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Antifungal modes of action of *Saccharomyces* and other biocontrol yeasts against fungi isolated from sour and grey rots

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Abstract

The aim of this study was to determine the putative modes of action of 59 viticultural yeasts (31 *Saccharomyces* and 28 non- *Saccharomyces*) that inhibited fungi isolated from sour and grey rot in grapes. Inhibition of fungal mycelial growth by metabolites, enzyme activities (laminarinases, chitinases), antifungal volatiles, competition for nutrients (siderophores, Niche Overlap Index (NOI)), inhibition of fungal spore germination and decreased germinal tube length and induction of resistance were assayed. Biofungicide yeasts were classified into "antifungal patterns", according to their mechanisms of action. Thirty isolates presented at least two of the mechanisms assayed. We propose that inhibition of fungal mycelial growth by metabolites, laminarinases, competition for nutrients, inhibition of fungal spore germination and decreased germinal tube length, and antifungal volatiles by *Saccharomyces* and non- *Saccharomyces* viticultural yeasts is used as putative biocontrol mechanisms against phytopathogenic fungi. Twenty-four different antifungal patterns were identified. Siderophore production (N) and a combination of siderophore production and NOI> 0.92 (M) were the most frequent antifungal patterns observed in the biofungicide yeasts assayed. Elucidation of these mechanisms could be useful for optimization of an inoculum formulation, resulting in a more consistent control of grey and sour rot with *Saccharomyces* and non- *Saccharomyces* biocontrol yeasts.

Keywords: grape, *Saccharomyces* biofungicides, possible action mechanisms, antifungal patterns.

1-Introduction

Most fruits are highly perishable products, especially during the postharvest period, and a major loss is caused by fungal pathogens (Spadaro and Gullino, 2004). *Botrytis cinerea* Pers. Fr., a ubiquitous fungal pathogen, causes "grey rot" in a large number of economically important agricultural and horticultural crops (Keller et al., 2003). It is the most common postharvest pathogen of grapes in most regions of the world, resulting in severe postharvest losses (Nally et al., 2012; Qin et al., 2010). "Sour rot" is an emerging grape disease affecting late ripening cultivars under postharvest conditions (Hashim-Buckey et al., 2008; Puelles Tamsec and Sepulveda Ramirez, 2012). This disease is associated with a wide variety of microorganisms including yeasts, bacteria and filamentous fungi (Barata et al., 2011; Nally et al., 2013).

Chemicals are the primary method to control grey rot, but treatments with these products are rapidly becoming inefficient (Calvo- Garrido et al., 2013a; Couderchet, 2003). Despite the increasing incidence of grape sour rot, there is a lack of chemical control strategies (Calvo- Garrido et al., 2013b). The use of biocontrol yeasts to manage decay of fruits has been studied in order to reduce or replace the use of synthetic fungicides (Droby et al., 2009; Wilson and Wisniewski, 1989; Liu et al., 2013). Mechanisms that have been reported to play a significant role in the biocontrol activity of non-*Saccharomyces* yeasts against fungi include: competition for nutrients and space (Bencheqroun et al., 2007; Droby et al., 1989; Liu et al., 2013), production of laminarinases and chitinases (Fan et al., 2002; Grevese et al., 2003; Masih and Paul, 2002), induction of host resistance (Droby et al., 2002; El-Ghauth et al., 2003), reduction in spore germination and decreased germ tube length (Zheng et al., 2005), and inhibition of fungal mycelial growth by diffusible and volatile metabolites (Huang et al.,

2011; Lutz et al., 2013). However, there are few reports about antifungal mechanisms of non-*Saccharomyces* against fungi isolated from viticultural environments (Castoria et al., 2001; Rabosto et al., 2006) and there are no reports at all regarding the mechanisms of action of *Saccharomyces* biofungicides against fungi isolated from grapes. Elucidation of these mechanisms could be useful for optimization of a biocontrol inoculum formula with *Saccharomyces* and non- *Saccharomyces* biocontrol yeasts, which would most likely result in a more consistent control of grey and sour rots. In order to reduce this information gap, the aim of the present study was to determine antifungal patterns based on possible mechanisms of 31 *Saccharomyces* and 28 non- *Saccharomyces* strains that previously inhibited fungi isolated from grey and sour rot grapes (Nally et al., 2012; 2013).

2- Materials and methods

2.1- Biocontrol yeasts

Fifty-nine biocontrol yeasts belonging to 10 genera and 16 species, previously isolated at our laboratory (Nally et al., 2012; 2013), were assayed for antifungal patterns. Forty-three strains showed antagonistic properties against fungi isolated from sour rot (Nally et al., 2013) and 16 yeasts reduced grey rot incidence (Nally et al., 2012) (**Tables 1 and 2**).

A loopful of pure isolated yeast was transferred to a 250 mL Erlenmeyer flask containing 100 mL of YEPD (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Dextrose, pH 4.5). Erlenmeyer flasks were agitated on a rotary shaker for 12 h. Yeast cells were pelleted by centrifugation, re-suspended in sterile distilled water and then centrifuged again. The resulting pellets were re-suspended in sterile distilled

water and the yeast concentration was adjusted to 10^6 cells/mL using a Neubauer Chamber (El-Ghaouth et al., 1998; Nally et al., 2012; 2013).

