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*original scientific paper*

# Biocontrol of Grey Mould Disease on Grape Caused by *Botrytis cinerea* with Autochthonous Wine Yeasts

*Peter Raspor*[*,#]*, Damjana Miklič-Milek, Martina Avbelj and Neža Čadež*

Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

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**Summary**

Biocontrol activities of different yeast species and strains isolated from grape/must/wine environments have been compared to those of commercially available antagonistic yeast species of *Candida oleophila*. A total of 591 yeast isolates were tested in a preliminary screening on agar to select isolates showing inhibitory effect against *Botrytis cinerea*, the plant pathogen causing grey mould disease on grape. Yeast species *Aureobasidium pullulans*, *Metschnikowia pulcherrima* and *Pichia guilliermondii* showed, on average, higher biocontrol activity than commercially used yeast *Candida oleophila*. Furthermore, these three species and *Saccharomyces cerevisiae*, which is potentially interesting biocontrol agent against grey mould of grapes, were selected for their inhibitory effects and assayed *in vitro* on different solid synthetic media for their antagonistic capacity towards *B. cinerea*. The results indicate that the composition of the medium had an impact on the biocontrol activity of yeast species and strains, as *Saccharomyces cerevisiae* showed the highest antagonistic activity against *B. cinerea* when tested on media with increased concentrations of glucose. The antagonistic activity of selected yeast strains was finally determined on wounded and sound grape berries of cultivars Rebula and Chardonnay for their ability to inhibit infection by *B. cinerea* moulds. Results suggest that antagonist yeasts with the potential to control *B. cinerea* on grape can be found among the microflora associated with the berries.

*Key words*: biocontrol agents, *Botrytis cinerea*, grey mould, antagonism, grapes, wine yeasts

## Introduction

*Botryotinia fuckeliania* (de Bary) Whetzel (teleomorph of *Botrytis cinerea* Pers. ex Fr.), the causal agent of grey mould of grapes, is responsible for significant economic losses in vineyards worldwide. The conidial infection on the one side and the development of microfissures on grape berries under moist conditions on the other, provide sites for fungal penetration and the release of the grape berry nutrients (*1*). By changing the biochemical properties of the grape berries, they alter the conditions of the growth of natural populations of yeasts before and after the harvest and during alcoholic fermentation (*2*). Chemical control and use of fungicides are the most effective way of preventing the occurrence of *Botrytis* disease (*3*). However, following an increased public health concern and fast development of resistance to novel fungicides by fungi, biocontrol has become an interesting alternative to conventional methods.

The main principles of biological control are defined as the use of living organisms, their products or the use of a biological process to control pest populations (*4*). Only twenty-five years ago, the seminal work by Wilson and Pusey (*5*) published the basic idea and principles of the biological control as an alternative to synthetic pesticide treatments. Since then a variety of microbial antagonistic microorganisms have been reported to control several different pathogens on fruits and vegetables (*6*) and even several commercially available products have been available (*7*). The first step in developing biocon-

*Corresponding author; Phone: ++386 1 320 3750; Fax: ++386 1 257 4092; E-mail: peter.raspor@bf.uni-lj.si

[#]There are teachers and teachers, from time to time you have a chance to have a professor. I was lucky. Professor Marić was one. It was an honour for me to be his student. I hope I will convey some of his message to my students.

trol agents is the isolation and screening process (*4*), and the best sources of antagonistic microorganisms are their natural environments in which they compete with plant pathogens (*8*). The microflora associated with grape berries consists mostly of yeasts (*9*,*10*) and various species of bacteria (*11*) that outcompete pathogens for nutrients and space (*12*). Yeasts possess several important properties which make them useful for biocontrol purposes: they do not produce allergenic spores or mycotoxins, as many mycelial fungi do, or antibiotics, which might be produced by bacterial antagonists (*13*). Some yeasts can colonize plant surfaces or wounds for long periods under dry conditions, and can produce extracellular polysaccharides that enhance their survival and restrict pathogen colonization sites (*12*,*14*). Therefore, the fruit surface is an excellent source of naturally occurring antagonists. Several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts. The main modes of actions of yeast biocontrol agents were considered competition for nutrients and space, production of cell wall-degrading enzymes, production of antifungal metabolites, induction of host resistance, and mycoparasitism (reviewed by El-Tarabily and Sivasithamparam (*13*)). In particular, nutritional competition has been reported to play a fundamental role in yeast--mould interactions (*15*).

