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Antagonistic Activity of Three Newly Isolated Yeast Strains from the Surface of Fruits

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Summary

Three *Metschnikowia pulcherrima* strains isolated from white grapes (strains designated SG1 and SG2) and cherries (strain CPM1) from Romania were tested for antagonistic activity against natural and reference yeasts, fungi and *E. coli*. The best results were obtained for SG2 strain against the following microbes isolated from natural environments: yeasts *Candida tropicalis* OT4 and *Candida albicans* Cc, and fungal strains *Botrytis cinerea* BCS and BCF1. Using 2 % sodium hydrogen carbonate and 1 or 2 % calcium chloride, antimicrobial activities of strains SG1 and SG2 were maintained or even enhanced, while strain CPM1 was less influenced. Antimicrobial tests revealed four types of colonies and halos of strains SG1, SG2 and CPM1 reflecting differences in iron immobilization under similar growth conditions. Antifungal activity and the level of pigment production increased at 25 °C, and the largest diffusible pigment area was obtained by strain SG2, which altered *Botrytis* conidia germination and induced modifications of mycelium. Competition for iron seems to be the major mechanism of antagonistic action of our strains, which is reduced/absent under iron chloride addition. Lowering of antifungal activity by heating or proteolytic treatment indicates that a protein might also be involved. The inhibitory activity of yeast supernatants was less important, suggesting a cell-related mechanism. When combined, strains SG1 and SG2 had synergistic activity, which was reduced in the presence of CPM1 due to possible production of an inhibitory compound.

Key words: antagonistic activity, *Metschnikowia pulcherrima*, antifungal mechanism, antimicrobial activity enhancement, biocontrol

Introduction

Fresh fruits and vegetables are subject to postharvest disease mainly due to the attack of various microorganisms, such as fungi, yeasts and bacteria. Therefore, a growing interest has been shown during the last years in the isolation and complete characterization of microorganisms that may act as possible antimicrobial agents (*1–4*).

Biological control of postharvest diseases of fruits and vegetables by microbial antagonists is well documented, but the majority of the information is related to bacterial and fungal antagonists. In the past ten years the interest in yeast antagonists has been increasing with the aim to isolate and test these species (*Candida saitoana*, *C. oleophila*, *C. sake*, *C. guillermondi*, *Debaryomyces hansenii*, *Metschnikowia fruticola*, *M. pulcherrima*, *Pichia anomala*, *Rhodotorula glutinis*, *etc.*) for their antimicrobial properties (*5*). The biocontrol abilities of various *M. pulcherrima* strains against different fungal strains like *Botrytis cinerea*, *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp. and *Rhizopus* spp. were studied regarding their mechanism

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of action and increase of effectiveness through various approaches, alone or in combination with different fungicides (*6–12*).

The three strains used in the present study (named SG1, SG2 and CPM1) had previously been analysed and identified as most probably belonging to *Metschnikowia pulcherrima* species (*13,14* and Csutak, unpublished results). The antifungal activity of SG1 and SG2 strains had also previously been tested against *Sclerotinia sclerotiorum, Fusarium culmorum, F. graminearum, Alternaria alternata* (*A. tenuis*) and *A. alternata* f. sp. *lycopersici* (*15*). Based on these results, we developed a series of antimicrobial tests and the results were correlated with those described in the literature for similar experiments on *Metschnikowia pulcherrima* natural isolates or standard strains.

Materials and Methods

Microbial strains

Three yeast strains were analyzed: two strains were isolated from the surface of white grapes in the Ialomita region, Romania, and named SG1 and SG2, and one strain was isolated from cherries from Pitesti, Romania, and named CPM1. The fruits (grapes and cherries) were collected aseptically and homogenized separately in 5 mL of sterile water and the samples of the resulting must were spread on YPD plates (1 % yeast extract, 1 % peptone, 2 % dextrose and 2 % agar).

