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YEASTS FROM XEROPHILIC ENVIRONMENTS REVEAL ANTIMICROBIAL ACTION AGAINST FRUIT PATHOGENIC MOLDS

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ABSTRACT

In the present study, we investigated the occurrence of yeast flora in a xerophilic environment, the desert region of Namibia, with the aim to isolate the yeast strains with antimicrobial activity toward plant pathogen molds. Using traditional culture-dependent methods, a total of 44 yeast strains belonging to 6 genera and 12 species were isolated. The yeast isolated from red berry samples revealed the widest biodiversity, while only *Cryptococcus albidus* strains were isolated from desert melon and leaf samples. A wide antimicrobial activity toward pathogenic molds was observed *in vitro* tests for the yeasts belonging to *Aureobasidium pullulans* (strains F32 and F60), *C. albidus* (S14) and *Saccharomyces cerevisiae* (RB24). This behavior was confirmed *in vivo* tests, where *A. pullulans* F32 and F60 completely counteracted *Penicillium digitatum* decay in lemon and orange, respectively, while *C. albidus* S14 showed a similar strong antimicrobial effect in both fruits. *S. cerevisiae* strain RB24 exhibited a widespread inhibitory activity against *Monilinia fructicola* on cherries.

PRACTICAL APPLICATIONS

To the best of our knowledge, the antimicrobial activity exhibited by selected yeasts from xerophilic environment could be used as a biological practice in postharvest as an alternative biotechnological approach to reduce the use of synthetic fungicides. Indeed, the biological control using antagonistic yeasts offers an alternative approach to combat pathogen molds on fruits and vegetables.

INTRODUCTION

The use of natural antimicrobial compounds produced by yeast to counteract undesired microorganisms has been studied for a long time. These have included investigations into yeast antimicrobial properties in several fields of application, such as the food, agricultural, medical and veterinary industries (Suzuki *et al.* 2001; Comitini *et al.* 2004; Chiquette 2009; Hatoum *et al.* 2012).

In the recent years, considerable effort has been made to introduce new technologies regarding the use of biological processes in the food and agricultural industries. Accordingly, there have been several reports on the inhibitory effects *in vitro* of killer yeast against plant pathogenic filamentous fungi (Comitini and Ciani 2010; Jamalizadeh *et al.* 2011; Platania *et al.* 2012). Other investigations have reported on antagonistic yeasts that have antimicrobial activities as potential candidates for biocontrol during preharvest and/or postharvest storage of fruit and vegetables (Masih *et al.* 2000; Mari *et al.* 2012).

In this context, investigations into new antimicrobial compounds produced by yeast are of great interest to reduce the phenomenon of fungicide resistance in plant pathogens toward chemical pesticides that have emerged particularly in the past few years. Indeed, the growing demand by consumers for agricultural products with fewer chemical additives and the resistance of pathogenic fungi to the more commonly used fungicides has led the industry and researchers to focus their attention toward biological agents to combat fruit decay. A lot of studies have underlined the significance of unconventional and extreme environments as a source of biodiversity for the isolation and selection of new useful microorganisms (Adams *et al.* 2006; Turchetti *et al.* 2011; Cardinali *et al.* 2012).

In this regard, an extreme environment such as a desert might be a rich source of this attractive and unexplored yeast. In this environment, annual precipitation ranges from 2 mm in the most arid regions to 200 mm at the escarpment, making the Namib the only true desert in southern Africa. Having endured arid or semi-arid conditions for roughly 55–80 million years, the Namib may be the oldest desert in the world. On this basis, yeasts isolated in this extreme habitat could have interesting and new antimicrobial metabolites. For instance, *Basidiomycetes* spp. and *Cryptococcus* spp. are considered to be interesting producers of a large number of secondary metabolites, such as antibacterial, antifungal, antiviral, cytotoxic and hallucinogenic compounds (Breheret *et al.* 1997; Marumoto *et al.* 1997). Moreover, great attention has been focused on the study of the yeast-like fungus *Aureobasidium pullulans*, which is used successfully in the control of *Botrytis cinerea*, *Penicillium expansum*, *Monilinia* spp. and *Rhizopus stolonifer* on apple, sweet cherry, grape, strawberry and peach (Schena *et al.* 2003; Bencheqroun *et al.* 2007; Mari *et al.* 2012).

