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ORIGINAL RESEARCH ARTICLE

Identification of grape berry indigenous epiphytic yeasts with *in vitro* and *in vivo* antagonistic activity towards pathogenic fungi

Peter Ayogu, António Teixeira, Hernâni Gerós* and Viviana Martins*

Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, Campus de Gualtar, Braga, Portugal

ABSTRACT

During their lifespan, grapes are exposed to conditions that can endanger plant health, including fluctuating climate and infection by pests and insects. In particular, attacks by filamentous fungi represent huge annual losses for grape farmers. Present concerns about rising antimicrobial resistance, fungicide prohibitions and shifting consumer tastes are driving the search for less harmful ways of preventing damage to grapevine by pathogenic fungi. In this study, the antagonist activity of yeasts isolated from grape berry surface microbiota was assessed by *in vitro* and *in vivo* assays on an assortment of grapevine fungal pathogens, including *Botrytis cinerea* and *Aspergillus niger*. The results show that *W. anomalus*, *A. pullulans*, *C. intermedia*, *R. glutinis*, *Z. meyerae* and *H. takashimae* were able to inhibit by 75 to 100 % mycelia growth and sporulation of the filamentous fungi *in vitro*. Strains of *M. pulcherrima* and *S. bacillaris* also showed 50 to 75 % efficacy in reducing mould growth. The strains were further assessed on incised leaves of 2-month-old grapevines for their ability to reduce disease incidence by the filamentous fungi. *W. anomalus*, *A. pullulans*, *M. pulcherrima*, *C. intermedia* and *R. glutinis* significantly reduced infection symptoms in the leaves, indicating their potential as effective biocontrol yeasts in the vineyard.

KEYWORDS: antagonistic activity, *Botrytis cinerea*, biocontrol, fungal pathogens, grape berry yeasts, *Vitis vinifera*, *Wickeramomyces anomalus*

*correspondence:
geros@bio.uminho.pt
vmartins@bio.uminho.pt

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INTRODUCTION

The surface of grape berries is naturally colonised by microbes (bacteria, fungi and yeasts) whose interactions with one another and the host play important roles in plant health and development (Barata *et al*., 2012). However, microbes are among the major causes of damage to grapes and many other fruits, and are thus associated with significant economic costs. Of these organisms, fungal pathogens account for greater yield losses at local and global scale (Rousseaux *et al*., 2014; Cordero-Bueso *et al*., 2017). Fungal pathogens originating from the soil include: *Aspergillus niger,* responsible for the production of ochratoxin A (OTA) in grapes and wine; *Botrytis cinerea,* which causes bunch rot and produces off-flavours in grape products; *Colletotrichum acutatum* and *C. nymphaeae,* which cause ripe rot and twig antrachnose; *Penicillium expansum* and *P. glabrum,* which cause post-harvest fruit rot and produce volatile compounds, such as geosmin, as well as mycotoxins, such as citrinin and patulin; and the oomycete *Plasmopara viticola,* which causes downy mildew (Rousseaux *et al*., 2014; Guginski-Piva *et al*., 2018; Cabañas *et al*., 2020; González-Fernández *et al*., 2020). The appearance in vineyards and presence on the aerial parts of grapes of this group of filamentous fungi can negatively affect vine vigour and development, with extensive damage to postharvest fruits prior to and during storage (Scott *et al*., 2022). They are naturally able to produce spores which are highly resistant to conventional pesticides and some other sanitary measures, making their eradication from the environment difficult (Cabañas *et al*., 2020; Cortesão *et al*., 2020).

Pest management in vineyards largely relies on the use of fungicide treatments. Common examples of synthetic fungicides that are massively used in the control of grapevine diseases are iprodione, fludioxonil, copper compounds and sulfur-based chemicals (Čadež *et al*., 2010; Buonassisi *et al*., 2017). However, the continuous application of chemical pesticides threatens the environment, leads to more fungicide-resistance strains and can also compromise the vineyard's natural defence network by damaging populations of natural predators (Christ and Burritt, 2013). A massive application of chemical fungicides can lead to the amount of pesticide residues on grapes at harvest time exceeding the permitted level set by national and international authorities (Commission Regulation (EC) 396/2005, 2005; Grimalt and Dehouck, 2016). High amounts of pesticide residues are a major concern in viticulture as they can influence the quality of grapes and wines and are capable of endangering consumer health. In this regard, the quest for alternative disease management strategies to those that involve the use of conventional chemicals is currently a big challenge for viticulturists all over the world.

Many yeasts that occur naturally on the surface of grape berries have important roles in winemaking (Fleet, 2003; Barata *et al*., 2012; Khan *et al*., 2020). Like other microbes, their abundance and composition on the grape berry can change over time to reflect the stage of ripening, pesticide application and health condition (Barata *et al*., 2012; Bokulich *et al*., 2014; Kántor *et al*., 2017). Most native yeasts living in plant tissues, such as *Aureobasidium pullulans* and *Metschnikowia pulcherrima,* can have beneficial effects without causing disease (Di Canito *et al*., 2021). However, the presence of some acid-tolerant vineyard native yeasts, including those that belong to the genera *Dekkera*/*Brettanomyces,* can be a source of concern in winemaking, as they can produce high amounts of acetic acid; they are thus considered as wine spoilage yeasts (Silva *et al*., 2004). It has been revealed that the presence and metabolic activities of some organisms on grape berry surfaces can antagonise other species within the microbial population, and that endospheric and phylospheric yeasts secrete bioactive molecules that can potentially be used as essential agrochemicals (Laman Trip and Youk, 2020); this knowledge has opened up new potential avenues for farmers in terms of the prevention and management of plant disease. Thus, given the possibility for optimising formulations that are more eco-friendly than conventional fungicides, the potential of native yeasts as biocontrol agents is being increasingly explored (Wang *et al*., 2018; Cabañas *et al*., 2020; Rodriguez Assaf *et al*., 2020; Agarbati *et al*., 2022; Maluleke *et al*., 2022).

