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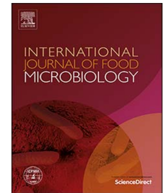

Isolation, identification and selection of antagonistic yeast against *Alternaria alternata* infection and tenuazonic acid production in wine grapes from Argentina

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ABSTRACT

Epiphytic isolates with yeast characteristics from grapes of the Malbec cultivar were obtained in order to find antagonists against *Alternaria alternata*. From a total of 111 isolates, 82% corresponded to the yeast-like organism *Aureobasidium pullulans* and the rest to the non-*Saccharomyces* yeasts *Hanseniaspora uvarum* (6.3%), *Metschnikowia pulcherrima* or spp. (5.4%), *Cryptoccocus laurentti II* (2.7%), *Starmerella bacilaris* or *Candida zemplinina* (2.7%) and *Rhodotorula* spp. (0.9%). The 22.4% (15 out of 67) of epiphytic yeasts and yeast-like organisms evaluated were able to reduce *A. alternata* infection from 0.0 to 4.4% when applied 2 h previous to pathogen inoculation on wounds of grape berries. From these selected strains, 14 out of 15 strains completely prevented *A. alternata* infection (0.0%), which implies potential for field application. All *Metschnikowia* (*pulcherrima* or spp.), *S. bacillaris* and almost all *H. uvarum* evaluated strains showed antagonist capability against *A. alternata*. Meanwhile, none of the lesser nutritional requirement strains belonging to *A. pullulans*, *Cr. laurenti II* and *Rhodotorula* spp. did. All the yeasts with capacity to prevent *A. alternata* infection also reduced tenuazonic acid (TA) production by 81.2 to 99.8%, finding TA levels similar to negative controls. Therefore, the epiphytic yeasts selected are promising as biological control agents against *Alternaria* infection and toxin production in grapes for winemaking.

1. Introduction

Wine is an ancient product that has become a synonym of culture and lifestyle worldwide being also associated with beneficial effects for human health (Artero et al., 2015). Argentina ranks 8th among wine producing countries with 8.8 MhL of production estimated during 2016 (OIV, 2016). In particular, the well-recognized winemaking region of DOC “San Rafael”, located in the west-centre of Argentina, has distinctive ecological features that allow the production of high quality wines, making viticulture one of the main activities (INV, 2016).

Development of undesirable filamentous fungi in wine grapes is a detrimental issue in the wine industry. Besides the significant yield losses and the alteration of chemical composition, due to rotting or secondary invader fungi, some of them have also the potential to produce mycotoxins harmful to wine consumers (Steel et al., 2013). *Alternaria* is the main component of wine grape mycobiota from different winemaking regions in Argentina and worldwide (Magnoli et al., 2003; Prendes et al., 2015; Rousseaux et al., 2014; Tančinová et al., 2015). During a previous study, *Alternaria alternata* strains isolated from Malbec wine grapes have demonstrated the *in vitro* ability to produce alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) and exhibited pathogenicity on wine grapes (Prendes et al., 2015). Also, the same toxins were produced in a synthetic nutrient media similar to grape composition under conditions normally found during wine grape ripeness in the field (Prendes et al., 2017a). In recent works, natural occurrence of TA was reported in different varieties of wine grapes and the ability of *A. alternata* strains to produce it directly on wine grapes was also confirmed (Fontana et al.,

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2016; Prendes et al., 2017b). Additionally, a number of previous studies have reported the natural occurrence of AOH, AME and TA on grape juices and wine (Broggi et al., 2013; Fan et al., 2016; López et al., 2016; Pizzutti et al., 2014; Scott et al., 2006). *Alternaria* toxins exposure has been linked to a great variety of adverse effects to both human and animal health (Dall'Asta et al., 2014) and its regulation on food is currently a concern (Alexander et al., 2011; Arcella et al., 2016).

Prevention of growth of mycotogenic fungi is the most effective strategy to control the presence of mycotoxins on foods (Hocking et al., 2007). Although synthetic fungicides are effective, their continued or repeated application has disrupted biological control by natural enemies of fungus and stimulated the development of resistant pathogen populations, leading to widespread outbreaks of diseases (Lemos Jr et al., 2016). The increasing concern over the adverse agronomical and environmental effects of synthetic fungicides brought to search new types of crop protection methods without or with reduced use of conventional fungicides. In that scenario, the biological control has gained attention as a promising area of development. *Alternaria* biocontrol should be done during grape development as a preventive strategy to avoid colonization of the wine grapes by toxicogenic species and, consequently, mycotoxin accumulation (Lee et al., 2015). Among potential candidates, epiphytic wine grape yeast seems promising, because they are naturally occurring, and have shown ability to colonize wound sites (Bai et al., 2008). Antagonistic yeasts have been used in the control of several rotting and toxicogenic fungi on table and wine grapes (Bleve et al., 2006; Karabulut et al., 2003; Lemos Jr et al., 2016; Nally et al., 2012, 2013; Ponsone et al., 2011; Raspor et al., 2010; Zahavi et al., 2000).

