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Virginia M. Pesce [a,b*,1], María C. Nally [a,b,1], Gabriela P. Carrizo [a], Cecilia Rojo [b,c], Beatriz A. Pérez [d], María E. Toro [a], Lucía I. Castellanos de Figueroa [b,e,f], Fabio Vazquez [a]

[a] IBT, Instituto de Biotecnología–Facultad de Ingeniería–Universidad Nacional de San Juan, Av. Libertador San Martín 1109 oeste (5400), San Juan, Argentina

[b] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina

[c] Centro de Estudios Enológicos, Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), San Martín 3853 (5507), Luján de Cuyo, Mendoza, Argentina

[d] IMyZA, Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolás Repetto y de los Reseros s/n (1686) Hurlingham, Buenos Aires, Argentina.

[e] PROIMI, Planta Piloto de Procesos Industriales Microbiológicos, Av. Belgrano y Pasaje Caseros (4000) Tucumán, Argentina

[f] FBQyF-UNT, Facultad de Bioquímica, Química y Farmacia, Ayacucho 455, (4000) Tucumán, Argentina

*Corresponding author. Tel.:+54 264 4211700; fax: +54 264 4213672.

E-mail address: virgi_pesce@yahoo.com.ar (V.M. Pesce).

[1] Equal contribution

ABSTRACT

Anthracnose is a fungal disease caused by *Colletotrichum* species, which mainly affects olive fruits during ripening. The use of yeasts from related and unrelated microenvironments in the control of plant pathogens has become an alternative to the use of synthetic fungicides. The objectives of the present study were to evaluate *in vitro* biocontrol strategies against *C. gloeosporioides*, in order to select native antagonistic yeasts isolated from different microenvironments, and to verify the *in vivo* biocontrol activity on ripe olive fruits. The potential human pathogenicity of the antagonistic yeasts was also considered. A total of 241 wild yeasts from diverse olive cultures and vitivinicultural microenvironments were assayed *in vitro* and *in vivo* for their antagonistic effect against the pathogen *C. gloeosporioides*. Ninety-two yeasts with *in vitro* characteristics of competitiveness such as niche overlap, dominance against the pathogen, reduction of fungal growth and production of antifungal metabolites were examined on ripe olive fruits. Nine biocontrol yeasts, three from fermenting grape must and six from olive fruits, leaves and branches *in vivo* reduced the incidence and severity of *C. gloeosporioides*. According to the 42°C test, which assays for pseudohyphal formation and phospholipase production, these biocontrol yeasts did not present a health risk to humans. Microenvironments with selective stress factors can be relevant sources of microorganisms with biocontrol purposes.

Keywords: *Colletotrichum gloeosporioides*; olive fruits; antagonistic yeasts; biocontrol strategies

1- INTRODUCTION

The olive (*Olea europaea* L.) is a plant species belonging to the Oleaceae family worldwide recognized as an important producer of olive oil and table olives. During the 2013-2014 harvest, Argentina produced 140,000 and 30,000 t of table olives and olive oil, respectively (International Olive Council, IOC, 2015).

In Argentina, the provinces of Catamarca and La Rioja are the main olive producers, covering 49% of the total olive culture area. The provinces of Mendoza and San Juan follow immediately, covering 41% of the total olive culture area (Gómez del Campo et al., 2010).

Anthracnose is a fungal disease of olives caused by *Colletotrichum* species such as *C. acutatum* and *C. gloeosporioides* and mainly affects mature fruits (Cacciola et al., 2012). *Colletotrichum* genus includes a large number of fungal species that cause high yield losses in numerous crops (Landum et al., 2016; Olivieria et al., 2005).

Anthracnose occurs in many olive-growing countries in the world, and in Argentina it has been reported in the provinces of Catamarca, La Rioja, Córdoba, Mendoza and San Juan (del Toro et al., 2011a; Docampo et al., 2015). Under favorable environmental conditions, the disease can devastate entire production fields. Docampo et al. (2010) described a 70% harvest loss of the "Arauco" olive in the Mendoza province caused by anthracnose.

Typical symptoms of olive anthracnose are fruit rot and, consequently, mummification. Mature drupes are most affected, but green fruits of susceptible cultivars may also be affected when environment conditions are favorable for the pathogen (Cacciola et al., 2012; del Toro et al., 2011a). Decay develops from quiescent infections that take place in the field reducing the production of table olives and the quality of commercial olive oil. In fact, oil

produced from affected olives has off-flavor, a reddish color and shows chemical alterations (Mincione et al., 2004; Trapero and Blanco-López, 2008).

In Argentina, copper oxychloride and carbendazim are the active ingredients of approved fungicides for preventive control of anthracnose in olive orchards (SENASA resolution: 934/2010) (del Toro et al., 2011b). The copper fungicide is preferred by many growers because of its long persistence and wide activity spectrum against fungal and bacterial diseases (del Toro et al., 2011a). However, heavy metals may have a negative effect on human health and the environment because they are immobile in soil, so they tend to accumulate and persist in agricultural soils for a long time. Elevated concentrations of copper are toxic and may result in a range of effects including a reduction in soil microbial biomass and a decrease in biological activity, resulting in fertility loss (Aikpokpodion, 2010). Eventually, high copper concentrations affect physical and chemical soil processes (Van Zwieten et al., 2004). Wang et al. (2009) suggest that residual carbendazim in soil, after successive treatments, may have toxic effects on soil bacteria communities, and therefore negatively affect soil microbial enzyme activities.