2.2- Fungi

Nine phytopathogenic fungi were assayed: *Aspergillus caelatus, Aspergillus versicolor, Aspergillus terreus, Aspergillus carbonarius, Rhizopus stolonifer, Penicillium commune, Ulocladium sp.* and *Fusarium oxysporum* were isolated from sour rot grapes (Nally et al., 2013), and *Botrytis cinerea* was isolated from grey rot grapes (Nally et al., 2012). All fungi were grown on Czapeck-Agar medium (30 g/L $NaNO_3$; 5 g/L KCl; 5 g/L $MgSO_4$ 7 H_2O; 0.1 g/L $FeSO_4$ 7 H_2O; 30 g/L Sucrose, 20 g/L Agar, pH 5.5) and incubated at 25 °C for 7 d. The spore concentration was adjusted to 10^4 spores/mL (Neubauer Chamber) (Nally et al., 2012; 2013).

2.3- Mechanisms of action of biofungicide yeasts

According to previous results, interacting pairs of biofungicide yeasts and controlled fungi (Nally el al., 2012; 2013) were selected in order to determine the possible antagonistic mechanisms (**Tables 1 and 2**).

2.3.1- Effects of metabolites from biofungicide yeasts on fungal mycelial growth inhibition

Inhibition of fungal mycelial growth was assayed according to the method by Castoria et al. (1997) with modifications. Twenty microliters of each biocontrol yeast (10^6 cells/mL) were streaked onto Petri dishes with YEPD- MB- Phosphate Citrate Buffer- Agar (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Dextrose- 0.01% Methylene Blue- 0.1 M Phosphate Citrate Buffer- 20 g/L Agar), pH 4. This medium

was previously inoculated with 100 µL of fungal spores (10^4 spores/mL) as a lawn. Plates were incubated at 25 °C for 5 d. If a particular yeast streak was surrounded by a clear halo, it was assumed to produce antifungal metabolites *in vitro*, and the halo diameter was measured (Santos et al., 2004). The assay was performed in triplicate and the experiment was repeated twice.

2.3.2- Chitinase and Laminarinase activities

Chitinolytic activity was determined by measuring the release of N-acetylglucosamine using the DNS method reported by Molano et al. (1977) with modifications. Yeasts were inoculated (100 µL, 10^6 cells/mL) in 125 mL Erlenmeyer flasks containing 25 mL of YNB (6.7 g/L), 0.1 M Sodium Acetate Buffer, pH 5.5 and colloidal chitin (10 g/L). Flasks were agitated on a rotary shaker (250 rpm) and incubated at 25 °C for 5 d in the dark. The culture was then centrifuged (10,000 rpm, 10 min) and the pellet was dried at 70 °C until constant weight. The cell-free culture filtrate (100 µL) was mixed with 900 µL of 0.1 M Sodium Acetate Buffer, pH 5.5, and supplemented with colloidal chitin (10 g/L). Colloidal chitin was prepared from shrimp shell chitin according to the methods provided by Roberts and Selitrennikoff (1988) and Zhang et al. (2011). Enzyme-substrate mixtures were incubated in a water bath at 37 °C for 7 h under shaking. All samples were supplemented with 1,500 µL of 3,5-dinitrosalicylic acid (DNS) and immersed in water at 100 °C for 10 min. When cooled down, samples were centrifuged at 10,000 rpm for 10 min. N-acetylglucosamine was quantified spectrophotometrically at 540 nm using an N-acetylglucosamine standard curve. One unit of chitinase (U) was defined as the amount of N-acetylglucosamine (µmoles) produced per g of yeast (dry weight) per min under the given assay conditions. Each treatment was carried out in triplicate and the experiment was repeated twice.

Laminarinase activity was determined by measuring glucose release using the DNS method by Zhang et al. (2011) with modifications. Biocontrol yeasts were inoculated (100 µL, 10^6 cells/mL) in 250 mL Erlenmeyer flasks with 50 mL of 0.1 M Sodium Acetate Buffer, pH 5.5, 6.7 g/L YNB and 2 g/L laminarin as substrate inducer. Flasks were agitated (250 rpm) at 25 °C for 7 d in the dark. Then, cultures were centrifuged (10,000 rpm, 10 min) and the cell-free culture filtrate was used to determine laminarinase activity. One hundred µL of sample and 900 µL of 0.1 M Sodium Acetate Buffer, pH 5.5 with 10 g/L laminarin were mixed. The enzyme-substrate mixtures were incubated at 45 °C for 30 min in a water bath. All samples were supplemented with 1,500 µL of DNS and incubated in a water bath at 100 °C for 10 min. When cooled down, samples were centrifuged at 10,000 rpm for 10 min, and absorbance was measured spectrophotometrically at 540 nm using a glucose standard curve. One unit of β-1,3 glucanase (laminarinase) (U) was defined as the amount of reducing sugar (glucose) (µmoles) released per g of yeast (dry weight) per min under the given assay conditions. The experiment was repeated 3 times to confirm reproducibility.

2.3.3- Antifungal volatiles

Production of antifungal volatiles was assayed as described by Huang et al. (2011) with some modifications. The ability of yeasts to produce antifungal volatiles was assayed in a sealed system, but the phytopathogenic fungus and its respective biocontrol yeast were not in physical contact. Plates containing 20 mL of Czapeck-Agar were centrally inoculated with an agar plug containing each fungus. At the same time, another plate containing 20 mL of YEPD- Agar was superficially inoculated with 100 µL of a suspension of 10^6 cells/mL of the biocontrol yeast. The covers of both inoculated plates were

removed, and the sets of double dishes were sealed using double layers of Parafilm® to assure closed chambers. Control sets of the closed double dish chambers were also prepared. The closed plates were incubated at 25 °C for 5 d. At the end of the assay, the fungal growth diameter was measured and the results were expressed as the percentage of fungal growth compared with the fungal control. Each treatment was carried out in triplicate and the experiment was repeated twice.