Standard strains used in this study were: yeast strains *Saccharomyces cerevisiae* 17/17 (killer sensitive [KIL-o]; Institute for Biomedical Investigations 'Alberto Sols', Madrid, Spain), *S. cerevisiae* S288C (R.K. Mortimer's Collection, part of the American Type Culture Collection (ATCC), Manassas, VA, USA), *Schizosaccharomyces pombe* 972h$^-$ (Institute of Cell Biology, University of Bern, Bern, Switzerland), *Candida parapsilosis* CBS 604 (Fungal Biodiversity Centre, Utrecht, The Netherlands), *C. albicans* ATCC 10231 (ATCC), *C. krusei* CMGB 94 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Bucharest, Romania) and a bacterial strain *Escherichia coli* DH5α (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest). The antimicrobial activity of SG1, SG2 and CPM1 strains was also tested in the presence of *C. albicans* Cc and *C. tropicalis* OT4 strains previously isolated from vaginal and oral infections, respectively (*16*). Yeast strains were maintained at –70 °C on YPG medium (1 % yeast extract, 1 % peptone and 2 % glucose) supplemented with 20 % glycerol. *Escherichia coli* DH5α was cultivated on Luria-Bertani medium (1 % Bacto^{TM} Tryptone, 0.5 % yeast extract and 1 % NaCl).

Antifungal experiments were performed using reference fungal strains from the microbial collection of the Faculty of Biotechnology, University of Agronomic Science and Veterinary Medicine, Bucharest, Romania, and from the collection of the Institute of Plant Protection, Bucharest, Romania: *Botrytis cinerea*, *Alternaria alternata*, *A. tenuissima*, *A. mali*, *Pythium* spp., *Sclerotium bataticola*, *Penicillium* spp., *P. chrysogenum*, *Aspergillus ochraceus*, *Fusarium oxysporum* var. *lycopersici*, *F. solani*, *F. moniliforme* and *Rhizoctonia* spp. Natural strains of *Fusarium solani*, *F. moniliforme*, *Aspergillus ochraceus* and five *Botrytis cinerea* strains isolated from different sources (strains BCF1 and BCP2 from strawberries, BCS and BCPP from grapes and Bclys from leaves) were also used. All fungal strains were maintained on potato dextrose agar (PDA) medium (Carl Roth GmbH & Co, Karlsruhe, Germany) and identified by morphophysiological analyses (Cornea, unpublished results).

Killer activity assay

Killer activity tests were performed by patching the colonies of freshly grown culture of SG1, SG2 and CPM1 strains on plates with killer medium (0.1 M phosphate citrate buffer, pH=4.8, 2 % glucose, 1 % yeast extract, 2 % agar and 0.03 % methylene blue) with an overlay of the sensitive yeast strains *S. cerevisiae* 17/17, *C. albicans* ATCC 10231, *C. parapsilosis* CBS 604, *C. krusei* CMGB 94, *C. albicans* Cc and *C. tropicalis* OT4. The plates were incubated for five days at 25 and 28 °C, and checked daily. When the colonies were surrounded by a clear blue zone, the assay was considered positive for killer activity.

Investigation and enhancement of antimicrobial activity against yeasts and E. coli

Inhibition of yeast and bacterial growth was tested on plates inoculated with a thin layer of cells of the test organism and patches of fresh cultures of SG1, SG2 and CPM1 strains. YPG agar (YPG plus 2 % agar) was used for *S. cerevisiae* S288C, *S. pombe* 972h$^-$, *C. albicans* ATCC 10231, *C. albicans* Cc, *C. parapsilosis* CBS 604 and *C. tropicalis* OT4 and Luria-Bertani agar for *E. coli* DH5α. Plates were incubated at 25 and 28 °C for standard yeast strains, at 28 °C for strains *C. albicans* Cc and *C. tropicalis* OT4, and at 37 °C for *E. coli* DH5α. The results were observed for five consecutive days. In order to further investigate the antimicrobial activity, mixtures of SG1, SG2 and CPM1 cultures in YPG were centrifuged and cell suspensions (10^8 cells/mL) were mixed with sodium hydrogen carbonate in final fractions of 0.1, 0.5, 1 and 2 %, or calcium chloride in final fractions of 1, 2 and 3 % (by mass per volume). Equal aliquots of mixtures were plated on Petri dishes floated with *C. albicans* Cc and *C. tropicalis* OT4 cultures (10^6 cells/mL). The plates were incubated at 28 °C and observed for five days.