In this study we hypothesise that the native yeast isolates of grape berries from vines in two Portuguese DOC regions, Vinhos Verdes and Douro, have biocontrol activity against six selected filamentous fungi that are relevant to viticulture, as they comprise the phytopathogenic fungi found on grapes (Kassemeyer and Berkelmann-Löhnertz, 2017; Echeverrigaray *et al.*, 2020). The six filamentous fungi were *A. niger*, *B. cinerea*, *C. acutatum*, *C. nymphaea*, *P. expansum* and *P. glabrum*. In our previous study, preliminary assays showed the ability of *A. pullulans* and *M. pulcherrima* to inhibit the growth of *A. niger in vitro* (Martins *et al*., 2022). Extensive studies that involve a vast array of indigenous strains are thus necessary in order to be able to design an effective foliar formulation to ensure sustainable agriculture. In the light of this, we combined different approaches, involving *in vitro* and *in vivo* assays in detached young leaves and young grapevines infected with spoilage filamentous fungi, to determine the biocontrol activities of the yeast isolates. The novel *in vivo* approaches would enable us to preliminarily assess the ability of yeast isolates to protect grapevine tissues from damage caused by filamentous fungi.

MATERIALS AND METHODS

1. Yeast strains and culture conditions

The native yeast strains used in this study had previously been isolated from the microbiota of grape berries (Martins *et al*., 2022) during two harvest seasons in two vineyards located in the DOC regions of Vinhos Verdes and Douro (Northern Portugal). Briefly, the isolation methodology consisted of platting freshly extracted grape juice in a solid Wallerstein differential medium, and isolating approximately 200 unique colonies (per vineyard) in a fresh medium after 5 days of incubation at 24 °C. Species identification was performed following Sanger sequencing of the rDNA internal transcribed

spacers (ITS) and the rDNA D1/D2 domain region applying previously optimised methods (Martins *et al*., 2022). The sequences were queried onto BLASTn (nucleotide BLAST – basic local alignment search tool) of NCBI (National Center for Biotechnology Information) and strain identification was based on the result with the highest score (sequence of the best match, determined through values of query cover, percentage identity and E value). The yeast strains were preserved as glycerol stocks in the Microbial Collection of the Biology Department of University of Minho (Portugal). The isolates were freshly grown in YPD medium for 3 days at 24 °C prior to carrying out the experiments. The most abundant strains of each species isolated per vineyard were selected to be tested for their antagonistic potential in the present study: *Candida intermedia* AUMC 10767, *Aureobasidium pullulans* LXH3, *A. pullulans* Y11, *Hanseniaspora uvarum* CBS:2584, *H. uvarum* PY-11, *Holtermanniella takashimae* NYG26-2, *H. festucosa* P40D005, *Metschnikowia pulcherrima* CBS:2256, *M. pulcherrima* Ipub65, *Rhodotorula glutinis* AD407, *R. glutinis* IFM 55305, *R. babjevae* CBS:6020, *Saccharomyces cerevisiae* AUMC 10229, *Starmerella bacillaris* IWBT-Y505, *Wickerhamomyces anomalus* HN1, *Zygoascus meyerae* 118, *Curvibasidium pallidicorallinum* CBS:9091, *Filobasidium magnum* bca-17, *F. stepposum* bca-379, *F. wieringae* CBS:8353, *Rhodosporidium babjevae* BD19, *Sporidiobolus metaroseus* AUMC 11218, *Sporobolomyces roseus* AUMC 11233, *S. ruberrimus* CBS:10230 and *Vishniacozyma victoriae* PYCC 6195. In addition, two strains were obtained from the Microbial Collection of the Biology Department of the University of Minho (Portugal), namely *Candida stellata* CBS 157 and *Saccharomyces bayanus* IGC 4569.

2. Pathogen mould strains and culture conditions

The following filamentous fungi used in the present study were retrieved from the Microbial Collection of the Biology Department of the University of Minho (Portugal): *Aspergillus niger* (DB E), *Penicillium expansum* (DB Pex), *Penicillium glabrum* (DB Pgl), *Colletotrichum nymphaeae* (DB Cony) and *Colletotrichum acutatum* (DB Coac). *Botrytis cinerea* (BC1) was kindly provided by Professor Philippe Nicot (French National Institute for Agriculture, Food and Environment, France). These moulds are phytopathogenic fungi found on grapes and they were selected based on the results of previous studies (Kassemeyer and Berkelmann-Löhnertz, 2017; Echeverrigaray *et al.*, 2020). The moulds were cultivated on Potato Dextrose Agar (PDA; MP Biomedicals, USA) at 25 °C for 14 days, in an 8 h dark/16 h light (200 µmol photons $m^{-2} s^{-1}$) photoperiod to induce sporulation prior to carrying out the experiments.

3. *In vitro* antagonistic activity

The *in vitro* antagonistic activity was determined on PDA by dual assay, as described by Parafati *et al*. (2015) with some modifications. The fungal spores were harvested from individual plates using 2 mL of 0.05 % Tween 20 solution. The conidia suspensions were filtered through 0.45 µm membranes to exclude mycelia and the spores counted with a haemocytometer (HBG Germany). Fungal conidial suspensions were adjusted to the desired concentrations (10^3, 10^5 or 10^7 spores/mL) with sterile distilled water. Similarly, freshly grown yeast cultures were diluted in sterile distilled water to the desired concentrations (10^4, 10^6 or 10^8 CFU/mL). Concentrations of yeasts and spores were selected based on previous studies (Parafati *et al*., 2015; Cabañas *et al*., 2020).