This work was carried out to find natural and suitable biofungicides that might be highly effective without having toxic effects and environmental impact, and to potentially substitute these for chemical agents. For this, we investigated a yeast community from a xerophilic environment: the Giant Playground region in Namibia, and after the isolation campaign, the yeast strains were identified and screened for their *in vitro* and *in vivo* antimicrobial activities toward the most common pathogenic filamentous fungi on fruits.

MATERIALS AND METHODS

Media and Mold Strains

The yeast and molds were cultivated and then stored at 4C both on YPD agar plates (prepared with 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 2% agar) and Potato Dextrose Agar (PDA) medium (Oxoid, Basingstoke, U.K.). Successively, collected purified yeast strains were lyophilized and conserved for a long time. Before use, the yeast was transferred into a 10-mL pre-sterilized YPD broth. The yeasts were seeded onto YPD agar plates and incubated at 25C for 48 h. The enumeration and macroscopic identification of the yeast isolated for the natural matrix were carried out using WL nutrient agar (Wallerstein Laboratory, Oxoid) as differential medium. The inhibition activities of the collected yeast were tested against *B. cinerea*, *Monilinia fructicola*, *Aspergillus carbonarius*, *Penicillium digitatum* and *Penicillium expansum* from the culture collection of the Department of Life and Environmental Sciences (Polytechnic University of Marche, Ancona, Italy).

Isolation and Identification of Yeast Strains

Samples from the microhabitats of the Giant Playground region in Namibia were collected during the summer period, from a desert area that is consists of sand seas near the coast, and from gravel plains and scattered mountain outcrops further inland. Each product collected from fruit, plants and soil were placed aseptically in sterile plastic bags and transferred in ice boxes (4C) to the laboratory for analysis, where the yeast occurrence was investigated.

The samples collected were homogenized using a Stomacher 400 Circulator (Seward, Worthing, U.K.) for 5 min and then serial dilution and standard viable plate counting were carried out. The yeast isolation and enumeration were performed in WL nutrient agar (Oxoid). Differently, the yeast population on the surface of the leaves sample was detected following the procedure described by Comitini and Ciani (2006).

The resulting yeast colonies were counted and then selected according to their macromorphological and micromorphological aspects; they were finally isolated in proportion to their frequencies (Martini *et al.* 1996). Representative colonies (10–15) were picked randomly from the plates. Isolates were purified by streak plating and subcultured onto the YPD medium for subsequent identification through the identification procedures.

For all of the yeast isolates, the DNA was extracted following the procedure proposed by Stringini *et al.* (2009). The isolates were identified using an internal transcribed spacer–polymerase chain reaction (ITS-PCR) procedure, using the primers ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′, and ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′. The PCR mixture and the thermocycling protocol conditions were applied as described by Esteve-Zarzoso *et al.* (1999). The amplified DNA was subsequently digested without further purification, using three restriction endonucleases: CfoI, HaeIII and HinfI (AB Fermentas Int., Inc., Burlington, Canada). The amplified products (ITS-PCR) and their restriction fragments were separated on a horizontal electrophoresis apparatus (Bio-Rad, Hercules, CA) in 1.5 and 2.5% (w/v) agarose gels, respectively, both in 0.5× Tris Borate EDTA (TBE) buffer. The gels were stained with ethidium bromide and visualized under UV light (UV source GelDoc 1000, Bio-Rad). The fragment sizes were estimated by comparisons with a DNA standard marker (GeneRuler 100-bp DNA Ladder, AB Fermentas Int., Inc.), and the restriction patterns were compared with those reported in the previously published studies (Esteve-Zarzoso *et al.* 1999; de Llanos Frutos *et al.* 2004; Arroyo-Lòpez *et al.* 2006). Strains with uncertain identification using the above method were taken through D1/D2 domain analysis, which identified specific nucleotide sequences of individual species (Kurtzman and Robnett 1998).

