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In vitro control of *Botrytis cinerea* and *Penicillium italicum* by antagonistic yeasts

Controle *in vitro* de *Botrytis cinerea* e *Penicillium italicum* por leveduras antagonistas

Renata Mori Thomé[1*]; Luiz Vitor Barbosa de Oliveira[2]; Ciro Hideki Sumida[3]; Maria Isabel Balbi-Peña[4]

Highlights:
Volatiles from *Hanseniaspora opuntiae*, *Lachancea thermotolerans*, *Pichia caribbica,* and *Pichia manshurica* had an antagonistic effect on the mycelial growth of *B. cinerea*.
Metabolites from *P. caribbica* and *P. manshurica,* which are diffusible in the medium, inhibited the mycelial growth of *B. cinerea*.
Yeast strains did not show an antagonistic effect against *P. italicum.*

Abstract

Gray mold in grapes (caused by *Botrytis cinerea*) and blue mold in citrus (caused by *Penicillium italicum*) are post-harvest diseases that cause major losses in these crops. The control of these diseases is based on the use of synthetic chemical fungicides. The increase in regulatory policies and demand to reduce the application of pesticides, due to harmful effects on the environment and humans, have led to the search for more ecofriendly alternatives, such as biological control agents. Thus, the present work aims to verify the antagonistic potential of four yeast strains, *Pichia caribbica* (CCMA 0759), *Hanseniaspora opuntiae* (CCMA 0760), *Pichia manshurica* (CCMA 0762), and *Lachancea thermotolerans* (CCMA 0763), against of *B cinerea* and *P. italicum*. To assess the antagonism of volatile compounds, Petri plates with two divisions containing potato-dextrose-agar (PDA) were used by placing a fungal mycelial disc and yeast suspension (3.0×10^6 cells mL^{-1}) on opposite sides of the plate. The colony diameter and mycelial growth rate index of the fungi were evaluated via comparisons with the control plate without yeast. For the evaluation of the antagonism of diffusible substances in the medium, yeasts were striated 3 cm from the center of the plates containing PDA. After 48 h, a mycelial disc of each phytopathogen was placed in the center of the plates. The colony growth, inhibition halo, and mycelial growth rate index were evaluated via comparisons with the control plate. All yeast strains showed an antagonistic effect on the mycelial growth of *B. cinerea* in both tests. In the volatile compounds test, *H. opuntiae*, *L. thermotolerans*, *P. caribbica,* and *P. manshurica* inhibited mycelial growth by approximately 82%, 75%, 72%, and 50%, respectively. In the antagonism test of the diffusible substances in the medium, *P. caribbica* and *P. manshurica* inhibited mycelial growth by 58% and 33%, respectively. However, these yeast strains did not show an antagonistic effect against *P. italicum*. Thus, all isolates demonstrated potential to be tested as biocontrol agents of gray mold in post-harvest grape fruits.
Key words: Antagonism. Biological control. Blue mold. Gray mold. Volatile compounds.

[1] Discente do Curso de Doutorado do Programa de Pós-Graduação em Agronomia, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: rmtagronomia@gmail.com
[2] Discente do Curso de Graduação em Agronomia, UEL, Londrina, PR, Brasil. E-mail: luizvito@outlook.com.br
[3] Prof. Dr., Departamento de Agronomia, Universidade Estadual de Londrina, UEL, PR, Brasil. E-mail: cirosumida@hotmail.com
[4] Profª Drª, Departamento de Agronomia, Programa de Pós-Graduação em Agronomia, UEL, Londrina, PR, Brasil. E-mail: mariabalbi@uel.br
[*] Author for correspondence