Therefore, the aims of this work were: i) to isolate and identify by molecular methods epiphytic yeasts and yeast-like organisms from wine grapes from the region DOC San Rafael; ii) to select yeast and yeast-like organisms able to control infection of *A. alternata* and mycotoxin production on grapes.

2. Material and methods

2.1. Isolation of epiphytic yeasts and yeast-like organisms

The yeasts and yeast-like organisms were isolated from Malbec wine grapes (*Vitis vinifera* L.) obtained from vineyards located in the DOC or DO (Denomination of Origin) San Rafael wine grape-growing region (Mendoza, Argentina) during 2011, 2012 and 2013 vintages. The geographical area selected for sampling is located between 34.3° and 34.8° S latitude, 67.4° and 68.5° W longitudes, corresponding to an altitude of 500 to 800 m a.s.l. During 2011 vintage, the field sampling comprised three rows of vines from a vineyard at the INTA (Argentina's National Institute of Agricultural Technology) Experimental Station of Rama Caída, in which a randomized trial of 6 blocks with 4 plants per block was used. An independent sample was taken from each block, at harvest time, containing grape bunches collected at 1.5 m from the ground from three out of the 4 plants (a bunch per plant) reaching approximately 0.3 kg. This sampling procedure was repeated during 2012 vintage. During 2013, 6 representative vineyards from DOC San Rafael were sampled. An independent sample was taken from each vineyard, at harvest time, containing grape bunches collected at 1.5 m from the ground from 9 plants located in three different parts of the vine (a bunch per plant) reaching approximately 1 kg. All samples were kept in plastic bags and immediately transferred to the laboratory.

Asymptomatic grape berries (15 in 2011, 30 in 2012 and 90 in 2013 vintages) were randomly selected from each sample, surface-disinfected with 1% (w/v) sodium hypochlorite solution for one min and rinsed in sterile distilled water three times and placed directly onto Dichloran Rose Bengal Chloramphenicol Agar (DRBC) plates and incubated at 25 °C for 7 days. To generate selection pressure, only colonies with yeast characteristics that prevailed in their coexistence with filamentous fungi after 7 days of incubation were selected. They were isolated, subcultured onto Yeast Extract Peptone Dextrose agar (YEPD) and stored at 4 °C.

2.2. Molecular identification of yeasts and yeast-like organisms

The isolated yeasts and yeast-like organisms were identified by restriction fragment length polymorphisms (RFLPs). Briefly, each isolate was streak-inoculated in YEPD agar, incubated at 28 °C for 48 h and a fresh colony was resuspended in 50 µL sterile distilled water placed in an Eppendorf tube. Tubes were placed in boiling water for 5 min, cooled by immersion in ice for 5 min and centrifuged 10,000 rpm × 10 min at 4 °C. Supernatants were then used to amplify the region between the 18S rRNA and 28S rRNA genes with the specific internal transcribed spacers ITS1 and ITS4 primers (White et al., 1990). This region contains the highly conserved region of ribosomal 5.8S, and a variable zone which is the region on the ITSs. The amplified genes were then treated with restriction enzymes *Cfo*I, *Hind*I, *Hae*III and *Dde*I for identification of yeasts at species level (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2006). The size of amplified and restriction fragments were then analyzed using YEAST-ID (www.yeast-od.org) to assign species.

2.3. Evaluation of antagonistic activity of the isolated yeasts and yeast-like organisms against *A. alternata* in a detached berries test

2.3.1. Preparation of fungal inoculum

The yeast and yeast-like strains were grown in Malt Yeast extract Glucose Peptone agar (MYGP) Petri dishes at 28 °C for 48–72 h. After incubation, a loopful of a pure isolated yeast or yeast-like strain was suspended in 1 mL of sterile distilled water in an Eppendorf tube. Yeast suspension was centrifuged at 6200 rpm × 5 min at 4 °C, supernatant was discarded and the pellet re-suspended in sterile distilled water twice, to eliminate remnant nutrients from the initial medium. Finally, the pellet was suspended in sterile distilled water and concentration was adjusted to 10^6 CFU/mL using a Neubauer chamber.

