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Running head: *R. fluviale* as biocontrol agent

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Evaluation of *Rhodosporidium fluviale* as biocontrol agent against *Botrytis cinerea* on apple fruit

Significance and Impact of the Study

Botrytis cinerea Pers: Fr, which causes gray mold of fruits and vegetables around the world, is difficult to control successfully because it is genetically variable and rapidly develops resistance to the chemicals commonly used for its control. The present study is a contribution to the biocontrol of this phytopathogen fungus. The evaluation of the native yeast *Rhodosporidium fluviale* as biocontrol agent and the elucidation of possible mechanisms of action, including the participation of nonviable cells of this yeast, have not been reported up to date.

Abstract

The aim of the present work was to evaluate the ability of the native yeast *Rhodosporidium fluviale* to control *Botrytis cinerea* on apple fruit and to study the possible mechanisms of action with the goal of improving the control of gray mold. For this, the influence of application time of the yeast was studied simulating preventive and curative effects. Also, the effect of nonviable cells of the yeast in the biocontrol was assessed. According to the results obtained, the following mechanisms of action of *Rhodosporidium fluviale* could be proposed: 1-competition for space, 2-direct interaction between antagonist and pathogen, 3- induction of β-1,3-glucanase in apple tissue, 4- Probable production of glucanase in the apple wounds and 5- antifungal action of cellular components, probably chitin, present in the wall of yeast cells that could be the explanation for the activity of nonviable cells.

Keywords: *Botrytis cinerea*; *Rhodosporidium fluviale*; Biocontrol mechanisms; Postharvest diseases; Gray mold

Introduction

In Argentina, around 900,000 tons of apple fruits are produced per year. The discarding of fruit due to diseases pre and post harvest exceeds 25% (Ministerio de Hacienda y Finanzas Públicas de Argentina 2016). The main causes of discarding in postharvest are fungal diseases, especially those caused by *Botrytis cinerea* and *Penicillium expansum* (Spadaro and Droby 2016). *Botrytis cinerea* Pers: Fr, which causes gray mold of fruits and vegetables around the world, is difficult to control successfully because it is genetically variable and rapidly develops resistance to the chemicals commonly used for its control (Pertot *et al*. 2017). Among the new strategies for the management of this fungus is the use of biocontrol agents, alone or in mixtures, or combined with different substances (Calvo *et al*. 2010; Parafati *et al*. 2015). A major goal in the development and implementation of successful biological control products is to increase the ability of antagonists to control postharvest diseases in a broader range of conditions and with minimal variability. The capacity to control preexisting infections inflicted during harvesting and transport, as is possible with synthetic fungicides, is also highly desirable.

The evaluation of a potential biological control agent should include the study of its mechanisms to control the pathogen. This knowledge will help to decide the best time for its application and also to strengthen its control mechanisms.

A biocontrol agent antagonizes a plant pathogen by competition for nutrients and space or by the production of enzymes or other compounds with antimicrobial action (Nunes 2012). Furthermore, a secondary mechanism of action is the ability of a biocontrol agent to induce resistance in the host plants. The β-1,3-glucanase (EC 3.2.1.39) is a protein of resistance, constitutively expressed in low concentration. This enzyme is involved in the defense mechanisms and its induction in fruit tissues, by different chemical and microbial elicitors, has been reported (Spadaro and Droby 2016; Romanazzi *et al*. 2016).

In this work, we evaluated the ability of the yeast *Rhodosporidium fluviale*, isolated in our laboratory from apple skin, to control *B. cinerea* on apple fruit. For this, the influence of timing of yeast application, and the production of enzymes or siderophores were studied. Also, the induction of β-1,3-glucanase, a resistance enzyme, in apple tissue was investigated. Taking into account that any method of preservation of a biocontrol agent reduces its viability (Navarta *et al.* 2014) the effect of nonviable cells in the biocontrol was assessed.