In the first assay, 5 µL of spore suspension were placed at the centre of a Petri dish containing PDA medium. Next, 5 µL of yeast cells were positioned on the periphery of the plate 25 mm from its centre. Control plates inoculated with only the mould were also prepared. Plates were incubated at 25 °C for up to 10 days prior to screening the antagonistic activity, which was evaluated by the radial growth of the mould in the presence or absence of the yeast. The assay was performed using three biological replicates for each combination of yeast and mould. The yeast strains showing antagonistic activity were selected for the second assay. It should be noted that all the yeasts grow well in PDA medium.

The second assay aimed at confirming the antagonistic activity of 14 selected yeasts, by embedding the yeast cells in the PDA medium. For this purpose, 5 mL of soft PDA (7 g/L agar) containing a final concentration of 10^6 CFU/mL of yeast cells were laid onto Petri dishes containing 10 mL of solid PDA. After the medium was allowed to solidify, 5 µL of fresh mould conidial suspensions at 10^5 spores/mL were inoculated at the centre of the medium. The plates were incubated at 25 °C and the antagonistic activity was expressed as the inhibition rate (%), which was calculated as follows: % I = , where RC represents the radial growth measurement in the control plate and RA is the radial mycelial growth of the mould in the presence of the yeast (Singh *et al*., 2017).

4. Plant culture conditions

Following the method described by Orsenigo *et al*. (2017) with modifications, grapevine seeds were germinated *in vitro* to obtain young grapevines (*Vitis vinifera* L.) for the experiment. Briefly, ripe grape berries were collected from cv. Sousão vineyards of the DOC Douro region and the seeds were separated from the pericarp by squashing the berries through metal sieves, cleaned under running water and then surface-dried on a thin layer of filter paper. The surface of the seeds was sterilised by incubating them in 70 % ethanol for 2 min and then in 50 % commercial bleach solution for 10 min. The seeds were then thoroughly washed three times with sterile distilled water in aseptic conditions. The sterilised seeds were sown in transparent re-sealable plastic boxes (17 x 12 x 10 cm) containing 1 % of distilled water agar and 10 mM of potassium nitrate (KNO_3), then stratified at 4 °C for three weeks in the dark. This was followed by incubation at 24 °C with an 8 h dark/16 h light (200 µmol photons $m^{-2} s^{-1}$) photoperiod. The germinated seeds were placed in glass vessels for plant culture, containing 1 % (w/v) agar and ½ strength basal Murashige and Skoog medium, and the seedlings were left to grow for 2 months until they had

developed three to four leaves for use in the experimental assays.

5. Antagonistic activity on detached young grapevine leaves

The effects of the yeast isolates on the mycelial growth of the tested moulds were assessed by inoculating yeast suspensions and fungal conidia successively in healthy young detached grapevine leaves, according to the procedure described by Lindsey III *et al*. (2017) with minor modifications. Detached sterile leaves were aseptically placed in the centre of Petri dishes containing 1 % water agar using sterile scissors and forceps. Briefly, the midrib of each leaf was gently incised on the adaxial axis with a sterile blade and 5 µL of yeast cell suspension (10^6 or 10^8 CFU/mL) was inoculated inside the incision. The plates were incubated at 25 °C for 1 h. Afterwards, 5 µL of fresh conidial suspension (10^7 spores/mL) of each mould was placed on the same spot. The plates were incubated for 7 days at 25 °C, in an 8 h dark/16 h light (200 µmol photons m^{-2} s^{-1}) photoperiod, and with 80 % humidity. At least three biological replicates were performed for each yeast-mould combination. The leaves inoculated with only the mould, only the yeast or only sterile distilled water were used as controls. After incubation, the disease incidence index was determined by visual scores on a 5 interval H-B scale of 0 - 4 (Horsfall and Barratt, 1945; Xu *et al*., 2004), which recognises lesion size relative to infected area, whereby 0 = no visible symptoms (0-5 % leaf area infected); 1 = trace symptoms (6-25 % leaf area); 2 = slight lesions (26-50 % leaf area); 3 = pale/yellowing (51-75 % leaf area); 4 = extensive deformation/loss of integrity (> 75 % leaf area).

6. Bioprotective activity on young grapevine plants

The *in planta* antagonistic activity was assessed in young healthy grapevines grown *in vitro*, as described in the previous sections. After gently incising the seedlings' midribs, 5 µL of yeast cells at 10^6 CFU/mL were inoculated into the lower internodes and flasks were incubated for 1 h, as described previously. The vines were then challenge-inoculated with 5 µL of the suspension of pathogenic moulds at 10^5 spores/mL. The disease incidence was evaluated after incubation at 25 °C for 10 days.

