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Biological control of the vineyard: new microbiological findings from CREA-VE

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Abstract. According to the FAO, 75.866 km^2 of the world is dedicated to grape cultivation. Grape production is mainly hindered by fungal infections, that can develop both in field and post-harvest. The main grape diseases include downy mildew, powdery mildew and grey mold. Moreover, other microbial pathogens contribute to the deterioration of grape being the causal agents of secondary rot, such as different species of the genera *Alternaria*, *Cladosporium*, *Penicillium*, and *Aspergillus*. Today, the common strategies to control these diseases are the use of agrochemicals during pre-harvest and the application of SO_2 generator pads during post-harvest. Considering the negative impact of chemical control systems on both the environment and human health, one of the main goals of research remains the development of alternative sustainable management strategies to reduce the use of agrochemicals in pre- and post-harvest. One promising alternative, for a safer and more effective control strategy, is the use of microbial fungicides based on Biological Control Agents (BCAs) such as bacteria, yeasts, and fungi. The CREA-VE of Turi (Apulia region, Southern Italy) has been dealing with finding new effective BCAs against downy mildew and grey mold for several years. We isolated different non-Saccharomyces yeast and bacteria strains respectively from berries and leaves of native Apulian table grape genotypes. Eight non-Saccharomyces yeast and five bacteria strains were selected for their *in vivo* ability to control grey mold and downy mildew, respectively. These promising BCAs were further characterized for their mechanisms of action through *in vitro* assays, their antagonistic activity against a wide range of fungal pathogens, and their safety to human health.

1. Introduction

According to the Food and Agriculture Organization (FAO), 75.866 km^2 of the world is dedicated to grape cultivation. About 71.0% of the world's grape production is destined for winemaking, 27.0% for consumption as fresh fruit and 2.0% as raisin [1]. Table grape is one of the most appreciated fruits by consumers all over the world and, according to the 2022-23 USDA (United States Department of Agriculture) report, its worldwide production is expected to increase from 1.2 million to 27.4 million tons [2], which represents a 7% year-on-year increase.

Grape production is mainly hindered by fungal infections, that can develop both in field and post-harvest. The main grape diseases include downy mildew (caused by *Plasmopara viticola*), powdery mildew (caused by *Erysiphe necator*), and grey mold (caused by *Botrytis cinerea*). Losses attributed to fungal decay in different countries range from 30 % to 50 % [3], and in some cases are even higher in some developing countries [4]. Today, the common strategies to control these diseases are the use of agrochemicals during pre-harvest and the application of sulfure dioxide (SO_2) generator pads during post-harvest. The FAO estimated that the average amount of pesticides used in agriculture worldwide has increased from 2.28 kg Ha^{-1} in 2005 to 2.69 kg Ha^{-1} in 2019 [5]. In recent years, the development of alternative approaches has been encouraged, aiming to reduce the use of pesticides by 50% before 2030 [6], to respond to public concerns regarding the risk of pesticide residues in food, the negative impact of these sub-stances on the environment, and the negative effects that excessive doses of SO_2 can have both on grapes and human health [7]. An additional reason to reduce the use of synthetic chemical fungicides against fungal rot

species is the fast, rapid, and relatively easy selection of resistant strains to single-site fungicides in fungal populations, caused by the continuous use of active substances with the same action mechanisms [8]. Concretely, fungicide resistant strains of grapevine pathogenic molds such as *B. cinerea* [9], *Penicillum expansum* [10] and *Aspergillus* spp. [11] have widely been documented.

Microbial fungicides, based on Biological Control Agents (BCAs), such as bacteria, yeasts, and fungi, represent a valid alternative to chemicals for a safer and more effective control strategy [12–13].

The CREA-VE of Turi (Apulia region, Southern Italy) has been dealing with finding new effective BCAs against downy mildew and grey mold for several years and in this work, we summarize the main results achieved regarding the identification of non-Saccharomyces yeast isolates effective against *B. cinerea* and bacterial strains effective against *P. viticola*.

2. Materials and methods

2.1. Yeast and bacterial isolation and culture conditions

The development of a BCA for pre- and/or postharvest disease is a long, costly, and interactive process that involves several steps, among which the choices made in the isolation step strongly influence the success of the selected microorganism under commercial conditions. For these reasons, starting from several scientific evidence that demonstrate a relationship between the microbial community and the disease tolerance of specific genotypes [14-17], we isolated 31 strains of non-Saccharomyces yeasts and 47 bacterial isolates, starting from grapes and leaves of both *V. vinifera* genotypes and varieties, respectively tolerant to gray mold and downy mildew. All the *V. vinifera* genotypes were obtained through the CREA-VE breeding program and cultivated in an experimental vineyard, located in Rutigliano (BA) (40°57'26.65''N, 17°00'31.34''E, 185 mt. a.s.l.).