Three *A. alternata* strains (5.5, 7.5 and 25.1) previously isolated from wine grapes from DOC or DO (Denomination of Origin) San Rafael (Mendoza, Argentina) during 2011 and 2012 vintage were used (Prendes et al., 2015, 2017a). Briefly, each *A. alternata* strain was placed separately on Potato-Carrot-Agar (PCA) medium Petri dishes and incubated at 20–25 °C during 7–10 days under cool-white fluorescent lamps with 8/16 light/dark cycle. After incubation, 4 mL of sterile water containing 0.05% (v/v) Tween 20 were poured into the dishes to remove the spores from the mycelium and the suspension was centrifuged at 13,000 rpm × 5 min at 4 °C. The supernatant was decanted and the spore pellet re-suspended in 1 mL of sterile 0.01% (v/v) Tween 20. Spore concentration was adjusted using a Neubauer chamber to 1.75×10^4 spores/mL for strains *A. alternata* 5.5 and 25.1 and 5×10^4 spores/mL for strain *A. alternata* 7.5, respectively. Those concentrations corresponded to the minimum spore concentration to reach 100% of grape infection after 5 days of incubation under 25 °C and 100% RH, defined as Minimum Infective Concentration (MIC) (Prendes et al., 2017b).

2.3.2. Grapes

Grape bunches of *Vitis vinifera* cv. Malbec were harvested during the commercial ripening period in a vineyard from DOC San Rafael (INTA Experimental Station - Rama Caída) and immediately transferred to the laboratory. Homogeneous bunches were selected according to size, shape, color, weight and absence of injuries (Nally et al., 2013). Healthy detached berries were surface disinfected with sodium hypochlorite solution (1%, v/v) for 1 min, rinsed in sterile distilled water and left to dry at room temperature.

2.3.3. Inoculation, incubation and preventive effect assessment

A single wound (three mm diameter and three mm deep) was made

at the equator of each berry using the tip of a sterile dissecting needle. 20 μL 10^6 CFU/mL of an evaluated yeast or yeast-like strain were pipetted into the wound. After 2 h, 20 μL of MIC of an *A. alternata* strain were poured into the wound (1.75×10^4 spores/mL for *A. alternata* 5.5 or 25.1 and 5×10^4 spores/mL for *A. alternata* 7.5). Treated berries were sterile air dried and placed in Petri dishes (8 grapes per dish) and incubated at 25° C and 100% RH during 5 days. At the end of the experiment, the incidence of *A. alternata* rot on each infected berry was calculated as follows: incidence (%) = (number of decayed wounds/ number of total wounds) × 100. A positive control of wounded berries with 20 μL of sterile distilled water and 20 μL of fungal spore suspension was included, as well as two negative controls: wounded berries with 40 μL of sterile distilled water and wounded berries with 20 μL of yeast suspension and 20 μL of sterile water. Each experiment used 8 berries per replicate and three replicates per treatment in a randomized complete block design. A reduction in disease incidence of 60% or more on each of the three *A. alternata* strains was considered a pre-selection criterion of antagonistic yeasts or yeast-like strains (Nally et al., 2013). Then, the experiment was repeated with pre-selected strains to select those which showed reproducibility of the results.

2.4. 26S rRNA gene sequence analysis of antagonistic yeasts

DNA extraction was carried out following a procedure previously described by Querol et al. (1992). Then, a fragment of 500–600 nucleotides, corresponding to the codifying region of RNAr 26S (large subunit) with D1 and D2 domains, was amplified using the Polymerase Chain Reaction (PCR) with NL1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Kurtzman and Robnett, 1998). Fragments were purified with QIAquick PCR Purification kit (Qiagen) according to manufacturer specifications and send to sequencing services (Genomic Unit, INTA Castelar, Hurlingham, Argentina). Obtained sequences were edited with MEGA6 software v2013 and comparisons with already published sequences available at GenBank database in NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/) were done using BLAST (Basic Local Alignment SearchTool).