7. Statistical analysis

The data were analysed using the GraphPad Prism software, version 6.00 for Windows (GraphPad Software, La Jolla California USA). The statistical differences among variables were evaluated with the Student's *t*-test. In the graphs, the asterisks indicate the degree of significance when comparing the growth of the moulds in the absence of yeasts as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

RESULTS

1. *In vitro* antagonistic yeast-mould interactions

Examples of the results of the *in vitro* plate antagonistic yeast-mould interactions are shown in Figure S1. *W. anomalus* inhibited the mycelia growth of *A. niger*, *B. cinerea* and *C. nymphaeae*, while *C. intermedia* and *R. glutinis* showed inhibition of *P. expansum*. Detailed results of all the combinations of yeast-mould interactions are shown in Table 1. Overall, *A. pullulans* LHX3, *C. intermedia* AUMC 10767, *W. anomalus* HN1, *M. pulcherrima* CBS:2256 and *R. glutinis* AD407 showed large spectrum inhibition, even at 10^4 CFU/mL, of the moulds at 10^5 spores/mL. In turn, *H. takashimae* NYG26-2, *S. cerevisiae* AUMC 10229 and *H. uvarum* PY-11 showed inhibition of specific moulds, essentially at 10^6 or 10^8 CFU/mL. Conversely, 8 yeasts (*F. magnum* bca-17, *F. stepposum* bca-379, *F. wieringae* CBS:8353, *H. festucosa* P40D005, *Rhodosporidium babjevae* BD19, *R. babjevae* CB S:6020, *S. metaroseus* AUMC 11218 and *S. roseus* AUMC 11233) were unable to inhibit mycelia growth of the tested moulds even at 10^8 CFU/mL. For the yeasts that were capable of inhibiting mould growth, a significant increase in the antagonistic activity was observed as the range of yeast concentrations increasing from 10^4 to 10^8 CFU/mL. In addition, *A. niger* and *B. cinerea* seemed to be more resistant to yeast activity than the remaining filamentous fungi, while *P. glabrum* was the most sensitive. Detailed interactions of yeast isolates with *A. niger* at different concentrations are graphically presented in Figure S2. The results showed that, in general, the antagonistic activity of the yeasts at 10^4 CFU/mL increased as the concentration of fungal conidia suspension decreased. However, the moulds (10^4, 10^6 and 10^8 spores/mL) completely outcompeted the yeast isolates (10^4, 10^6 and 10^8 CFU/mL) during co-inoculation at equal strengths (data not shown).

A second *in vitro* assay was performed with a group of 14 yeast strains that had been selected based on the results of the initial screening. This assay involved a test on yeast-embedded solid media with a drop of mould conidial suspensions in the centre of the Petri dish. Examples of representative interactions are shown in Figure 1. *R. glutinis* AD407 (10^6 CFU/mL) completely inhibited the growth and sporulation of *A. niger* (10^5 spores/mL), in contrast to its relative, *R. glutinis* IFM 55305. *W. anomalus* HN1 completely inhibited the mycelial growth of *B. cinerea* and *C. nymphaeae*, while *C. intermedia* AUMC 10767 caused complete inhibition of *C. acutatum* growth. *M. pulcherrima* CBS:2256 only partially inhibited the radial growth of *P. glabrum,* while the lowest antagonistic activity was observed in the interaction between *S. cerevisiae* AUMC 10229 and *P. expansum*.

The heatmap of Figure 2 shows the overall interactions of all the selected yeast strains with the filamentous fungi in growth experiments performed in triplicate. *W. anomalus* completely inhibited the growth of all the tested moulds. It was followed by *C. intermedia* AUMC10767, which

TABLE 1. *In vitro* antagonistic profile of yeast isolates at different concentrations against moulds (10^5 spores/mL), after 10 days of co-incubation in PDA medium, at 25 °C.

DOC region	Yeast species and strains	Moulds (10^5 spores/mL)																	
		A. niger			*B. cinerea*			*C. acutatum*			*C. nymphaeae*			*P. expansum*			*P. glabrum*		
	yeast concentration (CFU/mL) ->	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8
Vinhos Verdes	*Aureobasidium pullulans* LHX3	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+
	Candida intermedia AUMC 10767	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
	Candida stellata CBS 157	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+	+
	Hanseniaspora uvarum CBS:2584	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	-	+	+
	Metschnikowia pulcherrima CBS:2256	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Rhodotorula glutinis AD407	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Starmerella bacillaris IWBT-Y505	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	Saccharomyces bayanus IGC 4569	-	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
	Saccharomyces cerevisiae AUMC 10229	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	-	+	+
	Wickerhamomyces anomalus HN1	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
	Zygoascus meyerae 118	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
Douro	*Aureobasidium pullulans* Y11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
	Curvibasidium pallidicorallinum CBS:9091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	Filobasidium magnum bca-17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Filobasidium stepposum bca-379	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Filobasidium wieringae CBS:8353	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Hanseniaspora uvarum PY-11	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+
	Holtermanniella festucosa P40D005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Holtermanniella takashimae NYG26-2	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
	Metschnikowia pulcherrima Ipub65	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	Rhodosporidium babjevae BD19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rhodotorula babjevae CBS:6020	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rhodotorula glutinis IFM 55305	-	+	+	-	-	+	-	-	+	-	-	+	-	+	+	-	+	+
	Sporidiobolus metaroseus AUMC 11218	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sporobolomyces roseus AUMC 11233	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sporobolomyces ruberrimus CBS:10230	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vishniacozyma victoriae PYCC 6195	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-

Scores were expressed as (+) if yeast strains caused visible inhibition of mould growth, and (-) if yeasts did not substantially inhibit mould growth. Results are representative of 3 independent trials.

was less effective in inhibiting *A. niger*. Furthermore, high inhibitive activity (≥ 75 %) was observed in *A. pullulans* LHX3, *A. pullulans* Y11, *R. glutinis* AD4087, *R. glutinis* IFM55305, *C. intermedia* AUMC 10767, *Z. meyerae* 118 and *H. takashimae* NYG26-2. In addition, the strains of *M. pulcherrima* and *S. bacillaris* showed moderate inhibitive activity (50-75 %), whereas the least inhibitive activity was observed in *H. uvarum* PY-11 and *S. cerevisiae* AUMC 10229 (Figure 2 and Table S1).