2.3.4- Competition for substrates

2.3.4.1- Niche Overlap Index (NOI)

NOIs were evaluated according to the method by La Penna et al. (2004) with modifications. These values show coexistence/niche exclusion between biofungicide yeasts and grape fungi (Cavaglieri et al., 2004). Fungal mycelium discs (9 mm diameter) and yeast aliquots (20 µL, 10^6 cells/mL) were inoculated on separate plates. Each plate contained one carbon source (10 mM), YNB (Yeast Nitrogen Base) with 20g/L Agar, pH 5.5. The carbon sources assayed are present in grapes and represent the size of the niche (Hernandez Orte et al., 2003; Lee et al., 2009): proline, asparagine, rhamnose, alanine, melibiose, glutamic acid, tyrosine, raffinose, arginine, lysine, fructose, methionine, glycine, malic acid, tartaric acid and glucose. Plates were incubated at 25 °C for 14 d in the dark. At the end of the experiment NOI values were evaluated as follows: the number of carbon sources used by both microorganisms (yeast and fungus) divided by the total number of sources used by the fungus. NOI values > 0.90 represent occupation of the same niche (competitive exclusion) and scores < 0.90 represent occupation of separate niches (coexistence). Each treatment was carried out in triplicate and the assay was repeated twice.

2.3.4.2- Siderophores

Aliquots of 20 µL of yeast suspension (10^6 cells/mL) were inoculated on CAS-HDTMA-YNB- glucose-Agar medium. This medium contained the following constituents: 60.5 mg/L CAS (Chrome Azurol S), 72.9 mg/L HDTMA (Hexadecyltrimethylammonium Bromide), 30.24 g/L PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid)), 1 mM $FeCl_3$ 6 H_2O in 10 mM HCl, 6.7 g/L YNB, 20 g/L glucose and 20 g/L Agar, at pH 5. Plates were incubated at 25 °C in the dark for 5 d. Presence of siderophores will change the color of this medium from yellow to orange (Schwyn and Neilands, 1987; Vero et al., 2012). Three replicates per treatment were performed and the experiment was repeated twice.

2.3.5- Effects of yeasts on fungal spore germination and on germinal tube length (using low nutrient medium)

Inhibition of spore germination was assayed according to the method reported by Dal Bello et al. (2008) with modifications. Twenty-five µL of yeast suspension (10^6 cells/mL), 200 µL of 0.5 °Bx sterile grape must (low nutrient concentration) and 25 µL of fungal suspension (10^4 spores/mL) were inoculated on sterile excavated slides. The slides were placed in a sterile Petri dish with filter paper, previously moistened with sterile distilled water (80% RH), at 25 °C during 12 h in the dark. After this period, spore germination was observed under a light microscopy (Wild, Switzerland). The percentage of spore germination was estimated from the observation of 100 spores on each excavated slide. The efficacy of yeast-mediated inhibition of postharvest fungal spore germination was determined by comparing the number of germinated spores in the yeast-supplemented medium, with that obtained in the control assay (yeast solution replaced by water). Spores were considered germinated when the

germ tube length equaled or surpassed the length of the spore. Each treatment was performed in triplicate and the experiment was repeated twice.

2.3.6- Effects of yeasts to induce grape resistance against fungi

Homogeneous Redglobe berries were selected according to size (3 cm diameter), shape, color, weight and absence of injuries (Martínez- Romero et al., 2007). Before each assay, fruits were washed with sodium hypochlorite solution (1% active chlorine), rinsed with distilled water and left to dry at room temperature (Nally et al., 2013).

The biocontrol yeast (20 µL, 10^6 cells/mL) and each fungus (20 µL, 10^4 spores/mL) were individually inoculated in two grape wounds made on one berry (1 cm separation) (Droby et al., 2002). The berries were incubated in plastic bags at 25 °C for 5 d in the dark at 80 % RH. At the end of the assay, the severity of the fungal disease was measured with a caliber. The disease severity (%) was calculated as follows:

$$\% \text{ of Severity} = \frac{\text{Average lesion diameter in grape wound inoculated with fungus and yeast}}{\text{Average lesion diameter in negative control (fungus)}} \times 100$$

Three replicates per treatment were performed and each replicate consisted of 18 grapes. The experiment was repeated twice.

2.4- Antifungal formulae and antifungal patterns

Putative antifungal mechanisms are expressed with the following numbers: **1**: inhibition of fungal mycelial growth by metabolites; **2**: chitinase production; **3**: laminarinase production; **4:** antifungal

volatile production; **5**: inhibition of fungal spore germination; **6:** reduction in germinal tube length; **7:** Niche Overlap Index (NOI) > 0.92; **8:** siderophore production and **9:** induced resistance.

Antifungal formulae (Arabic numbers) were subsequently clustered in antifungal patterns (capital letters).

A matrix value of one or zero was assigned to express the presence or absence of antifungal mechanisms, respectively. Simple Matching (SM) coefficients were used to compute similarity between pairs (Lopes et al., 2006; Sokal and Michener, 1958).

2.5- Statistical analysis

Homogeneity of variance was determined using Levene's test. Prior to variance analysis, percentages were arcsine-square-root transformed. Data were submitted to one-way univariate analysis of variance (ANOVA, *SPSS* release 17.0 for Windows; SPSS Inc., Chicago, IL). The threshold for statistical significance was set at $p \leq 0.05$. In cases with statistical significance, Tukey's test was applied to separate means (Nally et al., 2013).