2.5. Evaluation of antagonistic activity of selected yeasts against tenuazonic acid production by A. alternata in a detached berries test

A single wound was made at the equator of each berry as described in Section 2.3.3. 20 μL of 10^6 CFU/mL of an antagonistic yeast previously selected were pipetted into the wound. After 2 h, 20 μL of MIC of *A. alternata* strain 7.5 or 25.1 (5×10^4 spores/mL or 1.75×10^4 spores/mL, respectively), which have shown TA production on grapes (Prendes et al., 2017b), were poured into the wound. Treated berries were sterile air dried and placed in Petri dishes (8 grapes per dish) and incubated at 25 °C and 100% RH during 24 days, optimum for TA production (Prendes et al., 2017b). A positive control of wounded berries with 20 μL of sterile distilled water and 20 μL of fungal spore suspension was included as well as two negative controls: wounded berries with 40 μL of sterile distilled water and wounded berries with 20 μL of yeast suspension and 20 μL of sterile water. Each experiment used 8 berries per replicate and three replicates per treatment in a randomized complete block design. At the end of the experiment, each sample (replicate) was ground in a laboratory mixer, an aliquot of 2.5 g in a 50 mL PTFE centrifuge tube was collected and stored at − 20 °C until mycotoxin extraction procedure. The experiment was repeated twice.

TA extraction was done following a high-throughput modified QuEChERS (quick, easy, cheap, effective, rugged and safe) method previously developed for TA in wine grapes (Fontana et al., 2016). Briefly, 2.5 mL of ultrapure water (acidified with 1% FA) were added to each sample (2.5 g). After slurring the sample with water, 5 mL ethyl acetate were added and the tube vigorously hand-shaken for 1 min. For phase separation, 4 g of $MgSO_4$ and 1 g of NaCl were added; the tube was shaken for 1 min and centrifuged for 10 min at 8000 rpm. Thereafter, the top layer was transferred to a tube containing 0.25 g anhydrous $CaCl_2$, vortexed for 30 s and centrifuged at 8000 rpm for 5 min. The supernatant was collected in a glass Khan tube and evaporated to dryness (SpeedVac concentrator). Finally, the residue was re-suspended in 0.5 mL mobile phase [MeOH: 0.1 M NaH_2PO4 (2: 1 v/v), adjusted to pH 3.2] and 20 μL were injected in the HPLC-MWD system. The working wavelength for TA was 279 nm. HPLC separations were carried out in a Kinetex XB-C_{18} column (4.6 mm × 150 mm, 5 μm) Phenomenex (Torrance, CA, USA) and TA mobile phase and running conditions were those described by Fontana et al. (2016). Samples were quantified by using a matrix-matched calibration. Limit of detection (LOD, signal-to-noise ratio 3) was 0.01 μg/g and the quantification limit (LOQ) was 0.05 μg/g.

2.6. Statistical analysis

To analyze preventive effect of antagonistic yeasts, multivariate analysis of variance (MANAVA) was done using Infostat software v2013 (FCA, Universidad Nacional de Córdoba, Argentina). When the analysis was statistically significant, Hotelling-Bonferroni test was used for separation of the means ($p < 0.05$). The percentage of TA reduction by antagonistic yeasts was performed by the Kruskal Wallis non-parametric analysis of variance followed by multiple comparison test, when significant differences were found ($p < 0.05$).

3. Results

3.1. Isolation and molecular identification of epiphytic yeasts and yeast-like organisms

One hundred eleven epiphytic isolates with yeast characteristics were obtained from Malbec grape berries at harvest time from 2011 (18), 2012 (27) and 2013 (66) vintages. All the 111 isolates were identified by PCR amplification of 5.8 ITS region at species level. The yeast-like organism *Aureobasidium pullulans* was predominant in terms of relative abundance (44.4% in 2011, 100% in 2012 and 84.8% in 2013) followed by *Hanseniaspora uvarum* (22.2% in 2011 and 4.5% in 2013), *Metschnikowia pulcherrima* (33.3% in 2011) and by *Cryptococcus laurentti II* or *Candida zemplinina* (both 4.5% in 2013) (Fig. 1). Besides, one isolate (1.5% in 2013) was assigned as *Rhodotorula* spp. because its morphological characteristics and its PCR-RFLP pattern partially match with several species of this genus (*Rhodotorula graminis*, *Rhodotorula acuta*, *Rhodotorula glutinis*). The different PCR-RFLP patterns obtained are shown in Table 1.

3.2. Antagonistic effect of epiphytic yeasts and yeast-like organisms against A. alternata and 26-S identity assignments

Sixty-seven epiphytic strains among the 111 isolates, corresponding to all yeast strains (7 of *H. uvarum*, 6 of *M. pulcherrima*, three of *C. laurentti II*, three of *C. zemplinina* and one of *Rhodotorula* spp.) and 47 out of 91 of the yeast-like strains (*A. pullulans*) were selected for evaluation of antagonistic activity against *A. alternata* in detached berries.