2. Antagonistic activity of native yeasts in detached young grapevine leaves and young grapevine plants

The biocontrol potential of selected yeast strains displaying antagonistic activity in the previous *in vitro* tests was assessed on detached young healthy grape leaves inoculated with the pathogenic moulds. Thus, 11 yeast strains were selected for this assay. Figure 3 shows the representative results of disease incidence reduction of the young leaves inoculated with combinations of selected yeasts and moulds. The results show that the moulds were able to infect the young leaves, producing various effects. In the leaf inoculated with *A. niger* only, sporulation and growth occurred all over the leaf surface, followed by an extensive breakdown of leaf integrity. Inoculation with only the yeast did not show any leaf damage. When *W. anomalus* HN1 was co-inoculated with *A. niger*,neither sporulation nor loss of leaf integrity occurred. The spores of *B. cinerea* germinated on the leaf and caused damage to the midrib zone, resulting in the partial collapse of the leaf tissues. The damage was however reduced in the challenge-inoculation with *A. pullulans* LXH3. *C. acutatum* caused bleaching of the leaf, with sporulation occurring only in the zone of infection. The disease incidence

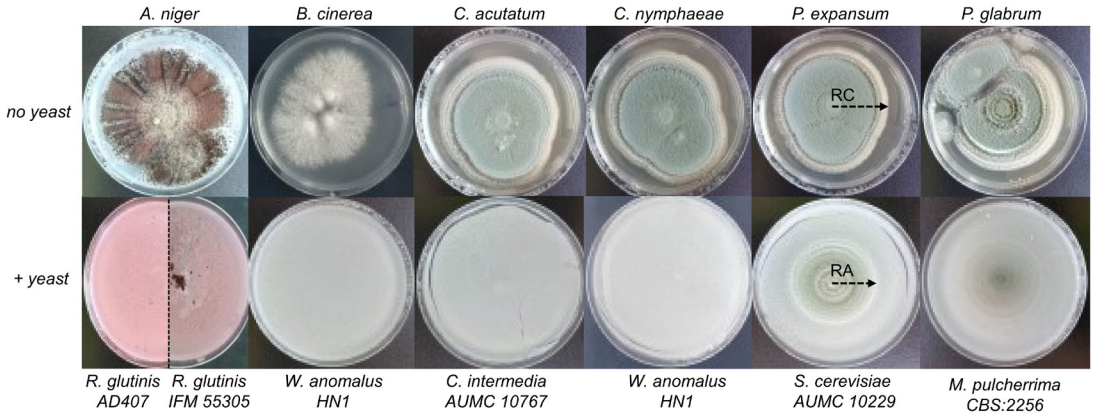


FIGURE 1. Interaction of yeasts embedded in PDA medium at 10^6 CFU/mL, with moulds inoculated at the centre of the plate at 10^5 spores/mL, after 10 days of incubation at 25 °C.

Images show examples of the most representative antagonistic activities, and arrows indicate the radius of mould growth in the absence (RC) or presence of yeasts (RA), that was used to calculate the percentage of inhibition, as detailed in Material and Methods.

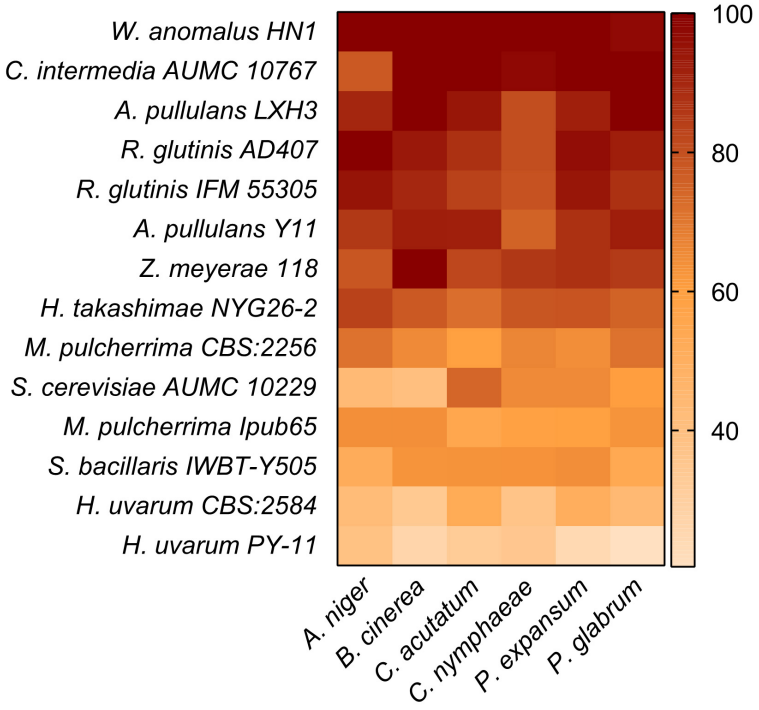


FIGURE 2. Heatmap of the interactions of selected yeast strains (10^6 CFU/mL) with the moulds (10^5 spores/mL), *in vitro*.

