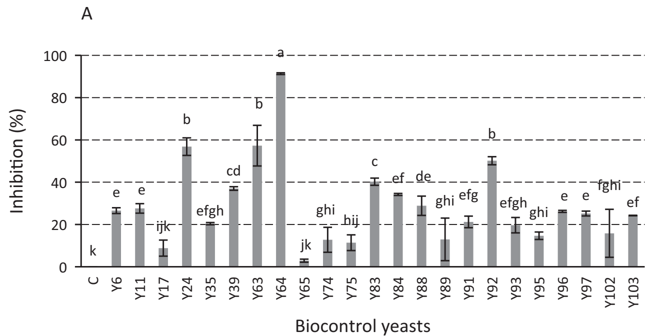
Volatile organic compound assay

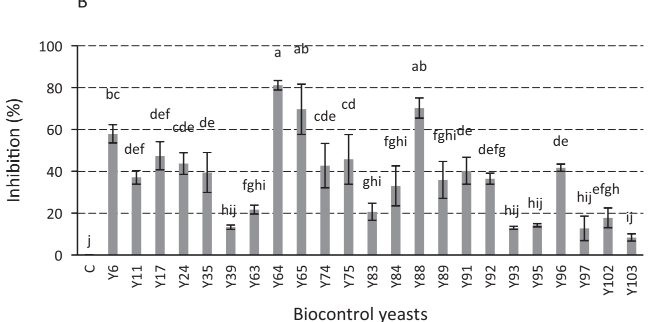
The production of VOCs as a mode of action against mould was investigated using the mouth-to-mouth assay. Most of the 23 yeasts produced VOCs that inhibited the growth of *B. cinerea* (Figure 3a), *P. expansum* (Figure 3b) and *A. alstroemeriae* (Figure 3c), but the level of growth inhibition varied among the yeasts. Yeast isolates Y64 (*P. kluyveri*), Y63 (*C. pyralidae*), Y24 (*M. guilliermondii*) and Y92 (*Zygosaccharomyces rouxii*) showed 91%, 57%, 56% and 50% growth inhibition activity against *B. cinerea*, respectively (Figure 3a). Against *P. expansum,* the highest growth inhibition was shown by *P. kluyveri* Y64, *M. guilliermondii* Y88 and Y65, with 81%, 70% and 69%, respectively (Figure 3b). The best performing yeasts against *A. alstroemeriae* were *P. kluyveri* Y64, *C. pyralidae* Y63 and *M. guillermondii* Y88, with 76%, 68% and 61% growth inhibition activity, respectively (Figure 3c).

Yeast isolate Y64 (*P. kluyveri*) showed the highest growth inhibition activity against all three moulds and was significantly better than the other yeast treatments during the VOC trial (Figure 3). While the opposite was observed during the diffusible metabolite assay (Figure 2). This strongly suggests that the mode of action of Y64 is linked to its ability to produce VOCs. The findings of this study are in agreement with Mewa-Ngongang *et al*. [2019b], who also reported on the ability of *P. kluyveri* and *C. pyralidae* to inhibit the growth of *B. cinerea* under *in vitro* conditions. Ruiz-Moyano *et al.* [2020] reported that *H. uvarum* also produced VOCs to control the growth of *B. cinerea* on fruits. Choińska *et al*. [2020] observed that *M. guilliermondii* produced VOCs to control the growth of *B. cinerea* and *P. expansum*, which is in agreement with the findings from this study. *Pichia kluyveri* showed the highest inhibition against *P. expansum*. Cordero-Bueso *et al*. [2017] also reported that VOCs produced by *P. kluyveri* exhibited antagonistic activity against *P. expansum.* This is the first report of VOCs from *P. kluyveri*, *C. pyralidae* and *M. guillermondii* inhibiting the growth of *A. alstroemeriae.* However, Al-Maawali *et al*. [2021] showed that VOCs produced by *M. guilliermondii* inhibited the mycelial growth of *A. alternata.*

Post-harvest application of biocontrol yeasts on apples

The yeasts were effective in preventing mould spoilage of apples and reducing decay considerably (Figure 4). The inhibition responses were yeast and mould species-dependent. *Meyerozyma guilliermondii* Y88 and *P. kluyveri* Y64 were effective in suppressing mould growth on apples, with 100% inhibition activity against *A. alstroemeriae.* The commercial fungicide (Captan) also provided 100% inhibition. *Candida pyralidae* Y63 showed 36% inhibition against *A. alstroemeriae,* which was significantly lower compared to the other treatments*.* This is the first report on growth inhibition activity of *P. kluyveri*, *C. pyralidae* and *M. guillermondii* against *A. alstroemeriae* on apples*.* However, Al-Rahbi *et al*. [2021]





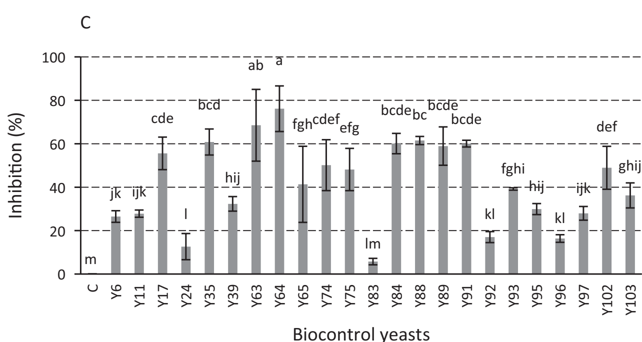


FIGURE 3. The growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the volatile organic compound production. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences (p<0.05). The negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