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Resumo

O mofo cinzento na uva (causada por *Botrytis cinerea*) e o bolor azul nos citros (causada por *Penicillium italicum)* são doenças de pós-colheita que causam perdas importantes nestas culturas. O controle destas doenças é baseado na utilização de fungicidas químicos sintéticos. O aumento das políticas regulatórias e a demanda para reduzir a aplicação de agrotóxicos, devido a efeitos prejudiciais ao meio ambiente e aos seres humanos, levam à procura de alternativas mais ecológicas como o uso de agentes de controle biológico. Desta forma, o presente trabalho teve por finalidade analisar o potencial antagônico de quatro cepas de leveduras, *Pichia caribbica* (CCMA 0759)*, Hanseniaspora opuntiae* (CCMA 0760)*, Pichia manshurica* (CCMA 0762) *e Lachancea thermotolerans* (CCMA 0763) no controle de *B. cinerea* e *P. italicum*. Para avaliar o antagonismo por compostos voláteis, foram utilizadas placas bipartidas contendo meio batata-dextrose-ágar (BDA) onde foram colocados um disco micelial dos fungos e uma suspensão de $3{,}0x10^6$ cél. mL^{-1} das leveduras em lados opostos da placa. Avaliou-se o diâmetro da colônia e índice de velocidade de crescimento micelial dos fungos, comparando-a com a placa controle sem a levedura. Para a avaliação do antagonismo de substâncias difusíveis no meio, as leveduras foram estriadas a 3 cm do centro de placas contendo meio BDA e após 48 h colocou-se um disco micelial de cada fitopatógeno no centro das mesmas. Avaliou-se o crescimento da colônia, a formação do halo de inibição e o índice de velocidade de crescimento micelial comparando-os com a placa controle. Todos os isolados apresentaram efeito antagônico no crescimento micelial de *B. cinerea* nos dois testes realizados. *H. opuntiae*, *L. thermotolerans*, *P. caribbica* e *P. manshurica* inibiram o crescimento micelial em aproximadamente 82%, 75%, 72% e 50%, respectivamente, no teste de compostos voláteis. *P. caribbica* e *P. manshurica* inibiram o crescimento micelial em 58% e 33%, respectivamente, no teste de antagonismo por substâncias difusíveis no meio. Contudo, nos testes com *P. italicum* as leveduras não apresentaram efeito antagônico. Deste modo, todas as cepas utilizadas apresentam potencial para ser testados no controle de mofo cinzento em uva.

Palavras-chave: Antagonismo. Bolor azul. Compostos voláteis. Controle Biológico. Mofo cinzento.

A large number of plant diseases are responsible for significant losses in crops with large socioeconomic impacts, causing annual losses estimated at $40 billion worldwide (Syed Ab Rahman, Singh, Pieterse, & Schenk, 2018). Today, diseases caused by fungi constitute 64-67% of the total globally reported diseases. Fungi account for yield losses of 20%, with a further 10% loss at the post-harvest level (Fisher, Hawkins, Sanglard, & Gurr, 2018). For decades, fungicides have been used to control fungi-induced diseases. However, the increase in global regulatory policies and demand to reduce the application of fungicides, due to possible harmful side effects to the environment and humans, have led to the search for new ecofriendly alternatives (Ferreira-Saab et al., 2018).

Fruits are crucial for human health and well-being, as they provide various essential nutrients, such as vitamins, antioxidant compounds, and minerals. Although the use of fungicides brings many advantages, such as better yields and quality, they are one of the most toxic, stable, and mobile substances in the environment. Fungicides can penetrate the tissues of fruits, appearing in the pulp and juice and, depending upon the properties of their active ingredients, may cause adverse side effects, from allergies to chronic diseases and cancer (Lozowicka, Hrynki, Kaczynski, Jankowska, & Rutkowska, 2016).

The grapevine is prone to fungal diseases that affect various stages of plant development, causing reduced productivity, affecting fruit aesthetics, and altering the final quality of the derivatives (Campos Nogueira, Ferrari, & Töfoli, 2017). The necrotrophic fungus *Botrytis cinerea* Pers. (1794), teleomorph *Botryotinia fuckeliana* (De Bary) Whetzel (1945), colonizes senescent or dead plant tissues, developing diseases, such as gray mold

and/or fruit rot (Martínez-Hidalgo, García, & Pozo, 2015). It has the ability to grow at low temperatures, almost at freezing point, which is one of the reasons why this pathogen becomes difficult to control (Parafati, Vitale, Restuccia, & Cirvilleri, 2015).

The typical symptom of grapevine disease is bunch rot, with the presence of a vast mass of gray-green spores, giving the berries a moldy appearance. Infection usually occurs when the plant blooms and the fungus remains latent until the fruit matures. Alternatively, direct infection occurs in ripe berries (Amorim, Spósito, & Kuniyuki, 2016). The infection of berries with conidia and mycelium produced in neighboring tissues or floral remains occurs mainly through wounds or cracks (Reglinski, Elmer, Taylor, Wood, & Hoyte, 2010).