In Vitro Biocontrol Assay

The yeast and sensitive mold strains were pre-grown on YPD agar and PDA for 48 h and for 7 days, respectively. Subsequent interactions between yeast strains and molds were tested on PDA buffered at pH 4.4 with citrate-phosphate buffer, to adjust the agar medium. Each yeast was abundantly streaked around the perimeter of each sterile Petri dish, leaving the center of the plate empty, and incubated at 25C for 48 h to allow growth. After this, a 25-mm^2 square plug of each mold mycelium was placed in the sterile center of the plate. A total of 220 combinations of 44 isolated yeasts and 5 molds were tested. The control was carried out by placing each mold alone in the plate.

The plates were incubated at 25C for 7 days. Inhibition was considered positive when there was a clear zone between the yeast strain and the mold under assayed, indicating a strong yeast antimicrobial effect against the mold growth (symbol ++). When the inhibition was moderate with 2 cm of mycelial growth around the plug, the symbol + was used, while when the inhibition was weak with only 3 cm of mycelial growth around the plug, the symbol ± was attributed. The symbol − indicated the absence of inhibition with the mold growth comparable to those exhibited in the control test (without yeast inoculum).

In Vivo Biocontrol Assay

Tarocco oranges, red globe grapes, Monachello lemons, common medlar, Giorgia cherries and Amiga strawberries coming from different supermarkets were used in the *in vivo* tests as natural matrix to confirm the interaction type between yeasts and molds achieved during *in vitro* test.

Selected undamaged fruit (10 fruits for each test) were placed in a solution of 2% sodium hypochlorite for 10 min to sterilize the surface and then rinsed with sterile de-ionized water and air dried under a sterile hood. The fruits were then cut with a scalpel to make from two to four 3-mm-deep and 3-mm-wide wounds, according to the size of the fruit. Each yeast suspension was adjusted until 2×10^7 of inoculated cells (about 20 µL of suspension), which were placed into each wound and allowed to dry. The control fruits were treated with 20 µL of sterile water only. After 1 day, 10^6 mold conidia in 20 µL suspension (absorbance, 0.1 at 420 nm, as determined using a spectrophotometer) was also inoculated into each wound and air dried. The artificial mold infection was made with a high concentration, simulating a strong rot decay to evaluate the effective inhibitory action of the antimicrobial yeasts. After inoculation, the fruits were placed into plastic bags to create a humid environment, with sterile wet paper positioned on the trays where the fruits were placed. The trays were stored at 25C after inoculation of the mold. The disease progression was assessed monitoring the presence and the dimension of the rot around the wound of each fruits, assigning three classes according to the percentage of mold infection reduction: (1) weak reduction, ca. 30% of mold reduction; (2) reduction, ca. 60% of mold reduction; and (3) strong reduction, ca. 90% of mold reduction. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Yeast Isolation, Identification and Enumeration

The results of the yeast enumeration as isolated from the collected samples are shown in Table 1. The abundance of the cultivable yeast varied from 7.9 cell/g in the leave sample to 26,900 cell/g in red berries. As expected, the isolation campaign carried out in a xerophilic environment like the Giant Playground desert of Namibia showed low yeast biodiversity, with the exception of the red berry samples. These samples (which contain high sugar content) showed the highest yeast number, similar to those reported in other studies (Tournas and Katsoudas 2005; Nyanga *et al.* 2007). In contrast, the desert melon sample showed a scarce colonization by yeast flora, probably due to the coriaceous exocarp that limits its colonization.