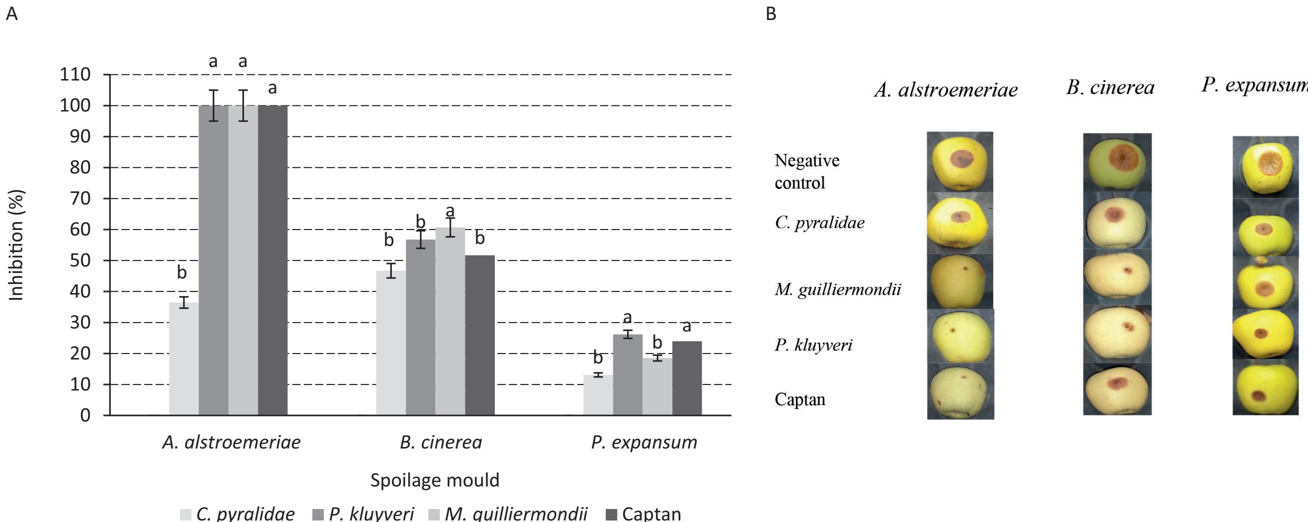


FIGURE 4. The growth inhibition activity expressed as a percentage (%) of *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Pichia kluyveri* Y64 against *Alternaria alstroemeriae*, *Botrytis cinerea* and *Penicillium expansum* during postharvest trials on apples (A). Values are means of five replicates and the standard deviations are also shown. The different letters indicate significant differences ($p<0.05$) between treatments. (B) Photographs of apples showing lesion diameters. Each set is a representative example of 25 apples. For the negative control treatments, the apples were only infected with the respective moulds, therefore no growth inhibition.

and Al-Maawali *et al*. [2021] showed that *M. guilliermondii* reduced the fruit rot lesions of *A. alternata* on strawberries and tomatoes by 68% and 50%, respectively.

Against *B. cinerea*, *M. guilliermondii* Y88 and *P. kluyveri* Y64 showed 61% and 57% inhibition, respectively, which was higher than the 52% obtained by the commercial fungicide (Figure 4a). *Candida pyralidae* Y63 inhibited *B. cinerea* growth by 47%, which was significantly lower than the other treatments. These findings are in agreement with those of Mewa-Ngongang *et al*. [2019b] who also reported on the antagonistic effects of *C. pyralidae* against *B. cinerea* on apples. Wang *et al*. [2018] reported that *M. guilliermondii* showed an antagonistic effect against *B. cinerea* isolates on grape berries, while Mewa-Ngongang *et al*. [2021], showed that *P. kluyveri* when applied preventively, was effective in suppressing *B. cinerea* growth by 95% on apples.

All the yeasts showed the lowest growth inhibition activity against *P. expansum* (Figure 4a). *Pichia kluyveri* Y64 exhibited the highest growth inhibition activity (26%) against *P. expansum* and performed slightly better than the commercial fungicide, which ensured 24% inhibition. The commercial fungicide displayed lower activity against *B. cinerea* and *P. expansum* than expected, which could be possibly attributed to the resistance of the specific moulds. Follow up studies should include more than one fungicide. *Meyerozyma guilliermondii* Y88 and *C. pyralidae* Y63 inhibited the growth of *P. expansum* by 19% and 13%, respectively. This study confirmed the findings of Cordero-Bueso *et al*. [2017], who reported that *P. kluyveri* exhibited antagonistic activity against *P. expansum*. In turn, Han *et al*. [2021] demonstrated that *M. guilliermondii* exhibited antagonistic activity against *P. expansum* on pears. This is the first report on the growth inhibition properties of *C. pyralidae* against *P. expansum* on apples. These observations on apples could be of great importance to the agricultural industry because these biocontrol yeasts can potentially be used as alternatives to chemical fungicides.

CONCLUSIONS

The cell suspensions of yeast strains *C. pyralidae* Y63, *M. guillermondii* Y88 and *Z. hellenicus* Y89 elicited the best antagonistic effects against *B. cinerea*, *P. expansum* and *A. alstroemeriae.* The production of VOCs by *P. kluyveri* was the mechanism of inhibition against *B. cinerea*, *P. expansum* and *A. alstroemeriae. Candida pyralidae* Y63, *M. guillermondii* Y88 and *P. kluyveri* Y64 were effective inhibitors of all three mould species on apples and their efficacy was comparable to the commercial fungicide. These yeasts can potentially be considered as alternatives to chemical fungicides. However, further research is needed to determine how to apply these yeast-based biocontrol agents and to establish the most effective minimum dosage or inhibitory concentration needed. The main VOCs and other possible compounds that are responsible for inhibition should be identified and the production process needs to be optimised. Future research should also investigate other mechanisms of action and the application of yeast-based biological agents on fruit for pre-harvest control of mould.

ACKNOWLEDGEMENTS

The authors would like to thank the Agricultural Research Council (ARC) and the National Research Foundation (NRF) of South Africa for funding and infrastructure. The opinions, findings and conclusions or recommendations expressed in this publication is that of the authors alone, and the NRF accepts no liability whatsoever in this regard. Thank you to Ms Louise Smit for culturing and supplying *Botrytis cinerea*. The authors extend their gratitude to all students, interns, technicians and research assistants for their individual contributions.

RESEARCH FUNDING

This work was supported by the Agricultural Research Council (ARC) and the National Research Foundation (NRF) of South Africa (Grant Numbers: 117833 and 122220).

CONFLICTS OF INTEREST

The authors hereby declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

The following are available online at http://journal.pan.olsztyn.pl/Control-of-Mould-Spoilage-on-Apples-Using-Yeasts-as-Biological-Control-Agents,147913,0,2.html; Yeast used in the study. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Meyerozyma guilliermondii* Y88 against *B. cinerea* (B) on yeast malt agar. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Pichia kluyveri* Y64 against *B. cinerea* (B) on yeast malt agar.

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Pol. J. Food Nutr. Sci., 2022, Vol. 72, No. 2, pp. 119–128
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/147913
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Control of Mould Spoilage on Apples Using Yeasts as Biological Control Agents

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Key words: mould spoilage, fungicides, postharvest control, biocontrol agents (BCAs), apples, yeast

Considerable quantities of fruit are lost during pre- and post-harvest stages due to mould spoilage. The aim of this study was to evaluate the antagonistic effect of selected yeasts against spoilage mould *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae*. One hundred and four yeast isolates were screened for antagonistic activity against *B. cinerea*, *P. expansum* and *A. alstroemeriae* using radial inhibition, dual and mouth-to-mouth plate assays. Sixty-seven out of 104 yeasts showed growth inhibition activity against *P. expansum*, while 36 yeasts inhibited *B. cinerea*, 47 yeasts inhibited *A. alstroemeriae,* but only 22 yeasts displayed inhibition activity against all three moulds. *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Zygoascus hellenicus* Y89 had the highest inhibition activity against all three moulds, when mode of inhibition was due to direct contact. Volatile organic compounds produced by *Pichia kluyveri* Y64, *C. pyralidae* Y63 and *M. guilliermondii* Y88 demonstrated the highest growth inhibition against all three moulds. These yeasts were also evaluated against all three moulds on apples. *P. kluyveri* Y64 displayed 100%, 57% and 26% growth inhibition against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively, on apples and performed slightly better than a commercial fungicide against *B. cinerea* and *P. expansum*. While *M. guillermondii* Y88 showed 100%, 60% and 18% inhibition on apples against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively. *P. kluyveri* Y64 and *M. guilliermondii* Y88 demonstrated potential as biofungicides and warrant further investigation.