In spite of the fact that yeasts possess a great deal of ideal biocontrol microorganism characteristics, currently only two antagonistic yeasts, *Candida oleophila* and *Cryptococcus albidus*, are commercially available. They appear under the trade names Aspire™ and YieldPlus™, respectively. Many more are in the advanced stages of commercialization (*16*,*17*).

The aim of this study is to determine whether autochthonous yeasts, present in grape/must/wine ecosystems, have biocontrol activity against plant pathogenic fungus *Botrytis cinerea*. For this purpose the biocontrol activity of different yeast species and strains isolated from grape/must/wine system were compared to that of yeast *Candida oleophila in vitro* on different solid media and *semi in vivo* on grape berries of two different grapevine (*Vitis vinifera*) cultivars.

## Material and Methods

### *Yeasts*

A total of 591 yeast strains were obtained from the Collection of Industrial Microorganisms, Ljubljana, Slovenia. The isolation sources of these strains were either grapes of several grapevine varieties (*Vitis vinifera* L.) or fermenting grape musts collected in vine-growing regions of Slovenia during the last fifteen years. These strains belonged to twenty-two different yeast species of twelve genera. The commercially used yeast species *Candida oleophila* Montrocher NRRL Y-2317$^T$ was obtained from the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, USA.

### *Plant pathogen*

*Botrytis cinerea* Pers. was isolated from grapes in Slovenia and is maintained in ZIM culture collection as strain ZIM F58. Two additional strains of geographically diverse origins were used, strain BC-3, ZIM F63 from France and ZIM F61 from Germany. All *B. cinerea* Pers. strains were maintained on potato dextrose agar (PDA) (Merck, Darmstadt, Germany).

### *Biocontrol activity in vitro*

The radial growth inhibition of *Botrytis cinerea* strains by antagonistic yeasts was tested according to the method of Spadaro *et al.* (*18*) with a few modifications. A loop of 3-day-old yeast cells grown on YM agar plates (0.3 % malt extract, 0.3 % yeast extract, 0.5 % bactopeptone, 1 % glucose, 2 % agar) at 26 °C was streaked on the NYDA agar plate (1 % nutrient broth (Oxoid, Cambridge, UK), 0.5 % yeast extract, 1 % glucose, 2 % agar (*19*)) 20 mm from the edge of the Petri plate (90 mm). In the middle, 32 mm from the yeast line and 32 mm from the opposite edge of the Petri plate, a single drop of 50 μL of *Botrytis cinerea* ZIM F58 conidial suspension in the concentration of $10^4$ conidia per mL was spread. The plates were incubated for 5 to 7 days at 25 °C and the mycelium growth was checked daily. When the mycelium reached the Petri plate edge, the following distances were measured: (*i*) the inhibition distance, which is defined as a gap between yeast line and mycelium of filamentous fungi; and (*ii*) the diameter of the mycelium of filamentous fungi towards the yeast line. The biocontrol activity in % was calculated as follows:

$$\text{Biocontrol activity} = [(\text{distance 1})/(\text{distance 1}+\text{distance 2})]\times 100 \quad /1/$$

The experiment was performed in triplicate.

The impact of different solid media on biocontrol activity of selected yeasts was also evaluated. Different synthetic media were tested: NYDA, YM agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose, 2 % agar), wine-yeast-peptone-dextrose agar (WYPDA: 0.5 % yeast extract, 1 % peptone, 20 % glucose, 2 % agar) and solid chemically defined must (CDM) composition published by Henschke and Jiranek (*20*). The plates were incubated at 25 °C and the first measurement was done when the mycelium reached the edge of the plate (max. 8 days), and later on day 15 and day 30. The experiment was carried out in triplicate and repeated twice.