The soil samples showed extensive colonization by molds and bacteria, and showed yeast colonization comparable to that of other investigations of desert environments (Jumpponen 2003; Anderson and Cairney 2004), while the leaf samples exhibited the lowest yeast numbers. However, the flowers and stems of *Euphorbia damarana* showed extensive yeast colonization, which was comparable with that found in other investigations (Brysch-Herzberg 2004; Sláviková *et al.* 2007; Pozo *et al.* 2011).

The results of the identification of the different yeast are given in Table 2. Almost all of the yeast strains were identified using the ITS-PCR method coupled with restriction

TABLE 1. QUANTITATIVE EVALUATION OF THE YEAST ISOLATED FROM EACH MATRIX SAMPLED

Sample source	Sample code	cfu* ($\times 10^3$/g)
Red berries	RB	26.9 ± 0.12
Desert melon (*Citrullus colocynthis*)*	DM	0.1 ± 0.08
Soil†	S	1.7 ± 0.24
Flowers	F	5.7 ± 0.42
Leaves	L	0.0079 ± 0.01
Euphorbia damarana	ED	0.2 ± 0.42

Data are means ± standard deviation.

* cfu, colony forming units.

† Extensive presence of molds and bacteria.

TABLE 2. SOURCES, CODES AND MOLECULAR IDENTIFICATION OF THE ISOLATED YEAST STRAINS

Sample source	Strain code	Identification
Red berries	RB2	*Cryptococcus albidus*
	RB6	*Kodamaea laetipori* (AY520398)*
	RB9	*Cryptococcus albidus*
	RB13	*Cryptococcus chernovii*
	RB17	*Candida solani* (DQ377642)*
	RB19	*Cryptococcus randhawai* (AJ876599)*
	RB22	*Cryptococcus albidus*
	RB23	*Cryptococcus kuetzingii* (AJ876599)*
	RB24	*Saccharomyces cerevisiae*
	RB31	*Aureobasidium pullulans*
	RB50	*Cryptococcus albidus*
	RB53	*Cryptococcus kuetzingii*
	RB54	*Cryptococcus kuetzingii*
Desert melon	DM59	*Cryptococcus albidus*
Soil	S1	*Cryptococcus aerius*
	S3	*Cryptococcus diffluens*
	S7	*Amauroascus volatilis* (AB075324)*
	S8	*Cryptococcus albidus*
	S14	*Cryptococcus albidus*
	S38	*Cryptococcus chernovii*
Flowers	F4	*Cryptococcus albidus*
	F5	*Cryptococcus albidus* (AY296054)*
	F10	*Cryptococcus albidus*
	F11	*Cryptococcus albidus* (AF335982)*
	F20	*Cryptococcus albidus*
	F21	*Cryptococcus albidus*
	F25	*Cryptococcus diffluens*
	F28	*Cryptococcus albidus*
	F32	*Aureobasidium pullulans*
	F34	*Cryptococcus amylolyticus*
	F46	*Aureobasidium pullulans*
	F51	*Aureobasidium pullulans*
	F55	*Cryptococcus kuetzingii*
	F58	*Aureobasidium pullulans*
	F60	*Aureobasidium pullulans*
	F62	*Cryptococcus albidus*
Leaves	L12	*Cryptococcus albidus*
Stems of *Euphorbia damarana*	ED27	*Cryptococcus albidus*
	ED37	*Cryptococcus kuetzingii*
	ED40	*Cryptococcus amylolyticus*
	ED41	*Cryptococcus albidus*
	ED47	*Cryptococcus diffluens*
	ED49	*Cryptococcus aerius*
	ED57	*Cryptococcus albidus*

* Yeast identified through D1/D2 domain analysis of 26 S gene. Accession numbers are shown in parentheses.

analysis, which gave incontrovertible profiles. However, in seven isolates, when the 5.8 S-ITS restriction profile did not match any of the published restriction patterns, the extracted DNA were amplified with D1/D2 primers.