Values represent the average percentage inhibition observed in 3 independent trials in PDA medium, after 10 days of incubation at 25 °C. Values were centred and scaled in the row direction to form virtual colours as presented in the colour key. Statistically significant growth inhibitions following Student's *t*-test are detailed in Table S1.

was significantly reduced when the leaf was co-inoculated with *A. pullulans* LXH3. *C. nymphaeae* infection was marked by a profuse bleaching of the entire leaf. However, the challenge-inoculation with *M. pulcherrima* CBS:2256 provided moderate protection against the damage. *P. expansum* infection was associated with the growth of spores in the midrib area, partial bleaching and loss of leaf integrity. Co-inoculation with *R. glutinis* AD407 showed a reduction in disease incidence. In the case of *P. glabrum*, sporulation at the site of the infection was observed, and leaf symptoms included bleaching and the partial collapse of the leaf. The results show that co-inoculation with *S. cerevisiae* AUMC 10229 was not effective in preventing sporulation and damage to the leaf.

Figure 4 shows details of the disease incidence of the leaves inoculated with each mould in the presence of the yeasts at 10^6 or 10^8 CFU/mL. The results show that *W. anomalus* HN1 provided the highest protection from infection by all the moulds, followed by *A. pullulans* LXH3, *M. pulcherrima*

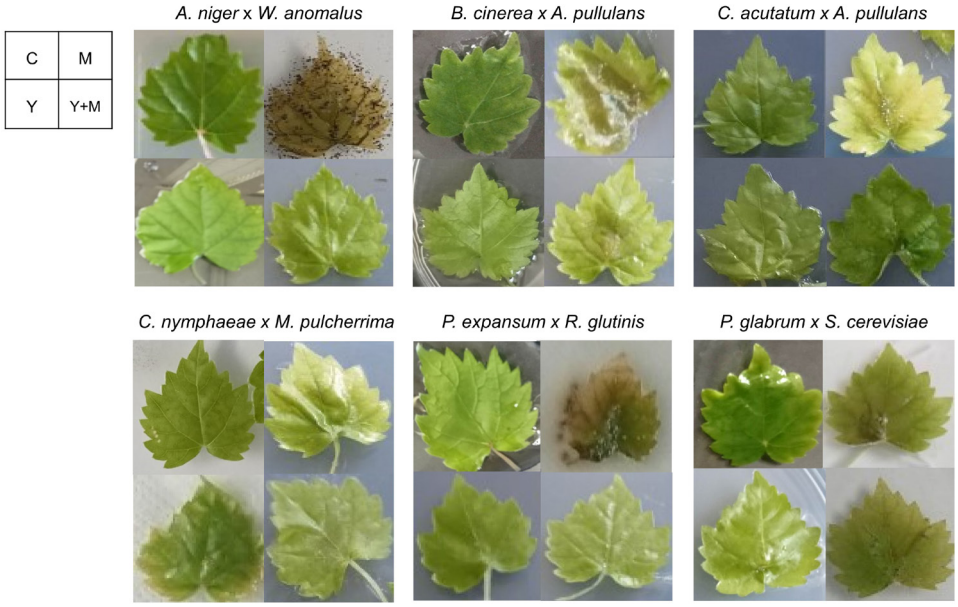


FIGURE 3. Disease incidence in wounded young grapevine leaves after challenge inoculation with yeasts and moulds, and incubation at 25 °C for 7 days.

For each interaction, images show a non-inoculated control leaf (C), a leaf inoculated with only the mould (M), a leaf inoculated with only the yeast (Y) and a leaf inoculated with both yeast and mould (Y+M). Yeasts were inoculated at 10^8 CFU/mL, except *W. anomalus* (10^6 CFU/mL) and moulds were inoculated at 10^7 spores/mL.

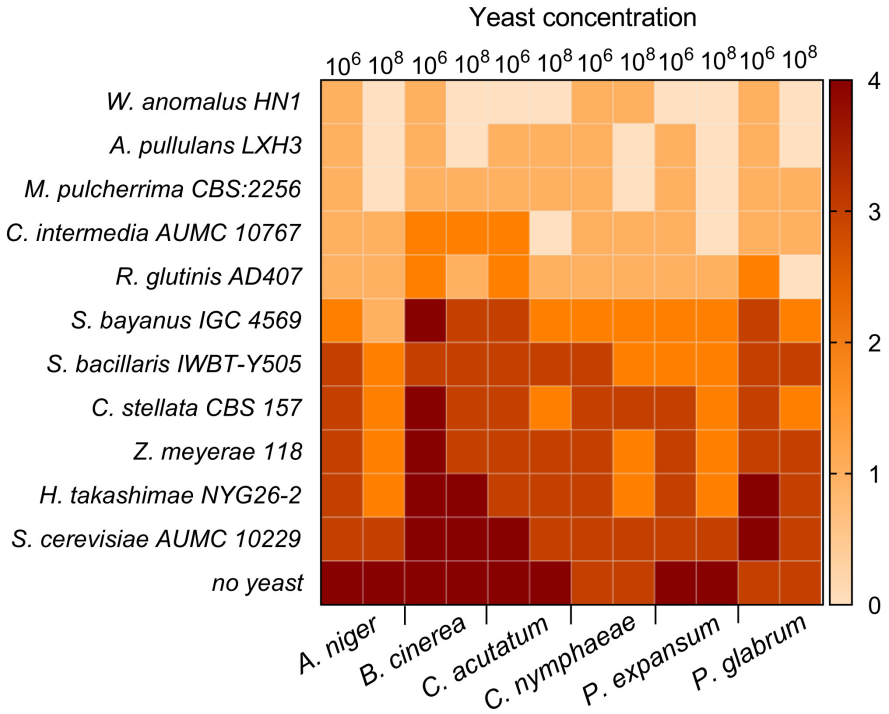


FIGURE 4. Heatmap of the disease incidence in young grapevine leaves after co-inoculation with yeasts (10^6 or 10^8 CFU/mL) and moulds (10^7 spores/mL).