The global trend appears to be shifting towards a reduction in chemical fungicides and hence there is a strong public and scientific desire to seek safer and eco-friendly alternatives to reduce losses due to decay (Janisiewicz and Korsten, 2002; Liu et al., 2013; Sharma et al., 2009). The use of microbial agents is one of the alternative approaches and it has been reported to be effective for control of a variety of rot pathogens in diverse crops (Droby et al., 2009). Several yeast species have proven to be efficient biological control agents (Droby et al., 2009; El-Tarabily and Sivasithamparam, 2006; Liu et al., 2013; Sharma et al., 2009; Wilson and Wisniewsky, 1989). Besides their antagonistic properties,

some yeasts play an important role during the lactic fermentation of olive fruits determining the safety, quality and flavor of the final product (Arroyo-López et al., 2012; Bonatsou et al., 2015). Therefore, yeasts are microorganisms with a great significance to the olive industry. However, biosafety of the antagonistic yeasts towards animals and humans is an important factor to be considered for commercialization of biocontrol agents (Liu et al., 2013; Nally et al., 2012, 2013).

Although the fruit surface microenvironment has proven to be the most desirable source for isolation of antagonistic yeasts against fungal fruit pathogens, antifungal microorganisms have also been isolated from unrelated microenvironments (Lopes et al., 2015; Parafati et al., 2015).

Jamalizadeh et al. (2011) explained the mechanism of action of biocontrol microorganisms as the strategy against a plant pathogen employed by the microorganism. Understanding of the mechanism of action is an important topic in the biofungicide production because it allows an increase in biocontrol performance (Spadaro and Droby, 2016) and helps develop criteria to select biocontrol agents (El-Tarabily and Sivasithamparam, 2006).

Competition for nutrients and space is considered the primary mode of action of yeasts against fungal pathogens (Spadaro and Droby, 2016). However, other mechanisms including cell wall-degrading enzymes, production of antifungal diffusible and volatile metabolites, induction of host resistance and mycoparasitism have also been reported to play a significant role in the biocontrol activity of yeasts (El-Tarabily and Sivasithamparam, 2006; Jamalizadeh et al., 2011; Parafati et al., 2015; Spadaro and Droby, 2016).

In a previous study, indigenous yeasts showed their ability to reduce the incidence of grey and sour rot on grapes (Nally et al., 2012, 2013). However, currently there is no information about the biocontrol ability of wild yeasts against phytopathogenic fungi in olive fruits. There exists only one study that has reported biocontrol characteristics of yeast-like fungi belonging to *Aureobasidium pullulans* against *C. acutatum* in olive fruits (Segura Pérez, 2003). Landum et al. (2016) showed that filamentous fungi isolated from olive leaves *in vitro* inhibited fungal growth of *C. acutatum*. To our knowledge, the contribution of yeasts to biocontrol *C. gloeosporioides* in olive fruits has not been examined yet.

The objectives of this work were: a- to select native antagonistic yeasts isolated from different microenvironments and *in vitro* assess their possible biocontrol strategies against *C. gloeosporioides*; b- to verify *in vivo* the biocontrol activity against *C. gloeosporioides* in ripe olive fruits; c- to determine the potential human pathogenicity of the antagonistic yeasts.

2- MATERIALS AND METHODS

2.1- Fungal pathogen and pathogenicity test in ripe olive fruits

C. gloeosporioides was obtained from the Plant Pathology Laboratory, IMYZA-INTA-Castelar (http://www.redmicrobios.com.ar/cientifico/altadecepas.php?action=4&id_cepa=40) and used throughout the study. The fungus was cultured on potato dextrose agar medium (PDA) (200 g/L potato infusion; 20 g/L dextrose, 20 g/L agar) for 10 days at 25°C. A conidial suspension was prepared by washing the fungus conidia with sterile distilled water

containing 0.05% (v/v) Tween 80, and the concentration of conidial suspension was quantified using a Neubauer Chamber.

In order to verify the pathogenicity of the fungal isolate, "Manzanilla de Sevilla" olive fruits, which were asymptomatic and homogeneous in maturity and size, were used for the experiment. Cacciola et al. (2012) reported that these fruits are susceptible to anthracnose. Fruits were surface-sterilized with 1% v/v commercial sodium hypochlorite, rinsed twice with sterile distilled water and ambient air-dried. Then, fruits were treated with 7% ethanol. A sterilized scalpel was used to make one wound per fruit (5 mm wide and 3 mm deep) on the equator, and aliquots of 20 µL of different concentrations of a conidial suspension (10^2 to 10^8 conidia/mL) were inoculated in each wound. Wounded fruits inoculated with sterile distilled water without the fungus were used as control. Three replicates of 10 fruits were used for each treatment. The wound-inoculated fruits and controls were stored at 25°C in darkness and at high humidity. After 5 days, the disease incidence was measured and data are presented as the percentage of fruits with disease symptoms for each treatment; the disease severity was recorded as the average size (cm) of the lesions. Experiments were repeated twice (Chanchaichaovivat et al., 2007).

The conidia concentration that showed the highest disease incidence and severity was used for *in vivo* biocontrol assays.

2.2- Native yeast isolates

Indigenous yeasts were isolated from different olive culture microenvironments in the San Juan province, Argentina: soil under the olive plant canopy, healthy olive fruits, olive leaves and branches, the brine of table olives and olive cake. One gram of sample of

healthy fruits, leaves and branches of olives was suspended in 50 mL of physiological solution and kept in a shaker for 10 min at laboratory temperature. Serial dilutions of 1 mL were streaked onto YEPD-agar medium (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Dextrose, 20 g/L agar; pH 4.5). Soil samples were taken from the top layer under the olive plant canopy (10 cm deep) and 1g was suspended in 50 mL of physiological solution. Samples of olive brine were taken aseptically and streaked onto YEPD-agar medium. The plates were incubated in darkness at 25°C during 4 days. Each colony with morphological different characteristics was re-streaked onto YEPD-agar medium to isolate single colonies. Yeast isolates were preserved in 30% glycerol at -80°C.

In addition to the indigenous olive culture yeasts, 59 autochthonous yeasts (31 *Saccharomyces* and 28 non- *Saccharomyces*) from vitivinicultural environments in the San Juan province were included in the tests, since they had shown antagonistic activity in previous studies against phytopathogenic fungi causing grey and sour rot in table grapes (Nally et al., 2012, 2013).