The 13 isolated yeasts collected from the red berry samples revealed the widest biodiversity, showing eight different species belonging to five genera: *Cryptococcus*, *Kodamaea*, *Candida*, *Saccharomyces* and *Aureobasidium*. On the contrary, in the desert melon and leaf samples, only *Cryptococcus albidus* was isolated. The stems of the *E. damarana* samples led to the isolation of only *Cryptococcus* genus strains belonging to five different species. *Cryptococcus* was the dominant genus in soil and flower samples, although the *Aureobasidium* (flowers) and *Amauroascus* (soil) genera were also found.

Our findings show that only *C. albidus* and *A. pullulans* are widely distributed through almost all of the samples, including in the rocky soil. This wide diffusion could be due to their richness in lipids, which probably improves cell survival under the arid and desiccating conditions that are typical of xerophilic environments. It is quite reasonable that most of the fungal cultures from desert soils form highly resistant spores and that the majority of the cultivated fungi were from more fertile regions, which were deposited at the sample sites as wind-borne spores (Conley *et al.* 2006).

Even if the number of mycological studies on desert soil is rather limited and the diversity of the microbes, including fungi, is lower compared to the soil of temperate or tropical regions, this extreme ecosystem represents a suitable *in situ* model to study the relationships between microbial biodiversity and related metabolic functions (Adams *et al.* 2006; Sterflinger *et al.* 2012).

In Vitro and *In Vivo* Antagonistic Activity

The *in vitro* antifungal activities of all of the isolated yeasts were determined in a simple plate test against five pathogenic molds that belong to *B. cinerea*, *M. fructicola*, *A. carbonarius*, *P. digitatum* and *P. expansum* (Table 3). Twenty of the 44 isolated from these unexplored xerophilic environment yeast strains did not show any inhibitory effects against the molds tested. On the other hand, inhibitory activity toward all of the tested molds was observed only for three yeast strains belonging to *A. pullulans* (F32, F60, F46). The other isolated strains showed inhibitory activities toward one or more of the pathogenic molds. The screening carried out here showed that a high number of strains isolated showed effective antimicrobial activities toward pathogenic molds.

On the basis of their wide and strong antimicrobial activity in the *in vitro* tests, seven of these isolated yeasts (strains F32, S14, F5, RB24, RB9, ED41 and F60) were selected and tested for their inhibitory activities *in vivo* on ripe and the undamaged fruit of grapes, lemons, oranges, medlar, strawberries and cherries. The strongest antifungal action was shown by *C. albidus* S14, *A. pullulans* F32 and F60 against *P. digitatum* in oranges and lemons, while *Saccharomyces cerevisiae* RB24 showed antifungal activity against *M. fructicola* on cherries (Fig. 1). A slightly lower inhibition