Values represent the average disease incidence observed in at least 3 independent trials, after 7 days of incubation at 25 °C. Values were centred and scaled in the row direction to form virtual colours, as presented in the colour key, and indicate the disease incidence index, as follows: 0 = no visible symptoms (0-5 % leaf area infected); 1 = trace symptoms (6-25 % leaf area); 2 = slight lesions (26-50 % leaf area); 3 = pale/yellowing (51-75 % leaf area); 4 = extensive deformation/loss of integrity (> 75 % leaf area).

CBS:2256, *C. intermedia* AUMC10767 and *R. glutinis* AD407. In particular, *R. glutinis* AD407 and *C. intermedia* AUMC 10767 provided less protection from infection by *B. cinerea* and *C. acutatum*. In turn, *S. cerevisiae* AUMC 10229 and *H. takashimae* NYG26-2 were the least effective in protecting the leaves from all the moulds. In general, at the concentration of 10^6 CFU/mL, a lower antagonistic yeast activity was observed, while no protection was observed at 10^4 CFU/mL (data not shown). It is worth noting that most of the yeast strains that provided greater protection in the leaf assay were amongst the strains observed to be highly antagonistic in the confirmatory *in vitro* assay (Figure 1), namely *W. anomalus* HN1, *C. intermedia* AUMC10767, *A. pullulans* LXH3, *A. pullulans* Y11, *R. glutinis* AD407 and *R. glutinis* IFM 55305.

Given the positive results obtained for the detached leaves, the assay was extended to young grapevine plants. The results show that six out of the 11 yeast strains (*C. intermedia*, *W. anomalus*, *A. pullulans*, *M. pulcherrima*, *R. glutinis* and *H. takashimae*) are able to protect the vines infected with the 105 spores/mL suspension of the fungal pathogens. Figure S3 shows a representative result of a positive bioprotective effect of *C. intermedia* on *P. expansum*. *H. uvarum* was used as a negative control, providing no protection from infection by *C. nymphaeae*.

DISCUSSION

The present study evaluated the biocontrol activity of grape berry native yeast against fungi pathogens *in vitro* and *in vivo*. As expected, some of the selected yeast strains were able to inhibit the growth of the selected moulds *in vitro*. In general, various yeast isolates, namely *W. anomalus* HN1, *A. pullulans* (strains LHX3 and Y11), *C. intermedia* AUMC 10767, *R. glutinis* (strains AD407 and IFM 55305), *M. pulcherrima* CBS:2256, *Zygoascus meyerae* 118 and *Starmerella bacillaris* IWBT-Y505, demonstrated high potential biocontrol activity against *A. niger, B. cinerea, C. acutatum, C. nymphaeae, P. expansum* and *P. glabrum*. The earlier discovery of certain yeast species and specific strains retaining high biocontrol potential against several microorganisms (Türkel *et al*., 2014) seems to have spurred an increasing demand for its exploration in the management of plant diseases. Some microorganisms can produce plant hormones, including auxins, gibberellins, cytokins and indole-3-acetic acid (IAA), as well as vitamins, antifungal and antibiotic compounds; some can also solubilise minerals, like phosphorus and other nutrients, and fixate nitrogen and degrade toxic chemicals (Nutaratat *et al*., 2014; Pérez-Montaño *et al*., 2014). The yeast-like fungi *A. pullulans* YA05 and the yeast *R. mucilaginosa* YR07 have been found to produce plant growth enhancement compounds, especially IAA, and were subsequently considered for inclusion into commercial plant-growth-promoting fertilisers (Ignatova *et al*., 2015).

The yeast-like *A. pullulans* demonstrated great biocontrol potential and its *in vitro* antagonistic activity increased with higher concentrations of the yeast cell suspension, but it decreased slightly as the fungal conidial suspension increased. Although previous studies have found *A. pullulans* strains FZ02a, Y1 and W32 to be highly inhibitory of the hyphal growth of *P. expansum, A. niger* and *B. cinerea* (Cordero-Bueso *et al*., 2017; Sukmawati *et al*., 2021; Maluleke *et al*., 2022), in our more expansive dual *in vitro* assays, *A. pullulans* (strains LHX3 and Y11) was also found to be highly inhibitory for *C. acutatum, C. nymphaeae* and *P. glabrum*. The yeast also significantly reduced the severity of disease on grape leaves caused by all the tested moulds, being highly efficient against *B. cinerea* and *C. acutatum*. Similar observations were made of the yeast regarding the protection it provided for young grapevine plants under controlled laboratory conditions. *A. pullulans* is among the predominant yeast-like fungal species found on the surface of grape berries at all stages of maturity (Renouf *et al*., 2005; Martins *et al*., 2021), and previous studies have shown that the L1 and L8 *A. pullulans* strains are involved in the biocontrol of *B. cinerea, C. acutatum* and *P. expansum* in grapes and other fruits (Mari *et al*., 2012; Agirman and Erten, 2020; Galli *et al*., 2021; Moura *et al*., 2021). In these strains, it has been shown that antagonistic activity is largely due to competition for nutrient and space and host defence induction (Di Francesco *et al*., 2017). Notably, the yeast-like fungi *A. pullulans* is also a microorganism with great biotechnological relevance given its capacity to produce enzymes, sugars and metabolites such as pullulan (poly-α-1,6-maltotriose), which underlies its biocontrol activity; this makes it a good candidate for its use in the production of edible films in food processing and pharmaceutical industries and for improving animal health (Mehta *et al*., 2017; Trinetta, 2017).