2.2.1- Yeast identification

Yeast isolates from olive cultures were first identified using conventional methods based on morphological and biochemical characteristics described by Barnett et al. (2007) and Kurtzman et al. (2011). Identification was corroborated by DNA sequencing of the D1/D2 domain of the large subunit of the 26S rRNA gene (Valente et al., 1999). PCR amplification was performed using the universal primer sets NL1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG -3') and NL4 (5'- GGT CCG TGT TTC AAG ACG G-3')

(Kurtzman and Robnett, 1991). DNA sequences were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

2.3- Characterization of native yeasts as biocontrol microorganisms

2.3.1- *In vitro* evaluation of antagonistic activity

Potential biocontrol yeasts were selected *in vitro* using four tests detailed below, according to modified protocols by Cavaglieri et al. (2004) and Nesci et al. (2005). All assays were carried out in triplicate and repeated twice.

2.3.1.1- Index of Dominance (ID)

The ID for each interacting species (native yeasts-plant pathogen fungi) was determined in dual cultures on Malt Extract Agar (MEA) medium containing 20 g/L malt extract, 1 g/L peptone, 20 g/L glucose, 20 g/L agar. Twenty microliters of each yeast suspension (10^8 cells/mL) were streaked in the center of the corresponding agar plate. 5-mm agar plugs of a *C. gloeosporioides* culture were inoculated at two equidistant points from the center and edge of the plate. Two controls were used: a- one Petri dish with a streak of each yeast; b- an agar plug with the fungus but without the yeast. Treatments and controls were incubated at 25°C in darkness for 5 days. Colonies were observed macroscopically and the type of interaction was expressed using numerical scores: 1/1 (mutual intermingling); 2/2 (mutual inhibition on contact); 3/3 (mutual inhibition at distance); 4/0 (dominance on contact); 5/0 (dominance at distance).

2.3.1.2- Niche Overlap Index (NOI)

Carbon sources used by the phytopathogenic fungus and yeasts were determined by assaying D-fructose, D-galactose, D-glucose, sucrose, inositol, mannitol, L-citric acid, L-malic acid, L-alanine, L-arginine and L-glycine as found in olive fruits (Galanakis, 2011; Manoukas et al., 1973; Marsilio et al., 2001; Nergiz and Ergönül, 2009).

Water Agar (WA) medium was used as basic medium containing 1.5% agar and a concentration of 10 mM of each C-compound. Ten microliters of starved yeasts (10^8 cells/mL) were poured on plates containing each carbon source. Agar plugs or disks of *C. gloeosporioides* were placed on plates containing different carbon sources. Yeasts with the pathogen without carbon source were used as controls. Plates were incubated at 25°C in darkness, and presence or absence of growth was recorded after 14 days. Niche overlap indices (NOIs) were calculated as the ratio between the number of carbon sources used in common (indigenous yeasts-*C. gloeosporioides*) and the total number of carbon sources utilized only by the pathogen. NOI values equal or above 0.9 (≥ 0.9) represent competition between species while scores below 0.9 (<0.9) represent occupation of separate niches (Wilson and Lindow, 1994).

2.3.1.3- Antifungal Activity (AA): effect of yeasts on growth rate of *C. gloeosporioides*

One hundred microliters (10^8 cells/mL) of each yeast were inoculated as a lawn on MEA medium. After solidification a disk of *C. gloeosporioides* was located in the center of the plate. Plates were incubated at 25°C in darkness for 14 days. Two radii measured at right angles to one another were averaged to find the mean radius for each fungal colony. Three replicates of each interaction assay were carried out. The growth rate (mm/day) was calculated with the following equation: $y = ae^{rt}$

with r being the fungal growth rate. Control plates only had the pathogen disk on solidified MEA.

2.3.1.4- Antibiosis (A)

Petri dishes containing MEA medium were inoculated with an agar plug of *C. gloeosporioides* in the center of the plate. Ten microliters of the different isolated yeasts were inoculated at a final concentration of 10^8 cells/mL in 5 mm diameter wells around the fungal inoculum. Control plates only contained the pathogen. Treatments and controls were incubated at 25°C in darkness for 5 days. The presence of an inhibition zone around the inoculation site was considered a positive result.

Native yeasts that showed at least two antagonistic activities in two *in vitro* tests were selected as biocontrol yeasts and further examined in *in vivo* experiments.

2.3.2- Biocontrol *in vivo* assay

Antagonistic isolates were assayed for their effect against *C. gloeosporioides* on harvested olive fruits. Ripe olive fruits were disinfected and wounded as mentioned above. Twenty microliters of each yeast suspension in sterile water (10^8 cells/mL) were applied to the wound, and the fruits were left for 2 h to allow the yeast cells to penetrate into the wound. After this period, 20 µL of *C. gloeosporioides* were placed in the same wound. This *Colletotrichum* concentration was chosen after the pathogenicity assay described above (100% infection and significant lesion size). Three controls were included: 1- wounded olive fruits with 20 µL of fungal conidial suspension and 20 µL of sterile distilled water; 2- wounded fruits with 40 µL of sterile distilled water; 3- wounded fruits with 20 µL of yeast

suspension and 20 µL of sterile water (Nally et al., 2012). The inoculated fruits were placed separately in sealed plastic bags and kept at 25°C and high humidity for 5 days. At the end of the storage period, disease was evaluated as disease incidence and severity. The assays included three replicates of 10 wounded olive fruits per treatment and each experiment was carried out twice.

A reduction in disease incidence of 50% or more was the threshold for selection of possible biocontrol yeasts (Bouzerda et al., 2003; Karabalut and Baykal, 2003).