Rot and/or mold caused by *Penicillium digitatum* (Pers.) Sacc. (1881), *P. italicum* Wehmer (1894), and *Geotrichum citri-aurantii* (Ferraris) EE Butler. (1988) are the main diseases that cause post-harvest economic damage in citrus, as they affect the quantity and quality of the citrus fruits, which diminishes their trade value (Moura, Moretto, Machado, & Kupper, 2019).

Molds cause soft rot in fruits. Soft rot begins with the appearance of small water-soaked areas on the surface of the fruit peel, which increase in size, eventually encompassing the entire fruit surface. The fungus develops a white mycelium on the tissue, which is covered by a dense mass of spores. The sporulant area has a blue, blue-green, and/or olive-green color (Bassanezi et al., 2016), depending upon the species of *Penicillium*. Directly applying fungicides to fruits efficiently controls mold. However, these fruits retain residues and may cause poisoning due to ingestion or the intoxication of workers at the time of fungicide spraying (Piati, Schneider, & Nozaki, 2011).

To reduce the application of pesticides for controlling diseases, the use of biological control agents, such as yeasts, has been proposed. These microorganisms do not present a health risk and can be found on the fruit surface (Coelho et al., 2007). They are ideal candidates for the biocontrol of phytopathogens in fruits, as they are not nutritionally demanding, multiply quickly, are resistant to adverse environmental conditions, and do not produce metabolites or mycotoxins that affect human health (Ruiz-Moyano et al., 2016). Concerning food security, the use of these microorganisms does not leave residues in the fruits that could be consumed (Kupper, Cervantes, Klein, & Silva, 2013).

Yeasts can also secrete antimicrobial compounds, such as killer toxins (Santos, Oliveira, Tonial, Yamaguchi, & Coelho, 2016). Killer toxins are acid proteins that are lethal to sensitive microbiota, causing cell death via the interruption of cell division by blocking DNA synthesis, the inhibition of the synthesis of the cell wall component β-1,3-glucan, and ion leakage caused by the formation of channels in the cytoplasmic membrane (Hatoum, Labrie, & Fliss, 2012).

The present work aims to evaluate the antagonistic potential of the yeasts *Pichia caribbica* (CCMA 0759), *Hanseniaspora opuntiae* (CCMA 0760), *Pichia manshurica* (CCMA 0762), and *Lachancea thermotolerans* (CCMA 0763) against *B. cinerea* and *P. italicum,* which are causal agents of gray mold in grapes and blue mold in citrus, respectively.

The maintenance of the yeasts, maintenance of the pathogenic fungi, and execution of the experiments were done at the Plant Pathology Laboratory of the Universidade Estadual de Londrina - UEL. The yeast strains belonged to the Agricultural Microbiology Culture Collection - CCMA at the Laboratory of Physiology and Genetics of Microorganisms of the Graduate Program in Agricultural Microbiology at the Universidade Federal de Lavras (UFLA). Yeasts were maintained on PDA (15 g L^{-1} of agar, 20 g L^{-1} of dextrose, and 200 g L^{-1} of potato) plates at 25ºC, with a photoperiod of 12/12 h. The fungus *P. italicum* was isolated from the surface of orange fruits that had the typical signs and symptoms of

blue mold. The isolate of *B. cinerea* belongs to the fungi collection of the Laboratory of Phytopathology - UEL. For the experiments, the yeasts and fungi were grown in YEPD (20 g L^{-1} of peptone, 10 g L^{-1} of yeast extract, 20 g L^{-1} of dextrose, and 20 g L^{-1} of agar) for 48-72 h and PDA for 7 days, respectively, at 25ºC.