INTRODUCTION

Fruits are commercially and nutritionally important commodities and play an important role in human health by supplying vitamins and minerals [Al-Hindi *et al*., 2011]. Globally, apple (*Malus domestica*) production increased from 75 to more than 85 million tonnes per annum from 2014 to 2020 [FAOstat, 2020]. South Africa is a relatively small apple grower in terms of global hectares and produces approximately 1.3 million tonnes each year, with a total value of more than 8 billion rands [Du Plessis, 2017]. Ninety-two percent of this income is generated by fresh fruit sales [Du Plessis, 2017]. Agricultural products are subject to mould spoilage before, during and after harvest, as well as during transportation and processing [Romanazzi *et al*., 2016; 2017]. Fruit losses due to spoilage mould pose several challenges to the agrifood industry [Parveen *et al*., 2016]. Each year, 25% of the total fruits produced is lost in industrialised countries and more than 50% in developing countries [Droby, 2005; Nunes, 2012]. During the pre- and post-harvest stages, considerable amounts of fruits including apples are lost due to mould diseases caused by *Botrytis cinerea* (grey mould), *Penicillium expansum* (blue mould), *Alternaria* spp. (necrotic leaf blotch), *Venturia inaequalis* (apple scab), *Cladosporium* spp. (Sooty spot) and *Colletotrichum gloeosporioides* (bitter rot) [Sharma *et al*., 2009].

Spoilage moulds need to be controlled to maintain the quality and abundance of fruit produced around the world [Mercier & Lindow, 2001]. Currently, fruit producers and exporters are using costly spraying programs incorporating synthetic chemicals, which are labour intensive and require the application of various classes of fungicides sprayed up to 20 times during the growing season. These practices, even at the lowest dose, can negatively affect the health of consumers,

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Submitted: 15 December 2021
Accepted: 31 March 2022
Published on-line: 30 May 2022

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the environment, and the taste and aroma of the food being preserved [Benito *et al*., 2009; Contarino *et al*., 2019; Oliveira *et al*., 2014]. Some mould can become resistant to fungicides as farmers use the chemicals regularly [Fernández-Ortuño *et al*., 2008].

The desire to minimise chemical residues and to offset rising prices of new synthetic chemicals is fostering the search for alternatives to synthetic chemical fungicides [Quaglia *et al*., 2011; Robiglio *et al*., 2011]. The recent trend is shifting towards safer and environmentally friendly alternatives for the control of post-harvest decay [Sharma *et al*., 2009]. Biological control using yeasts demonstrated great potential as an alternative to chemical fungicides [Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019a] and is more environmentally friendly and cost effective [Bonaterra et al., 2012]. In the last years, a considerable amount of microbial-based commercial products and patents have been developed worldwide in order to exploit the microbial strategies to counteract the growth of spoilage and/or pathogenic microorganisms in pre and post-harvest [De Simone *et al*., 2021].

Yeasts can be used as an alternative to synthetic chemicals because of their ability to compete for nutrients and space, their ability to grow faster than most fungal pathogens and the production of inhibitory growth compounds [Liu *et al*., 2013]. Yeasts, such as *Meyerozyma guilliermondii*, *Candida pyralidae* and *Hanseniaspora* species, have the ability to secrete extracellular metabolites, such as volatile organic compounds (VOCs), acetic acid, hydrogen sulphide and cell wall-degrading enzymes, which have antimicrobial properties against many fruit spoilage moulds [Al-Maawali *et al*., 2021; Cordero-Bueso *et al*., 2017; Grevesse *et al*., 2003; Han *et al*., 2021; Hua *et al*., 2014; Mewa-Ngongang *et al*., 2019b; Ruiz-Moyano *et al*., 2020; Zhou *et al*., 2018]. The aim of this study was to screen yeasts for growth inhibition activity against *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae* under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Culturing conditions and inoculum preparation

One hundred and four yeast isolates were obtained for evaluation from the ARC Infruitec-Nietvoorbij (the Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa), the Instituto Superior de Agronomia (Lisbon, Portugal), the Centraal Bureau voor Schimmelcultures (Utrecht, Netherlands), the Gulbenkian Institute of Science (Oeiras, Portugal) and the Council for Scientific and Industrial Research (Pretoria, South Africa) (Table S1 in the Supplementary Materials). Yeast selection was based on previous research and the fact that the yeasts were isolated from different fruits and environments. The yeasts were cultured on yeast malt agar (YMA) (1% glucose, 0.3% malt extract, 0.5% peptone, 2% bacteriological agar) for 2 days at 28°C. A wire loopful of each pure yeast colony on the plates was transferred into test tubes containing 5 mL of sterilised yeast malt broth (YMB) (Sigma-Aldrich, Saint Louis, USA) and incubated at 28°C for 2 days. Thereafter 1 mL of the culture was transferred to a sterile 2 mL microtube and centrifuged at 20,400×*g* for 5 min. The supernatant was discarded, and the pellet resuspended in 100 μL of sterile distilled water. Yeast cells were counted using a Neubauer haemocytometer (Sigma-Aldrich, Darmstadt, Germany) and a microscope (Euromex, Arnhem, Netherlands), at 400× magnification, in order to prepare the yeast inoculum (1×10^8 cells/mL).

For the apple bioassay, grape pomace extract was obtained by pressing "Chenin Blanc" grape pomace from the ARC Infruitec-Nietvoorbij research farm (Stellenbosch) at 200 kPa. The resultant grape pomace extract was frozen in a 25 L polypropylene bucket at −20°C. Prior to use, the grape pomace extract was thawed and diluted with sterile distilled water to a sugar concentration of 100 g/L. Yeast strains Y63 (*C. pyralidae*), Y88 (*M. guilliermondii*) and Y64 (*P. kluyveri*) from the ARC Infruitec-Nietvoorbij culture collection, were grown in 5 mL of YMB for 2 days at 28°C and then transferred to Erlenmeyer flasks containing 50 mL of sterile grape pomace extract broth (GPB), incubated at 28°C and agitated at 150 rpm, using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 2 days. The yeast cultures were then transferred to 500 mL of GPB and grown at 28°C for 24 h under agitation. The yeast inoculum of 1×10^8 cells/mL was used, as mentioned previously.

The fruit spoilage moulds, *B. cinerea* FFD 003–15, *P. expansum* C370V59 and *A. alstroemeriae* C370V51, were obtained from the fungal genebank and Post-harvest Pathology laboratory at ARC Infruitec-Nietvoorbij and cultured for 7 to 14 days at 25°C on potato dextrose agar (PDA, Merck, Johannesburg, South Africa). Spores were harvested by gently scraping them from the surface of the agar and rinsing with sterile distilled water to attain a volume of 50 mL of the spore suspension in a sterile 250 mL Schott bottle. Prior to the dilution with sterile distilled water, a haemocytometer and a microscope (400× magnification) were used to count the spores in the initial spore solution, as previously described. The inoculum was prepared by diluting the spore suspension to 1×10^5 spores/mL.