All positive controls (20 μL water + 20 μl MIC *A. alternata* strain) reached 100% of infection after 5 days of incubation and the 2 negative controls (40 μL water; 20 μL 10^6 CFU/mL yeast or yeast-like strain + 20 μL water) showed no visual infection (0%).

Fifteen out of 67 (22.4%) yeast strains were able to reduce grape infection by 60% for each of the three *A. alternata* strains (5.5, 7.5 and 25.1) in independent experiments ($p < 0.05$). All the *M. pulcherrima* (6), *C. zemplinina* (3) and most (6 of 7) of *H. uvarum* strains evaluated were selected for its antagonist activity. None of the *A. pullulans* (47), *Cr. laurentti II* (3) or *Rhodothorula* spp. (1) strains evaluated showed antagonistic activity (Table 2). The selected yeast strains were grouped

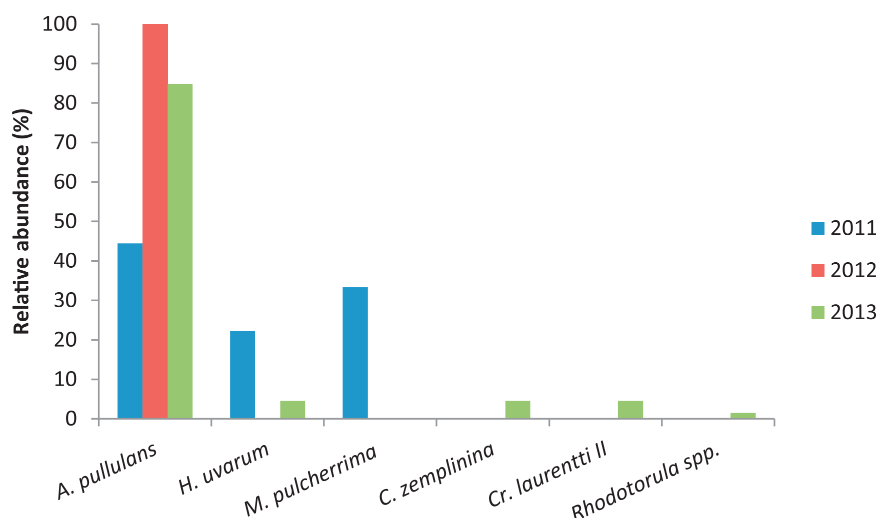

Fig. 1. Relative abundance (%) of each epiphytic yeast or yeast-like species found during 2011, 2012 and 2013 vintages in Malbec grapes from DOC San Rafael.

in two categories through Hotelling-Bonferroni test (MANAVA; $p < 0.05$) according to their antagonistic capability. Fourteen out of 15 strains reduced grape infection to 0% for each of the three *A. alternata* strains at its MIC, while only one strain (LP123.2) showed less efficiency.

In order to confirm the identity of the selected antagonistic yeast strains, the D1–D2 loop region of 26S rRNA gene was sequenced and compared with the available DNA sequence database. Strains LP124, LP125.1, LP8.1.1, LP126, LP8.2.1 and LP10.2.1 were conspecific with *H. uvarum* (Table 2). Strains LP6.4.1, LP8.5.1 and LP8.5.2 were conspecific with *C. zemplinina* as well as its obligate synonyms, *Starmerella bacillaris* (Table 2). However, there were discrepancies among the identity of strains LP131.2, LP132.1, LP128.2, LP125.2, LP122.2 and LP123.2 (Table 2). One strain (LP132.1) was conspecific with *M. pulcherrima*, while the rest showed high similarity (98–99%) with *M. pulcherrima* as well as with *M. fructicola*, reason why they were designated as *Metschnikowia* spp.

3.3. Antagonistic activity of selected yeasts against tenuazonic acid production

TA levels found in berries inoculated with water plus A. *alternata* (positive controls) were 21.8 ± 2.8 μg/g and 13.0 ± 1.3 μg/g for *A. alternata* strain 7.5 and 25.1, respectively. Negative controls (water alone; antagonist yeast + water) showed not quantifiable levels of TA (< 0.05 μg/g). Table 3 shows the percentage of TA reduction with respect to TA levels obtained in the positive control (water + *A. alternata* strain) achieved by the addition of antagonistic yeasts.

All the selected antagonistic epiphytic yeasts strains (15) were effective in reducing TA presence in grape berries infected with *A. alternata* strains (7.5 or 25.1) under optimum conditions for the metabolite yield (100% RH, 25° C, 24 incubation days).