Results and Discussion

Effect of *Rhodosporidium fluviale* on conidial germination of *Botrytis cinerea*

The ability of yeast *R. fluviale* to prevent the colonization of the pathogen (preventive effect) or to control the pathogen already installed (curative effect) was evaluated by observing its effect on the germination of conidia of two strains of *B. cinerea*. The selected strains, BNM 0527 and BNM 0528, have different pathogenicity characteristics. Despite the fact that the growth rate of *B.cinerea* BNM 0528 is greater than that of strain BNM 0527 (Sansone *et al*.2011), the latter is the most pathogenic. According to Sansone *et al*. (2011), the highest pathogenicity of *B.cinerea* BNM 0527 is related to the higher production of polygalacturonase and laccase, which are attack enzymes of this pathogen.

Results of the inhibition of germination are shown in Table 1. In general, the BNM 0527 strain was the most inhibited by *R. fluviale*. According to the percentage of germinated conidia at 23 h, the preventive effect of viable cells was higher than the curative one for both *Botrytis* strains.

In the case of the preventive effect, the strong inhibitory effect on germination could be due to a mechanism of competition for space (Spadaro and Droby 2016) taking account that the specific growth rate of *R. fluviale* was 0.46 h^{-1} while that of *B. cinerea* was 0.13 h^{-1}. Also, it could be postulated a direct interaction yeast/pathogen as another mechanism for controlling *B. cinerea*.

According to Figures 1b and 1c, this mechanism appears to be more effective for the preventive than curative effect. On the other hand, because siderophores were not detected in the culture supernatant of *R. fluviale*, the competition for iron was discarded as a mechanism of control.

The results of the inhibition of germination, in the case of the curative effect, showed a greater efficiency of non-viable cells for *B.cinerea* BNM 0527. Perhaps, the presence of chitin in the wall of the yeast, classified as a Basidiomycete (Spencer and Spencer 1997), exposed by the thermic treatment was the cause of its capacity for antagonizing the phytopathogen. FTIR studies of nonviable cell suspensions showed the presence of chitin (data not shown), and various authors have demonstrated that this compound for its antifungal activity may be useful in the control of postharvest fungal diseases (Lu *et al*. 2014; Fu *et al*. 2016). *B.cinerea* 0528 was also inhibited by non-viable *Rhodosporidium* cells but it seemed less sensitive to them than the BNM 0527 strain.

Effectiveness of *Rhodosporidium fluviale* for controlling *Botrytis cinerea* on apple

Figure 2 shows the results of simulation of preventive and curative effects, using viable and nonviable yeast cells, in apple at 15°C. This temperature was chosen because the optimal germination temperature of the *B.cinerea* conidia is around 15-20ºC and the mycelium requires a humid and moderately cold environment (18 to 23ºC) to grow and expand the infection (Droby and Lichter 2004). For preventive effect, in the case of *B. cinerea* BNM 0527, the percentages of severity reduction reached by viable yeast cells were 55% and 75% at 5 and 10 days, respectively. While with *B. cinerea* BNM 0528, the reduction reached 55% at 10 days. On the other hand, the reduction in the severity of the lesion by non-viable cells at 10 days was 53% for strain BNM 0527 and 20% for the other strain.

The results of the curative effect are also shown in Figure 2. There, it can be observed that for the strain BNM 0527 the reduction of decay was 48% at 10 days using viable cells, and there were no significant differences with the treatments of non-viable cells. Reduction of decay was

minor for BNM 0528 strain and reached 35% and 25% with viable cells and nonviable cells respectively.

Botrytis control was more effective when yeast was present prior to infection (preventive effect) than when infection was already in place (curative effect). In general, *B. cinerea* BNM 0527 was more controlled than *B. cinerea* BNM 0528 and this fact was independent of the type of treatment (viable or non-viable cells) and the time of application (preventive or curative effect).