2.3.3- *In vitro* virulence factors of biocontrol yeasts

To verify the presence or absence of phenotypic traits related to human pathogenicity in native yeasts, *in vitro* assays were carried out according to de Llanos et al. (2006) and Nally et al. (2012), with minimal modifications. Strains with antifungal activity in ripe olive fruits were assayed for specific enzyme activities, invasive growth and growth at elevated temperature to examine human pathogenicity traits. All tests were carried out in triplicate and repeated twice.

Candida albicans ATCC10231, a human pathogen, was used as positive control.

Two enzymatic activities, phospholipase and protease, were assayed because they are human pathogenicity determinants. Phospholipase activity plays a significant role in damaging cell membranes (Mayser et al., 1996) and protease activity facilitates colonization and invasion of host tissues via degradation of physiological substrates such as albumin, immunoglobulins and skin proteins (Silva et al, 2012). Yeast phospholipase production was assayed on Egg Yolk medium containing 70.62 g/L Sabouraud Dextrose Agar (Difco), 63.6 g/L NaCl, 0.6 g/L $CaCl_2$, 20 g/L agar and 10% (v/v) sterile egg yolk

(Sigma Aldrich). Ten microliters of each yeast suspension (10^8 cells/mL) were poured on the plates, which were then incubated at 30°C in darkness for 7 to 10 days.

Protease activity was determined by inoculation of each isolate on Petri dishes with agar medium containing 0.2 g/L $MgSO_4.7H_2O$, 2.5 g/L K_2HPO_4, 5 g/L NaCl, 0.2 g/L Yeast Extract, 20 g/L glucose, 2.5 g/L bovine serum albumin (BSA), 20 g/L agar, pH 5. The dishes were incubated at 37°C in darkness for 4 days. Phospholipase and protease activity were visualized as the area of precipitation around each yeast colony.

Human pathogenic yeasts are able to form pseudohyphae to adhere to host cells in order to facilitate the invasion of human tissue (de Llanos et al., 2006). Yeast isolates were cultured in Petri dishes containing YEPD-agar medium. Plates were first incubated at 30°C in darkness for 3 days, and then at 25°C for two additional days. After the incubation period, sterile distillated water was used to remove all the cells from the agar surface. Cells growing below the agar indicated surface adhesion. Pseudohyphal formation of antagonistic yeasts was also examined. Culture medium to assay pseudohyphal growth contained the following ingredients: 3.5 g/L ammonium sulfate; 1.5 g/L asparagine; 1 g/L glucose, 0.85 g/L potassium phosphate monobasic; 0.15 g/L potassium phosphate dibasic; 0.5 g/L magnesium sulfate; 0.1 g/L sodium chloride, 0.1 g/L calcium chloride; 20 g/L agar (Kurtzman et al., 2011). Plates were incubated at 25°C in darkness for 7 days.

Yeast growth at elevated temperature (42°C) is also an important characteristic of human pathogenic strains (de Llanos et al., 2006). Suspensions of isolated yeasts (10^8 cells/mL) were poured on YEPD agar medium and incubated at 42°C in darkness for 3 days. Plates inoculated with yeasts and incubated at 25°C were used as positive control. Yeast growth at 42°C was considered a positive result.

2.4- Statistical analysis

Matrix values (1 or 2) were assigned to absence or presence according to *in vitro* antifungal activity tests. For cluster analysis, statistical InfoStat software professional version 2011 (FCA-UNC, Córdoba, Argentina) was used. Different clusters represent diverse antifungal patterns of indigenous yeasts and they are identified with roman numbers.

Disease incidence and severity were analyzed by ANOVA, using statistical SPSS Inc., Chicago, IL, version 17.0 for Windows. The means were compared by Tukey's test at 5% significance (Nally et al., 2013).

3- RESULTS

3.1- Native yeast isolates

One hundred and forty two (142) yeast colonies were isolated from olive culture environments, mainly from the fruit surface (68). Twenty eight (28) were isolated from leave surface, 18 from olive branches and also 18 from table olive brine. The remaining 10 colonies were isolated from soil and olive cake (**Table 1**).

All yeasts could be classified into 10 genera (*Aureobasidium*, *Cryptococcus*, *Candida*, *Debaryomyces*, *Dekkera*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Trichosporon* and *Wickerhamomyces*), belonging to 28 taxonomically distinct groups at species level (**Table 1**). *A. pullulans* (50), *Cryptococcus albidus* (14) and *Debaryomyces hansenii* (12) are the species with the highest number of isolates from the olive environments (**Table 1**).

3.2- Pathogenicity of the fungal strain in mature olive fruits

Pathogenicity of *C. gloeosporioides* was assayed on ripe olive fruits. Disease incidence of the pathogen was between 10% ± 5.77 and 100% ± 0 (means ± standard deviation). Lesion diameters varied between 1.51 ± 0.03 and 1.86 ± 0.12 cm for the different concentrations assayed (10^2 to 10^8 conidia/mL). For fungal concentrations higher than 10^5 conidia/mL, no significant differences in incidence and lesion diameter ($p>0.05$) were observed. Therefore, 10^5 conidia/mL was the pathogen concentration used for *in vivo* assays.

3.3- *In vitro* antifungal activity of native yeasts evaluating different competitive strategies

The antagonistic activity of 201 native yeasts (142 from olive environments and 59 from vitivinicultural environments) was evaluated using four plate culture tests. Ninety two (92) of them showed at least two different strategies to biocontrol *C. gloeosporioides*; 45 were isolated from vitivinicultural environments (**Fig. 1**) and 47 from olive environments (**Fig. 2**).

Results of the *in vitro* tests varied according to the interacting species (native yeast-plant pathogen). The main interactions between antagonistic yeasts and *C. gloeosporioides* were as follows: 66 isolates demonstrated niche overlap (NOI ≥ 0.9), 63 yeasts evidenced dominance and fungal growth inhibition in dual culture (ID ≥ 3), 59 reduced the growth rate of the pathogen (AA ≥ 80%), and 34 isolates presented diffusible inhibitory metabolites on agar plates (A). Statistical analysis revealed 11 different antifungal patterns (**Fig. 1**), which were related to the different antifungal activities observed *in vitro*: AA (36 isolates), ID (27 isolates), NOI (26 isolates) and A (23 isolates).