3- Results and Discussion

3.1- Putative antifungal modes of action:

3.1.1- In vitro inhibition of fungal mycelial growth by yeasts

Inhibition of fungal mycelial growth by yeasts on plate media suggests synthesis and secretion of suppressive substances into the medium in the presence of the fungi (Korres et al., 2011). Previous

studies have indicated that mycelial inhibition on plates could be mediated by killer toxins (Bleve et al., 2006; Santos and Marquina, 2004; Santos et al., 2004; Walker et al., 1995) as well as non-proteinaceous inhibitory molecules (Golubev, 2006; Kulakovskaya et al., 2005). In the present work, 4 *S. cerevisiae* isolates inhibited *B. cinerea* on plates **(Table 1)**. Mycelial growth of *A. terreus* was inhibited by two isolates (*P. membranifaciens* BPm6 and *S. cerevisiae* BSc109), and *A. carbonarius* by *C. sake* BCs198 **(Tables 1 and 2)**. To the best of our knowledge, this is the first report on inhibitory activity of these non- *Saccharomyces* genera against the two fungi on plates. There are only few reports about *Saccharomyces* isolates inhibiting *B. cinerea* growth on plates (Santos and Marquina, 2004; Santos et al., 2004; Parafati et al., 2015), but there are no *in vitro* studies of *Saccharomyces* isolates against *A. terreus*, *F. oxysporum* and *R. stolonifer*. In the present study, some of the yeast genera that inhibited fungi such as *Saccharomyces* (Ramon Portugal et al., 1997), *Candida* (da Silva et al., 2008) and *Pichia* (Barandica et al., 1999) have been cited as *in vitro* killer toxin producers.

3.1.2-Laminarinases and chitinases

Laminarinase and chitinase enzymes may be involved in the degradation of fungal walls (Jijakli and Lepoivre, 1998). In the present work, 15 yeasts belonging to different genera secreted laminarinases in liquid medium with laminarin as substrate: *Saccharomyces* (8), *Pichia* (1), *Candida* (2), *Debaryomyces* (1), *Kluyveromyces* (1) and *Issatchenkia* (1). *S. kluyveri* BSk11 produced significantly more laminarinase activity in liquid medium (276.73 U/g of dry yeast) than the other yeasts assayed, which showed enzymatic activity values between 29.93 and 202.54 U/g dry yeasts **(Tables 1 and 2)**. Biofungicides belonging to *Candida* (Fan et al., 2002; Saligkarias et al., 2002) and *Pichia* (Chanchaichaovivat et al., 2008; Fan et al., 2002; Grevesse et al., 2003; Jijakli and Lepoivre, 1998;

Masih and Paul, 2002) produced laminarinase activity under similar conditions (liquid medium and laminarine as inductor). To our knowledge, this is the first publication on *Debaryomyces, Kluyveromyces*, *Issatchenkia* and *Saccharomyces* biofungicides as laminarinase producers.

None of the biocontrol yeasts produced chitinases in liquid medium (colloidal chitin substrate) **(Tables 1 and 2).** This activity could be evaluated over grape pruine because solid medium is probably more suitable for the production of these enzymes (Rattanakit et al., 2002; Suresh and Chandrasekaran, 1999; Viniegra-González et al., 2003).

3.1.3-Antifungal volatiles

Inhibition of fungal pathogens by volatile compounds produced by yeasts has been previously reported by authors such as Bruce et al. (2004), Fialho et al. (2010) and Masoud et al. (2005). In the current study, 8 yeast isolates produced antifungal volatiles **(Tables 1 and 2).** Volatiles produced by *Saccharomyces* yeasts significantly inhibited fungal mycelial growth between 5.96 % and 26.32 % and volatiles produced by non- *Saccharomyces* between 8.69 % and 45.48 %. None the *R. stolonifer* isolates assayed was inhibited by volatiles **(Tables 1 and 2)**. Fungal mycelial growth of *B. cinerea, A. versicolor, A. caelatus* and *F. oxysporum* was inhibited by *Saccharomyces* isolates and *P. comune*, *A. carbonarius* and *A. terreus* by non- *Saccharomyces* strains. All yeast species that inhibited *Aspergillus* genera belonged to the *Saccharomycetales* order **(Tables 1 and 2)**. Our results are in agreement with findings by several other researchers. Masoud et al. (2005) found that biofungicide strains belonging to *Saccharomycetales* produced volatiles (2 phenyl ethyl acetate, acetate, ethyl acetate, isobutyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl alcohol and 2-pentanone) that inhibited growth of *Aspergillus* isolates.

3.1.4-Competition for substrates:

3.1.4.1-Niche Overlap Index (NOI)

Usually, from a biological control perspective, the ability to occupy the same niche indicates a potential for effective competitiveness. High NOI scores represent low coexistence and a stronger competition for carbon sources (Cavaglieri et al., 2004). Nine non- *Saccharomyces*-fungus interactions and 6 *Saccharomyces*-fungus interactions showed NOI values between 0.92 and 1. The yeasts belonged to *S. cerevisiae* (5) **(Table 1)**, *T. delbrueckii* (3), *C. sake* (2), *D. vanrijiae* (1), *C. catenulata* (1), *C. famata* (1) and *Sch. pombe* (1) **(Table 2)**. These results suggest that the microorganisms assayed were able to successfully assimilate a wide variety of carbon sources like mono- and di-saccharides, making these nutrients unavailable to fungi and allowing rapidly proliferation of yeasts (competitive exclusion) (Bautista- Rosales et al., 2014; Spadaro et al., 2010).

This is most likely the first report on NOI scores regarding biofungicide yeasts-phytopathogenic fungi from grapes.