TABLE 3. ANTIFUNGAL ACTIVITIES OF THE ISOLATED YEAST AGAINST PATHOGENIC MOLDS IN THE *IN VITRO* ASSAY

Strain code	Species	*Botrytis cinerea*	*Monilinia fructicola*	*Aspergillus carbonarius*	*Penicillium expansum*	*Penicillium digitatum*
S1	*Cryptococcus aerius*	–	–	–	+	–
RB2	*Cryptococcus albidus*	–	–	–	–	–
S3	*Cryptococcus diffluens*	–	–	–	–	–
F4	*Cryptococcus albidus*	–	–	–	–	–
F5	*Cryptococcus albidus*	+	+	–	–	±
RB6	*Kodamaea laetipori*	–	–	–	–	–
S7	*Amauroascus volatilis*	–	–	–	–	–
S8	*Cryptococcus albidus*	–	–	–	–	–
RB9	*Cryptococcus albidus*	+	+	±	–	–
F10	*Cryptococcus albidus*	–	–	–	±	–
F11	*Cryptococcus albidus*	–	+	–	–	±
L12	*Cryptococcus albidus*	–	+	–	–	±
RB13	*Cryptococcus chernovii*	–	–	–	–	–
S14	*Cryptococcus albidus*	+	–	+	±	+
RB17	*Candida solani*	±	–	–	–	+
RB19	*Cryptococcus randhawai*	–	–	–	–	–
F20	*Cryptococcus albidus*	–	–	–	–	–
F21	*Cryptococcus albidus*	–	±	–	–	–
RB22	*Cryptococcus albidus*	–	+	–	–	–
RB23	*Cryptococcus kuetzingii*	–	–	–	–	–
RB24	*Saccharomyces cerevisiae*	±	+	±	–	±
F25	*Cryptococcus diffluens*	–	–	±	±	–
ED27	*Cryptococcus albidus*	±	+	–	–	±
F28	*Cryptococcus albidus*	–	–	–	–	–
RB31	*Aureobasidium pullulans*	–	+	–	–	+
F32	*Aureobasidium pullulans*	±	++	±	+	+
F34	*Cryptococcus amylolyticus*	–	–	–	–	–
ED37	*Cryptococcus kuetzingii*	–	–	–	–	–
S38	*Cryptococcus chernovii*	–	–	–	–	–
ED40	*Cryptococcus amylolyticus*	–	–	–	–	+
ED41	*Cryptococcus albidus*	+	–	–	–	+
F46	*Aureobasidium pullulans*	±	+	±	±	±
ED47	*Cryptococcus diffluens*	–	–	–	–	–
ED49	*Cryptococcus aerius*	–	–	–	–	–
RB50	*Cryptococcus albidus*	–	–	–	–	–
F51	*Aureobasidium pullulans*	–	–	±	±	–
RB53	*Cryptococcus kuetzingii*	±	+	–	–	+
RB54	*Cryptococcus kuetzingii*	±	+	–	–	–
F55	*Cryptococcus kuetzingii*	–	+	–	–	+
ED57	*Cryptococcus albidus*	–	+	–	–	+
F58	*Aureobasidium pullulans*	–	–	–	±	±
DM59	*Cryptococcus albidus*	–	+	–	+	±
F60	*Aureobasidium pullulans*	+	++	+	±	++
F62	*Cryptococcus albidus*	–	+	–	–	–

–, no inhibition; ±, weak inhibition; +, inhibition; ++, strong inhibition.

was exhibited by ED41 strain toward *P. digitatum* in orange and by F5 and F32 toward *M. fructicola* on cherries. Also, *C. albidus* strain RB9 was effective against *B. cinerea* on strawberries as well as *S. cerevisiae* strain RB24 against *A. carbonarius* in grape. Weak antifungal activity was shown by *C. albidus* F5 against *M. fructicola* on medlar and *A. pullulans* F32 toward *B. cinerea* on grapes and strawberries (Table 4).

DISCUSSION

Biological control using antagonistic yeasts offers an alternative approach to combat pathogenic mold on fruit and vegetables, and thus to reduce, or even replace, synthetic fungicides (Janisiewicz *et al.* 2000; Sharma *et al.* 2009). The first steps in the discovery and development of new biocontrol agents are the isolation and screening processes