Radial growth inhibition assay

The radial growth inhibition assay was applied as described by Núñez *et al*. [2015], with some modifications. In brief, a 5 mm mycelial disk, obtained from a 7-day old mould culture, was placed at the centre of a fresh YMA plate using a sterile cork borer. Subsequently, 15 μL of the yeast cells suspension (1×10^8 cells/mL) was spotted 25 mm away from the mycelial disk. Four different yeast isolates were spotted per plate and incubated at 25°C for 7 days. All yeast treatments had three replicates. The control plates only contained the 5 mm diameter mycelial disk of the respective mould. Results were recorded as (-) no activity, (+) mild activity, (++) medium activity, (+++) strong activity. Positive (+) growth inhibition results were observed by the presence of C-shaped growth around the yeast colonies, as shown in Figure 1. Yeasts with strong inhibition activity (+++) had the biggest C-shaped inhibtion zone and the distance between the yeast colony and the mould growth exceeded 10 mm. Yeasts with medium inhibtion activity (++) had a smaller C-shaped zone and the distance between the yeast colony and the mould growth was between 6 and 9 mm. For yeasts with mild

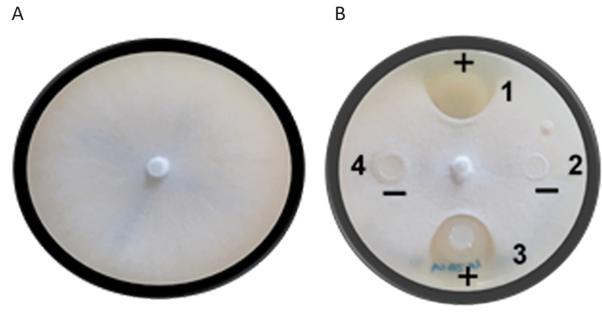


FIGURE 1. An example of the radial growth inhibition assay results. *Botrytis cinerea* growth (A) and antagonistic effect of selected yeast isolates *Candida pyralidae* Y63 (1), *Pichia kluyveri* Y64 (2), *Meyerozyma guilliermondii* Y88 (3) and *Debaryomyces hansenii* Y8 (4) against *B. cinerea* (B) on yeast malt agar. The positive sign (+) represents growth inhibition activity and the negative sign (-) represents no inhibition. This is a representative example of three replicates.

activity (+), the distance between the yeast and mould colony was less than 6 mm and no activity (-) meant that no mould growth inhibition was observed.

Diffusible metabolites assay

The dual assay described by Chen *et al*. [2018] was used to evaluate 23 yeasts (Table 1). Only those yeasts that showed growth inhibition activity against all three mould species during the radial growth inhibition assay were evaluated further. The yeast Y64 (*Pichia kluyveri*) was used in a previous study by Mewa-Ngongang *et al.* [2019a,b] and was included as the reference strain. Similar to the radial growth inhibition assay, a 5 mm mycelial disk was placed at the edge of the YMA plate. Subsequently, 20 μL of the yeast suspension (1×10^8 cells/mL) was spotted 40 mm away from the mycelial disk (Figure S1 in the Supplementary Materials) and incubated at 25°C for 9 days. The negative control (C) plates contained only the 5 mm diameter mycelial disk of the respective mould. All treatments had three replicates. The percentage inhibition was calculated as fungal radial growth inhibition (FRGI) using the following mathematical expression:

$$FRGI = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (1)$$

with D_0 representing the horizontal growth average of the fungal colony on the negative control plates and D_t representing the horizontal growth average of the fungal colony on the yeast treated plates (Figure S1 in the Supplementary Materials), as described by Núñez *et al.* [2015].

Volatile organic compound (VOCs) assay

To assess the effect of VOCs produced by the 23 yeasts used in relation to their growth inhibition potential against fruit spoilage organisms, the mouth-to-mouth assay described by Medina-Córdova *et al*. [2016] was used. Two YMA plates facing each other were sealed with laboratory film, per experimental repeat. The bottom plate was spread with 100 μL of the yeast suspension (1×10^8 cell/mL), while the top plate contained a 5 mm mould mycelial disk placed at the centre. The negative control treatment (C) only contained the 5 mm diameter mycelial disk in the centre of the plate, while no yeast was spread on the second plate. The plates were incubated at 25°C for 7 days. All treatments had three replicates. The VOC inhibition activity (VOCIA) was calculated using the following mathematical expression [Núñez *et al*., 2015]:

$$VOCIA = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (2)$$

with D_0 representing the average diameter of the fungal colony on the negative control plates and D_t representing the diameter of the fungal colony on the yeast treated plates, as shown in Figure S2 in the Supplementary Materials.

TABLE 1. Yeasts selected for the dual and mouth-to-mouth assays on yeast malt agar.

Yeast code	Species
Y6	*Aureobasidium melanogenum*
Y11	*Debaryomyces hansenii*
Y17	*Hanseniaspora occidentalis*
Y24	*Meyerozyma guilliermondii*
Y35	*Rhodotorula dairenensis*
Y39	*Meyerozyma guilliermondii*
Y63	*Candida pyralidae*
Y64	*Pichia kluyveri**
Y65	*Meyerozyma guilliermondii*
Y74	*Torulaspora delbrueckii*
Y75	*Saccharomyces cerevisiae*
Y83	*Brettanomyces lambicus*
Y84	*Debaryomyces hansenii*
Y88	*Meyerozyma guilliermondii*
Y89	*Zygoascus hellenicus*
Y91	*Zygosaccharomyces rouxii*
Y92	*Zygosaccharomyces rouxii*
Y93	*Zygosaccharomyces microellipsoides*
Y95	*Zygosaccharomyces florentinus*
Y96	*Zygosaccharomyces fermentati*
Y97	*Zygosaccharomyces bisporus*
Y102	*Candida magnolia*
Y103	*Saccharomyces cerevisiae*

*Used as reference yeast.

Post-harvest application using apple bioassay

The post-harvest biocontrol efficacy assay was performed on the apple cultivar "Panorama Goldens" and sixteen treatments were applied (Table 2). Each treatment had five replicates. Each replicate consisted of a rectangular fruit-packaging box containing five apples. Ethanol (70%, *v/v*) was sprayed on the apples to eradicate any microorganisms on the surface and allowed to dry completely before wound infliction.

TABLE 2. Treatments applied on apples during postharvest biocontrol trials*.