To assess the antagonistic activity against phytopathogenic fungi, yeasts were streaked orthogonally from the center of a Petri dish containing PDA. Five plates were used for each yeast and fungus. After incubation at 25ºC for 48 h, mycelial discs (6 mm in diameter) of the fungi were inoculated onto the plates, 3 cm away from the yeast. Control plates, only inoculated with the fungi, were also prepared (Parafati et al., 2015). At the end of the incubation period, the reduction in mycelial growth was calculated in relation to the growth of the control using the following formula:

$$\mathrm{IMG}(\%) = \frac{(\mathrm{DC} - \mathrm{DT})}{\mathrm{DC}}\ x\ 100$$

where IMG (%) represents the inhibition of mycelial growth, DC is the fungus colony diameter in the control treatment, and DT is the fungus colony diameter in the presence of yeast. The mycelial growth rate index (MGRI) was calculated using the following equation:

$$\mathrm{MGRI} = \sum \frac{(\mathrm{D} - \mathrm{Dp})}{\mathrm{N}}$$

where D is the current average diameter of the colony, Dp is the average diameter of the colony on the previous day, and N is the number of days after incubation. The experiments were repeated three times.

To determine the production of volatile compounds, the methodology of Rezende, Fialho, Brand, Blumer and Pascholati (2015) with modifications was adopted. *B. cinerea* or *P. italicum* were cultivated simultaneously with the yeast using Petri plates with two divisions, to prevent the non-volatile exudates produced by the yeast from entering into contact with the fungi through the culture medium. A 6 mm diameter mycelial disk of *B. cinerea* or *P. italicum* was placed on one side of the plate containing PDA. Meanwhile, on the other side, 50 µl of a yeast suspension (3.0×10^6 cells m L^{-1}) was streaked on the surface of the medium with a Drigalski loop. The plates were immediately sealed with plastic film and incubated for 4 days at 25ºC with a 12/12 h photoperiod. Daily measurements of the diameter of the *P. italicum* and *B. cinerea* colonies were performed in two perpendicular directions for the MGRI and IMG calculations.

The normality of the data and homogeneity of variances were verified by the Shapiro-Wilk and Bartlett tests, respectively. Data were submitted to an analysis of variance (ANOVA) with a significance level of 5%. IMG data of *P. italicum* were transformed for analysis by $\sqrt{(x+0,5)}$. When treatment effects were significant, means were separated by the Tukey test ($p \leq 0.05$). Statistical analyses were performed using SISVAR software.

The volatile compounds of the CCMA0759, CCMA0760, CCMA0762, and CCMA0763 strains reduced the mycelial growth of *B. cinerea,* differing statistically from the control (Table 1). All yeasts strains affected the final diameter of the colony and speed of the mycelial growth of *B. cinerea*, but this decrease was greater in confrontation against CCMA0760, CCMA0763, and CCMA0759. These yeast strains inhibited *B. cinerea* mycelial growth by 72-82%, demonstrating their potential as biocontrol agents of gray mold in the post-harvest of grapes through volatiles.

In the antagonism test of diffusible substances in the medium, the CCMA0759, CCMA0760, CCMA0762, and CCMA0763 strains showed an antagonistic effect on the mycelial growth of *B. cinerea,* which was verified through the MGRI, IMG, and inhibition halo of mycelial growth. For the IMG, all strains differed from the control. However, only the CCMA0759 strain showed a growth inhibition of *B. cinerea* that was greater than 50% (Table 2).

Table 1
Colony diameter on the third day, mycelial growth rate index (MGRI), inhibition of mycelial growth (IMG) of the fungus *Botrytis cinerea* (Bc) exposed to volatile compounds from *Pichia caribbica* (*CCMA* 0759), *Hanseniaspora opuntiae* (CCMA 0760), *Pichia manshurica* (CCMA 0762) and *Lachancea thermotolerans* (CCMA 0763) strains co-cultivated in Petri plates with two divisions

Treatments	Colony diameter on the 3rd day (cm)	MGRI (cm day^{-1})	IMG (%)
Bc (control)	3,97 a	1,52 a	0,00 c
Bc + *Pichia caribbica* CCMA 0759	1,10 c	0,31 c	72,20 a
Bc + *Hanseniaspora opuntiae* CCMA 0760	0,71 c	0,16 c	82,19 a
Bc + *Pichia manshurica* CCMA 0762	2,00 b	0,67 b	49,62 b
Bc + *Lachancea thermotolerans* CCMA 0763	1,00 c	0,26 c	74,76 a
CV (%)	17,6	14,8	12,5

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 2
Colony diameter on the fourth day, mycelial growth rate index (MGRI), inhibition of mycelial growth (IMG) and inhibition halo of *Botrytis cinerea* (Bc) co-cultivated with *Pichia caribbica* (CCMA 0759), *Hanseniaspora opuntiae* (CCMA 0760), *Pichia manshurica* (CCMA 0762) and *Lachancea thermotolerans* (CCMA 0763)