All the 15 antagonistic yeasts reduced TA with respect to positive control: water + *A. alternata* strain 7.5 by 86–100%; water + *A. alternata* 25.1 by 81–100%. There were no significant differences among the different yeasts tested (Kruskal Wallis, $p > 0.05$). It was noticeable that at the end of the experiment, no visible fungal growth was observed in all treatments where antagonistic yeasts had been applied.

4. Discussion

Most of the epiphytic strains with yeast characteristics isolated from Malbec grape berries of different vintages (2011, 2012 and 2013) were identified as the "yeast-like" *A. pullulans* species (82%), considered as "true" yeast by some authors (Baffi et al., 2011; Clavijo et al., 2010; Nisiotou and Nychas, 2007; Prakitchaiwattana et al., 2004; Raspor et al., 2006). According to its physiological characteristics, it integrates with basidiomycete oxidative yeasts and some lactic acid bacteria an oligotrophic group favored by poor nutrient environments like healthy grape berries (Barata et al., 2012). Several studies have also demonstrated the predominance of this group of microorganisms on grape berry surfaces when direct plating method of isolation were used instead of inoculation of juice fermentation (Baffi et al., 2011; Barata et al., 2011, 2012; Clavijo et al., 2010; Nisiotou and Nychas, 2007; Prakitchaiwattana et al., 2004; Raspor et al., 2006; Sabate et al., 2002; Subden et al., 2003).

The rest of the epiphytic yeast species isolated in the present work: *H. uvarum* (6.3%), *M. pulcherrima* or spp. (5.4%), *Cr. Laurentti II* (2.7%), *C. zemplinina* (2.7%) and *Rhodotorula* spp. (0.9%) are all non-*Saccharomyces* yeasts that agree with the reported yeasts isolated from wine grape berries surface from different regions worldwide (Baffi et al., 2011; Barata et al., 2008a, 2008b; Chavan et al., 2009; Clavijo

Table 1
Different patterns obtained from the amplification product and restriction length size of the isolates with yeast characteristics from 2011, 2012 and 2013 vintages.

Species	N° of isolates 2011/2012/2013	AP[a] (bp)	Restriction lengths (pb)			
			Cfo I[b]	Dde I[c]	Hae III[d]	Hinf I[e]
Aureobasidium pullulans	8/27/56	600	100 + 180 + 190	ND	150 + 450	130 + 180 + 290
Hanseniaspora uvarum	4/0/3	775	100 + 320 + 340	80 + 100 + 170 + 300	775	160 + 200 + 370
Metschnikowia pulcherrima	6/0/0	400	95 + 100 + 205	ND	100 + 280	190 + 200
Cryptococcus laurentti II	0/0/3	550	260 + 290	ND	100 + 375	270 + 270
Candida zemplinina	0/0/3	460	56 + 103 + 105 + 196	ND	460	225 + 235
Rhodothorula spp.	0/0/1	660	240 + 320	ND	660	150 + 230

[a]AP 5.8S-ITS-amplified product size, [b,c,d,e]Restriction enzymes used.
ND, not determined.

Table 2
Antagonistic capability of selected epiphytic yeast strains from Malbec wine grapes during 2011, 2012 and 2013 vintages against *Alternaria alternata*.

Origin	Strain	Species		% of infection[c]			
		PCR-RFLP[a]	26S[b]	5.5	7.5	25.1	[d]
2011	LP123.2	*M. pulcherrima.*	*Metschnikowia* spp.	4.4 ± 0.7	2.0 ± 2.8	0.0 ± 0.0	A
2011	LP122.2	*M. pulcherrima.*	*Metschnikowia* spp.	0.0 ± 0.0	2.2 ± 3.1	0.0 ± 0.0	B
2011	LP125.2	*M. pulcherrima.*	*Metschnikowia* spp.	1.9 ± 2.7	0.0 ± 0.0	2.0 ± 2.8	B
2011	LP128.2	*M. pulcherrima.*	*Metschnikowia* spp.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2011	LP131.2	*M. pulcherrima.*	*Metschnikowia* spp.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2011	LP132.1	*M. pulcherrima.*	*M. pulcherrima*	1.9 ± 2.6	0.0 ± 0.0	2.1 ± 2.9	B
2011	LP124	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2011	LP125.1	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2011	LP126	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP8.1.1	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP8.2.1	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP10.2.1	*H. uvarum*	*H. uvarum*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP6.4.1	*C. zemplinina.*	*C. zemplinina* o *S. bacillaris*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP8.5.1	*C. zemplinina*	*C. zemplinina* o *S. bacillaris*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B
2013	LP8.5.2	*C. zemplinina*	*C. zemplinina* o *S. bacillaris*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	B

[a] Species assignment by amplification and restriction length polymorphisms method (PCR-RFLP).
[b] Species assignment by sequencing of 26S region.
[c] Average and standard deviation from 2 independent experiments, in the percentage of infected grapes caused by corresponding *A. alternata* strain (5.5, 7.5, 25.1) evaluated at its MIC with preventive effect of selected yeast.
[d] Different letters represent significant differences ($p < 0.05$) according to Hotelling-Bonferroni test.