Antifungal tests

After the incubation of SG1, SG2 and CPM1 strains at room temperature for five days, yeast colonies (white and pink-purple colonies) were isolated and tested for antifungal activity against *Botrytis cinerea*. Tests were performed in two ways. First, agar discs containing fungal pathogen were placed in the middle of the PDA or YPDA plate and incubated for 24 h at 25 °C. Then, the yeast isolates were streaked 10 mm from the edges of the growth mycelia. The plates were incubated at 25 or 28 °C for seven days and the inhibition was checked daily. In another test, PDA plates were flooded with conidial suspensions of several fungal pathogens, and the yeast isolates were streaked on each plate.

Tests for investigating the mechanism of antifungal activity

In order to investigate the mechanism of antifungal activity, yeast strains and filamentous fungi were plated on PDA/YPDA or PDA/YPDA supplemented with 10 μg/mL of $FeCl_3$. Cocultivation effect of yeast strains against fungi was examined using three variants of liquid media: YPD, PDA (Carl Roth GmbH & Co.) and YEMS (3 % yeast extract, 0.5 % mannitol, 0.5 % L-sorbose) (*17*). Two combinations of strains were used: SG1+SG2 and SG1+SG2+CPM1. After 48 h of cultivation the suspensions were centrifuged, and the supernatants were sterilized by filtration. For each experimental variant, the supernatant was heat inactivated (incubation at 100 °C for 15 min) or treated with 1 mg/mL of trypsin or sodium hydrogen carbonate (1 %, by mass per volume). Samples of 20 μL of each treated or untreated supernatant and cellular sediment were placed 1.5 cm from the edge of the fungal colony and incubated at 25 °C for seven days.

Results

Production of killer toxins

Tests for assesing the killer activity of *Metschnikowia pulcherrima* strains SG1, SG2 and CPM1 against reference strains were performed at 25 and 28 °C as indicated in similar experiments (*3,18*), while those for assesing the killer activity against the natural strains *C. albicans* Cc and *C. tropicalis* OT4 were performed only at 28 °C, since these strains grew poorly at lower temperatures. Results registered after three and five days of incubation are comprised in Table 1. All three strains, SG1, SG2 and CPM1, were highly active against *C. parapsilosis* CBS 604 at 25 °C forming clear halos starting from the third day of incubation. When plated at 28 °C, positive results were recorded only after five days, with the exception of CPM1 strain, which tested negative. On *C. albicans* ATCC 10231 plates, weak killer toxin production was observed. In the case of *C. krusei* CMGB 94, SG1 strain formed wide halos when incubated at 25 °C for 3 days and small halos at 28 °C, while CPM1 had no killer activity. No activity was recorded against killer sensitive strain *S. cerevisiae* 17/17. In contrast, clear, wide halos were observed on *C. tropicalis* OT4 and *C. albicans* Cc within five days of incubation.

Antimicrobial activity against yeasts and E. coli

We tested SG1, SG2 and CPM1 strains for antimicrobial activity against several yeast strains and *E. coli* DH5α. The growth of *E. coli* DH5α was tested at 37 °C and was not altered by any of the three strains. The tests performed at 25 and 28 °C against reference yeast strains revealed no growth inhibition of *C. albicans* ATCC 10231, as the three strains formed pink colonies (Fig. 1a). For *S. cerevisiae* S288C the results were similar after five days of incubation both at 25 and 28 °C, and diffuse pink halos of about 2 mm were observed surrounding SG1 (Fig. 1b) and SG2. When tested against *S. pombe* 972h⁻ substrate, all three strains formed colonies with thin dark purple edges without growth reduction halos (Fig. 1c).

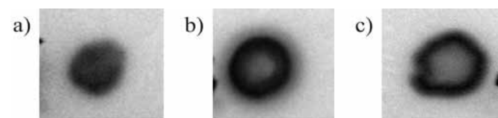

Fig. 1. Types of colonies of strain SG1 observed in the presence of: a) *Candida albicans* ATCC 10231 – uniform colonies without inhibition zone, b) *Saccharomyces cerevisiae* S288C – colonies surrounded by difuse halos, and c) *Schizosaccharomyces pombe* 972h⁻ – colonies with dark edges and no halo

When tested against *Candida krusei* CMGB 94, strain CPM1 proved to be the least active, forming small inhibition zones. An interesting case was *C. parapsilosis* CBS 604, where dark pink colonies and growth inhibition zones were observed surrounding the colonies of all three yeast strains at 28 °C; in contrast, at 25 °C strain SG2 was more active than CPM1.