### *Biocontrol activity semi in vivo*

Grape berries of Rebula cultivar (*Vitis vinifera* cv. Rebula) and Chardonnay (*Vitis vinifera* cv. Chardonnay) were collected at harvest time (September 2005) in the Primorska vine-growing region, Slovenia. Ten healthy and undamaged grape berries were carefully cut off from grape bunches with their pedicels. They were surface--sterilized with 2 % sodium hypochlorite for 2 min and rinsed with sterile distilled water. The grapes were inoculated with the plant pathogen *Botrytis cinerea* and yeast antagonists by either their spraying on the grape berries or by injection through a calibrated wound of 0.4 mm in diameter. Spraying was applied from a distance of approx. 20 cm with two sprayer squeezes (1.6 mL) of yeast cell suspension containing $10^8$ CFU/mL, and one sprayer squeeze (0.8 mL) of *B. cinerea* conidial suspension containing $10^4$ conidia per mL. The wound infection was performed by inoculating 20 μL of yeast cell suspension ($10^8$ CFU/mL), followed by the inoculation of 10 μL *B. cinerea* spore suspension ($10^4$ spore per mL) into the

wound with a sterile needle. The *B. cinerea* conidial suspension was applied either simultaneously or 24 hours later. Each sample contained 10 berries and was reproduced with three replicates for each yeast isolate. The samples were incubated for 5 days at 25 °C in sterile Petri plates. The results obtained are the mean of three independent experiments. The control groups of grape berries were treated either with yeast cell suspension only or with *B. cinerea* spore suspension only. As a negative control the sterile physiological solution was either sprayed (2.4 mL) or injected (20 μL and 10 μL) into the grape berries.

After incubation, the degree of grape berry decay was evaluated according to the modified Unterstenhöffer scale by which the levels of susceptibility ranged from 1 (0 % decay) to 5 (>50 % decay) (*21*). From this, the degree of infection (%) was calculated using the Townsend-Heuberger Eq. 2 (*21*), where *n* is the number of grape berries on one infection level, *v* is the infection level, *i* is the number of the highest infection level, and *N* is the number of all inspected grape berries.

$$\text{Degree of infection} = \left[ \frac{\sum_{0}^{i} (n \times v)}{i \times N} \right] \times 100 \qquad /2/$$

*Statistical analysis of the data*

The significance of differences between the samples was determined by single-factor analysis of variance (ANOVA; Microsoft Excel 2002).

## Results

*In vitro biocontrol activity of different yeast species*

Five hundred and ninety-one yeast isolates belonging to twenty-two yeast species/genera were tested for their potential antagonism against *Botrytis cinerea* strain ZIM F58 *in vitro*. The antagonistic capability was assessed by measuring the gap between the yeast strains and the edge of *Botrytis cinerea*'s colony edges compared to the diameter of fungal colony (Fig. 1) on NYDA medium after five to seven and 30 days of incubation at 25 °C. As a positive control, the commercially available yeast species *Candida oleophila* was used. Data showed that the yeasts could be categorized into three groups according to their ability to inhibit radial fungal growth in comparison with the biocontrol activity (BCA) of *Candida oleophila*, which showed biocontrol activity of 24.1 % after seven days and remained at that level until the 30th day of incubation. The first group comprised highly antagonistic species of *Aureobasidium pullulans*, *Metschnikowia pulcherrima*, *M. reukaufii* and *Pichia guilliermondii*, which showed biocontrol activity of 41.5, 39.1, 30.3 and 32.1 %, respectively, after seven days of incubation. Even though their biocontrol activity declined gradually by prolonged incubation of 30 days, the value did not fall under 25 %. The second group was characterized by its high biocontrol activity after seven days of incubation, but their biocontrol activity declined during prolonged incubation

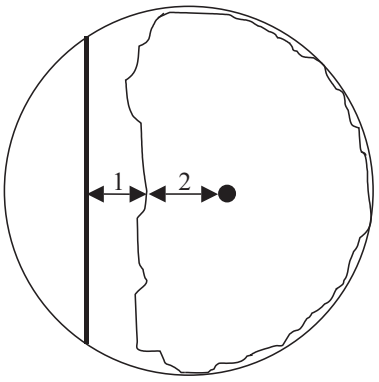


**Fig. 1.** Biocontrol activity measurement *in vitro*. Yeast strain was inoculated on the line and conidial suspension of *B. cinerea* was placed on the dot. After incubation (1), the inhibition distance, distance between the yeast line and the radial growth of *B. cinerea*, and (2) the radial growth of *B. cinerea* mycelium towards the yeast line were measured

period. This group included species of *Bulleromyces albus*, *Candida diversa*, *C. glabrata*, *C. norvegica*, *C. vini*, *Pichia kluyveri* and *P. membranifaciens*, which showed more than 24 % of biocontrol activity after seven days of incubation and this value declined down to only 7.6 % of biocontrol activity for *Bulleromyces albus* after 30 days of incubation. The third group inhibited the mycelial growth only moderately after seven days and by the end of incubation period, they had none or only negligible activity against *B. cinerea*. Most of these species are either predominant residents of grape berry microflora (*e.g. Cryptococcus* spp., *Rhodotorula* spp., *Sporidiobolus* sp., *Sporobolomyces* sp., *Hanseniaspora* sp. (*10*,*22*)) or leading species of wine fermentations (*e.g. Saccharomyces* spp., *Candida zemplinina* (*23*,*24*)).