2.1.1. Isolation of non-*Saccharomyces* yeasts

Seven *V. vinifera* genotypes were selected for their high bunch compactness and different degree of tolerance to grey mold infections. Apparently healthy berries sampled from each genotype were placed in a full-page microperforated-filter blander bag and manually crushed to obtain grape juice ready for the isolation protocol. Appropriate dilutions of grape juice were aseptically plated on WLN-Cm, amended with chloramphenicol 50 mgL^{-1} to avoid bacterial growth. Plates were incubated for three days at 25°C. Well-developed yeast colonies were grouped, based on their color and morphology. Representative colonies for each group were selected, grown on liquid YPD and then stored at -80°C in liquid YPD with 30% (v/v) of glycerol.

2.1.2. Isolation of bacterial strains

Three table grape varieties were selected based on their tolerance ('Dawn seedless' and 'Argentina') and susceptibility ('Blush') to powdery mildew. Ten grams of leaves, divided into old and young leaves, were collected from each variety. The leaves were chopped and shaken in flaks containing 100 mL of Ringer's solution, to dissolve the microbial component present both on the surface and inside the leaves. Appropriate dilutions of leaf solution were aseptically planted on King's B culture media (KB), a semi-selective substrate for bacteria. Plates were incubated for three days at 25°C. Well-developed bacteria colonies were grouped based on their color and morphology. Representative colonies for each group were selected, grown on liquid Nutrient Broth (NB) and then stored at -80°C in liquid NB with 15% (v/v) of glycerol.

2.2. Screening for antagonistic activity

2.2.1. Yeasts vs. B. cinerea

The antagonistic activity of 31 non-Saccharomyces yeast isolates against *B. cinerea* was evaluated by performing two consecutive *in vivo* experiments. In both assays, mature 'Red Globe' grape berries were collected from healthy bunches, preserving their pedicels. Their surface was sterilized by dipping in sodium hypochlorite (3.5% active chlorine) solution for 5 min, washed in sterile water two times and then air dried. Artificial wounds were performed along the berry equatorial area. Thirty grape berries for each yeast isolate were placed in three plastic boxes and each wound was inoculated with 20 µL drop of yeast cells suspension at the concentration of 1.5×10^7 $CFUmL^{-1}$. Thirty grape berries inoculated with 20 µL of sterile water were used as control. After 48 hours of incubation at 25°C each wound was inoculated with 20 µL of a conidia suspension of *B. cinerea* at the concentration of 1×10^5 conidia mL^{-1}. The Disease Severity (DS) was evaluated five days after pathogen inoculation and incubation at 25°C by using an empirical 0-to-4 rating scale, in which 0 = no visible symptoms; 1 = sporulation covering 5–10% of the wound surface; 2 = sporulation covering 10–25% of the wound surface; 3 = sporulation covering 25–50% of the wound surface; 4 = sporulation covering more than 50% of the wound surface. The average disease severity was calculated for each plastic box by using McKinney's formula [18] and the effectiveness (%) of each yeast strain to control disease severity was calculated using the following formula [19]:

$$E(\%) = (1 - T1/C1) \times 100 \quad (1)$$

where:

T1 = the average grey mold severity detected in treated grape berries

C1 = the average grey mold severity detected in untreated grape berries.

2.2.2. Bacteria vs. P. *viticola*

Healthy leaves of susceptible cultivar 'Cabernet Sauvignon' were used to perform a leaf disk assay. The fermentation broth of 16 bacterial strains were prepared in LB medium in a shaker at 180 rpm for 48 hours at 25°C and diluted to 10^7 CFU mL^{-1} with sterile LB. A total of 21 leaf disks (seven for each replicate) of 1.0 cm diameter were soaked in 100 mL of the prepared bacterial suspensions for 30 min. The same number of leaf disks soaked in sterile LB were used as controls. All the leaf disks were then transferred to Petri dishes with two discs of absorbent paper moistened with 5.0 mL of sterile water. After 48 hours of incubation at 25°C, the abaxial surface of each leaf disk was inoculated with 50 µL of *P. viticola* inoculum at the concentration of 10^5 sporangia mL^{-1}. Inoculated leaf disks were subsequently kept overnight in the dark at 21°C for 4 hours and then subjected to a 16-h photoperiod.