The highest antimicrobial activities were obtained against *C. tropicalis* OT4, with 2-mm growth inhibition zones surrounding SG1 and SG2 colonies starting even from the third day of incubation (Fig. 2a). When plated

Table 1. The killer activity of *Metschnikowia pulcherrima* strains SG1, SG2 and CPM1 recorded after three and five days of incubation

Plated strain	Incubation temperature	Killer activity after 3 days of incubation			Killer activity after 5 days of incubation		
	°C	SG1	SG2	CPM1	SG1	SG2	CPM1
Candida parapsilosis CBS 604	28	–	–	–	+++	+++	–
	25	+++	+++	+++	+++	+++	+++
C. albicans ATCC 10231	28	+	+	+	++	++	++
	25	–	–	–	+	+	+
C. krusei CMGB 94	28	++	++	–	+++	+++	–
	25	+++	–	–	+++	+++	+++
Saccharomyces cerevisiae 17/17	28	–	–	–	–	–	–
	25	–	–	–	–	–	–
C. albicans Cc	28	–	–	–	+++	+++	+++
C. tropicalis OT4	28	–	–	–	+++	+++	+++

–=no activity, +=weak, ++=medium activity, +++=strong activity

against *C. albicans* Cc, SG1 and SG2 strains remained the most active, but formed smaller halos than in the case of *C. tropicalis* OT4 (Fig. 2b).

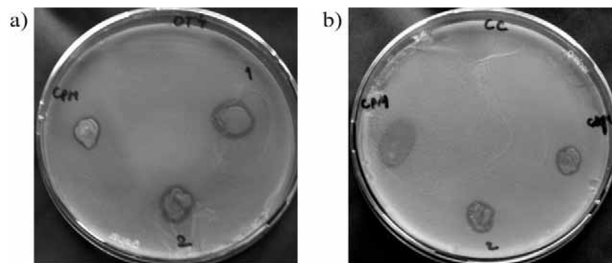

Fig. 2. Antimicrobial activity of SG1, SG2 and CPM1 strains recorded after 5 days of incubation against: a) *Candida tropicalis* OT4 and b) *Candida albicans* Cc

Enhancement of antimicrobial activity

The presence of $NaHCO_3$ or $CaCl_2$ as additives did not increase significantly the antimicrobial activity of SG1, SG2 and CPM1 strains. However, *C. tropicalis* OT4 seemed to be more sensitive to the antimicrobial activity of SG1 and SG2 in the presence of 2 % $NaHCO_3$ or 2 % $CaCl_2$, while the activity of CPM1 remained the same in the presence of 0.1 % $NaHCO_3$. Growth inhibition zones were obtained on *C. albicans* Cc plates when SG1 and SG2 cultures were used in mixture with 2 % $NaHCO_3$ or 1 % $CaCl_2$ and when CPM1 culture was mixed with 0.5 % $NaHCO_3$.

Assesing the antifungal activity

The antifungal activity of strains SG1, SG2 and CPM1 was detected against natural isolates of *Fusarium solani*, *F. moniliforme*, *Aspergillus ochraceus*, five *Botrytis cinerea* strains: strains BCF1 and BCP2 isolated from strawberries, BCS and BCPP from grapes and Bclys from leaves, and reference strains *Penicillium chrysogenum*, *Alternaria alternata*, *A. tenuissima*, *A. mali*, *Rhizoctonia* spp. and *Fusarium oxysporum* var. *lycopersici*.

Antifungal activity was increased when the plates were incubated at 25 °C and the level of pigment (shown as a purple ring surrounding the yeast colony) was also increased. The clearest areas of inhibition were observed after three days of incubation, the results being influenced by the growth abilities of fungal strains during the same period of time. Thus, for *Rhizoctonia* spp., the results were observed within the first 48 h, while for *Penicillium* the results were observed after 3 to 4 days of incubation.

Differences in antifungal potential were also detected among the three yeast strains studied. The best results were obtained for strains SG2 and SG1, which were able to inhibit most of the fungal strains except *Sclerotium* spp., in contrast with CPM1 strain, which presented the best activity against all *Botrytis* strains.