Although the biocontrol activity was mostly species-specific, there were some exceptions when the strains of the same species showed significant deviations. The inhibition distances for 24 *Aureobasidium pullulans* strains ranged from 7.3 to 13.0 mm, for 46 *Metschnikowia pulcherrima* strains they varied from 6 to 14.7 mm, for 56 *Saccharomyces cerevisiae* strains from 2 to 10.3 mm and for seven strains of *Pichia guilliermondii* from 6.7 to 14.3 mm. The distances were measured after seven days of incubation on NYDA solid medium.

The strains *A. pullulans* ZIM 2098, *M. pulcherrima* ZIM 2055 and *P. guilliermondii* ZIM 624, which were detected as strong antagonists, and strain *S. cerevisiae* ZIM 2180, which is a potentially interesting biocontrol agent against grey mould of grapes, were examined for their biocontrol activity on different solid media.

*In vitro biocontrol activity on different solid media*

Because the main mechanism of antagonism of yeasts involved in the biological control of fungal plant pathogens is competition for nutrients (*25*), five different solid growth media for yeasts were examined. The biocontrol activity of the three strong antagonist yeast strains, one *Saccharomyces cerevisiae* strain and a control strain of *Candida oleophila* NRRL Y-2317$^{T}$ on different media is presented in Fig. 2. The commercially available biocontrol yeast *C. oleophila* was able to inhibit the growth of *B.*

*cinerea* in the range of 16 to 34 % on all five different media after seven days of incubation (Fig. 2a) and between 8 and 24 % after 30 days on all media except on YM medium (0 %) (Fig. 2b). Other yeast strains showed similar biocontrol activities on synthetic NYDA, YPDA and YM media that contain low amounts of glucose (1 %) (Fig. 2a). However, the yeast strains *A. pullulans* ZIM 2098 and *P. guilliermondii* ZIM 624, with comparable or higher biocontrol activity than *C. oleophila* on the NYDA medium ($p=0.1$ and $p<0.05$, respectively), showed lower activity against *B. cinerea* on media containing similar concentrations of sugars as grape juice (20 % glucose, WYPDA and CDM media; $p<0.05$, except *A. pullulans* on WYPDA, $p=0.12$) (Fig. 2a). Nevertheless, *M. pulcherrima* ZIM 2055 inhibited the growth of *Botrytis cinerea* equally or better than *C. oleophila* NRRL Y-2317$^T$ on all five solid media ($p<0.1$, except on YPDA, $p=0.29$). Interestingly, *S. cerevisiae* ZIM 2180 exhibited extremely high biocontrol activities of 64 and 82 % after seven days of incubation and 51 % (16 mm of inhibition zone) and 74 % (24 mm of inhibition zone) after 30 days on WYPDA and CDM solid media containing high concentrations of glucose, respectively. On the contrary, on NYDA medium, which was introduced as a useful medium for determining the biocontrol activities of yeasts (*19*), and on YM medium, *S. cerevisiae* ZIM 2180 did not exhibit any antagonistic activities against *Botrytis cinerea*.

After prolonged incubation, the inhibition zone between the line of all antagonistic yeast species and mycelia of *B. cinerea* was generally reduced on all media with an exception of NYDA medium (Fig. 2b; $p<0.05$ for the biocontrol activities above zero). However, this inhibition was less significant for *S. cerevisiae* on media containing high concentrations of glucose, WYPDA and CDM ($p<0.05$ and $p=0.11$, respectively).