The ability of bacterial isolates to control downy mildew was evaluated five days post inoculation. The severity of disease on each leaf disc was assessed as percentage of the surface affected by sporulation compared to the total leaf disc surface. Then the following 0-5 scale was created based on the minimum and maximum values, first, second and third quartiles: 0 = no visible downy mildew development, 1 = 0-0.7%, 2 = 0.71-3.8%, 3 = 3.81 – 8.6%, 4 = 8.61 – 35.2%, 5 > 35.2% leaf area affected. The average disease severity and the effectiveness (%) of each bacterial isolates to control disease severity was calculated as described above.

2.3. Characterization of the mechanism of action

2.3.1. Yeasts vs. B. *cinerea*

Three different *in vitro* assays were performed to obtain further information regarding the mechanisms of action of five selected non-Saccharomyces yeast strains. In particular, the first one was carried out with the Cellophane Agar Layer (CALt) technique [20] to evaluate the yeasts' ability to produce fungistatic diffusible substances; the second one with the sandwich dual culture technique [21], to evaluate the ability to produce fungistatic Volatile Organic Compounds (VOCs). Both experiments were performed as reported by Marsico et al. 2021 [22]. Finally, based on the results collected in the previous two experiments, three non-Saccharomyces yeast strains were selected and evaluated for their ability to produce lytic enzymes (lipase, esterase, β-1,3-glucanase, chitinase, protease and pectinase) by streaking each yeast strains onto specific grow media, both in presence and absence of *B. cinerea*. (I) Lipase activity was evaluated on tributyrin agar medium (pH = 6) [23]; after the incubation for five days at 25 °C, a clearer zone around the yeast colonies expressed the lipase activity. (II) Esterase activity was also tested following the indications of Buzzini and Martini, (2002) [23], using a solid medium (pH = 6.8) containing 10 g L^{-1} of TWEEN 80, 10 g L^{-1} of peptone, 5 g L^{-1} of NaCl, 0,1 g L^{-1} of $CaCl_2 \cdot 2H_2O$ and 6,8 g L^{-1} of Agar [24]; after the incubation periods of five days at 25 °C, a clearer zone around the isolates deter-mined the esterase activity [25]. (III) β-1,3-glucanase solid medium (pH = 7) was prepared using 5.0 g L^{-1} of glucan (Tokyo Chemical Industry, Tokyo, Japan), 6.7 g L^{-1} of Yeast Nitrogen Base (YNB) and 15.0 g L^{-1} of Agar; after the incubation period of 72h at 25 °C the plates were covered with 0,6 g L^{-1} of Congo Red and left to rest at 25 °C for 90min; once removed the excess dye, the capacity to hydrolyze glucan was evaluated by assessing a yellow-orange zone around the colonies [26]. (IV) Chitinase solid medium was prepared following the second method reported by Roberts and Selitrennikoff (1988) for the addition of colloidal chitin [27], and the method of Souza et al. (2009) for the mineral salts [28]; detection of extracellular chitinase activity was assessed after an incubation period of seven days at 25 °C by the observation of a clearer zone around the inoculum zone. (V) Protease activity was evaluated following Strauss et al. (2001) indications: YPDA was amended with 20 g L^{-1} of casein (pH =7) and left to incubate for seven days at 25 °C; a clearer zone around the isolates expressed the ability to degrade casein [29]. (VI) Pectinase activity was evaluated on solid medium (pH = 7) containing 10.0 g-1 of citrus pectin, 6,7 g L^{-1} of Yeast Nitrogen Base (YNB) and 15 g L^{-1} of Agar; after 72h of incubation at 25 °C, the plates were flooded with 10.0 g L^{-1} of hexadecyltrimethylammonium bromide: degradation of pectins was evaluated through observation of a clearer zone around the colonies [23]. For each combination yeast strain/growth media, six replicates (plates) were realized: three plates in which the yeast alone was streaked to form a square in the center of the plate, and three plates in which a 9-mm mycelial disc of *B. cinerea* was placed in the center of the square formed with the yeast streak.