Mechanism of antifungal activity

The antifungal activity of all three yeast strains was increased on PDA, compared with YPDA. This effect was very clear for SG2 and CPM1 strains against *Fusarium oxysporum* var. *lycopersici* (Fig. 3). A possible expla-

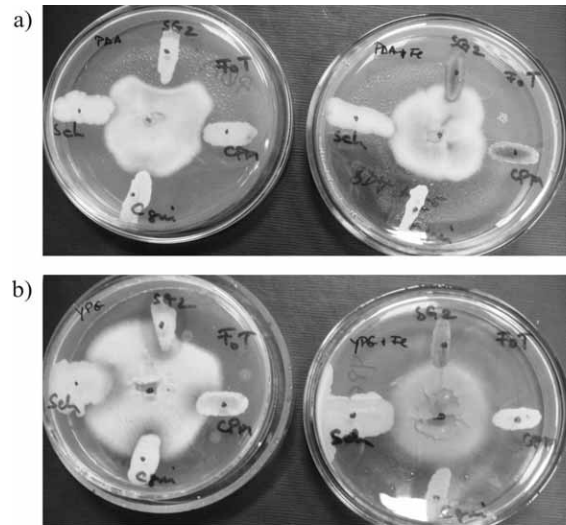

Fig. 3. The influence of the culture medium on inhibitory activity of yeast strains against *Fusarium oxysporum* var. *lycopersici*: a) PDA/PDA+$FeCl_3$ and b) YPDA/YPDA+$FeCl_3$

nation resides in the presence of an increased concentration of iron in YPDA compared with PDA.

The inhibition of the growth of fungal strains was inactivated by the presence of iron, suggesting that the main mechanism of antifungal activity consists in the competition for this nutrient. Moreover, on PDA plates supplemented with $FeCl_3$, yeast cells accumulated iron and colonies grew pink-purple without any pigmented ring around them (Fig. 4).

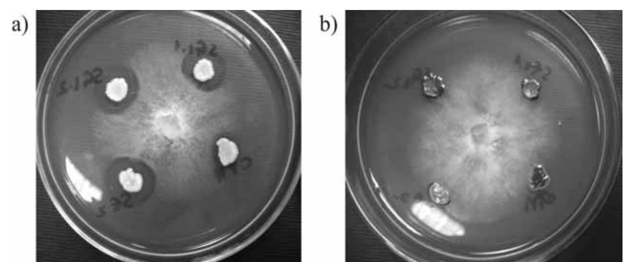

Fig. 4. Antifungal activity of yeast strains against *Botrytis cinerea* BCPP: a) clear inhibition area around yeast colonies cultivated on PDA compared to b) PDA+$FeCl_3$

The effect of incubation temperature was also investigated and it was shown that the inhibitory activity (estimated as the size of inhibitory area compared to untreated fungal colony) was higher at 25 °C. For this reason, all the other tests were performed at this temperature.

The influence of strain cocultivation and growth medium composition was also examined using all three yeast strains and a combination of SG1 and SG2. It was shown that the inhibitory activity against *B. cinerea* BCS and BCF1 strains was slightly increased in the presence of both SG1 and SG2 compared to their individual activities. We observed that antifungal activity depended on the strain (BCS was more sensitive) and on the culture medium: the largest inhibition zone was detected when the yeasts were cultivated in YEMS and PD broth (PDB). It was interesting to observe the reduction of the

inhibition zone when strain CPM1 was added to the mixture of SG1 and SG2 strains.

In experiments with yeast suspensions obtained by filtration of yeast cocultures on different growth media (YDP, YEMS and PDB) subsequently inactivated by heat or protease treatment, the antifungal activity was reduced but not totally absent (Fig. 5).

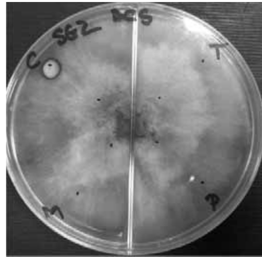

Fig. 5. Effect of heat (T) and protease treatment (P) on the activity of SG2 strain against *Botrytis cinerea* BCS, compared to cells (C) and untreated culture filtrate (M), after 3 days of incubation

When the filtrates were treated with sodium hydrogen carbonate, the inhibitory activity was maintained but it depended on the culture medium, with the best results obtained on YEMS broth. However, the clearest and largest inhibition zones were observed when yeast cell sediments were used, compared to the untreated or treated filtered suspension (Fig. 6).