### *Biocontrol activity on grape berries*

The inhibitory effect of previously determined antagonistic yeast strains and *S. cerevisiae* was further examined on wounded and sprayed grape berries by coinoculation of the yeasts with *Botrytis cinerea* test strains in various combinations. As shown in Fig. 3, the degree of infected grape berries was quantified by the modified Unterstenhöffer scale and calculated using the Townsend-Heuberger equation (*21*). The degree of infection was tested on Rebula (Figs. 3a and b) and Chardonnay (Figs. 3c and d) grapevine cultivars of *Vitis vinifera*, which were chosen for their sensitivity and resistance to *B. cinerea* infection, respectively. When only yeasts were injected into or sprayed at grape berries at $10^8$ CFU/mL, an elevated degree of infection compared to control occurred. However, *Candida oleophila* NRRL Y-2317, *Metschnikowia pulcherrima* ZIM 2055 and *Pichia guilliermondii* ZIM 624 decreased the degree of infection on grape berries of cv. Rebula when applied with a sprayer (Fig. 3b; $p\leq0.15$). *S. cerevisiae* ZIM 2180, which showed a good biocontrol activity on WYPDA and CDM media, slightly inhibited the growth of *B. cinerea* on grape berries although this effect was not statistically significant. When conidia of *B. cinerea* were applied by needle 24 hours after the yeasts into and on the grape berries of the cv. Rebula, the degree of infection decreased significantly compared to simultaneous infection for both cultivars ($p<0.05$). However, this difference was less significant when the conidia were applied by spraying on cv. Chardonnay.

The degree of grape berry infection depended also on the *B. cinerea* strains used. When *B. cinerea* ZIM F58, isolated from grapes in Slovenia, was applied with a needle, the infection degree was 54.2 and 73.2 % on grape berries of cv. Chardonnay or on cv. Rebula, respectively. The German strain ZIM F61 caused only 21.2 and

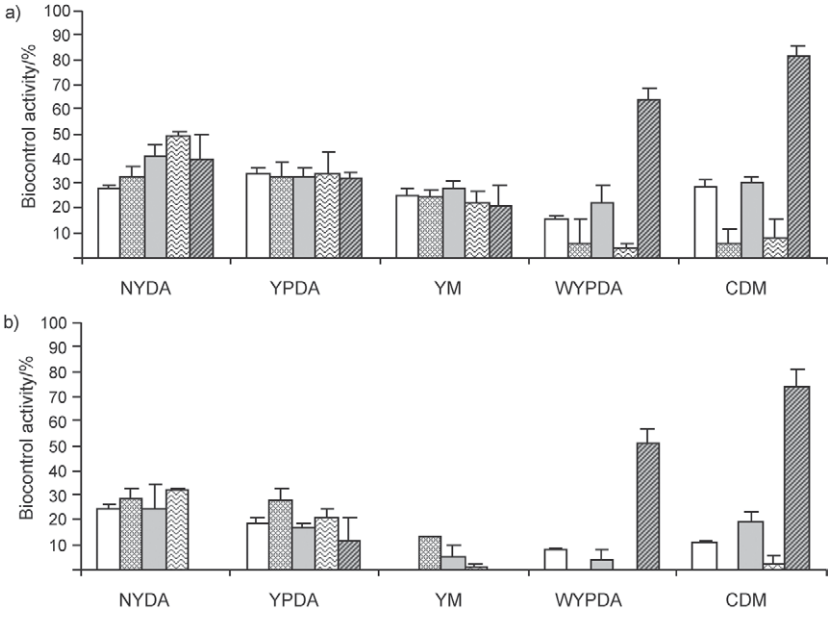


**Fig. 2.** Mean biocontrol activity (in %) of selected yeast strains against *B. cinerea* ZIM F58 growth on different solid media measured after: a) 7 days and b) 30 days of incubation at 25 °C. Bars represent standard errors of three parallel measurements and two independent repetitions

□ *Candida oleophila* NRRL Y-2317, ▨ *Aureobasidium pullulans* ZIM 2098, ▩ *Metschnikowia pulcherrima* ZIM 2055, ▧ *Pichia guilliermondii* ZIM 624, ▨ *Saccharomyces cerevisiae* ZIM 2180