Treatment	Treatment description
Treatment 1	Sterile distilled water (Control)
Treatment 2	*Botrytis cinerea*
Treatment 3	*Penicillium expansum*
Treatment 4	*Alternaria alstroemeriae*
Treatment 5	*B. cinerea* and *Candida pyralidae* Y63
Treatment 6	*P. expansum* and *C. pyralidae* Y63
Treatment 7	*A. alstroemeriae* and *C. pyralidae* Y63
Treatment 8	*B. cinerea* and *Meyerozyma guilliermondii* Y88
Treatment 9	*P. expansum* and *M. guilliermondii* Y88
Treatment 10	*A. alstroemeriae* and *M. guilliermondii* Y88
Treatment 11	*B. cinerea* and *Pichia kluyveri* Y64
Treatment 12	*P. expansum* and *P. kluyveri* Y64
Treatment 13	*A. alstroemeriae* and *P. kluyveri* Y64
Treatment 14	*B. cinerea* and Captan
Treatment 15	*P. expansum* and Captan
Treatment 16	*A. alstroemeriae* and Captan

*Apples were incubated in rectangular fruit packaging boxes and five boxes (replicates) were used per treatment, with each box containing five apples.

Apples were uniformly wounded (approximately 5 mm diameter and 3 mm deep), with a sterile cork borer. After 15 min, 15 μL of sterile purified water was inoculated into the wound of the blank treatment, while the other treatments received 15 μL of the respective mould spore suspension (1×10^5 cells/mL) and then 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, *N*-trichloromethylthio-4-cyclohexene-1,2--dicarboximide, common name Captan (800 g/kg; Universal Crop Protection (Pty) Ltd, Kempton Park, South Africa) at a concentration of 0.5 g/L, was introduced into the wound. The negative control treatments were only infected with *B. cinerea*, *P. expansum* or *A. alstroemeriae* and not treated with yeast or the commercial fungicide. Treated apples were incubated at ±20°C for 7–20 days at a relative humidity of 80%. Growth inhibition results were characterised by the absence of mould development. Lesion diameters were measured, and percentage growth inhibition was calculated and analysed statistically to determine the effectiveness of the treatments.

Yeast identification

Twenty-four isolates that showed growth inhibition activity were identified during this study (Table 3). Yeast DNA was extracted using the method described by Lõoke *et al*. [2011]. Yeast identification to species level was performed by amplification of the 5.8S-internal transcriber spacer (ITS) ribosomal region, using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [Mitchell *et al*., 1994]. PCR reaction mixture (50 μL)

TABLE 3. Species identity and growth inhibition activity* of the yeasts screened against selected mould on yeast malt agar.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y1	*Rhodotorula dairenensis*	***	+	−	++
Y2	*Hanseniaspora uvarum*	***	+	−	−
Y3	*Hanseniaspora uvarum*	91	−	+++	−
Y5	*Saccharomyces uvarum*	***	+	−	+
Y6	*Aureobasidium melanogenum*	99	++	+++	++
Y7	*Aureobasidium melanogenum*	***	−	++	+++
Y8	*Debaryomyces hansenii*	***	+	−	−
Y10	*Saccharomyces uvarum*	***	+	−	+
Y11	*Debaryomyces hansenii*	***	+	++	++
Y12	*Rhodotorula dairenensis*	***	++	−	++
Y13	*Hanseniaspora opuntiae*	99	−	++	−
Y14	*Saccharomyces uvarum*	97	+	−	+++
Y15	*Hanseniaspora uvarum*	***	−	−	+
Y16	*Hanseniaspora uvarum*	***	+	−	−
Y17	*Hanseniaspora occidentalis*	89	++	+++	+++
Y18	*Debaryomyces hansenii*	98	−	+	+
Y19	*Hanseniaspora uvarum*	98	+	−	+
Y20	*Hanseniaspora uvarum*	99	+++	−	−
Y21	*Debaryomyces hansenii*	***	+++	−	−
Y24	*Meyerozyma guilliermondii*	***	++	++	+++
Y25	*Hanseniaspora uvarum*	93	+++	++	−
Y26	*Hanseniaspora uvarum*	***	+	−	−
Y27	*Hanseniaspora uvarum*	***	−	−	+
Y30	*Candida oleophila*	***	+	−	+
Y31	*Candida oleophila*	92	+	+	−
Y32	*Candida oleophila*	***	+	−	−
Y34	*Candida oleophila*	99	+	−	−
Y35	*Rhodotorula dairenensis*	99	++	+++	++
Y36	*Candida oleophila*	***	++	−	−
Y37	*Candida oleophila*	99	++	−	−
Y38	*Hanseniaspora uvarum*	99	+	−	+

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y39	*Meyerozyma guilliermondii*	90	+	+++	+++
Y43	*Hanseniaspora guilliermondii*	99	+	−	−
Y45	*Zygosaccharomyces bailii*	***	+	−	−
Y47	*Hanseniaspora opuntiae*	99	++	−	−
Y50	*Candida stellimalicola*	97	+	−	+++
Y51	*Pichia kudriavzevii*	100	++	−	−
Y53	*Rhodotorula dairenensis*	***	−	−	+++
Y54	*Hanseniaspora guilliermondii*	***	+	−	−
Y55	*Pichia kudriavzerii*	94	+	−	−
Y56	*Pichia fermentans*	***	−	−	+
Y57	*Hanseniaspora valbyensis*	***	−	+	+
Y58	*Saccharomyces cariocanus*	***	+	−	−
Y61	*Dekkera anomala*	***	+	−	+
Y62	*Dekkera anomala*	***	−	+	−
Y63	*Candida pyralidae*	***	+++	+++	+++
Y65	*Meyerozyma guilliermondii*	98	+	+++	++
Y67	*Brettanomyces lambicus*	***	+	+	−
Y69	*Zygosaccharomyces bailii*	***	−	−	++
Y70	*Lanchancea thermotolerans*	***	−	−	+
Y71	*Torulaspora delbrueckii*	***	+	−	−
Y72	*Metschnikowia pulcherrima*	***	−	+	−
Y73	*Lanchancea thermotolerans*	***	+	−	−
Y74	*Torulaspora delbrueckii*	93	++	+++	++
Y75	*Saccharomyces cerevisiae*	***	+	+	++
Y76	*Zygosaccharomyces bailii*	***	+	−	−
Y78	*Meyerozyma guilliermondii*	98	+	−	−
Y79	*Pichia kluyveri*	***	+	−	−
Y80	*Zygoascus hellenicus*	***	+++	−	−
Y81	*Meyerozyma guilliermondii*	***	+	−	+
Y82	*Meyerozyma guilliermondii*	***	+	−	+
Y83	*Brettanomyces lambicus*	***	+	+++	+++