Fig. 6. Activity of yeast strains against *Botrytis cinerea* BCS: left: culture filtrate of SG1+SG2 mixture cultivated in untreated (S) or treated (t°=heat treatment, P=protease treatment, B=sodium hydrogen carbonate) PD broth; right: the activity of the cell sediments obtained in YPD (I), YEMS (II) or PD broth (III)

Microscopic analyses revealed modifications of fungi due to the antimicrobial activity of the yeast strains: inhibition of sporulation, cytoplasm retraction, cell deformation and fragmentation or lysis of mycelium. The degree of cell modifications depends rather on the fungal strain than on the antagonistic yeast. Increased damage of the cells was observed in *Fusarium oxysporum* var. *lycopersici* in the presence of SG2 and in *Botrytis cinerea* in the presence of SG1 strain (Fig. 7).

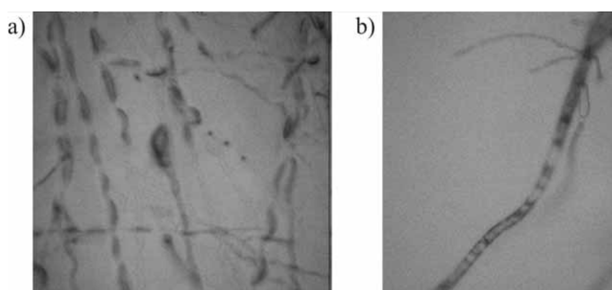

Fig. 7. Modifications of: a) *Fusarium oxysporum* var. *lycopersici* due to SG2 activity and b) *Botrytis cinerea* due to SG1 activity

It was also observed that the inhibitory effect was maintained even after 10 to 21 days of incubation and the targeted fungi were not able to grow over the inhibitory area surrounding the yeast colony. This fact suggests that the inhibitory agent is stable during time and that it has a fungicidal effect.

Discussion

Killer and antimicrobial activities

Although for most of the described species (*S. cerevisiae, Williopsis mrakii, Ustilago maydis, Pichia pastoris*) killer activity resides in producing a toxin which is lethal to the cells of sensitive strains belonging to the same or different genera/species (*19*), for *M. pulcherrima* this behaviour seems to be most probably related to the pulcherrimin production rather than to the presence of a true killer system. However, experiments performed on various yeast strains revealed a possible killer-type activity of *M. pulcherrima* strains (*3*,*18*,*20*), which correlates with our results on reference strains *C. parapsilosis* CBS 604, *C. krusei* CMGB 94 and *C. albicans* ATCC 10123, and *C. albicans* Cc and *C. tropicalis* OT4 strains isolated from human infection. Data concerning the lack of sensitivity of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* strains were also observed by Janisiewicz *et al.* (*3*) and Sipiczki (*21*).

The antimicrobial tests revealed four types of colonies and halos corresponding to SG1, SG2 and CPM1 strains: pale pink colonies with no halo (*C. albicans* ATCC 10231), colonies with difuse pink halos (*S. cerevisiae* S288C), colonies with dark purple edges and no halo (*S. pombe* 972h^{-}) and colonies with clear growth inhibition zones (*C. parapsilosis* CBS 604, *C. krusei* CMGB 94, *C. albicans* Cc and *C. tropicalis* OT4). The formation of pulcherriminic pigment by *M. pulcherrima* is due to the ability of a precursor liberated by the cells to immobilize ferric ions from the media (*22*,*23*). This mechanism of iron sequestration seems to be responsible for the antagonic activity of *M. pulcherrima* through the inhibition of the growth of other potential competitor cells. Sipiczki (*21*) described wide pale halos appearing at low iron concentration, while halo size diminished when the iron concentration was increased and it completely disapears at high iron concentration when dark red colonies appeared. Until now, the extensive work done on *S. cerevisiae* has revealed six ways in which this yeast can acquire iron (*24*). Comparatively, *C. albicans* has three iron uptake pathways (*25*,*26*), while in *S. pombe* iron assimilation is highly dependent on its availability in the growth medium (*27*).