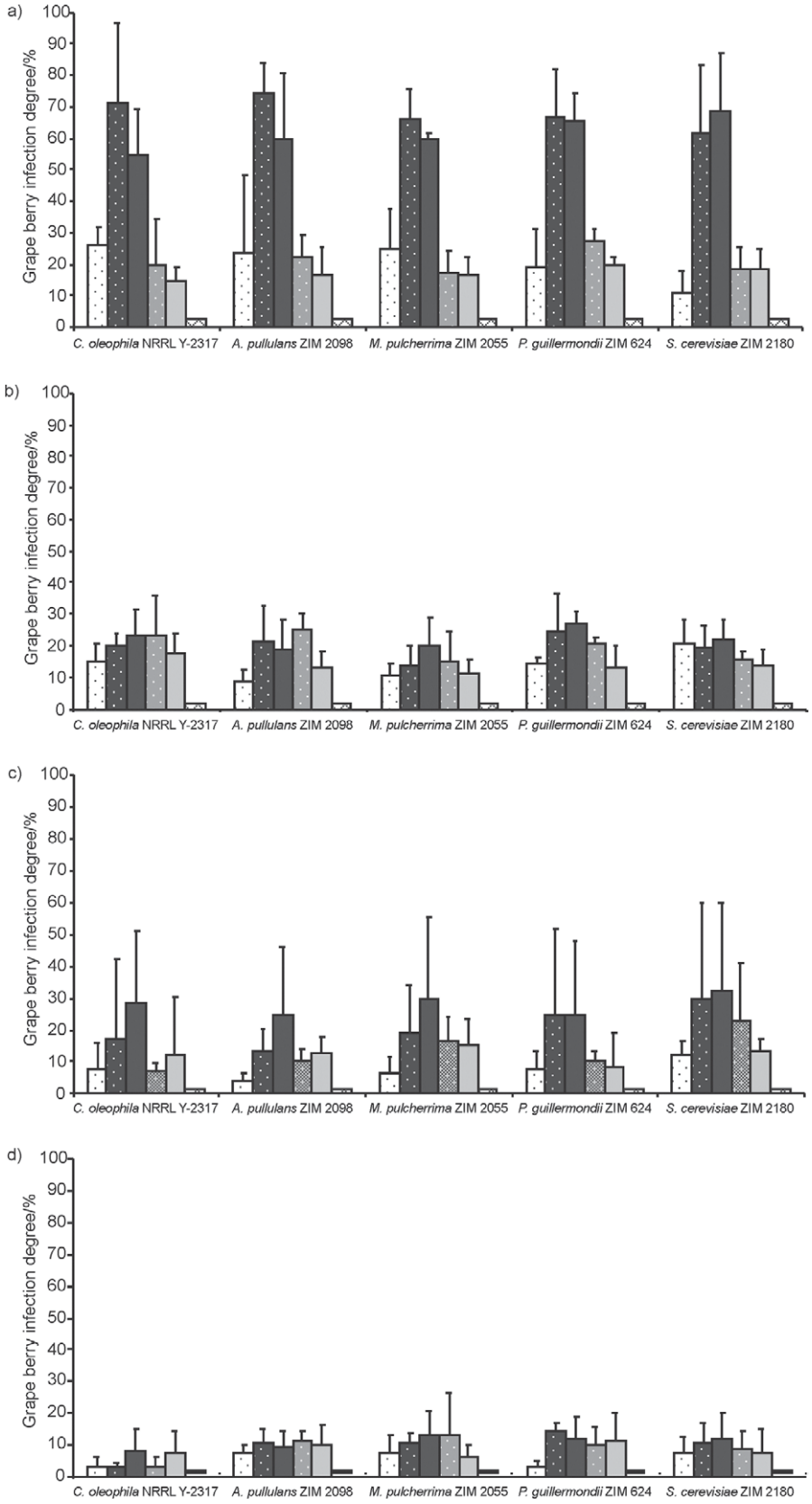


**Fig. 3.** Grape berry infection degree as a result of yeast strain biocontrol activity against three strains of *B. cinerea* on wounded and sprayed grape berries of two cultivars: a) grape berries cv. Rebula infected by needle, b) grape berries cv. Rebula infected by spraying, c) grape berries cv. Chardonnay infected by needle, d) grape berries cv. Chardonnay infected by spraying
□ yeast cell suspension, ▩ yeast cell and *B. cinerea* conidia suspensions applied simultaneously, ■ *B. cinerea* conidial suspension, ▨ yeast cell suspension applied at the beginning and *B. cinerea* spore suspension applied 24 hours later; ▨ solely *B. cinerea* spore suspension applied 24 hours later, ⊠ negative control

49.7 % infection degree, and the strain ZIM F63, isolated in France, showed 8.5 and 64.2 % infection degree on grape berries cv. Chardonnay and cv. Rebula, respectively. Similar results were obtained when different *B. cinerea* were applied by spraying (data not shown).