2.3.2. Bacteria vs. P. *viticola*

Five bacterial isolates (BLG_B2, BLG_B4, BLG_B5, BLG_B6 and DAG_B5) were selected based on their different antagonistic activity against *P. viticola.* An *in vitro* leaf assay were performed to study their effect on gene expression of two *P. viticola* effectors. For each bacterial isolates, five healthy leaves of susceptible cultivar 'Cabernet Sauvignon' were sterilized in a 10% sodium hypochlorite solution for 30 seconds, washed twice with distilled water and left to dry. Each leaf was sprayed with the suspension of the bacteria inoculum [10^7 CFU mL^{-1}] and placed in a sterilized plate containing agar at 1%. A *P. viticola* 10^6 sporangia mL^{-1} inoculum was sprayed on the abaxial surface of leaves, after 48h of incubation. Five leaves only sprayed with *P. viticola* and five leaves only sprayed with bacterial suspensions were used as positive and negative mock, respectively. Leaves were collected from plates at two different time points, 24 hours and 6 days after the inoculation of *P. viticola*, frozen with liquid nitrogen and stored at -80°C. Three biological replicates from each bacterial treatment and time point were used to perform total RNA extraction and then first strand complementary DNA (cDNA) synthesis. Finally, a gene expression analysis of the *P. viticola* effectors *PvRxLR28* and *PvRxLR67* was performed through qPCR.

For each biological replicate two technical replicates were executed. Values related to gene expression were calculated using the method described by Hellemans et al. [30].

2.4. Effectiveness against a wide range of pathogens

The spectrum of antagonistic activity of five yeast strains and three bacterial isolates was assessed against five phytopathogens: *Alternaria alternata*, *Penicillum digitatum*, *Penicillum glabrum*, *Cladosporium* sp. and *Aspergillus niger*. The effectiveness of five yeast strains was assessed using a wounded berries assay, performed as described in the paragraph 2.2.1.

The spectrum of activities of three bacterial isolates was assessed on PDA plates by dual culture method. Plates measuring 9.0 cm in diameter, each containing 15-20 mL of PDA medium, were used. 20 µL of a bacterial suspensions [10^7 CFU mL^{-1}] of the antagonistic strains BLG_B2, BLG_B4 and BLG_B5 were straked at the center of the PDA plates. After 48h of incubation at 25°C, two pathogen discs (9 mm in diameter) were placed equidistant from the center of plate. Plates inoculated only with the pathogens were used as control. Each combination antagonist/pathogen was replicated five times. Following the inoculation, plates were incubated at 25°C in the dark. The antagonistic activity was expressed as the inhibition rates against mycelia growth compared to the control. It was calculated by the formula:

$$PI(\%) = (1 - R1/R2) \times 100 \qquad (2)$$

where R1 and R2 were the mycelial radial growth of the pathogen in the control and in the presence of the antagonist, respectively.

2.5. Safety to human health

Selected yeast and bacteria isolates were tested for their ability to produce hemolysis and a consequently possible, deleterious action on human red blood cells (erythrocytes) [31]. 20 µL of solution of each yeast and bacteria were plated on a dextrose (1%)-enriched blood agar plate with a sterile loop. Six replicates for each isolate were set up. Three plates were incubated at 25°C (optimal temperature for yeast and bacteria growth) and three plates were incubated at 37°C (human body temperature). After five days of incubation, β-haemolysis was observed by a clear zone around the yeast and bacteria colony, indicating erythrocyte breakage; α- haemolysis or partial haemolysis was represented by a colour change to dark-green, indicating a reduction of red blood cells' haemoglobin to methaemoglobin. Non-alteration over the medium (γ-haemolysis) indicates no damage to erythrocytes [32].

3. Results

3.1. Yeast and bacterial isolation and culture conditions

Several yeast colonies were collected from the seven selected new *V. vinifera* genotypes and then divided into 16 different groups based on the morphotype expressed on WL Nutrient agar. One-to-three yeast colonies from each of the 16 groups were random selected, resulted in a total of 31 yeast strains.

All bacteria isolates obtained from leaves of 'Argentina', 'Blush' and 'Dawn seedless' varieties were divided into 37 different group based on their expressed morphology. One-to-two bacteria colonies from each group were random selected, resulted in a total of 47 bacteria strains.

3.2. Screening for antagonistic activity

3.2.1. Yeasts vs. B. *cinerea*

Among the 31 yeasts strains only 10, named 'N22_I4', 'OLB_9_BR', 'N22_I3', 'N20_9B', 'AxAR4', 'S13_I6', 'OLB_9.1_VL', 'N22_I1', 'CxM5' and 'OLB_6', showed an effectiveness greater than 60.0% and therefore selected for the subsequent analysis (Figure 1).