3.1.4.2-Siderophores

Ferric iron (Fe^{3+}) is biologically important as this ion is a constituent of cytochrome and other heme or non-heme proteins. In addition, it is a co- factor in various fungal enzymes (Macagan et al., 2008; Meziane et al., 2005). Some yeasts can produce low molecular weight, iron-chelating ligands or siderophores under iron-deficient conditions (Wang et al., 2009) and siderophore production decreases with increasing iron concentration in the medium (Calvente et al., 1999). In the present

study, 16 yeast isolates belonging to the genus *Saccharomyces* **(Table 1)** and 13 isolates belonging to non- *Saccharomyces* genera (4 *Torulaspora,* 5 *Candida,* 1 *Dekkera*, 1 *Pichia,* 1 *Kluyveromyces,* 1 *Debaryomyces*) **(Table 2)** demonstrated siderophore production in an iron-deficient medium. Grape berries contain few iron ions (Conde et al., 2007) and their concentration depends on several factors, with the soil where the grapes are produced being the most important one (Galani-Nikolakaki et al., 2002). The iron concentration in grapes measured by Byrne et al. (1983) is approximately 5 times lower than the concentration used in the present study (10 mM $FeCl_3$). Consequently, in wounded grapes, our yeasts should be able to produce siderophores and ''seize'' Fe^{3+}, thus making this ion unavailable to other microorganisms such as filamentous fungi (Calvente et al., 1999). Ismail et al. (1985) found that clinical pathogenic *Candida* isolates produced siderophores when grown in a deferrated medium at 37 °C. Currently, *Torulaspora*, *Dekkera*, *Pichia*, *Kluyveromyces*, *Debaryomyces* and *Saccharomyces* genera are not considered siderophore producers.

3.1.5-Inhibition of fungal spore germination (IFSG) and decrease in germinal tube length (DGTL)

Spore germination of *A. terreus* was significantly inhibited by 7 biocontrol yeasts (4 *Saccharomyces*, 1 *Torulaspora*, 1 *Kluyveromyces*, 1 *Candida*) **(Tables 1 and 2).** *I. orientalis* BIo148 significantly reduced *P. comune* spore germination *(p <0.04),* and *Saccharomyces* yeasts inhibited fungal spores of *A. versicolor, B. cinerea* and *R. stolonifer*. Yeasts that significantly inhibited fungal spore germination also decreased the fungal germinal tube length, with the exception of *S. cerevisiae* BSc149, BSc62 and BSc169. *S. cerevisiae* BSc81 significantly decreased the germinal tube length of *B. cinerea* but did not inhibit fungal spore germination **(Table 1).**

Some substances such as β-1,3-glucanases (Jijakli and Lepoivre, 1998) and antifungal volatiles like isoamyl alcohol (Ando et al., 2012) produced by biofungicide yeasts belonging to the same order have been reported to inhibit spore germination in yeast-fungus co-cultures. Other biofungicide yeasts that do not belong to *Saccharomycetales* have been reported to produce siderophores (Calvente et al., 2001), fatty acid esters (Urquhart and Punja, 2002) and cyclic depsipeptides (Xiaoping et al., 2007) that inhibited fungal spore germination.

None of the biocontrol yeasts assayed in this study inhibited spore germination of *A. caelatus*, *Ulocladium* sp., *F. oxysporum* and *A. carbonarius* **(Tables 1 and 2)**, suggesting the absence of *in vitro* production of secondary toxic metabolites. In our study, all biofungicide species that inhibited spore germination of different fungal species belonged to the *Saccharomycetales* order.

3.1.6-Effects of yeasts on induced resistance against fungi

None of the biocontrol yeasts assayed (20µl, 10^6 cells/mL) inhibited fungi at distance in grape wounds **(Tables 1 and 2).** A plausible explanation may be that a higher yeast biomass concentration is needed to induce resistance in harvested fruit (10^8 cells/mL) (Droby et al., 2002).

3.2-Antifungal Patterns- Formulae

The use of antagonistic yeasts is a promising method to reduce and even replace chemical fungicides in the control of grey rot (Calvo- Garrido et al., 2013a; Nally et al., 2012) and sour rot (Calvo- Garrido et al., 2013b; Nally et al., 2013) in grapes. Most of the reports dealing with biocontrol mechanisms focus on single biofungicide yeasts and/or a single mechanism of fungal disease suppression (Bar-Shimon et al., 2004; Droby et al., 2002; Saravanakumar et al., 2008). There are only few examples of

different biocontrol yeasts in literature, describing more than one control mechanism (Bautista-Rosales et al., 2014; Lutz et al., 2013; Vero et al., 2012). In the present work, analysis of individual antifungal mechanisms of 59 autochthonous yeasts revealed that 30 isolates presented at least two antifungal mechanisms, but none presented all the antifungal mechanisms assayed. Four yeasts (1 *Saccharomyces* and 3 non- *Saccharomyces*) showed four mechanisms of action against three different fungi **(Tables 1 and 2)**. The use of these yeasts may offer considerable advantage over synthetic fungicides. The use of biocontrol isolates that possess multiple mechanisms to inhibit pathogens reduces the risk of resistance (Janisiewicz and Korsten, 2002). Fourteen of the 59 yeast isolates did not present any antifungal mechanism. These results suggest that there may exist other factors like the production of certain enzymes (N-acetyl-b-D-glucosaminidase (Nagase)) and/or the ability to form film that could be responsible for fungus control (Bautista-Rosales et al., 2014; Vero et al., 2012).