According to this information, we assume that our results reflect mainly the differences in the ability of yeast strains to use iron when cultivated on the same medium (YPGA) under identical conditions. *S. cerevisiae* S288C is the most efficient in depleting iron, as pink halos formed due to low iron concentration around SG1,

SG2 and CPM1 colonies. *S. pombe* 972h$^-$ has a weaker ability of using iron and, finally, *C. albicans* ATCC 10231 is the least adapted to iron consumption under the given growth conditions. The absence of the halo in the case of *C. albicans* and *S. pombe* seems to be explained by the decreased ability of the two yeast species to immobilize iron from the culture medium (YPGA), which represents a step in the formation of pulcherrimin.

The growth inhibition zones formed easily by SG1, SG2 and CPM1 strains in the presence of natural isolates *C. tropicalis* OT4 and *C. albicans* Cc might be explained by the iron depletion mechanism of the three *Metschnikowia* strains. The growth of the isolated natural *Candida* strains is negatively influenced by the reduced iron concentration in media, a fact already observed during other studies on *Candida* pathogenic strains (*28*).

For the enhancement of antimicrobial activity, we used as a starting point the information from literature describing tests performed on fungi (*Penicillium expansum, Botrytis cinerea, Aspergillus niger, Alternaria* sp. and *Colletotrichum acutatum*) with mixtures of $NaHCO_3$ or $CaCl_2$ and yeasts (*Metschnikowia fructicola, Cryptococcus laurentii, Trichosporon pullulans, Rhodotorula glutinis* and *Pichia membranifaciens*). Results show an increase of antifungal activity of yeasts generally by the addition of 2 % $NaHCO_3$ or 2 % $CaCl_2$.

Positive killer and antimicrobial activities of SG1, SG2 and CPM1 when plated against *C. tropicalis* OT4 and *C. albicans* Cc indicate that the addition of $NaHCO_3$ or $CaCl_2$ could enhance the response of the three strains against natural isolates. Our results are similar to those from literature (*8,29–32*), since a slight increase and the maintenance of antimicrobial activity of SG1 and SG2 strains was observed when using 2 % $NaHCO_3$ or 1 and 2 % $CaCl_2$, respectively. The antagonism of CPM1 strain was less influenced by the addition of $NaHCO_3$ or $CaCl_2$, supporting our previous observations concerning a reduced killer/antimicrobial activity of this strain when compared to SG1 and SG2.

Antifungal activity

According to Royse and Ries (*33*), *in vitro* microbial interactions result in one of the following: production of a zone of inhibition, contact inhibition or no inhibition. During our experiments only two aspects were recorded for the interactions between *M. pulcherrima* SG1, SG2 or CPM1 yeast strain and the fungal strains: formation of an inhibition zone or no inhibition. These aspects are related to the variation of the biocontrol potential of the three *M. pulcherrima* strains and they confirm previous results (*34,35*), those from our killer and antimicrobial tests, as well as those from other similar experiments (*3,6,11*). Thus, the strongest inhibition was produced by the strain SG2, followed by strains SG1 and CPM1. Moreover, the susceptibility of the fungal strains belonging to the same species is different. For example, the most sensitive strains of *B. cinerea* were BCS (isolated from grapes) and BCF1 (isolated from strawberries).

The mechanisms of activity of SG1, SG2 and CPM1 strains against various fungal pathogens were studied *in vitro*, with competition for iron being the major mechanism of antifungal activity for all our strains. This is in accordance with the data from literature (*7,21*); while strong red halos were observed around the colonies cultivated on PDA, no halo was observed on PDA supplemented with $FeCl_3$ and, instead, a concentration of the pigment inside yeast cells was detected. The inhibitory activity was reduced or even absent in the presence of iron in YPDA or PDA supplemented with $FeCl_3$. The size of the inhibitory area surrounding the yeast colonies corresponds to the pigmented halo and reflects the ability of the strains to produce pulcherrimin. Strain SG2 produced the largest area of diffusible pigment, in contrast to CPM1. On the coloured inhibition area, *Botrytis* conidia did not germinate (even after 10 days of incubation) and mycelial modifications were observed, as it was also shown by El-Ghaouth *et al.* (*36*) for *B. cinerea* treated with *Candida saitoana.* Similar aspects were described by Sipiczki (*21*) and they demonstrate that iron starvation and/or the ring of pulcherrimin elicit complex physiological changes in the fungal cells.