Yeasts isolated from different olive environments exhibited 9 antagonistic patterns (**Fig. 2**): 40 isolates used NOI, 36 isolates ID, 23 isolates AA and 11 isolates A.

3.4- *In vivo* biocontrol tests (ripe olive fruits)

Several yeasts showed a decrease in fruit rot caused by *C. gloeosporioides* during biocontrol assays. Nine isolates (3 from vitivinicultural environments and 6 from olive environments) reduced the disease incidence in olive fruits with 50% to 90% (**Table 2**). Two isolates, *C. tropicalis* Bo13b from olive leaves and *W. anomalus* Bo156 from olive stems, showed the highest antifungal activity in ripe olive fruits, reducing the disease incidence with 90% ($p < 0.05$) (**Table 2**). Olive isolate *Cr. albidus* Bo86 also showed high biocontrol activity, reducing the anthracnose incidence in olive fruits with 76.7% (**Table 2**). All antagonistic yeasts, except for *W. anomalus* Bo107, showed significantly lower disease severity in mature olive fruits than the control treatment (fruits inoculated only with the pathogen) (**Table 2**). *W. anomalus* Bo156, a biocontrol yeast isolated from the olive phyllosphere, showed highest biocontrol activity, inhibiting the anthracnose incidence by 90% and disease severity by 70.54%

3.5- Human pathogenicity traits of biocontrol yeasts

All antagonistic yeasts isolated from olive environments showed at least one phenotypic characteristic associated with pathogenicity in humans (**Table 3**). The Table also shows vitivinicultural yeasts previously assayed (Nally et al., 2012; 2013). Three olive culture isolates, *C. tropicalis* Bo13b, *P. kudriavzevii* Bo108 and *W. anomalus* Bo107, were

considered the most virulent because they showed three human pathogenicity factors: phospholipase production, invasive growth and pseudomycelium formation (**Table 3**).

4- DISCUSSION

Anthracnose is considered the most devastating disease of olive fruits worldwide (Cacciola et al., 2012). Biocontrol through microorganisms has become a non-hazardous alternative to chemical control of fungal diseases in numerous crops (Droby et al., 2009), but there are no studies about antagonistic microorganisms against *Colletotrichum* species causing anthracnose in olive fruits. Microorganisms naturally present on the surface of fruits or vegetables are the most common source of antagonists against fungal fruit diseases (Janisiewicz and Korsten, 2002). However, some antagonists have been isolated from closely fruit related sources like the phyllosphere, roots and soil or even diverse unrelated sources such as sea water, Antarctic soil and fermentation environments (Cubaiu et al., 2012; Janisiewicz and Korsten, 2002; Liu et al., 2013; Nally et al., 2012, 2013; Raspor et al., 2010). The current study assayed antagonistic properties of native yeasts isolated from olive environments (soils, branches, leaves, olive cake, olive fruits) and vitivinicultural sources (fermenting must, table grapes).

In vitro screening provides preliminary information about the possible mechanisms of action used by each strain to control fungi (Capdeville et al., 2007; Nally et al., 2012). Therefore, appropriate *in vitro* assaying is important to define the strategy to select biocontrol agents against fungal plant pathogens (Zheng et al., 2011).

Most of the selection techniques of biocontrol agents are based on detection of antifungal diffusible metabolites (antibiosis) in dual culture, determining inhibition of vegetative fungal growth (Balouiri et al., 2016; Capdeville et al., 2007; Chanchaichaovivat et al., 2007; El-Tarabily and Sivasithamparam, 2006; Landum et al., 2016; Lima et al., 2012; Nally et al., 2012; Rosa-Magri et al., 2011). However, other researchers consider competition for nutrients and space the most important mode of action of antagonistic yeasts against fungal pathogens (Droby et al., 1989; Spadaro and Droby, 2016). Therefore, selection of biocontrol microorganisms in the present study was accomplished through four different tests: ID, A, AA and NOI. This allowed assessment of diverse strategies to inhibit growth of *C. gloeosporioides*, contributing to a better understanding of the probable mechanisms of action such as competition for limiting resources and antibiosis. To our knowledge, this is the first report that focuses on the screening of indigenous yeasts with different potential antagonistic strategies against a phytopathogenic fungus causing rot in olive fruits.

The results from the NOI, ID and AA assays suggest that the main mode of action employed by indigenous yeasts against *C. gloeosporioides* was competition. NOI values showed that most yeasts were able to compete for nutrients present in olive fruits. Antagonistic yeasts were also able to compete, reducing fungal growth as revealed by the ID and AA results. Our results are in agreement with Spadaro and Droby (2016) who suggested that yeasts can successfully compete *in vitro* with the pathogen for limiting nutrients, inhibiting its growth. In addition, they confirmed that yeasts usually have an advantage in competition for space because of their rapid growth.

Yeasts that showed different *in vitro* inhibition strategies were assessed for their efficacy to reduce the incidence and severity of anthracnose *in vivo*. Although Landum et al. (2016) conducted an *in vitro* screening of microorganisms against *C. acutatum*, causal agent of anthracnose in olive fruits, the authors only evaluated the production of volatile and non-volatile compounds by phyllosphere filamentous fungi and they did not verify their antifungal activity *in vivo*. Hence, the present study is the first that reports on the antagonistic activity of indigenous yeasts against *C. gloeosporioides* assayed in wounded ripe olive fruits.