Twenty-four different antifungal patterns (capital letters A through X in **Tables 1 and 2**) were characterized among the yeasts assayed according to individual antagonistic mechanisms. Nineteen different antifungal patterns could be distinguished among *Saccharomyces* biofungicides **(Table 1)**, while non- *Saccharomyces* yeasts showed 13 different antifungal patterns **(Table 2)**; both yeast groups shared 8 patterns (antifungal patterns C, E, H, N, O, M, S and H).

Twelve yeasts showed a single antifungal pattern (►symbol in **Tables 1 and 2**). “Siderophore production (N)” and “Siderophore production together with NOI > 0.92 (M)” were the most frequent antifungal patterns detected in the biofungicide yeasts (17.77 and 11.11%, respectively) **(Tables 1 and 2).** All yeast isolates that presented M or N patterns belonged to the *Saccharomycetales* order. These antifungal patterns are related to competition for substrates, something that several authors consider as the main mode of action of biocontrol yeasts (Liu et al., 2013; Vero et al., 2012).

In the present study, yeasts that inhibited two fungi showed different antifungal patterns **(Tables 1 and 2)**, except for *D. anomala* BDa143 that did not show any of the mechanisms assayed. *S. cerevisiae* BSc31 and BSc140 both inhibited *B. cinerea*, showing the same antifungal pattern (A); *S. cerevisiae* BSc64, BSc68 and BSc92 presented the N antifungal pattern against the same fungus **(Table 1)**. Other biofungicide yeasts presented a very specific yeast strain/pathogenic fungus antagonistic mechanism. However, the mode of action of these antagonistic yeasts varied from species to species and from isolate to isolate, and also depended on the fungal pathogen **(Tables 1 and 2).** Our results are in accordance with a recent report by Lutz et al. (2013) who found that not all biofungicide yeasts belonging to the same species (*Cryptococcus albidus, Pichia membranifaciens, Cryptococcus victoria)* presented the same antifungal mechanisms in pears.

Our results contribute to the aim to use antifungal patterns as a fingerprinting tool to differentiate biofungicide yeast strains and they could also be important to resolve eventual controversies related to the legal protection of these yeasts for their commercialization (Buzzini et al., 2007).

4-Conclusions

It can be concluded that the production of laminarinases, antifungal volatiles or growth-inhibiting metabolites, inhibition of fungal spore germination and decreased germinal tube length as well as competition for carbon sources (NOI) and/or iron (siderophores) could play an important role in the interactions between biofungicides (both *Saccharomyces* and non- *Saccharomyces*) and fungi isolated from grey and sour rot grapes. *Saccharomyces* yeasts presented a higher number of antifungal patterns than non- *Saccharomyces* yeasts. The present study is the first report demonstrating the putative modes of action of *Saccharomyces* viticultural biofungicides. Twenty-four different antifungal

patterns were identified in viticultural biofungicide yeasts. Further research on the mode of action of biocontrol yeasts to control postharvest fungal diseases of grapes is necessary, particularly to elucidate the specific antifungal mechanisms at molecular and proteomic level, and to determine the effect of a combination of different yeasts on the control of fungi. This information should be taken into account for further studies, especially when deciding formulation, large scale production and modes of application of these biofungicides in vineyards.

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Table 1. Antifungal biocontrol mechanisms by ***Saccharomyces***. All yeasts were previously reported as biocontrol yeasts in Nally et al. (2012; 2013). Different lower case letters represent significant differences ($p \leq 0.05$, ANOVA, Tukey's Test, SPSS). Statistical analyses were performed with three independent yeast trials for each mechanism.

Antagonistic interactions		Antifungal mechanisms										
Biofungicide	**Inhibited fungus**	**FMGl (1)**	**Ch (2)**	**L (3)**	**V (4)**	**IFSG (5)**	**DGTL(6)**	**NOIs (7)**	**S (8)**	**IR (9)**	**Antifungal mechanism formulae**	**Antifungal Pattern (codes)**
S. cerevisiae BSc5	*B. cinerea*	0	0	82.52 e	▲ a	28.7 b	51.16 b	0.76	0	▲ a	3,5,6	S
S. cerevisiae BSc16	*B. cinerea*	0.19 a	0	0	▲ a	▲ a	▲ a	0.92	1	▲ a	1,7,8	Q►
S. cerevisiae BSc31	*B. cinerea*	0.21 a	0	0	▲a	▲ a	▲ a	0.92	0	▲ a	1,7	A
S. cerevisiae BSc47	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.46	0	▲ a	-	-
S. cerevisiae BSc49	*B. cinerea*	0	0	68.98 d	▲ a	▲ a	▲ a	0.92	1	▲ a	3,7,8	U►
S. cerevisiae BSc56	*B. cinerea*	0	0	31.05 a	▲ a	▲ a	▲ a	0.56	0	▲ a	3	C
S. cerevisiae BSc61	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.38	0	▲ a	-	-
S. cerevisiae BSc64	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.69	1	▲ a	8	N
S. cerevisiae BSc68	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.68	1	▲ a	8	N
S. cerevisiae BSc81	*B. cinerea*	0	0	0	▲ a	▲ a	30.23 c	0.53	0	▲ a	6	K►
S. cerevisiae BSc92	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.84	1	▲ a	8	N
S. cerevisiae BSc121	*B. cinerea*	0.18 a	0	0	▲ a	▲ a	▲ a	0.76	1	▲ a	1,8	B►
S. cerevisiae BSc140	*B. cinerea*	0.22 a	0	0	▲ a	▲ a	▲ a	0.76	0	▲ a	1	A
S. cerevisiae BSc175	*B. cinerea*	0	0	0	80.95 b	▲ a	▲ a	0.53	1	▲ a	4,8	H
S. cerevisiae BSc203	*B. cinerea*	0	0	0	▲ a	3.73 c	37.2 c	0.46	1	▲ a	5,6,8	W
S. cerevisiae BSc109	*A. terreus*	0.24 a	0	29.93 a	▲ a	▲ a	▲ a	0.53	1	▲ a	1,3,8	O
S. cerevisiae BSc110	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	0.46	0	▲ a	-	-
S. cerevisiae BSc115	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	0.84	0	▲ a	-	-
S. cerevisiae BSc149	*A. terreus*	0	0	0	▲ a	16.06 c	95.55 a	0.76	0	▲ a	5	I►
S. cerevisiae BSc172	*A. terreus*	0	0	0	▲ a	10.03 c	89.88 b	0.38	1	▲ a	5,6,8	W
S. cerevisiae BSc187	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	0.76	0	▲ a	-	-