Table 3
Percentage of tenuazonic acid reduction[a] by antagonistic yeast applied 2 h previous to pathogen inoculation (*Alternaria alternata* strain 7.5 or 25.1) on grape berries followed by incubation at 25 °C for 24 days.

Antagonistic yeast		*Alternaria alternata* strain	
Strain	Species	7.5	25.1
LP123.2	*Metschnikowia* spp.	96.8 ± 3.9	96.8 ± 4.2
LP122.2	*Metschnikowia* spp.	99.6 ± 0.0	99.8 ± 0.2
LP125.2	*Metschnikowia* spp.	98.8 ± 1.7	99.5 ± 0.8
LP128.2	*Metschnikowia* spp.	97.4 ± 1.5	96.2 ± 5.2
LP131.2	*Metschnikowia* spp.	95.2 ± 4.4	96.4 ± 4.9
LP132.1	*M. pulcherrima*	97.9 ± 2.4	97.6 ± 3.3
LP124	*H. uvarum*	98.8 ± 0.0	99.2 ± 1.2
LP125.1	*H. uvarum*	99.9 ± 0.0	99.9 ± 0.0
LP126	*H. uvarum*	88.6 ± 14.4	99.3 ± 1.0
LP8.1.1	*H. uvarum*	99.2 ± 1.1	95.7 ± 6.0
LP8.2.1	*H. uvarum*	99.6 ± 0.4	92.0 ± 11.2
LP10.2.1	*H. uvarum*	98.1 ± 2.7	98.6 ± 1.9
LP6.4.1	*S. bacillaris*	93.8 ± 8.0	99.0 ± 0.9
LP8.5.1	*S. bacillaris*	86.2 ± 8.5	81.2 ± 19.9
LP8.5.2	*S. bacillaris*	89.6 ± 4.5	89.4 ± 4.8

[a] With respect to TA production obtained by positive control (water + *A. alternata* strain).

et al., 2010; Combina et al., 2005; Fleet et al., 2002; Li et al., 2010; Nisiotou and Nychas, 2007; Prakitchaiwattana et al., 2004; Raspor et al., 2006; Renouf et al., 2005; Sabate et al., 2002; Subden et al., 2003). *Cr. laurentti II* as well as *Rhodotorula* spp. are basidiomycete oxidative yeasts that share their niche with *A. pullulans* (Barata et al., 2012). Meanwhile, *H. uvarum*, *C. zemplinina* and *M. pulcherrima* (weak apiculate and fermentative or weakly fermentative yeasts) have been included in a copiotrophic group with higher nutritional requirements that need high availability of nutrients (Barata et al., 2012). This condition is present in mature grape berries at harvest, which release juice by exosmosis and other process (Barata et al., 2012).

To our knowledge, this is the first report of the antagonistic capability of epiphytic yeasts against *A. alternata* on wine grape berries. The searching of antagonists in the same ecological niche of target fungus and by using selection pressure during isolation via the enforced coexistence of isolates with filamentous fungi during incubation for 7 days in DRBC media, led to the obtainment of 15 (out of 67) antagonists. Also, the use of the Minimum Infective Concentration of fungal target in the present work (1.75×10^4 spores/mL for strains *A. alternata* 5.5 and 25.1 and 5×10^4 spores/mL for strain *A. alternata* 7.5) is important, since effective biocontrol depends on the appropriate ratio among the antagonist and the target fungus (Schisler et al., 2011).

The levels of protection reached during the present work match the recommended for successful antagonists in field application (Chalutz and Droby, 1998). Most of the antagonistic yeasts (14 out of 15), applied at a doses of 20 µL in a concentration of 10^6 CFU/mL in the wound 2 h previous to pathogen inoculation, completely prevented *A. alternata* infection on Malbec grape berries with the three pathogenic strains employed.

Some previous works, with a similar selection methodology as ours but in table grapes, have reported antagonistic yeasts against diverse fungal pathogens. Nally et al. (2013) reported yeast strains from different winemaking environments that decreased fungal infection by 60% of fungi from the complex of sour rot on table grapes (*Aspergillus caelatus*, *Aspergillus carbonarius*, *Aspergillus terreus*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Penicillium comune*, *Rhizopus stolonifer* and *Ulocladium* spp.). Zahavi et al. (2000) found epiphytic yeasts and yeast-like organisms isolated from table and wine grape berries able to reduce 60% of *B. cinerea* infection.