In the present study nine wild yeasts were able to control anthracnose when mature olive fruits were artificially inoculated. *C. tropicalis* Bo13b, *Cr. albidus* Bo86, *P. kudriavzevii* Bo91, *P. kudriavzevii* Bo108, *W. anomalus* Bo107 and *W. anomalus* Bo156 were isolated from olive environments, mainly from the fruit surface. Our results agree with other findings that showed that *P. kudriavzevii* (*Issatchenkia orientalis*) and *W. anomalus* were antagonists of anthracnose caused by *Colletotrichum* species in other crops (Campos-Martínez et al., 2016; Chanchaivovat et al., 2007; Chi et al., 2015; Lima et al. 2013). Our study is the first that reports the use of *P. kudriavzevii* and *W. anomalus* as biocontrol agents against *C. gloeosporioides* in olive fruits. This is also the first time that *Cr. albidus* showed biocontrol activity against *C. gloeosporioides* species. Nevertheless, one product based on *Cr. albidus* (Yield Plus, Anchor Yeast, South Africa) has already been registered for commercial use against postharvest fungal rot of citrus fruit and pome fruits (Droby et al., 2003). This product shows the potential of this species as biocontrol agent of fungal diseases.

The highest reduction in anthracnose incidence on ripe olive fruits was produced by *C. tropicalis* Bo13b and *W. anomalus* Bo156, both isolated from aerial parts (leaves and branches). This demonstrates that other segments of the plant such as leaves and branches could also be sources for biocontrol microorganisms of fungal pathogens of fruits. An explanation could be that the phyllosphere is a continuously fluctuating physical environment and may thus be considered an extreme environment (Fonceca and Inácio, 2006). In the phyllosphere microorganisms grow with limited available resources. Survival also depends on their ability to cope with diverse environmental stress conditions like fluctuating water availability, heat, osmotic stress, and exposure to solar UV radiation (Jacobs and Sundim, 2001; Mercier and Lindow, 2000). The phyllosphere has previously been reported to be an appropriate source for antagonists, as it may share part of the resident microbiota on fruits but it can also contain other microorganisms (Janisiewicz and Korsten, 2002). The current study documented that all yeast species isolated from the phyllosphere were also found in olive fruits, with the exception of two species, *C. tropicalis* and *C. parapsilosis*. In general, most research is focused on microorganisms isolated from the fruit surface to control fungal diseases on fruits, whereas only few studies have focused on the potential of microorganisms as control agents from closely related microenvironments. Rosa-Magri et al. (2011) showed that *Torulaspora globosa* isolated from leaves was able to inhibit *Colletotrichum sublineolum* and *Colletotrichum graminicola*, causal agents of anthracnose in sorghum and maize. Campos-Martínez et al. (2016) observed that *W. anomalus,* isolated from phyllosphere, inhibited *C. gloeosporioides* and *C. acutatum* on avocado fruits.

In the present study, three vitivinicultural yeasts isolated from fermenting must showed antagonistic properties against *C. gloeosporioides* in olive fruits: *P. membranifaciens* BPm6, *S. chevalieri* BSch25 and *T. delbrueckii* BTd126. According to *in vitro* assays, these enological yeasts used several competitive strategies to reduce fungal growth. The results suggest that competition for limiting resources and antibiosis could be involved in control of anthracnose in olive fruits.

Wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeast taxa. Microorganisms involved in winemaking are also exposed to challenging conditions like competition for substrates, and osmotic and ethanol stress (Querol et al., 2003). Growth of antagonists in stressful environments can deeply affect their survival and effectiveness (Rangel, 2011; Sui et al., 2015). Consequently, enological yeasts may have a competitive advantage (Querol et al., 2003). Furthermore, several researchers have informed that certain enological yeasts produce mycocins (killer toxins) that inhibit other microorganisms, which enhances their competitive strategy (Barata et al., 2012; Villalba et al., 2016). Other researchers have reported that these killer toxins can inhibit growth of plant pathogenic fungi (Lima et al., 2012; Santos and Marquina, 2004; Spadaro and Droby, 2016; Walker et al., 1995). In the current study, *P. membranifaciens* and *S. chevalieri* showed *in vitro* antibiosis as a strategy against *C. gloeosporioides*. This result indicates that inhibition of fungal growth by these enological yeasts could be mediated by killer toxins. To our knowledge, this is the first study that reports on enological yeasts as biocontrol agents against *C. gloeosporioides.*

Our results also suggest that stressful environments such as fermenting grape must and the plant phyllosphere are possible sources of biocontrol agents of fungal plant pathogens, that to date have been very little studied.

Many criteria have to be taken into account to select a biocontrol microorganism (Liu et al., 2013; Wilson and Wisniewski, 1989). One of the most important ones is their potential toxicity to immunosuppressed humans and animals (Liu et al., 2013; Wilson and Wisniewski, 1989). *C. albicans* is the main pathogenic yeast, but other *Candida* species like *C. tropicalis* and other yeast genera have emerged lately as opportunistic pathogens (Miceli et al., 2011). The ability to grow at body temperature is one of the most important characteristics that should be taken into account regarding biosafety of biocontrol microorganisms (Liu et al., 2013; Vero et al., 2013). Although this ability seems to be associated with virulence, it is not the sole determining factor (de Llanos et al., 2006). Other fungal properties are also frequently associated with human pathogenesis: growth at 42°C, pseudohyphal growth and phospholipase activity (de Llanos et al., 2006). These pathogenic characteristics are very seldom assayed and evaluated in the search for biocontrol yeasts. In the current study, olive culture yeasts presented at least one phenotypic trait associated with pathogenicity in humans. *C. tropicalis* Bo13b, *P. kudriavzevii* Bo108 and *W. anomalus* Bo107 showed three pathogenic factors: phospholipase production, invasive growth and pseudomycelium formation, all determining potential pathogenicity to humans. Despite the reported use of *C. tropicalis* in many biotechnology applications (Amprayn et al., 2012; Angumeenal and Venkappayya, 2013; Martínez-García et al., 2009), this species is considered an emerging human pathogen (Miceli et al., 2011; Silva et al., 2012). According to de Llanos et al. (2006) the ability to

grow at 42°C, pseudohyphal growth, and high levels of phospholipase activity are fungal properties associated with pathogenesis, and these tests should be carried out as preventive screening to detect potential virulent yeasts. None of the native yeasts assayed in the present study showed these three virulent characteristics at the same time. Nevertheless, further research is required to ensure safety of these antagonistic yeasts to animals and humans, especially the species reported as opportunistic human pathogens.