S. cerevisiae BSc206	*A. terreus*	0	0	32.8 a	▲ a	18.62 c	39.32 c	0.53	0	▲ a	3,5,6	S
S. cerevisiae BSc123	*P. comune*	0	0	0	▲ a	▲ a	▲ a	0.68	1	▲ a	8	N
S. cerevisiae BSc128	*P. comune*	0	0	60.13 c	▲ a	▲ a	▲ a	0.56	1	▲ a	3,8	E
S. cerevisiae BSc114	*R. stolonifer*	0	0	0	▲ a	▲ a	▲ a	0.45	0	▲ a	-	-
S. cerevisiae BSc22	*Ulocladium sp.*	0	0	202.54 g	▲ a	▲ a	▲ a	0.86	1	▲ a	3,8	E
S. cerevisiae BSc62	*A. versicolor*	0	0	0	83.19 b	15.8 c	94.44 a	0.81	0	▲ a	4,5	F►
S. cerevisiae BSc119	*A. caelatus*	0	0	0	73.68 b	▲ a	▲ a	0.92	0	▲ a	4,7	G►
	Ulocladium sp	0			▲ a	▲ a	▲ a	0.86		▲ a	-	-
S. cerevisiae BSc169	*A. terreus*	0	0	0	▲ a	5.5 c	▲ a	0.92	1	▲ a	5,7,8	X►
	Ulocladium sp.	0			▲ a	▲ a	▲ a	0.93		▲ a	7,8	M
S. kluyveri BSk11	*R. stolonifer*	0	0	273.67 h	▲ a	30.58 b	21.27 b	0.63	1	▲ a	3,5,6,8	T
	F. oxysporum	0			94.04 b	▲ a	▲ a	0.6		▲ a	3,4,8	R►
S. chevalieri BSch25	*A. caelatus*	0	0	0	▲ a	▲ a	▲ a	0.5	1	▲ a	8	N
Saccharomyces: positive isolates / total		5/31	0/31	8/31	4/31	8/31	6/31	5/31	16/31	0/31		
Control	*B. cinerea*				▲ a	▲ a	▲ a			▲ a		
Control	*A. terreus*				▲ a	▲ a	▲ a			▲ a		
Control	*A. versicolor*				▲ a	▲ a	▲ a			▲ a		
Control	*A. carbonarius*				▲ a	▲ a	▲ a			▲ a		
Control	*A. caelatus*				▲ a	▲ a	▲ a			▲ a		
Control	*F. oxysporum*				▲ a	▲ a	▲ a			▲ a		
Control	*Ulocladium sp.*				▲ a	▲ a	▲ a			▲ a		
Control	*P. comune*				▲ a	▲ a	▲ a			▲ a		
Control	*R. stolonifer*				▲ a	▲ a	▲ a			▲ a		

References:

FMGI (1) FUNGAL MYCELIAL GROWTH INHIBITION: fungal growth inhibition halo (cm)
Ch (2) CHITINASE ACTIVITY: chitinase activity (U/g dry yeast)
L (3) LAMINARINASE ACTIVITY: laminarinase activity (U/g dry yeast)
V (4) ANTIFUNGAL VOLATILES: fungal diametric growth (%)
IFSG (5) INHIBITION OF FUNGAL SPORE GERMINATION: germinated spores (%)

DGTL (6) DIMINUTION IN GERMINAL TUBE LENGTH: length of germinal tube (%)
NOI (7) NICHE OVERLAP INDEX: NOI values > 0.92 (competitive exclusion)
S (8) SIDEROPHORE: (0) non-siderophore producer, (1) siderophore producer
IR (9) INDUCED RESISTANCE: disease severity in wounds (%)

Antifungal formulae were established using numbers between 1 and 9, separated by commas. The ► symbol represents patterns comprising one yeast. Grey boxes represent positive mechanisms and (**–**) represents absence of mechanisms. The ▲ symbol represents 100 %.

Table 2. Antifungal biocontrol mechanisms by **non-** ***Saccharomyces***. All yeasts were previously reported as biocontrol yeasts in Nally et al. (2012; 2013). Different lower case letters represent significant differences ($p \leq 0.05$, ANOVA, Tukey's Test, SPSS). Statistical analyses were performed with three independent yeast trials for each mechanism.