## Discussion

The best sources of antagonistic microorganisms are their natural environments in which they compete with naturally colonized microflora among which are also plant pathogens or spoilage microorganisms. For that reason we systematically screened for sources of antagonistic microorganisms which inhabit grape berry and compete with plant pathogens. We evaluated 591 strains for their biocontrol activities against *Botrytis cinerea.* For this purpose, 21 yeast species/genera isolated from grape/must/wine environments in Slovenia were used. The results of this study demonstrated that among microflora associated with grapes, there are some antagonistic yeasts which are able to control *Botrytis cinerea.* Even more, we found out that our yeasts were able to suppress causal agent of grey mould disease better than the commercially available *Candida oleophila*. Particularly efficient were found strains of *Aureobasidium pullulans, Metschnikowia pulcherrima, M. reukaufii* and *Pichia guilliermondii*. Strains of yeast-like fungus *A. pullulans* (*26,27*), pigment-producing yeast *M. pulcherrima* and its close relative *M. reukaufii* (*15,28*), as well as opportunistic human pathogen *P. guilliermondii* (*29*) had already been shown to possess the biocontrol activity against different plant pathogens or spoilage moulds on table grapes. Even though *Saccharomyces cerevisiae* exhibited low antagonistic effect against *B. cinerea* on general synthetic media used for testing biocontrol activity, the strain ZIM 2180 showed high biocontrol activity on media which simulate grape juice. *S. cerevisiae* strains were already found to possess biocontrol activities by Suzzi *et al.* (*30*). The mechanism of *S. cerevisiae*'s antagonism is probably based on the production of killer toxins. Nevertheless, our study has confirmed the observations of other authors that the biocontrol activity is a strain-dependant characteristic (*15, 30,31*) due to high variance among the tested strains of the same species (Table 1).

The results of our study show that the composition of solid medium has a strong impact on the biocontrol activity of yeast strains. The biocontrol activity of *C. oleophila* ZIM 2276, *A. pullulans* 2098, *M. pulcherrima* ZIM 2055 and *P. guilliermondii* ZIM 624 on NYDA and YPDA media gave very similar results although NYDA medium contains NaCl (2 g/L). On YM medium, which has a similar composition as YPDA but with added malt extract, the yeasts showed reduced biocontrol activities. Since competition for nutrients is one of the major modes of action, the exogenous substances such as amino acids or carbohydrates enhance biocontrol capability of antagonists against fungal pathogens (*32*). Moreover, salt additives such as calcium chloride, calcium propionate, sodium carbonate, sodium bicarbonate or potassium metabisulphite can be used in combinations with microbial antagonists (*33*). Similarly, specific medium composition requirements were reported to be responsible for the production of volatile alkaline compounds (*e.g.* ammonia) by yeast colonies (*34*), Avbelj and Raspor (unpublished results). Volatile compounds were shown to mediate the inter-colony signal but could have a possible antagonistic effect and potential use in the biocontrol of pathogens.

Table 1. Number of total yeast strains tested in the study and their biocontrol activity against *Botrytis cinerea* on NYDA medium measured after 7 and 30 days of incubation

| Yeast species | No. of strains | Biocontrol activity±S.D./% | |
|---|---|---|---|
| | | 7 days | 30 days |
| ***Candida oleophila*** | 1 | **24.1** | **24.1** |
| *Aureobasidium pullulans* | 24 | 41.5±4.9 | 40.2±5.6 |
| *Bulleromyces albus* | 10 | 25.7±7.7 | 7.6±10.5 |
| *Candida diversa* | 2 | 32.2±1.6 | 21.4±9.8 |
| *Candida glabrata* | 13 | 34.9±6.2 | 14.1±13.2 |
| *Candida norvegica* | 2 | 24.7±0.01 | 24.7±0.01 |
| *Candida zemplinina* | 16 | 18.1±4.2 | 6.7±5.5 |
| *Candida vini* | 2 | 32.5±0.05 | 16.4±7.3 |
| *Cryptococcus* spp. | 97 | 10.6±5.9 | 0.9±2.6 |
| *Debaryomyces hansenii* | 37 | 17.6±9.8 | 5.6±8.5 |
| *Hanseniaspora uvarum* | 75 | 2.9±5.1 | 0 |
| *Metschnikowia pulcherrima* | 46 | 39.1±6.5 | 33.1±5.2 |
| *Metschnikowia reukaufii* | 4 | 30.3±2.3 | 27.9±2.9 |
| *Pichia guilliermondii* | 7 | 32.1±10.7 | 27.9±14.7 |
| *Pichia kluyveri* | 10 | 24.9±9.1 | 20.7±10.3 |
| *Pichia membranifaciens* | 11 | 24.2±9.2 | 19.4±7.6 |
| *Rhodotorula aurantiaca* | 10 | 4.6±3.6 | 0.1±0.3 |
| *Rhodotorula glutinis* | 62 | 20.1±6.9 | 5.4±6.8 |
| *Saccharomyces bayanus* | 4 | 16.4±5.1 | 0.0±0.0 |
| *Saccharomyces cerevisiae* | 56 | 18.9±7.9 | 8.1±7.9 |
| *Sporidiobolus pararoseus* | 29 | 10.8±8.4 | 1.2±3.3 |
| *Sporobolomyces roseus* | 74 | 7.3±7.0 | 1.2±4.5 |
| Total | 592 | | |