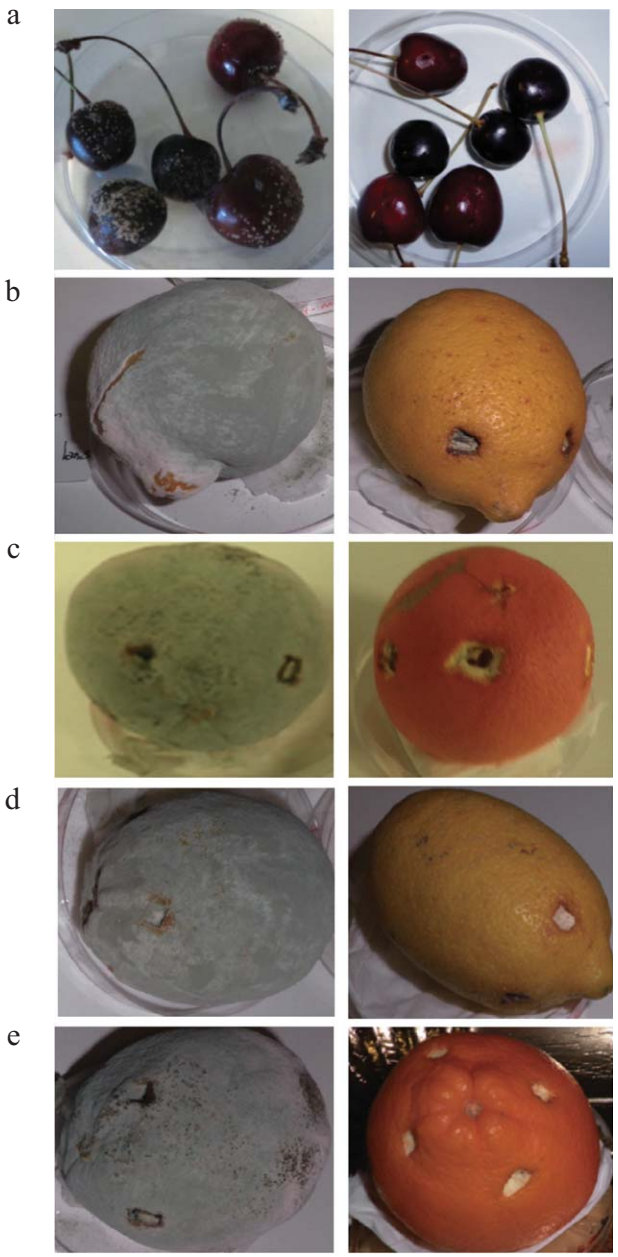

FIG. 1. *IN VIVO* TEST OF FUNGICIDAL ACTIVITY OF YEAST STRAINS THAT EXHIBITED INHIBITORY ACTIVITY

Representative photographs taken after 7 days of incubation at 25C and the images on the left report fruits infected with only molds, those on the right report the fruits treated with antimicrobial yeasts: (a) *Saccharomyces cerevisiae* strain RB24 toward *Monilinia fructicola* in cherries; (b) *Aureobasidium pullulans* strain F32 toward *Penicillium digitatum* in lemon; (c) *A. pullulans* strain F60 toward *P. digitatum* in orange; (d) *Cryptococcus albidus* strain S14 toward *P. digitatum* in lemon; (d) *C. albidus* strain S14 toward *P. digitatum* in orange.

carried out in natural environments. In this regard, we carried out an isolation campaign from a xerophilic ecosystem, the Giant Playground desert of Namibia, with the aim to found a wide yeast biodiversity as a useful source of antimicrobial compounds.

The yeast occurrence and variability evaluated using traditional culture-dependent methods revealed the presence of 6 genera between 44 yeast isolated strains, including *Amauroascus*, *Aureobasidium*, *Candida*, *Cryptococcus*, *Kodamaea* and *Saccharomyces*. In particular, the *Cryptococcus* genus was present with seven different species.

The results of the screening showed that most of the isolated strains exhibited an effective antimicrobial activity toward pathogenic molds tested. In particular, *C. albidus* strain S14 isolated from the soil showed a strong inhibitory activity against *B. cinerea*, *A. carbonarius* and *P. digitatum*, in both the *in vitro* and the *in vivo* assays on oranges and lemons. Accordingly, a previous study indicated *C. albidus* as an antimicrobial species toward *B. cinerea* and *P. expansum* infections in pear fruits (Lutz *et al.* 2013). However, for the first time, the results of this work indicate that *C. albidus* strain can inhibit the growth of *P. digitatum*, both in lemons and in oranges, representing an attractive possibility to develop and apply an antifungal formulation in citrus.