Antagonistic interactions		Antifungal mechanisms										
Biofungicide	**Inhibited fungus**	**FMGI (1)**	**Ch (2)**	**L (3)**	**V (4)**	**IFSG (5)**	**DGTL (6)**	**NOIs (7)**	**S (8)**	**IR (9)**	**Antifungal mechanism formulae**	**Antifungal Pattern (codes)**
C. catenulata BCc180	*R. stolonifer*	0	0	0	▲ a	▲ a	▲ a	1	0	▲ a	7	L
C. catenulata BCc185	*A. terreus*	0	0	111.16 f	▲ a	15.37 b	22.47 c	0.53	1	▲ a	3,5,6,8	T
	A. versicolor	0			▲ a	▲ a	▲ a	0.68		▲ a	3,8	E
C. famata BCf210	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	0.92	1	▲ a	7,8	M
C. rugosa BCr182	*A. caelatus*	0	0	0	▲ a	▲ a	▲ a	0.57	0	▲ a	-	-
C. sake BCs54	*A. versicolor*	0	0	0	▲ a	▲ a	▲ a	0.62	0	▲ a	-	-

C. sake BCs186	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	1	1	▲ a	7,8	M
C. sake BCs192	*P. comune*	0	0	0	91.31 b	▲ a	▲ a	0.68	1	▲ a	4,8	H
C. sake BCs198	*A. carbonarius*	0.82 b	0	0	61.13 c	▲ a	▲ a	1	1	▲ a	1,4,7,8	P►
C. versatilis BCv222	*P. comune*	0	0	29.93 a	86.41 b	▲ a	▲ a	0.87	0	▲ a	3,4	D
D. anomala BDa143	*Ulocladium sp.*	0	0	0	▲ a	▲ a	▲ a	0.6	0	▲ a	-	-
	A. terreus	0			▲ a	▲ a	▲ a	0.53		▲ a	-	-
D. anomala BDa84	*A. caelatus*	0	0	0	▲ a	▲ a	▲ a	0.57	1	▲ a	8	N
D. vanrijiae BDv179	*A. terreus*	0	0	0	▲ a	▲ a	▲ a	0.92	1	▲ a	7,8	M
D. vanrijiae BDv197	*A. terreus*	0	0	63.26 c	54.52 c	▲ a	▲ a	0.61	0	▲ a	2,3	D
I. orientalis Blo148*	*P. comune*	0	0	29.93 a	▲ a	65.82 b	39.06 c	0.56	0	▲ a	3,5,6	S
	A. terreus	0				▲ a	▲ a	0.69		▲ a	3	C
K. marxianus BKm128	*A. terreus*	0	0	0	▲ a	10.51 c	89.88 b	0.84	0	▲ a	5,6	J►
K. marxianus BKm145	*A. terreus*	0	0	42.17 b	▲ a	▲ a	▲ a	0.61	1	▲ a	3,8	E
P. membranifaciens BPm6	*A. terreus*	0.8 b	0	83.04 e	▲ a	▲ a	▲ a	0.38	1	▲ a	1,3,8	O
P. membranifaciens BPm113	*R. stolonifer*	0	0	0	▲ a	▲ a	▲ a	0.45	0	▲ a	-	-
Sch. pombe BSchp67	*B. cinerea*	0	0	0	▲ a	▲ a	▲ a	0.92	0	▲ a	7	L
S. roseus BSr157	*P. comune*	0	0	0	▲ a	▲ a	▲ a	0.75	0	▲ a	-	-
T. delbrueckii BTd136	*Ulocladium sp.*	0	0	0	▲ a	▲ a	▲ a	0.33	0	▲ a	-	-
T. delbrueckii BTd152	*A. terreus*	0	0	0	▲ a	31.37 b-c	67.41 b-c	0.92	1	▲ a	5,6,7,8	V►
T. delbrueckii BTd156	*A. caelatus*	0	0	0	▲ a	▲ a	▲ a	0.64	1	▲ a	8	N
T. delbrueckii BTd161	*A. versicolor*	0	0	0	▲ a	▲ a	▲ a	0.75	1	▲ a	8	N
T. delbrueckii BTd211	*F. oxysporum*	0	0	0	▲ a	▲ a	▲ a	0.6	0	▲ a	-	-
T. delbrueckii BTd125	*P. comune*	0	0	0	▲ a	▲ a	▲ a	0.93	1	▲ a	7,8	M
T. delbrueckii BTd126	*P. comune*	0	0	0	▲ a	▲ a	▲ a	0.93	0	▲ a	7	L
T. delbrueckii BTd129	*P. comune*	0	0	0	▲ a	▲ a	▲ a	0.62	0	▲ a	-	-
Non- *Saccharomyces*: positive isolates / total		2/28	0/28	6/28	4/28	4/28	4/28	9/28	13/28	0/28		
Control	*B. cinerea*				▲ a	▲ a	▲ a			▲ a		

Control	*A. terreus*				▲ a	▲ a	▲ a			▲ a		
Control	*A. versicolor*				▲ a	▲ a	▲ a			▲ a		
Control	*A. carbonarius*				▲ a	▲ a	▲ a			▲ a		
Control	*A. caelatus*				▲ a	▲ a	▲ a			▲ a		
Control	*F. oxysporum*				▲ a	▲ a	▲ a			▲ a		
Control	*Ulocladium sp.*				▲ a	▲ a	▲ a			▲ a		
Control	*P. comune*				▲ a	▲ a	▲ a			▲ a		
Control	*R. stolonifer*				▲ a	▲ a	▲ a			▲ a		

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Highlights

- We determined presumptive antifungal action modes of *Saccharomyces* yeasts.
- Laminarinases, fungal micelial inhibiter metabolites, antifungal volatiles and competition for substrates were detected.
- Twenty four different antifungal patterns were characterized among yeasts.
- Thirty yeasts presented at least two antifungal mechanisms.