Treatments	Colony diameter on the 4th day (cm)	MGRI (cm day^{-1})	IMG (%)	Inhibition halo (cm)
Bc (control)	2,64 a	1,03 a	0,00 d	0,00 c
Bc + *Pichia caribbica* CCMA 0759	1,10 d	0,46 d	58,14 a	1,39 a
Bc + *Hanseniaspora opuntiae* CCMA 0760	2,09 bc	0,78 bc	20,39 bc	0,54 bc
Bc + *Pichia manshurica* CCMA 0762	1,76 c	0,69 c	33,17 b	0,79 b
Bc + *Lachancea thermotolerans* CCMA 0763	2,17 b	0,85 b	17,59 c	0,41 bc
CV (%)	9,57	9,19	28,68	47,0

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Tests for the antagonism of volatiles and diffusible substances (data not shown) of yeast strains against *P. italicum* showed the absence of antagonistic effects, except for a 30% reduction in the MGRI caused by the volatiles of strain CCMA0762.

Ruiz-Moyano et al. (2016) studied the effects of two yeast strains isolated from fig, *Hanseniaspora opuntiae* and *Metschnikowia pulcherrima,* against *Penicillium expansum, B. cinerea,* and *Monilinia laxa.* When *H. opuntiae* was tested, the authors verified a reduction in the mycelial growth of 45.2%, 53.4%, and 58.2%, respectively. In comparison, when *M. pulcherrima* was tested, mycelial growth reduced by 63.7%, 61.1%, and 66.09%, respectively.

Heling et al. (2017) tested the effects of *Saccharomyces cerevisiae* and *S. boulardii* on *Colletotrichum musae*, the causal agent of anthracnose in banana, and verified that volatile compounds from these yeasts reduced the number of fungal conidia. These strains also caused inhibition halos of 0.28 and 0.44 cm, respectively, showing that these yeasts produce metabolites with antagonistic properties against *C. musae*.

In a study with 24 yeast isolates, Oliveira, Rabelo, Portes and Coelho (2011) reported that

22 isolates reduced the development of *B. cinerea* isolated from strawberries. Fifteen of these isolates showed antagonism by nutrient competition and only two strains showed antagonism by nutrient competition and antibiosis, which was verified by the formation of inhibition halos with diameters of 28.88 and 29.50 mm.

Strains of *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus* showed antagonism against *Colletotrichum gloeosporioides*, with a reduction in the mycelial growth of 70% and 50%, respectively, when compared to the control treatment (Lima, Gonçalvez, Brandão, Rosa, & Viana, 2013).

A study by Kupper et al. (2013) on the antagonistic activity of 6 isolates of *S. cerevisiae* on the mycelial growth of *P. digitatum*, showed that although all the tested isolates affected the development of the fungus, the ACB-CR1 and ACB-K1 isolates showed the best results, causing inhibitions of the pathogen colony of 42% and 47%, respectively.

Lahlali, Hamadi, El Guilli and Haissam Kikakli (2011), testing *Pichia guilliermondii* strain Z1 at a concentration of 1×10^8 CFU mL^{-1}, obtained the suppression of *P. italicum*, reducing the incidence of citrus blue mold by 85%. According to these authors, the effectiveness of a yeast as a biological control agent over a phytopathogen is dependent upon the concentrations of the antagonist and inoculum of the pathogen. In our experiments, the antagonism of non-volatile compounds was performed without quantifying the yeast concentration and the volatile test was performed with a yeast suspension concentration of 10^6, which may have been too low to exert antagonism against *P. italicum*.

Under the experimental conditions, our results indicate that the yeast strains tested (CCMA 0759 *Pichia caribbica*, CCMA 0760 *Hanseniaspora opuntiae*, CCMA 0762 *Pichia manshurica*, and CCMA 0763 *Lachancea thermotolerans*) showed *in vitro* antagonistic effects against *B. cinerea,* but they did not produce volatile substances or diffusible substances in the medium with significant antagonism against *P. italicum*. Therefore, these yeast strains should be tested as biocontrol agents of gray mold in the post-harvest of grapes.

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