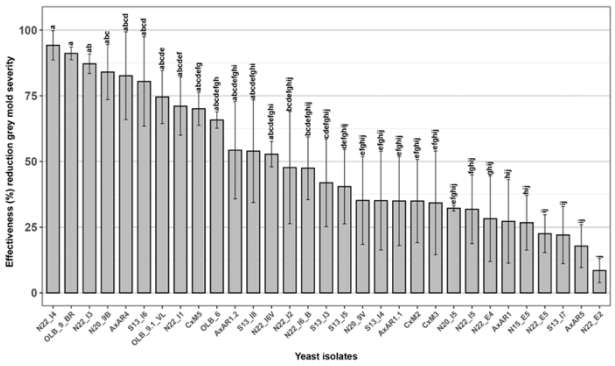

Figure 1. Effectiveness of 31 yeast isolates against Botrytis cinerea bunch rot. *In vivo* antagonistic activity of 31 yeast isolates to inhibit grey mold decay on wounded grape berries.

Data are presented as a percentage reduction of disease severity (McKinney Index) compared to the untreated control. The columns labeled with different letters are statistically significant according to Tukey's test ($p<0.05$)

The ten most effective yeast isolates, selected in the first preliminary tests, were used to perform a further in vivo antagonism assessment aimed to confirm their effectiveness against grey mold of table grapes. An efficacy greater than 60.0% was confirmed for the five yeast strains 'OLB_9.1_VL', 'N22_I1', 'OLB_9_BR', 'S13_I6' and 'N22_I3' (Figure 2), that were then selected for further characterization studies.

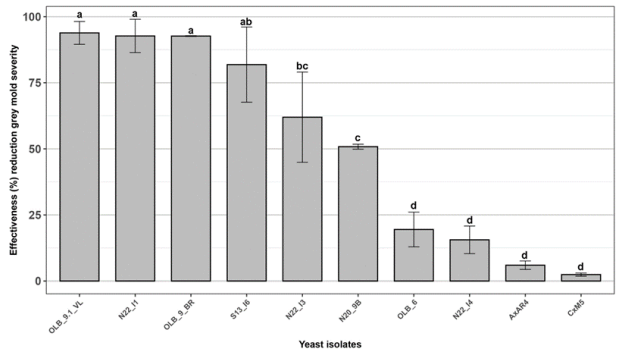

Figure 2. Effectiveness of ten yeast isolates against Botrytis cinerea bunch rot. In vivo antagonistic activity of ten yeast isolates in inhibiting grey mold decay on wounded grape berries.

Data are presented as a percentage reduction of disease severity (McKinney Index) compared to the untreated control. The columns labeled with different letters are statistically significant according to Tukey's test ($p<0.05$).

In particular, the analysis of the nuclear ribosomal internal transcribed spacer 2 (ITS2) region of the yeast DNA, identified two *Starmerella bacillaris* ('N22_I1' and 'S13_I6'), one *Hanseniaspora uvarum* ('OLB_9_BR') and one *Aureobasidium pullulans* ('OLB_9.1_VL').

3.2.1 Bacteria vs. P. viticola

The 16 bacteria strains tested showed a different degree of efficiency against Downy mildew (Figure 3). In particular, five bacteria strains, named 'BLG_B1.3', DAG_B4_1.1, 'BLG_B1_1.1', 'BLG_B1_1.2' and 'BLG_B4' showed the highest effectiveness, ranging between 45.7 and 66.0%. However, eight bacteria strains, named 'BLG_B5', 'DAG_B6_3.1', BLG_B6', 'DAG_B5', 'DAG_B2.2', 'DAG_B1.3', 'BLG_B1.2' and 'DAG_B4_1.2', showed an intermediate level of effectiveness, between 16.3 and 36.2%. Finally, only three isolates, named 'BLG_B2', 'DAV_B1_1.1.1' and 'DAV_B1_1.1', found to be ineffective to control Downy mildew of grapevine.

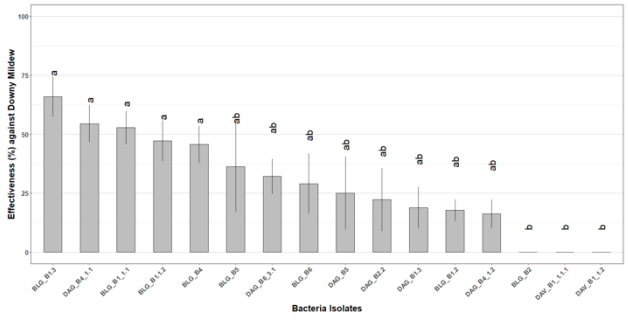

Figure 3. Effectiveness of 16 bacteria isolates in inhibiting Downy mildew on leaf disc assay.