Observation of the interactions by Electron Microscopy

Figure 3 shows electron microscopy images of apple tissue samples took after 48 h of the inoculation with *R. fluviale* and *B. cinerea* BNM 0527. Figure 3a shows host-antagonist interaction while Figure 3b correspond to images of apple wounds inoculated with the antagonist and the pathogen (host-pathogen-antagonist interaction). This last evidences a predominance of the yeast grouped and fixed to the *Botrytis* hyphae. These electron micrographs confirm the competition by space and direct interaction as a mechanism of action.

β-1,3-glucanase activity in apple tissue

Figure 4 shows the results of β-1,3-glucanase activity in apple tissue. At five days of incubation, in apples treated with viable *R. fluviale* cells, the activity of glucanase was 27 UE.g^{-1} tissue (f.w.). This activity was significantly higher than activities recorded for the other treatments with exception of antagonist plus pathogen treatment. In this last case, activity reached values of 50 and 35 UE.g^{-1} tissue (f.w.) with *B. cinerea* BNM 0527 and *B. cinerea* BNM 0528 respectively.

At ten days of storage, all treatments increased the enzyme activity in the apple tissue. The glucanase activity in apples treated with viable *R. fluviale* cells was close to 50 UE.g^{-1} tissue (f.w.) and the activity values obtained with the mixtures *R. fluviale-B. cinerea* BNM 0527 and *R. fluviale-B.*

cinerea BNM 0528 were 65 and 44 UE.g^{-1}tissue (f.w) respectively. There was not a difference between treatments with nonviable cells or water.

While the results obtained in the treatments with non-viable cells and water showed an induction of glucanase in the apple tissue in response to the injury, the glucanase production by the antagonist could be an explanation of the results obtained with the viable cells and the mixtures. Despite the negative result obtained in the "in vitro" test to evaluate the production of glucanase by *R. fluviale*, it cannot be ruled out that the yeast produces this enzyme in the wounds of the apple. Perhaps the medium provided by the apple tissue is more favorable for the production of glucanase than the medium used in the in vitro test. The high activity of glucanase caused by the treatment with the mixture of the viable *Rhodosporidium cells* and the BNM 0527 strain is also remarkable. Zhang et al (2016) reported similar results when co-inoculated *Streptomyces rochei* A1 and the pathogen *Botryosphaeria dothidea* in apple fruit.

In conclusion, we demonstrated that *R. fluviale* has potential as a biological control agent against *B. cinerea.* Viable and nonviable cells of *R. fluviale* had the ability for reducing the severity of the decay produced by both strains of *B. cinerea*; however, the BNM 0527 strain was more effectively controlled.

According to the results obtained, the following mechanisms of action of *Rhodosporidium fluviale* could be proposed: 1-competition for space, 2- direct interaction between antagonist and pathogen, 3- induction of β-1,3-glucanase in apple tissue, 4- Probable production of glucanase in the apple wounds and 5- antifungal action of cellular components, probably chitin, present in the wall of yeast cells that could be the explanation for the activity of nonviable cells.

Another interesting conclusion is about the timing of application of the antagonist. Perhaps the best results could be obtained with the application of *R. fluviale* in the preharvest, because not only this could help the competition but also favor the stimulation of defense mechanisms of the host.

Materials and Methods

Microorganisms

Two strains of *Botrytis cinerea* (BNM 0527 and BNM 0528) isolated from apple rot were used in this study. For their molecular identification, it was required the service of molecular identification of Macrogen (Korea), which amplified the ITS1 and ITS4 regions using primers 5'(TCCGTAGGTGAACCTGCGG) 3` and 5'(TCCTCCGCTTATTGATATGC) 3'.

The *B. cinerea* strains were deposited in the National Bank of Microorganisms (WDCM938) of the Facultad de Agronomia, Universidad de Buenos Aires (FAUBA), Argentina.

As biocontrol agent was used *Rhodosporidium fluviale*, isolated from the skin of apples, and identified biochemically. For the molecular identification, Macrogen (Korea) amplified the 26SRNA sequence using the primers LR0R 5' (ACC CGC TGA ACT TAA GC) 3' and LR7 5 '(TAC TAC CAC CAA GAT CT) 3'.