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y84	*Debaryomyces hansenii*	***	++	+++	++
Y85	*Pichia kluyveri*	98	+	−	+
Y87	*Meyerozyma guilliermondii*	***	+	−	−
Y88	*Meyerozyma guilliermondii*	***	+++	+++	+++
Y89	*Zygoascus helenicus*	***	+++	+++	+++
Y90	*Zygosaccharomyces bailii*	***	+	−	++
Y91	*Zygosaccharomyces rouxii*	***	+	+++	++
Y92	*Zygosaccharomyces rouxii*	***	++	+	++
Y93	*Zygosaccharomyces microellipsoides*	***	+	+++	++
Y94	*Zygosaccharomyces cidri*	***	+	−	−
Y95	*Zygosaccharomyces florentinus*	***	+	+++	++
Y96	*Zygosaccharomyces fermentati*	***	+	++	++
Y97	*Zygosaccharomyces bisporus*	***	+	+	++
Y98	*Zygosaccharomyces bisporus*	***	++	+	−
Y99	*Brettanomyces bruxellensis*	***	−	+	−
Y100	*Brettanomyces bruxellensis*	***	−	+	−
Y101	*Brettanomyces lambicus*	***	−	+	+
Y102	*Candida magnoliae*	***	+	+	++
Y103	*Saccharomyces cerevisiae*	95	+	++	++
Y104	*Saccharomyces cerevisiae*	***	+	−	+
Y105	*Meyerozyma guilliermondii*	***	+	−	−

*(-) no growth inhibition, (+) mild activity: inhibition zone less than 6 mm, (++) medium activity: inhibition zone 6 to 9 mm, (+++) strong activity: inhibition zone exceeded 10 mm.
**Percentage similarity compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool.
***Not identified during this study, previously identified yeasts.

contained 5 μL of SuperTherm Taq buffer, 0.2 μL of SuperTherm Taq polymerase (Separation Scientific SA (Pty) Ltd, Johannesburg, South Africa), 1.5 μL of 25 mM $MgCl_2$, 1 μL of 2.5 mM deoxynucleotide (dNTP) solution, 3 μL of each primer (2.5 mM), 0.5 μL of bovine serum albumin (BSA), 5 μL of template DNA (100 ng/μL) and 30.8 μL of sterile dH_2O. The PCR conditions used were: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were submitted to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for Sanger sequencing. The sequenced fragments were then compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nih.gov/BLAST).

Statistical analyses

Growth inhibition data were subjected to the analysis of variance (ANOVA) using XLSTAT software (Version 18.07.39157, Addinsoft, New York, NY, USA) and the general linear model (GLM) procedure of SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). Fisher's least significant difference (LSD) values were calculated at the 5% probability level (p=0.05) to facilitate comparison between treatment means.

RESULTS AND DISCUSSION

Radial growth inhibition assay and yeast identification

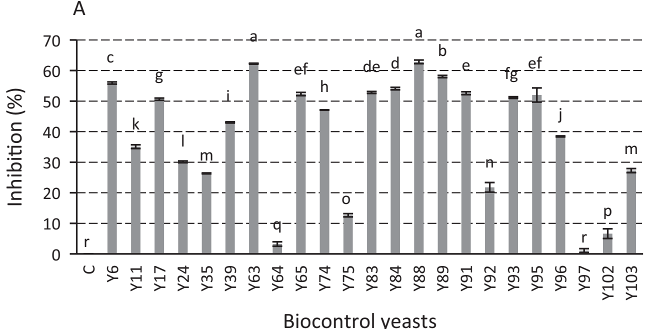
Out of the 104 yeasts tested, 83 showed growth inhibition activity against the selected mould species. Sixty-seven yeasts showed growth inhibition activity against *P. expansum*, 47 against *A. alstroemeriae,* 36 against *B. cinerea*, and 22 yeasts showed inhibition activity against all three moulds (Table 3). Most of the yeasts that showed growth inhibition activity against at least one mould species belonged to the genus *Hanseniaspora*, with *Hanseniaspora uvarum* being the predominant species. However, of the 22 yeasts that showed activity against all three mould species, most of the isolates belonged to the genus *Zygosaccharomyces* (Table 1). Only 24 of the 83 isolates that showed growth inhibition activity were identified during this study (Table 3). The identities of the other 59 isolates that showed growth inhibition activity were already known.

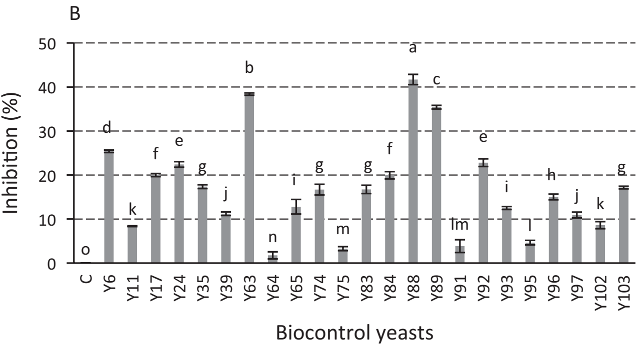
Yeasts can inhibit the growth of mould in different ways, such as the ability to grow faster than the spoilage mould by rapidly colonising surfaces, competition for nutrients or by production of growth inhibition compounds [Banjara *et al*., 2016; Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019b].

Diffusible metabolites assay

The 22 selected yeast strains and the reference strain (Y64) showed varying levels of antagonistic effects against *B. cinerea, P. expansum* and *A. alstroemeriae* (Figure 2). In general, the selected yeasts showed the highest inhibition activity against *B. cinerea* (39% mean inhibition) and lower activity against *A. alstroemeriae* (31% mean inhibition) and *P. expansum* (17% mean inhibition).

Y88 (*Meyerozyma guilliermondii*), Y63 (*Candida pyralidae*) and Y89 (*Zygoascus hellenicus*) exhibited the highest growth inhibition activity against *B. cinerea*, with 63%, 62% and 58%, respectively (Figure 2a). Y88, Y63 and Y89 showed significantly higher inhibition activity than the other 20 yeast treatments. Yeast Y64 (*Pichia kluyveri*), which was selected as the reference yeast, showed low inhibition activity (3%) against *B. cinerea*. Yeasts Y88, Y63 and Y89 also exhibited the highest growth inhibition activity against *P. expansum*, with 42%, 38% and 35%, respectively, and were significantly better than the other yeast treatments (Figure 2b). The reference yeast strain (Y64) showed 2% inhibition against *P. expansum*. The same three yeasts (Y88, Y63 and Y89) also exhibited the highest inhibition activity against *A. alstroemeriae*, with 41%, 37% and 35%, respectively (Figure 2c). The reference yeast (Y64) showed 7% inhibition activity against *A. alstroemeriae*. *Meyerozyma guilliermondii* strain Y88 had the highest inhibition activity against all three





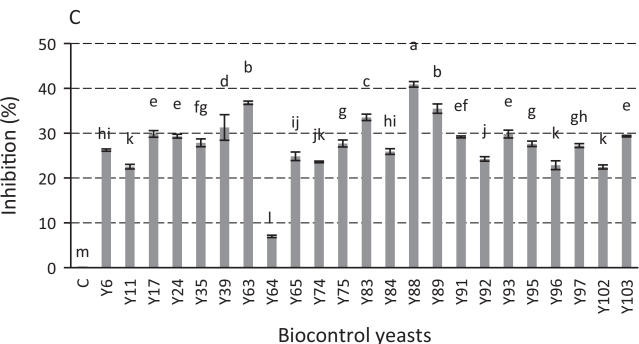


FIGURE 2. Growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the diffusible metabolites assay results. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences (p<0.05) between treatments. The plates of negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

mould species. This is the first report of growth inhibition activity of the *M. guilliermondii* species against *A. alstroemeriae.* Al-Rahbi *et al*. [2021] and Al-Maawali *et al*. [2021] reported that *M. guilliermondii* had an antagonistic effect against *Alternaria alternata* under *in vitro* conditions. In turn, Wang *et al*. [2018] reported that *M. guilliermondii* exerted antagonistic effects against two strains of *B. cinerea*, while inhibition of *P. expansum* growth by *M. guilliermondii* was reported by Han *et al.* [2021].