Data are presented as a percentage reduction of disease severity (McKinney Index) compared to the untreated control. The columns labeled with different letters are statistically significant according to Tukey's test ($p<0.05$).

3.3. Characterization of the mechanism of action

3.3.1. Yeasts vs. B. *cinerea*

To characterize the mechanism of action of the five yeast strains, selected for their effectiveness against Botrytis bunch root, two *in vitro* experiments were conducted. Results showed that *S. diversa* strain 'N22_I3' significantly reduced the *in vitro* growth of the fungus in both in CALt (Figure 4a) and VOCs (Figure 4b) experiments, by 35.1 and 80.1% respectively. On the other hand, the *St. bacillaris* 'N22_I1' significantly reduced the mycelium growth of *B. cinerea* in CALt experiment by 55.1% (Figure 4a), while *A. pullulans* 'OLB_9.1_VL' significantly reduced the mycelium growth of the fungus in VOCs experiment by 69.4% (Figure 4b). *St. bacillaris* strain 'S13_I6' and *H. uvarum* strain 'OLB_9_BR' did not significantly reduce the daily growth of *B. cinerea* in both experiments.

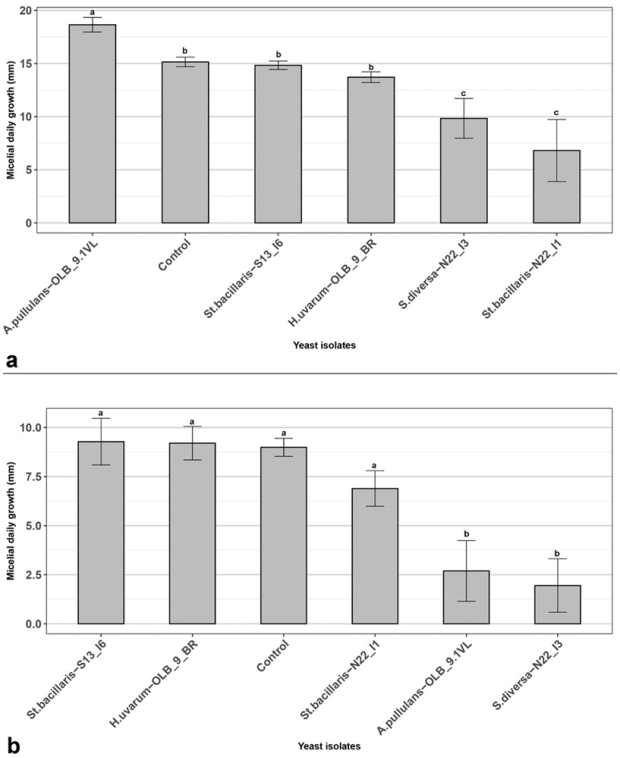

Figure 4. In vitro assays (a) Cellophane agar layer technique (CALt); (b) Sandwich dual colture for Volatile Organic Compounds (VOCs).

Data are presented as the mean of five replicates with standard deviation (vertical bars). Columns labeled with different letters are statistically significant according to Tukey's test ($p<0.05$)

To further characterize the specific enzymatic activity of diffusible or volatile substances produced by *St. bacillaris* strain 'N22_I1', *S. diversa* 'N22_I3' and *A. pullulans* 'OLB_9.1_VL', an *in vitro* experiment was performed, using selective substrates both in presence (P) and in absence of pathogen (W.P.), able to detect the ability of the yeast strain to produce specific lytic enzymes. Based on collected data, reported in Table 1, *A. pullulans* 'OLB_9.1_VL' and *S. diversa* 'N22_I3' yeasts strains showed lipase activity only when they were in the presence of the pathogen. Additionally, *A. pullulans* 'OLB_9.1_VL' showed also protease activity in the presence of the pathogen and esterase activity when not in contact with the pathogen. Finally, *St. bacillaris* strain 'N22_I1' was unable to produce the tested lytic enzymes, both in presence and in absence of pathogen.

Table 1. Yeasts were tested on media in the presence of the pathogen (P.) and without the pathogen (W.P.) on plates. Isolates able to hydrolyse the compound are indicated by (+); those not able by (-); and isolates that did not grow on the specific substrate are indicated by (N.G.).