Culture media and growth conditions

B. cinerea strains were cultured on Potato Dextrose Agar (Sigma Chemical Co, St. Louis) at 14 ºC. The concentration of conidia suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1 x 10^5conidia ml^{-1}.

R. fluviale was grown in potato dextrose broth (PDB) for 24 h at 28 °C, and then cells were harvested by centrifugation at 12,000 rpm for 15 min, washed and suspended in sterile distilled water. Cell concentration was adjusted to 1 x 10^6 cells ml^{-1}. For assays with nonviable cells, the suspension was heat-treated at 100 °C for 10 min

In vitro* assessment of the ability of *Rhodosporidium fluviale* for controlling *Botrytis cinerea

The assays of inhibition of conidia germination were performed using apple dextrose broth (ADB). For ADB, 200 g of apple were cut into 1 cm cubes and boiled with 1000 ml of distilled water for 20 minutes, mashed and squeezed through a muslin bag. Dextrose (10 g) was dissolved in the above extract and was made up to 1000 ml with distilled water. The pH was adjusted to 5.6 before sterilizing.

To assess the preventive effect, 200 µl of ADB and 200 µl of a suspension of *R. fluviale* were placed in 1.5 ml Eppendorf tubes. The mixture was incubated for two hours, and then 200 µl of the conidial suspension of *B. cinerea* (BNM 0527 or BNM 0528) were added.

To study the curative effect, 200 µl ADB and 200 µl of a conidial suspension of *B. cinerea* (BNM 0527 or BNM 0528) were put in 1.5 ml Eppendorf tubes. The mixture was incubated for five hours, and then 200 µl of the yeast suspension were added. In the control tubes, yeast suspension was replaced by sterile distilled water.

The Eppendorf tubes were maintained at 15° C, and samples of 50 µl were taken at 7 h and 23 h. Samples were put on microscope slides and 100 conidia per slide were evaluated. The preparations were observed with a light microscope (Olympia) at a magnification x 500 and the percentage of germinated conidia was calculated. The experiments were performed in triplicate.

For assessment the interaction between the yeast and the *B. cinerea* hyphae, the Eppendorf tubes were incubated to complete 48 h and their content was examined with a light microscope (Olympia) and recorded with a camera (Moticam 1SP 1.3 mp).

Growth kinetics of *B. cinerea* and *R. fluviale* were also made in ADB. For this, 100 ml of culture medium contained in 1000 ml Erlenmeyer flasks were inoculated with 10 ml of *R. fluviale* (1 x 10^6 cells ml^{-1}) or *B. cinerea* (1 x 10^5conidia ml^{-1}). The cultures were maintained on a rotary shaker (140 rpm) at 20°C. The assays were performed in triplicate.

Fruits

In all assays, Red Delicious apple fruit previously stored at 0 °C for six months was used. Before the assays, firmness, sugars, and pH were evaluated and the maturity parameters determined on fruit were 9,7 ± 0,91 $Kg.cm^{-2}$ of firmness, 15,48 ± 0,48 °Brix and pH 3,7 ± 0,07.

Effectiveness of *Rhodosporidium fluviale* for controlling *Botrytis cinerea* on apple

The effectiveness of *R. fluviale*, (viable and nonviable yeast cells) for controlling *B. cinerea* (BNM 0527 or BNM 0528) was evaluated on apples at 15°C.