Two solid media with grape juice-like composition were included in our experiment. On WYPDA and CDM media containing 200 g/L of glucose and different concentrations of macro- and micro-elements, some strains of *S. cerevisiae* showed high biocontrol activity. These observations might be connected to *S. cerevisiae* tolerance of environmental stresses, such as high sugar content and the ability to produce ethanol in the presence of oxygen (Crabtree effect), but further studies should be performed. However, ethanol was found to control postharvest diseases of grapes caused by *B. cinerea* in concentrations greater than 10 % (*35,36*). Additionally, *S. cerevisiae* showed higher biocontrol activity on CDM medium compared to WYPDA medium, which could be due to $CaCl_2$ (0.44 g/L) content since calcium salts were found to enhance biocontrol capability of antagonistic yeasts. Another reason for higher biocontrol activity could also be nitrogenous compounds in CDM medium, which according to Sharma *et al.* (*33*) considerably enhance the efficacy of microbial antagonists.

Because *in vitro* testing often does not correlate with *in situ* biocontrol results (*37*), a *semi in vivo* biocontrol

activity assay was developed to test antagonistic yeast strains against different strains of *B. cinerea* on grape berries of Rebula and Chardonnay grapevine cultivars. Grape berries of cv. Rebula were more susceptible to *B. cinerea* infection compared to grape berries of cv. Chardonnay. The differences found in the degree of *B. cinerea* infection of grape cultivars can be explained by physical and chemical properties of grape berries (*38*). The ability to lower the infection degree on table grapes has been published for yeast species *M. pulcherrima* (*15*), *A. pullulans* (*26,27*) and *P. guilliermondii* (*29*). It is interesting to mention that in our case, when yeasts were applied on grape berries together with *B. cinerea*, the degree of infection was higher than in the control group treated only with *B. cinerea*. However, we did not test their strains. Twenty years ago, yeast species *Hanseniaspora uvarum*, *Saccharomycopsis vini*, *Kluyveromyces lactis*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii* were found highly invasive, causing grape decay (*39*), but today, at least for some strains, their important role in wine fermentation has been established.

So far, studies for preventing *B. cinerea*-incited diseases have mostly been carried out on table grapes with the intention of prolonging their shelf life and not so much for preventing the occurrence and development of this disease in vineyards. Our results point out that *Saccharomyces cerevisiae* has a potential as a biocontrol agent against *B. cinerea* diseases on grapes, but further studies should be performed. A special attention should be paid to the fact that *S. cerevisiae* is isolated from natural environments with extreme difficulties (*9,40,41*). The future investigations, therefore, should be orientated towards microorganisms that are highly and consistently effective under the field conditions.

## Conclusions

The main conclusions of our current work can be summarized as follows: (*i*) some yeast species/strains isolated from vine/grape/must ecosystems in Slovenia inhibited the growth of filamentous fungus *B. cinerea*; (*ii*) yeast species *A. pullulans*, *M. pulcherrima*, *P. guilliermondii* have been found as biocontrol active; (*iii*) the composition of solid medium affects the biocontrol activity of yeast species; and (*iv*) *Saccharomyces cerevisiae* inhibited the growth of *B. cinerea* by 51 % on WYPDA and by 74 % on CDM solid medium. After a book written by Carson (*42*) was published, new ideas have started to flow into the field of environmental treatments. Our work illustrates that further investigations of biocontrol activity of wine yeast against fungi is needed.

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