Yeast Strains	Extracellular Lytic Enzymes Activity											
	Lipase		Esterase		β-1,3-glucanase		Chitinase		Protease		Pectinase	
	P.	W.P.	P.	W.P.	P.	W.P.	P.	W.P.	P.	W.P.	P.	W.P.
S. diversa N22_I3	+	-	-	-	-	-	-	N.G.	-	-	-	-
St. bacillaris N22_I1	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	-	-	-	-
A. pullulans OLB_9.1_VL	+	-	-	+	-	-	-	-	+	-	-	-

3.3.2. Bacteria vs. P. *viticola*

Analysis of *P. viticola* gene expression suggests that among the five tested bacteria isolates, only three of them (BLG_B2, BLG_B4 and BLG_B5), were able to reduce the expression levels of the early effector *PvRxLR28*. The impairing effect also remained for the late effector *PvRxLR67* whose expression levels were reduced, although to a lesser extent than the early effector. Also, BLG_B4 effect on gene expression impairment was more evident than the one of the other two bacteria isolates (Figure 5).

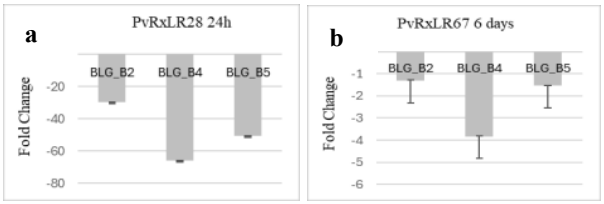

Figure 5. Fold change in gene expression levels of P. viticola effectors PvRxLR28 (a) and PvRxLR67 (b) at 24h and 6 days after inoculation with *P. viticola* of leaves previously *in vitro* treated with bacteria inocula.

3.4. Effectiveness against a wide range of pathogens

The wounded berries assay, performed to evaluate the spectrum of antagonistic activity of five yeast strains, showed that all the yeast isolates resulted effective in reducing the tested target fungal pathogens (Table 2).

Table 2. Antagonistic activity against various fungal pathogens by selected yeast strains in wounded berries assay.

Target fungal pathogens	Inhibitory activity				
	N22_I1	N22_I3	OLB_9.1_VL	OLB_9_BR	S13_I6
A. niger AS19	++++	++++	+++	++++	++++
A. arborescens AS9	++++	++++	++++	++++	++++
P. glabrum AS14	++++	++++	++++	++++	++++
P. digitatum AS13	++++	+++	++++	++++	++++
Cladosporium spp. AS3	++++	++	++++	++++	++++

+, ++, ++ and ++++ represent relative inhibition rates against the severity of disease on wounded berries to the extent of 50%-60%, 61%-70%, 71%-80% and > 80% respectively. – represents no effectiveness.

Conversely, the *in vitro* test conducted with the dual culture technique showed a variability in the efficacy of the bacterial isolates in reducing the mycelial growth of the tested fungi. Bacterial strain BLG_B5 reduced the mycelia growth of *A. niger* AS19 and *P. digitatum* AS13 by 55.7% and 53.0%, respectively. Bacterial strain BLG_B4 reduced the mycelial growth of *P. digitatum* AS13 by 82.4% compared to the control (Table 3).

Table 3. Antagonistic activity against various fungal pathogens by selected bacteria *in vitro.*

Target fungal pathogens	Inhibitory activity		
	BLG_B2	BLG_B4	BLG_B5
A. niger AS19	-	-	+
A. arborescens AS9	-	-	-
P. glabrum AS14	-	-	-
P. digitatum AS13	-	++++	+
Cladosporium spp. AS3	-	-	-

+, ++, ++ and ++++ represent relative inhibition rates against the mycelia growth of each fungal colony on PDA medium to the extent of 50%-60%, 61%-70%, 71%-80% and > 80% respectively. – represents no effectiveness.

3.5. Safety to human health

The previous five yeast strains and three bacterial isolates were investigated for their ability to lyse the red blood cell membrane through haemolysin production.

As shown in Table 4, none of the yeasts can grow on blood agar at the human body temperature (37°C), and only two yeasts (*S. diversa* N22_I3 and *A. pullulans* OLB_9.1_VL) grow at the temperature of 25°C. No clear or brown areas were detected around the colony of *S. diversa* N22_I3, suggesting the inability of this yeast to produce haemolysin (γ-haemolysis). Differently, a clear area was detected around the colonies of *A. pullulans* OLB_9.1_VL grown on blood agar, and this highlights the ability of the yeast strain to degrade the red blood cell membrane (β – haemolysis).

Differently, the tested bacterial isolates showed ability to produce haemolysin (α – haemolysis). In particular, a clear area was detected around the colony of BLG-B4 growth on blood agar both at 37°C and 25°C. Differently, BLG_B2 was able to produce haemolysin only at the room temperature (Table 4).