Washed and disinfected apples were wounded on three opposite sides, with the tip of a disinfected 10-penny nail (3 mm diameter, 3 mm deep). For evaluating the preventive effect, an aliquot (20 µl) of suspension of *R. fluviale* at 1 x 10^6 cell ml^{-1} (viable or nonviable cells) was put into each wound site. Sterile distilled water was used as a control. Two hours later, 20 µl of a suspension of *B. cinerea* BNM 0527 or *B. cinerea* BNM 0528 (1 x 10^5conidia ml^{-1}) were inoculated. For assessing the curative effect, *B. cinerea* suspension was put into the wounds and two hours later the *R. fluviale* cells suspension was inoculated. The wounds were examined after five and ten days and the lesion

diameter (∅) was recorded. There were three replicates of three apples per treatment and the experiment was repeated twice. The results were expressed as follow:

% reduction in decay severity = [(∅ control -∅ treatment)/ ∅ control] x 100.

***In vivo* assessment of interactions by using electron microscopy**

For observing the interactions in apple tissues by electron microscopy, apple wounds were inoculated with the following suspensions: 1) 20 µl of *B. cinerea* BNM 0527, 2) 20 µl of *R. fluviale* viable cell, 3) 20 µl of *R. fluviale* viable cell plus 20 µl of *B. cinerea* BNM 0527. The apples were incubated at 15 ° C for 48 h. After this time, samples from the wound and healthy tissue were taken. Samples were fixed and dehydrated by the technique described by Chan and Tian (2005) and were observed in a scanning electron microscope (SEM, LEO 1450 VP)

Assessment of the production of siderophore and β-1,3-glucanase by *Rhodosporidium fluviale*

Production of siderophore by *R. fluviale* was investigated in solid medium according the methodology developed by Calvente *et al* (2001).

To assess the production of glucanase by *R. fluviale* the yeast was cultured in a medium contained yeast extract (1.5 g l^{-1}) and cell wall preparations (CWP) of *B. cinerea* (1 %) as the carbon source (Bar-Shimon *et al*. 2004). For the assay, 50 ml of culture medium contained in 250 ml Erlenmeyer flasks were inoculated with 5 ml of *R. fluviale* (1 x 10^6 cells ml^{-1}). The cultures were maintained at 15 °C on a rotary shaker (140 rpm) for 120 h. After this time, the cultures were centrifuged for 15 min at 12000 rpm using a Sorvall SS-3 centrifuge. Supernatants were used to determine the extracellular glucanase.

Activity of β-1, 3-glucanase in apple tissue

To assess the activity of β-1,3-glucanase, the apples were wounded with the tip of a disinfected 10-penny nail (3 mm diameter, 3 mm deep) on three opposite sides of each fruit. Wounds were inoculated with viable *R. fluviale* cells, nonviable *R. fluviale* cells, *B. cinerea*, viable *R. fluviale* cells plus *B. cinerea*, and nonviable *R. fluviale* cells plus *B. cinerea*. The treatments were carried out by using the two *Botrytis* strains. Apples were stored at 15°C during five and ten days.

For determining glucanase activity, a tissue sample (1 g) was suspended in 2 ml of 50 mM sodium acetate buffer, pH 5 and was homogenized using a homogenizer IKA Ultra- turra T 50 with steel blade at 3000 rpm. The homogenate was put into Eppendorf tubes and was centrifuged at 12000 rpm for 15 minutes using a refrigerated centrifuge at 4 ° C, (Presvac EPF-12). The supernatant obtained was used for the assay of β-1,3-glucanase activity. The assay was performed according Kulminskaya *et al* (2001). By definition, one unit of the β-1,3-glucanase activity was the amount of enzyme that catalyzes the release of 1 µM glucose $minute^{-1}$. The β-1,3-glucanase activity was expressed as Units per gram of fresh tissue ($UE.g^{-1}$ tissue f.w.). There were three replicates of three apples per treatment and the experiment was repeated twice.

Statistical analysis

The data were analyzed by means of the analysis of variance (ANOVA) using Statistical Software InfoStat, 2008. The statistically significant differences (at $p < 0.05$) were analyzed by the Test of Multiple Range of Duncan.

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Conflict of Interest

We have no conflict of interest to declare.