Antifungal activity against *Fusarium, Rhizoctonia, Penicillium* and *Alternaria* was more variable: after an initial inhibition of the growth, the fungi extend their colonies over the pigmented halo near yeast colonies. According to Sipiczki (*21*), this transitory inhibition could be due to the degradation of pigment or to the release of iron from degraded fungal cells. Moreover, for most fungal pathogens tested in our experiments the growth is stopped before reaching the yeast cells, suggesting that besides the competition for nutrients, the antifungal activity could be related to diffusible agents other than pulcherrimin. The chemical nature of these toxic metabolites is still unclear, but chitinases and killer-like proteins are probable inhibitors (*10*). This could also explain the reduced inhibitory activity of SG1, SG2 and CPM1 strains against *B. cinerea* when incubated at 28 °C. In this case, the size of the inhibition area was reduced to approx. 75 % at 28 °C compared to the area detected at 25 °C. Nevertheless, the tests performed by plating the three strains against various reference and natural yeast strains did not reveal a clear difference between the results obtained at 25 and 28 °C. One possible explanation could reside in a lower sensitivity of yeasts, compared to fungi, to antimicrobial activities of other microorganisms, including SG1, SG2 and CPM1 strains.

The antifungal activity was totally eliminated after heating or proteolytic treatment of the filtered supernatant of the yeast culture, supporting the previous idea that a protein might also be involved in antifungal activity, a fact also mentioned by de Ingeniis *et al.* (*37*) in the case of *Pichia anomala*.

The activity of supernatants was lower than that of the cells, suggesting that the inhibitory activity could also be due to a cell-related mechanism. Similar conclusions were presented by Spadaro *et al.* (*7*) and Zhang *et al.* (*11*).

It is interesting to notice that SG1 and SG2 strains had synergistic activity when used in combination. The addition of strain CPM1 to the mixture was followed by a decrease of the inhibitory activity indicating a possible production of a compound that partially inactivates SG1 and SG2. The filtered supernatants of the mixtures cultivated in YEMS broth treated with various agents (heat, protease or sodium hydrogen carbonate) determined the inhibition of growth of fungal pathogens. This activity

was temporary: after five days of inhibition fungal growth restarted, but at a lower rate compared to the activity of the yeast cells. The sodium hydrogen carbonate added to the mixtures did not increase the inhibitory activity of the yeasts, which was similar to the results obtained by Karabulut *et al.* (*8*) with *Metschnikowia fructicola*. The maintained inhibitory activity of the heat-treated suspension and protease-treated samples implies that when the yeast strains are cocultivated in YEMS broth, the inhibitory compounds are more resistant, probably due to the presence of other cross-protective compounds. Similar results have recently been presented by Spadaro *et al.* (*17*), who observed an increased efficacy of *M. pulcherrima* grown in YEMS against *B. cinerea* and *Penicillium expansum*. These results suggest that for an efficient biocontrol, total yeast suspensions containing cells and supernatant need to be used, and the inhibitory activity (fungicide and fungistatic) is related both to cells and to secretion compounds present in the supernatant.

Conclusions

Our study deals with the antagonistic activity of three yeast strains, SG1, SG2 and CPM1, most probably belonging to *Metschnikowia pulcherrima*, previously isolated from the surface of fruits. The appearance of colonies of different colours (from pink to dark pink or with purple edges) correlates with inhibitory activity and with the iron concentration in the media, suggesting an iron immobilization mechanism similar to pulcherrimin formation. Since the antifungal activity of supernatants was reduced compared to cell sediments, we believe that a cell-related mechanism might also be involved. Heat and proteolytic treatments inhibited the antagonism of all three strains, pointing to an alternative protein-like action. On the contrary, during the same treatments in YEMS medium, cocultivated SG1 and SG2 suspension remained active, implying synergism and the necessity of specific conditions for an efficient biocontrol. The high killer and antimicrobial activities observed mainly for SG1 and SG2 strains against *Candida* isolates from human infections and their maintenance or slight enhancement in the presence of $NaHCO_3$ and $CaCl_2$ might represent an interesting starting point for further biomedical investigations.

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