Also, *A. pullulans* strains F32 and F60, which were both isolated from flowers, showed a remarkable antimicrobial activity against *P. digitatum* and *M. fructicola* in both *in vitro* and *in vivo* tests. In this regard, a number of investigations have demonstrated the antagonistic effects of *A. pullulans* for fruit and vegetables postharvest (Janisiewicz *et al.* 2000; Bencheqroun *et al.* 2007; Zhang *et al.* 2012). Specifically, several studies demonstrated the broad spectrum of action of *A. pullulans* yeast that can reduce blue mold and other filamentous pathogenic fungi, including *B. cinerea*, *Monilinia laxa*, *Alternaria solani*, *R. stolonifer*, *A. carbonarius* and *Aspergillus niger* (Castoria *et al.* 2001; Schena *et al.* 2003; De Curtis *et al.* 2012). Our results confirmed the wide antimicrobial activity of *A. pullulans*, which showed antagonistic effects on different fruit and toward different pathogenic molds. Indeed, the efficacy of the *A. pullulans* F32 and F60 strains was seen for grapes, lemons, oranges, strawberries and cherries, and against *B. cinerea*, *P. digitatum* and *M. fructicola*.

Among the isolated yeasts in the red berry matrices, there was *S. cerevisiae* RB24 strain that showed an actual broad inhibitory action toward the entire molds tested, with the exception of *P. expansum*. In particular, the results obtained *in vivo* test showed a strong antimicrobial activity of *S. cerevisiae* RB24 strain in cherries toward *M. fructicola* as a sensitive target. Previous applicative works showed an effective antagonistic behavior of *S. cerevisiae* strains on fruits; however, the inhibitory action was demonstrated

TABLE 4. FUNGICIDAL ACTIVITY OF THE SELECTED ISOLATED YEAST WITH *IN VIVO* ANTIMICROBIAL ACTIVITY, TESTED AGAINST MOLD-INFECTED FRUIT

Antimicrobial yeast code	Inoculated mold	Fruit	Inhibition intensity*
F32	*Botrytis cinerea*	Grape	±
	Botrytis cinerea	Strawberry	±
	Penicillium digitatum	Lemon	++
	Monilinia fructicola	Cherry	+
S14	*Penicillium digitatum*	Lemon	++
	Penicillium digitatum	Orange	++
F5	*Monilinia fructicola*	Medlar	±
	Monilinia fructicola	Cherry	+
RB24	*Monilinia fructicola*	Cherry	++
	Aspergillus carbonarius	Grape	+
RB9	*Botrytis cinerea*	Strawberry	+
ED41	*Penicillium digitatum*	Orange	+
F60	*Penicillium digitatum*	Orange	++

* The inhibition intensity was evaluated assigning three classes according to the percentage of mold infection reduction: ±, weak reduction, 30% of mold reduction; +, reduction, 60% of mold reduction; ++, strong reduction 90% of mold reduction.

only against *Botrytis* spp. on grape and apple (Alavifard *et al*. 2010; Raspor *et al*. 2010). In all cases, the authors demonstrated that the highest antagonistic activity of *S. cerevisiae* was seen on substrate with high concentrations of glucose, as well as our RB24 strain that was isolated from sweet fruits. Moreover, the use of *S. cerevisiae*, defined as GRAS microorganism, could represent a safe alternative to chemical fungicides for postharvest disease control.

CONCLUSION

The broad antimicrobial spectrum found in the yeast strains isolated in this work provides evidence to propose the *A. pullulans* F32 and F60 strains, the *C. albidus* S14 strain and the *S. cerevisiae* RB24 strain for use as biological controls to reduce chemical fungicides in the control of postharvest diseases caused by fungal pathogens. However, further studies on the mode of action of these bioactive microorganisms are needed to establish the specific modalities for a possible applicative use during pre- or postharvest phases. After that, the technology to produce and test the bioactive compounds will be developed, both on laboratory and on a larger scale.

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