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Table 1

Effect of *Rhodosporidium fluviale* (viable or nonviable cells) on conidial germination of *Botrytis cinerea* BNM 0527 and *Botrytis cinerea* BNM 0528 at 15°C

Conidial Germination (%)		
	7 h	23 h
***B. cinerea* BNM 0527**		
Control	25 ± 6,0 d	51 ± 5,6 e
Preventive Effect – viable cells	3 ± 0,69 a	8 ± 2,1 a
Curative Effect - viable cells	16 ± 0,33 c	23 ± 5,3 b
Preventive Effect –nonviable cells	11 ± 4,33 bc	13 ± 3,12 a
Curative Effect – nonviable cells	10 ± 3,65 b	12 ± 3,3 a
***B. cinerea* BNM 0528**		
Control	44 ± 3,6 f	67 ± 5,3 f
Preventive Effect – viable cells	6 ± 1,5 ab	13 ± 3 a
Curative Effect- viable cell	39 ± 5,3 ef	37 ± 5,3 c
Preventive Effect – nonviable cells	28 ± 2,9 d	42 ± 3,9 cd
Curative Effect – nonviable cells	35 ± 4,2 e	48 ± 6,3 de

Means in the same column followed by the same letters are not significantly different at p <0.05.

Legends to Figures

Figure 1

In vitro* interaction between *Rhodosporidium fluviale* and *Botrytis cinerea

Light microscope micrographs of *B. cinerea* control (a), preventive effect (b) and curative effect (c)

Figure 2

Preventive and curative effects using viable and nonviable yeast cells of *Rhodosporidium fluviale* against *Botrytis cinerea* BNM 0527 and BNM 0528

For the preventive effect (**PE**) an aliquot (20 µl) of *R. fluviale* suspension (viable or nonviable cells) was put into each wound site, and after two hours, 20 µl of a suspension of *B. cinerea* was inoculated. For the curative effect (**CE**), the conidial suspension was inoculated before inoculation with the yeast. ( ) **PE**, viable cells+BNM0527; ( ) **PE**, nonviable cells+BNM0527; ( )**CE**, BNM0527+viable cells; ( )**CE**, BNM0527+nonviable cells; ( )**PE**, viable cells+BNM0528;

( ) **PE**, nonviable cells+BNM0528; ( ) **CE**, BNM0528+viable cells; ( ) **CE**, BNM0528+ nonviable cells. Apples were stored at 15°C for 5 days (5d) and 10 days (10d). Means with the same letters within the same set of columns are not statistically different at $p<0.05$.

∅ control: BNM 0527: 14,32 mm (5d), 53 mm (10d); BNM 0528: 9,8 mm(5d) , 44,26 mm (10d).

Figure 3

***In vivo* interactions between *Rhodosporidium fluviale, Botrytis cinerea,* and apple tissue**

SEM micrographs of apple wound inoculated with *R. fluviale* after 48 h of incubation (a) and apple wound inoculated with *R. fluviale and B. cinerea* BNM 0527 after 48 of incubation (b) (SEM, LEO 1450 VP)

Figure 4

Activity of β-1,3-glucanase in apple tissue

The following treatments were applied to apple wounds: 1) 40 µl of sterile distilled water, 2) 40 µl of viable *R. fluviale* cells, 3) 40 µl of nonviable *R. fluviale* cells, 4) 40 µl of *B. cinerea,* 5) 20 µl of viable *R. fluviale* and after 2 hours, 20 µl of *B. cinerea*, 6) 20 µl of nonviable *R. fluviale* and after 2 hours, 20 µl of *B. cinerea*.

(▨) Healthy Tissue; (▨) Water; (▨) *R. fluviale* viable cells; (▨) *R. fluviale* nonviable cells; (▨) BNM0527; (■) *R. fluviale* viable cells+BNM0527; (▨) *R. fluviale* nonviable cells+BNM0527; (▨) BNM0528; (▭) *R. fluviale* viable cells+BNM0528; (▨) *R. fluviale* nonviable cells+BNM0528

Means with the same letters within the same set of columns are not statistically different at $p<0.05$.