Candida pyralidae Y63 was the second-best performing yeast against all three moulds. This is in agreement with the findings of Mewa-Ngongang *et al*. [2019b], who reported the antagonistic effects of *C. pyralidae* against the germination of *B. cinerea* spores under *in vitro* conditions. This is the first report of the growth inhibition properties of *C. pyralidae* against *P. expansum* and *A. alstroemeriae,* and of the growth inhibition properties of *Z. hellenicus* against *B. cinerea, P. expansum* and *A. alstroemeriae.* Nally *et al.* [2012] and Mewa-Ngongang *et al*. [2019b] also reported that different yeast species showed growth inhibition activity at different levels against fruit spoilage mould, which is in agreement with the findings of the current study.

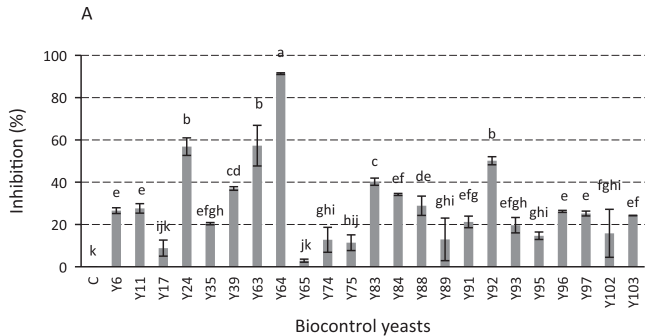
Volatile organic compound assay

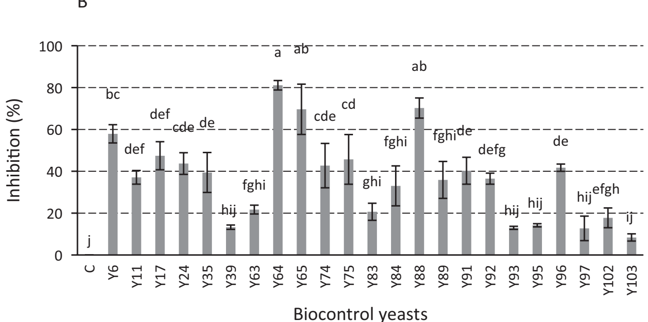
The production of VOCs as a mode of action against mould was investigated using the mouth-to-mouth assay. Most of the 23 yeasts produced VOCs that inhibited the growth of *B. cinerea* (Figure 3a), *P. expansum* (Figure 3b) and *A. alstroemeriae* (Figure 3c), but the level of growth inhibition varied among the yeasts. Yeast isolates Y64 (*P. kluyveri*), Y63 (*C. pyralidae*), Y24 (*M. guilliermondii*) and Y92 (*Zygosaccharomyces rouxii*) showed 91%, 57%, 56% and 50% growth inhibition activity against *B. cinerea*, respectively (Figure 3a). Against *P. expansum,* the highest growth inhibition was shown by *P. kluyveri* Y64, *M. guilliermondii* Y88 and Y65, with 81%, 70% and 69%, respectively (Figure 3b). The best performing yeasts against *A. alstroemeriae* were *P. kluyveri* Y64, *C. pyralidae* Y63 and *M. guillermondii* Y88, with 76%, 68% and 61% growth inhibition activity, respectively (Figure 3c).

Yeast isolate Y64 (*P. kluyveri*) showed the highest growth inhibition activity against all three moulds and was significantly better than the other yeast treatments during the VOC trial (Figure 3). While the opposite was observed during the diffusible metabolite assay (Figure 2). This strongly suggests that the mode of action of Y64 is linked to its ability to produce VOCs. The findings of this study are in agreement with Mewa-Ngongang *et al*. [2019b], who also reported on the ability of *P. kluyveri* and *C. pyralidae* to inhibit the growth of *B. cinerea* under *in vitro* conditions. Ruiz-Moyano *et al*. [2020] reported that *H. uvarum* also produced VOCs to control the growth of *B. cinerea* on fruits. Choińska *et al*. [2020] observed that *M. guilliermondii* produced VOCs to control the growth of *B. cinerea* and *P. expansum*, which is in agreement with the findings from this study. *Pichia kluyveri* showed the highest inhibition against *P. expansum*. Cordero-Bueso *et al*. [2017] also reported that VOCs produced by *P. kluyveri* exhibited antagonistic activity against *P. expansum.* This is the first report of VOCs from *P. kluyveri*, *C. pyralidae* and *M. guilliermondii* inhibiting the growth of *A. alstroemeriae.* However, Al-Maawali *et al*. [2021] showed that VOCs produced by *M. guilliermondii* inhibited the mycelial growth of *A. alternata.*

Post-harvest application of biocontrol yeasts on apples

The yeasts were effective in preventing mould spoilage of apples and reducing decay considerably (Figure 4). The inhibition responses were yeast and mould species-dependent. *Meyerozyma guilliermondii* Y88 and *P. kluyveri* Y64 were effective in suppressing mould growth on apples, with 100% inhibition activity against *A. alstroemeriae.* The commercial fungicide (Captan) also provided 100% inhibition. *Candida pyralidae* Y63 showed 36% inhibition against *A. alstroemeriae,* which was significantly lower compared to the other treatments*.* This is the first report on growth inhibition activity of *P. kluyveri*, *C. pyralidae* and *M. guillermondii* against *A. alstroemeriae* on apples*.* However, Al-Rahbi *et al*. [2021]





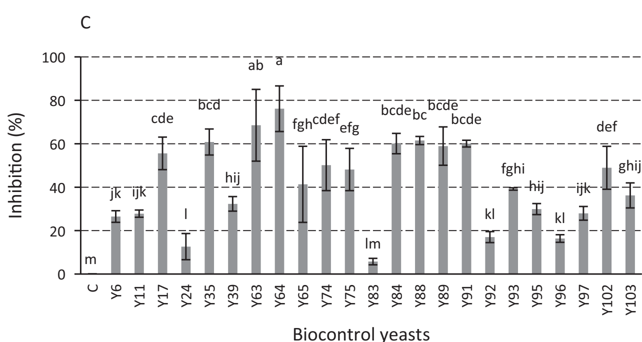


FIGURE 3. The growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the volatile organic compound production. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences (p<0.05). The negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