As well, the present work is the first report on yeast strains of *H. uvarum* and *S. bacillaris* (or *C. zemplinina*) with antagonistic activity against *Alternaria* and the first report on antagonistic strains of *Metschnikowia* spp. against *Alternaria* on grape berries for winemaking.

Identification of antagonists by PCR-RFLP method in almost all cases was confirmed by 26S. However, PCR-RFLP method did not have the adequate sensibility to discriminate among *M. pulcherrima* and *M. fructicola* because only 2.2% of substitutions separate them (11 from 499 nucleotides positions shared) (Kurtzman and Droby, 2001). This fine but elemental difference makes complex the species assignment within *Metschnikowia* genus even through 26S approach. On the other hand, *S. bacillaris* assignation as an obligated synonym of *C. zemplinina* is relatively recent (Duarte et al., 2012) and is not yet incorporated on PCR-RFLP yeast database.

There are previous reports on antagonistic activity of *S. bacillaris*,

Metschnikowia spp. and *H. uvarum* from viticulture environments against different fungal pathogens on grapes. Karabulut et al. (2003) applied *M. fructicola* to table grapes to control post-harvest incidence of *B. cinerea*, *Alternaria* spp. and *A. niger* rots. Bleve et al. (2006) reported antagonist activity of epiphytic *M. pulcherrima* strains from Negroamaro wine grapes against *A. carbonarius* and *A. niger*. Raspor et al. (2010) found a *M. pulcherrima* strain isolated from wine environments with the ability to reduce *B. cinerea* infection on Rebula and Chardonnay wine grapes. Liu et al. (2010) found an antagonistic epiphytic *H. uvarum* strain to *B. cinerea* on Kyoho wine grapes. Moreover, *S. bacillaris* strains from fermenting must of overripe grape berries showed antagonistic activity against *B. cinerea* on table grapes (Lemos Jr et al., 2016).

There are also reports on biocontrol of *A. alternata* with antagonistic yeasts but in other fruits like sweet cherries and cherry tomatoes (Tian et al., 2004; Wang et al., 2008).

Several studies have concluded that biocontrol is a strain-dependent characteristic but independent of the species (Suzzi et al., 1995). However, the idea of biocontrol as an attribute derived from physiological requirements of the antagonist on a particular substrate has not been analyzed previously. All the strains of *Metschnikowia pulcherrima*, *Metschnikowia* spp. and *S. bacillaris* and almost all of the *H. uvarum* (5 out of 6), belonging to the copiotrophic group on wine grapes, showed antagonistic effects against *A. alternata* on this substrate. Meanwhile, *A. pullulans*, *Cr. laurenti II* and *Rhodotorula* spp., included in a group of lower nutritional requirements on wine grapes did not show antagonistic ability towards *A. alternata*. These findings suggest a positive correlation between higher nutritional requirements and antagonistic ability of the evaluated organisms. Perhaps a higher nutritional requirement implies a more competitive mechanism, one of the widest used by antagonists (Liu et al., 2013).

On the other hand, the control of fungal growth by antagonistic yeasts does not necessarily indicate hindrance of mycotoxins production. Several fungicides have shown stimulating effects on mycotoxin production, possibly as a consequence of induced stress on toxicogenic strains (Dalcero et al., 1995; Ramirez et al., 2004). In the present work, all antagonistic yeasts selected (15) were able to protect wine grapes from *A. alternata* infection as well as diminishing TA production under the most favorable conditions for its biosynthesis (25 °C, 24 days of incubation, 100% RH).

There are no previous works about antagonistic yeasts that control *Alternaria* toxins production. However, several studies have demonstrated the ability of antagonistic yeasts to reduce *Aspergillus* species growth and ochratoxin A (OTA) production on wine grapes (Cubaiu et al., 2012; Dimakopoulou et al., 2008; Kapetanakou et al., 2012; Ponsone et al., 2011; Zhang et al., 2016).

The selected antagonistic epiphytic yeasts seem to be promising for the biological control of *Alternaria* on wine grapes. It would be necessary to determine the influence of different environmental factors on their effectiveness, the possible mechanisms involved in biocontrol, as well as their effects on the downstream process of vinification. Those future trends would allow us to choose the best antagonists to compose a biofungicide for wine grapes.

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