In the present study, native strains of *M. pulcherrima* showed moderate antagonistic activity *in vitro,* but effective protection against infection *in vivo,* especially for infection by *A. niger, C. nymphaeae* and *P. expansum*. Accordingly, previous studies have reported that *M. pulcherrima* UMY15 showed significant inhibition of *P. expansum*, but that it was less effective against *A. niger* (Türkel *et al*., 2014). In another study, Parafati *et al*. (2015) showed that *M. pulcherrima* MPR3 was highly active in the control of *B. cinerea*. It has been shown that its antagonistic activity largely depends on its iron immobilising pigment pulcherrimin (Sipiczki, 2006; Türkel and Ener, 2009; Kántor *et al*., 2015).

In the *in vitro* assays of the present study, *R. glutinis* AD40s7 showed more efficient antagonistic activity than *R. glutinis* IFM 55305. The antagonistic activity of *R. glutinis* AD407 was also significant in the grape plant assay in the present study. Previous studies have shown that *R. glutinis* significantly inhibited the growth of *B. cinerea* and *P. expansum in vitro* and *in vivo* (Zhang *et al*., 2008, 2010). The biocontrol performance of *R. glutinis* against various post-harvest diseases in some fruits has also been found to be positive, and its biocontrol activity is thought to rely on the ability to attach to hyphae or conidia of phytopathogenic fungi (Li *et al*., 2016), which is likely strain-dependent. However, the mechanisms

involved in its biocontrol activity are not yet fully understood. In a study by Zhang *et al*. (2010), high antagonistic activity of *R. glutinis* towards *B. cinerea* was observed when salicylic acid was added, suggesting a lower yeast performance in the absence of this metabolite. As well as the production of high amounts of pigment (Hernández-Almanza *et al*., 2014), it is also speculated that the ability of the yeast to secrete phenolic compounds, such as gallic acid, benzoic acid, catechin, caffeic acid and ferulic acid, in the presence of NaCl (1–5 %) or H_2O_2 (1–5 mM) could promote its biocontrol activity (Salar *et al*., 2013).

In other interactions, *W. anomalus* HN1 and *C. intermedia* AUMC 10767 were also highly antagonistic towards the six selected filamentous fungi. In particular, while *W. anomalus* was found to completely inhibit the growth of all filamentous fungi *in vitro*, it was observed that *C. intermedia* did not completely inhibit the growth of *A. niger* in the same conditions. The results of the *in vivo* assays show that *W. anomalus* was the most active in reducing the severity of the disease caused by the moulds on the young leaves, while *C. intermedia* was not as effective. Previous studies have shown that *C. intermedia* C410 and *W. anomalus* Y934 were able to antagonise *B. cinerea* (Huang *et al*., 2011; Maluleke *et al*., 2022); our dual *in vitro* and *in vivo* approaches have shown these yeasts to have broader biocontrol activity against several other moulds.

Though *S. bacillaris* IWBT-Y505 significantly outcompeted *A. niger, C. acutatum, C. nymphaeae, P. expansum* and *P. glabrum in vitro*, the yeast isolate did not inhibit the mycelia growth of *B. cinerea*. However, a previous study has shown that other strains of this species can be highly antagonistic towards *B. cinerea* (Lemos Junior *et al*., 2016). Conversely, though *H. uvarum* strains CBS:2584 and PY-11 did not significantly inhibit mould mycelia growth in our study, the strain CAMB9A was found to be up to 50 % effective against *B. cinerea* and *P. expansum in vitro* (Cordero-Bueso *et al*., 2017).

In a concerted effort to work beyond *in vitro* approaches to prevent the postharvest damage of grape in storage conditions, the biocontrol activity of some local microbial strains have been evaluated *in vivo* using table grapes as substrate, with measurable success (Nally *et al*., 2012; Parafati *et al*., 2015; Wang *et al*., 2018). The results of the present study confirm that detached young grape leaves and grapevine plants can be used for an initial evaluation of the ability of yeast isolates to protect grapevine tissues from damage caused by filamentous fungi.

The results of the present study support the increasing research on emerging strategies for growing crops with reduced use of agrochemicals. In particular, the high biocontrol activity of native strains of *W. anomalus* and *A. pullulans* was demonstrated. However, the mechanisms of the antagonistic effects of yeasts towards phytopathogens is still a subject for exploration, because it is not always possible to translate encouraging *in vitro* results into *in vivo*, especially field conditions. Although yeasts are not known to produce any toxic metabolites, thus making them biologically safe (Kowalska *et al.*, 2022), further knowledge of their biocontrol mechanisms would be necessary in order for them to be considered by regulatory agencies as valuable tools for addressing the challenge. The inhibitory activity of the yeast isolates - which could be due to their ability to secrete compounds that are toxic to the pathogens in these models - indicates their biocontrol and plant growth-promoting potential (Di Francesco *et al*., 2021). Furthermore, for grape growers, the foliar application of yeast cells or their formulations could potentially boost grapevine productivity and contribute to sustainable agriculture and the production of high-quality wine.

CONCLUSION

Despite being a species of high adaptability, the grapevine is still vulnerable to substantial infestation by fungal pathogens, leading to losses of tons of grapes and thus affecting both farmers and consumers. Preventive methods include using pest-resistant rootstocks and other *Vitis* species, or applying large quantities of broad-spectrum pesticides, but these have yet to attain full consumer acceptance due to safety concerns. The most recent alternative approach relies on the use of biological control agents, which could protect grapevines from pathogens without causing damage to the crops, the environment or the consumer. In the present study, we explored the antimicrobial activity and plant protective potential of vineyard native yeasts. Further studies addressing the inhibition mechanisms underlying yeast antagonistic activity will be a step forward to the application of these microorganisms in the field.

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