In conclusion, this research is the first step towards environmentally friendly alternatives for the chemical control of anthracnose in olive orchards. Native yeasts isolated from different olive and vitivinicultural environments showed potential antifungal activity against *C. gloeosporioides,* causal agent of anthracnose on ripe olive fruits. Most of the yeasts with antagonistic properties against *C. gloeosporioides* in ripe olive fruits were isolated from the fruit surface and other closely related microenvironments. In addition, unrelated fruits environments with selective stress factors can be relevant sources of microorganisms with biocontrol purposes. Further research is necessary to elucidate the mechanisms of action of the antagonistic yeasts to control *C. gloeosporioides* in olive fruits and to assure safety of the biocontrol yeasts.

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Table 1: Species and number of isolated yeasts from different olive environments: olive fruits, branches and leaves, soil, olive cake and table olive brine.

Table 2: Effect of antagonistic yeasts on disease incidence (%) and severity (cm) of *C. gloeosporioides* on mature olive fruits after incubation at 25°C in darkness for 5 days. Different letters within the same column denote a significant difference ($p<0.05$) according to Tukey's test.

Table 3: Virulent characteristics observed in biocontrol yeasts.

Fig. 1. Vitivinicultural yeast clades according to antifungal patterns during *in vitro* assays (ID, A, AA and NOI). Different roman numbers correspond to different antifungal patterns (Clusters).

References:

I: A, NOI; **II**: ID, A; **III**: ID, A, NOI; **IV**: ID, NOI; **V**: ID, AA, NOI; **VI**: ID, A, AA, NOI; **VII**: A, AA, NOI; **VIII**: AA, NOI; **IX**: ID, A, AA; **X**: ID, AA; **XI**: A, AA.

Fig. 2. Olive culture yeast clades according to antifungal patterns during *in vitro* assays (ID, A, AA and NOI). Different roman numbers correspond to different antifungal patterns.

References

I: ID, A; **II**: ID, AA; **III**: A, NOI; **IV**: A, AA, NOI; **V**: AA; NOI; **VI**: ID, AA, NOI; **VII**: ID, NOI; **VIII**: ID, A, AA, NOI; **IX**: ID, A, NOI.

Species	Fruit	Branches	Leaves	Soil	Olive cake	Brine	TOTAL
Aureobasidium pullulans	26	7	14	2	-	1	**50**
Candida ishiwadae	1	-	-	-	-	1	**2**
Candida magnoliae	1	-	1	-	-	-	**2**
Candida membranifaciens	1	-	-	1	-	1	**3**
Candida parapsilosis	-	-	1	-	-	-	**1**
Candida saitoana	1	-	-	-	-	-	**1**
Candida sake	3	-	-	1	-	-	**4**
***Candida* sp.**	1	-	-	-	-	2	**3**
Candida tropicalis	-	-	1	-	-	-	**1**
Candida versatilis	1	-	-	-	-	-	**1**
Cryptococcus albidus	7	2	-	1	-	4	**14**
Cryptococcus humícola	5	-	-	-	-	-	**5**
Cryptococcus laurentii	1	-	-	-	-	2	**3**
Debaryomyces hansenii	5	1	4	-	-	2	**12**
Debaryomyces nepalensis	1	-	-	-	-	-	**1**
Debaryomyces occidentalis	1	-	1	-	-	1	**3**
Dekkera anomala	-	1	-	-	-	-	**1**
Dekkera bruxellensis	1	-	-	-	-	-	**1**
Kluyveromyces lactis	-	2	-	-	-	-	**2**
Kluyveromyces marxianus	1	-	-	-	-	-	**1**
Pichia angusta	2	-	1	-	-	1	**4**
Pichia guilliermondii	1	1	-	-	-	2	**4**
Pichia holstii	-	-	-	-	-	1	**1**
Pichia kudriavzevii	3	-	-	-	-	-	**3**
Rhodotorula glutinis	2	1	3	-	1	-	**7**
Rhodotorula mucilaginosa	1	-	2	-	3	-	**6**
Trichosporon pullulans	1	-	-	-	-	-	**1**
Wickerhamomyces anomalus	1	3	-	-	1	-	**5**
TOTAL	**68**	**18**	**28**	**5**	**5**	**18**	**142**

References:

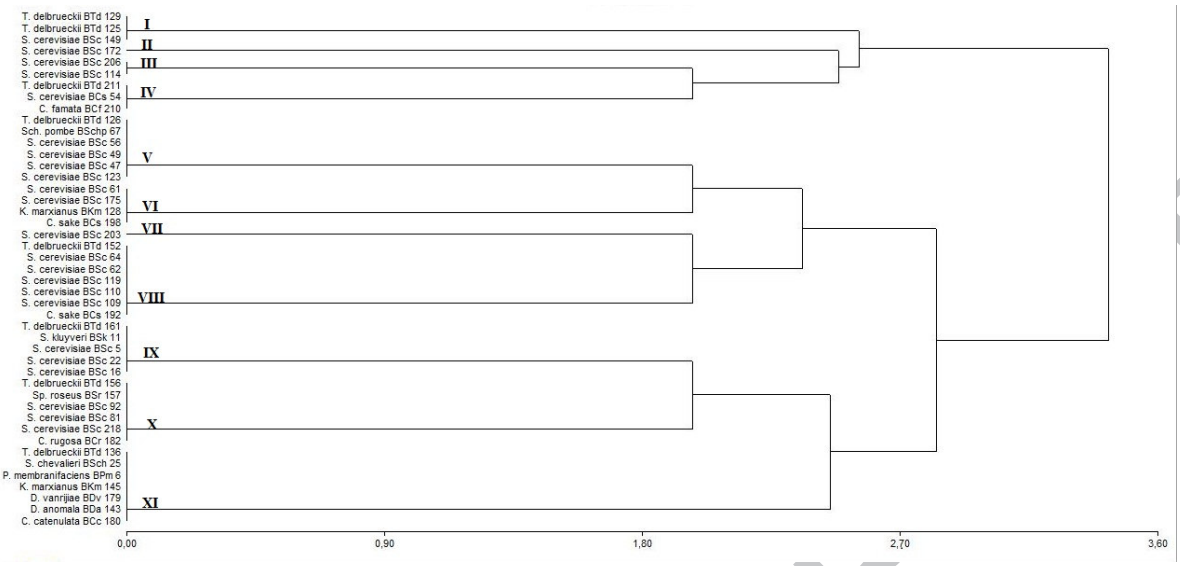
- = Absence of isolates

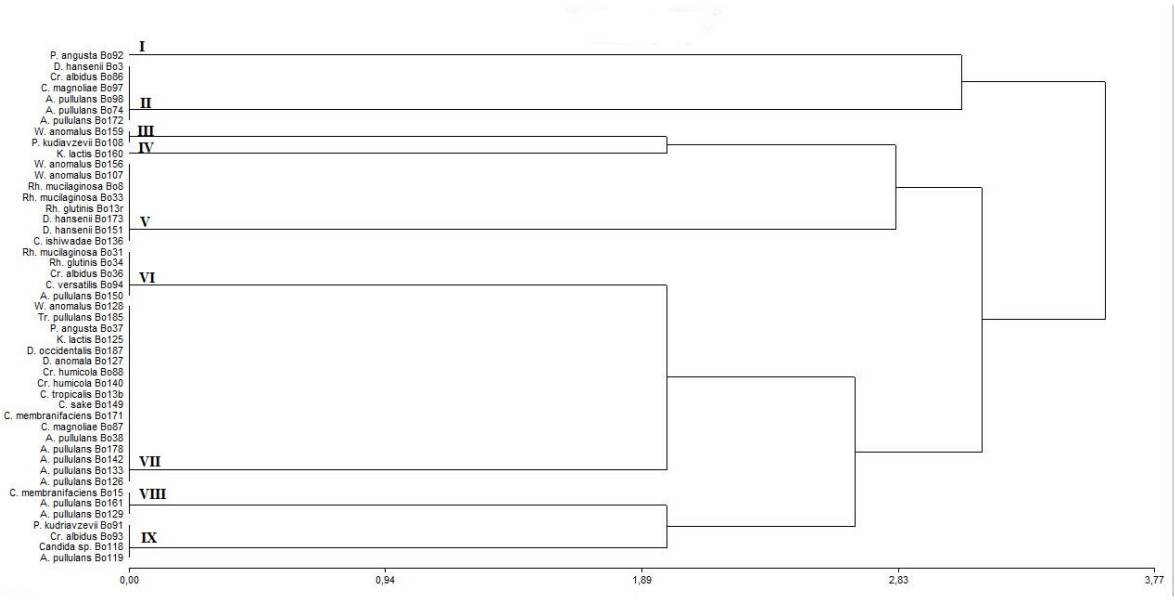
Antagonistic yeasts	Yeast origin	Disease Incidence (%)	Disease Severity (cm)
P. membranifaciens BPm6*	FGM	50 ± 5.7 (b)	1.22 ± 0.04 (bc)
S. chevalieri BSch25*	FGM	50 ± 11.5(b)	1.25 ± 0.05 (bc)
T. delbrueckii BTd126*	FGM	36.6 ± 33 (ab)	1.22 ± 0.04 (bc)
C. tropicalis Bo13b**	OL	10 ± 5.7 (a)	0.98 ± 0.04 (b)
Cr. albidus Bo86**	OF	23.3 ± 3.3 (ab)	0.97 ± 0.05 (b)
P. kudriavzevii Bo91**	OF	46.6 ± 6.6 (ab)	1.45 ± 0.05 (cd)
P. kudriavzevii Bo108**	OF	30 ± 5.7 (b)	1.3 ± 0.07 (cd)
W. anomalus Bo107**	OF	40 ± 11.5 (ab)	1.73 ± 0.05 (de)
W. anomalus Bo156**	OS	10 ± 0 (a)	0.55 ± 0.06 (a)
CONTROL (*C. gloeosporioides*)	-	100 ± 0 (c)	1.86 ± 0.07 (e)

References:
*Indigenous vitivinicultural yeasts
**Indigenous olive culture yeasts
FGM Fermenting grape must
OF Olive fruits
OL Olive leaves
OS Olive stems

Antagonistic yeast species	Phospholipase production	Protease production	Invasive growth	Pseudomycelium formation	Growth at 42°C	Total
C. tropicalis Bo13b [a]	+	-	+	+	-	3
Cr. albidus Bo86[a]	+	-	-	+	-	2
P. kudriavzevii Bo91[a]	-	-	-	+	-	1
P. kudriavzevii Bo108[a]	+	-	+	+	-	3
W. anomalus Bo107[a]	+	-	+	+	-	3
W. anomalus Bo156[a]	+	-	-	-	-	1
P. membranifaciens BPm6[b*]	-	-	-	-	-	0
S. chevalieri BSch25[b*]	+	-	-	-	-	1
T. delbrueckii BTd126[b*]	-	-	-	-	-	0
C. albicans ATCC10231	+	+	+	+	+	5

References:
+ positive activity; - negative activity
[a] olive culture yeasts
[b] vitivinicultural yeasts
[*] human pathogenicity test results previously reported (Nally et al., 2012; 2013)





Highlights

Biocontrol yeasts showed different strategies to reduce *Colletotrichum in vitro*

Environments with selective pressure were relevant sources of antagonistic yeasts

Native yeasts were effective for controlling *C. gloeosporioides* in ripe olive fruits