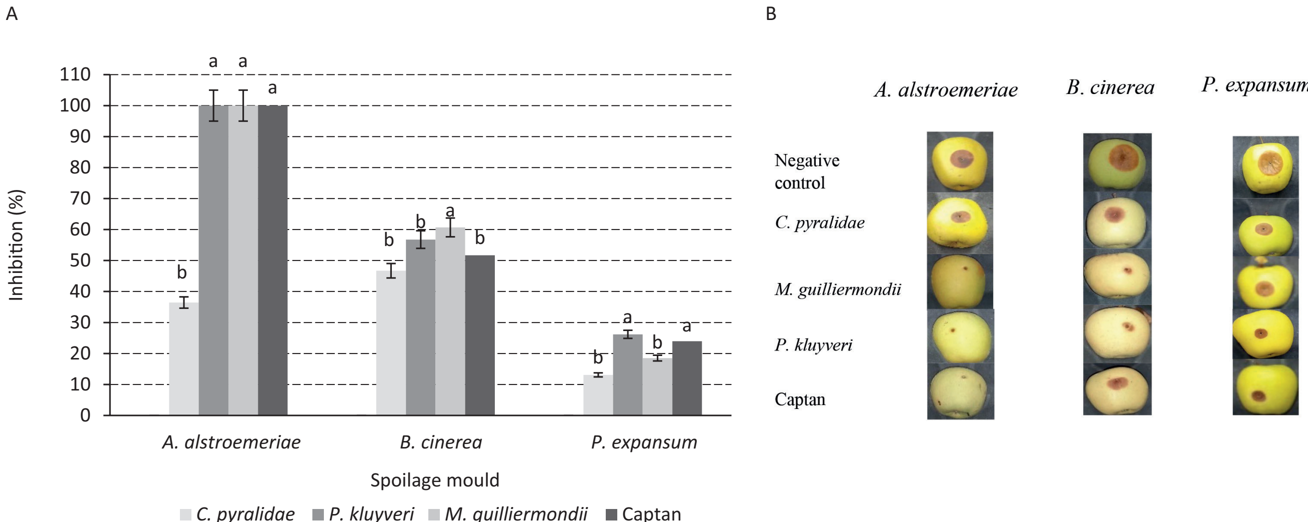


FIGURE 4. The growth inhibition activity expressed as a percentage (%) of *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Pichia kluyveri* Y64 against *Alternaria alstroemeriae*, *Botrytis cinerea* and *Penicillium expansum* during postharvest trials on apples (A). Values are means of five replicates and the standard deviations are also shown. The different letters indicate significant differences ($p<0.05$) between treatments. (B) Photographs of apples showing lesion diameters. Each set is a representative example of 25 apples. For the negative control treatments, the apples were only infected with the respective moulds, therefore no growth inhibition.

and Al-Maawali *et al*. [2021] showed that *M. guilliermondii* reduced the fruit rot lesions of *A. alternata* on strawberries and tomatoes by 68% and 50%, respectively.

Against *B. cinerea*, *M. guilliermondii* Y88 and *P. kluyveri* Y64 showed 61% and 57% inhibition, respectively, which was higher than the 52% obtained by the commercial fungicide (Figure 4a). *Candida pyralidae* Y63 inhibited *B. cinerea* growth by 47%, which was significantly lower than the other treatments. These findings are in agreement with those of Mewa-Ngongang *et al*. [2019b] who also reported on the antagonistic effects of *C. pyralidae* against *B. cinerea* on apples. Wang *et al*. [2018] reported that *M. guilliermondii* showed an antagonistic effect against *B. cinerea* isolates on grape berries, while Mewa-Ngongang *et al*. [2021], showed that *P. kluyveri* when applied preventively, was effective in suppressing *B. cinerea* growth by 95% on apples.

All the yeasts showed the lowest growth inhibition activity against *P. expansum* (Figure 4a). *Pichia kluyveri* Y64 exhibited the highest growth inhibition activity (26%) against *P. expansum* and performed slightly better than the commercial fungicide, which ensured 24% inhibition. The commercial fungicide displayed lower activity against *B. cinerea* and *P. expansum* than expected, which could be possibly attributed to the resistance of the specific moulds. Follow up studies should include more than one fungicide. *Meyerozyma guilliermondii* Y88 and *C. pyralidae* Y63 inhibited the growth of *P. expansum* by 19% and 13%, respectively. This study confirmed the findings of Cordero-Bueso *et al*. [2017], who reported that *P. kluyveri* exhibited antagonistic activity against *P. expansum*. In turn, Han *et al*. [2021] demonstrated that *M. guilliermondii* exhibited antagonistic activity against *P. expansum* on pears. This is the first report on the growth inhibition properties of *C. pyralidae* against *P. expansum* on apples. These observations on apples could be of great importance to the agricultural industry because these biocontrol yeasts can potentially be used as alternatives to chemical fungicides.

CONCLUSIONS

The cell suspensions of yeast strains *C. pyralidae* Y63, *M. guillermondii* Y88 and *Z. hellenicus* Y89 elicited the best antagonistic effects against *B. cinerea*, *P. expansum* and *A. alstroemeriae.* The production of VOCs by *P. kluyveri* was the mechanism of inhibition against *B. cinerea*, *P. expansum* and *A. alstroemeriae. Candida pyralidae* Y63, *M. guillermondii* Y88 and *P. kluyveri* Y64 were effective inhibitors of all three mould species on apples and their efficacy was comparable to the commercial fungicide. These yeasts can potentially be considered as alternatives to chemical fungicides. However, further research is needed to determine how to apply these yeast-based biocontrol agents and to establish the most effective minimum dosage or inhibitory concentration needed. The main VOCs and other possible compounds that are responsible for inhibition should be identified and the production process needs to be optimised. Future research should also investigate other mechanisms of action and the application of yeast-based biological agents on fruit for pre-harvest control of mould.

ACKNOWLEDGEMENTS

The authors would like to thank the Agricultural Research Council (ARC) and the National Research Foundation (NRF) of South Africa for funding and infrastructure. The opinions, findings and conclusions or recommendations expressed in this publication is that of the authors alone, and the NRF accepts no liability whatsoever in this regard. Thank you to Ms Louise Smit for culturing and supplying *Botrytis cinerea*. The authors extend their gratitude to all students, interns, technicians and research assistants for their individual contributions.

RESEARCH FUNDING

This work was supported by the Agricultural Research Council (ARC) and the National Research Foundation (NRF) of South Africa (Grant Numbers: 117833 and 122220).

CONFLICTS OF INTEREST

The authors hereby declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

The following are available online at http://journal.pan.olsztyn.pl/Control-of-Mould-Spoilage-on-Apples-Using-Yeasts-as-Biological-Control-Agents,147913,0,2.html; Yeast used in the study. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Meyerozyma guilliermondii* Y88 against *B. cinerea* (B) on yeast malt agar. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Pichia kluyveri* Y64 against *B. cinerea* (B) on yeast malt agar.

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