Table 4. Effect of haemolytic action of the five yeast and two bacterial strains plated on blood agar at 25°C and 37°C.

Microorganisms	25°C	37°C
St. bacillaris N22_I1	No growth	No growth
S. diversa N22_I3	γ-haemolysis	No growth
St. bacillaris S13_I6	No growth	No growth
A. pullulans OLB_9.1_VL	β-haemolysis	No growth
H. uvarum OLB_9_BR	No growth	No growth
BLG_B2	α – haemolysis	γ-haemolysis
BLG_B4	α – haemolysis	α – haemolysis
BLG_B5	No growth	No growth

4. Discussion and Conclusion

In our study, five of 31 yeast strains (about 16%) and five of 16 bacterial strains (about 31.0%) resulted effective to inhibit grey mold and powered mildew, respectively. In a leaf disk assay performed by Vecchione et al. [33], 47 out 1,700 microorganisms (about 3.0%) were able to reduce the grapevine powered mildew. In the same way, Zangh et al. [13] screened a total of 239 endophytic bacterial strains, isolated from the surface-sterilized grapevine leaves, for their ability to control grapevine downy mildew. Only two isolates (about 1.0%) showed distinctive inhibition to grapevine downy mildew. In the same way, Nunes et al. [34] tested in 'Blanquilla' pears the activity of 247 bacteria and yeasts, isolated from the fruit and leaf surface, against *P. expansum* showing that only the 2.0% inhibited decay by 50.0% or more. These contrasting results might be related to the adopted selection protocol, as isolating our yeast and bacteria from bunches and leaves of *V. vinifera* genotypes showing tolerance to *B. cinerea* and *P. viticola*, respectively. These data can also represent a confirmation of our previous of coevolution hypothesis of microorganisms within the growing area, to the extent of involving the genotype of the host plant [22]. Moreover, this hypothesis is supported also by other studies on Arabidopsis [35], suggesting a relationship between microbial communities in the phyllosphere and susceptibility to leaf pathogens. Exploiting this coevolution process could, in our opinion, represent a valid alternative strategy for a more rapid selection of microorganisms with antagonistic action.

An effective biocontrol agent is generally able to control a disease development by adopting several mechanisms of action that often work in concert. The *in vitro* tests performed in this work confirmed the presence of different mechanisms of biocontrol for some of the selected yeast strains. *St. bacillaris* strain 'N22_I1' and *S. diversa* strain 'N22_I3' significantly inhibited the mycelium growth of the pathogen in the cellophane-agar assays, letting us hypothesize the production of fungistatic diffusible substances as a further mechanism of biocontrol action. In addition, *S. diversa* strain 'N22_I3' as well as *A. pullulans* strain 'OLB_9.1_VL' significantly inhibited the mycelial growth of *B. cinerea* in the sandwich dual-culture assay, suggesting the production of volatile organic compounds as an inhibitory mechanism of action. Finally, *S. bacillaris* S13_I6 and *H. uvarum* OLB_9_BR resulted unable to significantly reduce the in vitro growth of *B. cinerea*, suggesting a mechanism of action mainly by nutritional and spatial competition.

To be considered a good BCA, a microorganism must possess some features, such as effectiveness against a wide range of pathogens and with long shelf-life and safe to human health [36]. In controlled conditions, the selected yeast strains resulted effective in inhibiting different fungal pathogens, except for *A. pullulans* 'OLB-9.1_VL', which was unable to control *A. niger* infections. On the other hand, the selected bacterial isolates (BLG_B4 and BLG_B5), appear to have a specific biocontrol action against *P. viticola*, which resulted more evident in the early stages of the infection as demonstrated by their ability to significantly reduce the expression of early pathogenicity gene. In fact, while the bacterial isolate BLG_B4 showed a high ability to contain the mycelial growth of *P. digitatum,* bacterial isolate BLG_B5 was found to be weakly effective in controlling the *in vitro* growth of *A. niger* and *P. digitatum*. This differences in efficiency

between yeast and bacterial strains could be related to the fact that the antagonistic yeasts were isolated from the carpophere, that represent the specific microenvironment of the test fungal target.

Human health risk assessment is a prerequisite for the application of a microorganism as biological control agents. In this work, we used erythrocytes to evaluate the potential toxicity of the studied antagonistic strains, which has previously been proposed as a useful biological model to study potential human health risks. None of the selected yeast strains and only the bacterial isolate BLG_B5 resulted unable to grow on blood agar at the human body temperature (37°C), suggesting the inability of these microorganisms to produce haemolysin (γ-haemolysis).

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