



Research Paper

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

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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 in the mentioned commodity. For this, we evaluated the influence of timing of yeast application and studied the production of the enzyme β -1,3-glucanase in apple tissues as a response to the presence of both, pathogen and antagonist. Taking into account that any method of preservation of biocontrol agent provokes death of cells, we assessed the effect of nonviable cells in the biocontrol. The results showed the existence of three possible mechanisms of action of *R. fluviale* in the control of *B. cinerea*: 1-Competence for nutrients and space that favored more the preventive effect than the cure of a preexisting infection; 2- The strong induction of β 1,3 glucanase enzyme in apple tissue, and 3-The action of cellular components, probably chitin, present in the yeast cell wall that could be the explanation for the activity of non-viable cells.

Keywords: *Botrytis cinerea*, *Rhodosporidium fluviale*, biocontrol mechanisms, Post-harvest diseases, Gray mold.

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INTRODUCTION

One of the most important plant pathogens is *Botrytis cinerea* Pers: Fr, which causes gray mold of fruits and vegetables around the world. This fungus is difficult to control successfully with fungicides because it is genetically variable and rapidly develops resistance to the chemicals commonly used for its control (Pertot et al., 2017). These facts have made necessary the permanent search for new strategies for the management of this fungus, especially the search for environmentally friendly methods. An alternative is the use of biocontrol agents, alone or in mixtures, or combined with different substances (Sharma et al., 2009). However, the application of the biological control is not universal because its results are not as consistent as results of chemical control. A major goal in the development and implementation of successful biological control products is to increase the ability of antagonists to control post-harvest diseases in a broader range of conditions and with minimal variability. The capacity to

control pre-existing infections inflicted during harvesting and transport, as is possible with synthetic fungicides, is also highly desirable.

Among the tools for improvement of biological control are the knowledge of the pathogen and the biological control agent (BCA) physiologies, the mechanisms of attack and control and the interactions between the pathogen, the antagonist (BCA) and the host (Nunes, 2012; Spadaro and Droby, 2016). Therefore, the evaluation of a potential biological control agent should include assessment of its ability to control pre-existing infections and 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 the production of enzymes or other compounds with antimicrobial action (Punja and Utkhede, 2003; Janisiewicz et al., 2010; Sansone

et al., 2011; Pertot et al., 2017). Furthermore, a secondary mechanism of action is the ability of a biocontrol agent to induce resistance in the host plants (El Ghaouth et al., 2003; Xu et al., 2008; Spadaro and Droby, 2016; Romanazzi et al., 2016).

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 (Cota et al., 2007) and its induction in fruit tissues by different chemical and microbial elicitors has been reported (Tian et al., 2007; Ge et al., 2010).

In recent times, *Rhodosporidium* species appeared as potential biocontrol agents. For example, Lu et al. (2014) reported the control of *Penicillium expansum* on apple by *Rhodosporidium paludigenum*.

In this work, the ability of the yeast, *Rhodosporidium fluvial* isolated in our laboratory from apple skin to control *B. cinerea* on apple fruit was evaluated and a study on the interaction between the pathogen, *B. cinerea*, yeast and the host apple fruit and the goal of improving the control of gray mold in the mentioned commodity was carried out. For this, the influence of timing of yeast application and the production of the enzyme β -1,3-glucanase in apple tissues was studied. Taking into account that any method of preservation of biocontrol agent kills a certain number of cells (Navarta et al., 2014), the effect of non-viable cells in the biocontrol was studied.

MATERIALS AND METHODS

Micro-organisms

In this study, two strains of *B. cinerea* (BNM 0527 and BNM 0528) isolated from apple root in our laboratory and characterized for pathogenicity and resistance to the fungicide iprodione was used (Sansone et al., 2011). For their molecular identification, the service of molecular identification of Macrogen (Korea), which amplified the ITS1 and ITS4 regions using primers 5'(TCCGTAGGTGAACCTGCGG) 3' y 5'(TCCTCCGCTTATTGATATGC) 3' was required.

The *B. cinerea* strains were deposited in the National Bank of Micro-organisms (WDCM938) of the Facultad de Agronomía, Universidad de Buenos Aires (FAUBA), Argentina. As biocontrol agent, *R. fluviale*, isolated in our laboratory from the skin of apples and identified biochemically was used. 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

Both *B. cinerea* strains on Potato Dextrose Agar (Sigma Chemical Co, St. Louis) at 14°C was cultured and

maintained on the same medium (PDA) at 4°C. For conidial production on PDA, the incubation temperature was between 20 to 25°C until mycelium apparition and the cultures kept at 15°C for inducing sporulation. After a week, the formation of aggregates was avoided by first harvesting the conidia and suspending it in 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80. The concentration of conidia suspension was determined using a Neubauer chamber and adjusted with sterile distilled water to 1×10^5 conidia ml⁻¹.

R. fluviale was grown in potato dextrose broth (PDB) for 24 h at 28°C, and thereafter, the cells were harvested, washed and suspended in sterile distilled water; cell concentration was adjusted to 1×10^6 cells ml⁻¹. For assays with non-viable cells, the suspension was treated using heat at 100°C for 10 min.

Culture medium for assessment of inhibition of conidia germination

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

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

200 μ l each of ADB and a suspension of *R. fluvial* were placed in 1.5 ml Eppendorf tubes to assess the preventive effect. The mixture was incubated for two hours and 200 μ l of the conidial suspension of *B. cinerea* added (BNM 0527 or BNM 0528).

To study the curative effect, 200 μ l of ADB and 200 μ l of a conidial suspension of *B. cinerea* was put (BNM 0527 or BNM 0528) in 1.5 ml Eppendorf tubes and the mixture incubated for five hours and thereafter, 200 μ l of the yeast suspension was added; yeast suspension was replaced in the control tubes by sterile distilled water. The Eppendorf tubes were maintained at 15°C and a sample of 50 μ l taken at 7 and 23 h, respectively. The sample was placed on microscope slides and 100 conidia per slide evaluated. The preparations with a light microscope (Olympia) at a magnification \times 500 were observed and the percentage of germinated conidia calculated; these experiments were performed in triplicate.

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

The effectiveness of *R. fluvial* was evaluated (viable and

non-viable yeast cells) for controlling *B. cinerea* (BNM 0527 or BNM 0528) on apples.

In all assays, Red Delicious apple fruit previously stored at 0°C for six months was used and before the assays, firmness, sugars and pH of the fruit evaluated; firmness was measured using manual Penetrometer (Fruit Pressure tester, FT 327). Sugars contained were determined using a handheld refractometer (ZGRB-32ATC) and pH measured using a Cole-Parmer pH meter.

The 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, we pipetted an aliquot (20 µl) of suspensions of *R. fluviale* at 1×10^6 cell ml⁻¹ (viable or non-viable cells) into each wound site and sterile distilled water used as the control. Two hours later, 20 µl of a suspension of *B. cinerea* BNM 0527 or *B. cinerea* BNM 0528 (1×10^5 conidia ml⁻¹) 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.

In either of the two cases, the wounds were examined after five and ten days of incubation at 15°C and the experiment repeated twice. The results were expressed as the percent of the reduction in decay severity and calculated from lesion diameter (Ø) as: % Reduction in decay severity = $[(\text{Ø control} - \text{Ø treatment}) / \text{Ø control}] \times 100$.

Electron microscopy

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 and 3) 20 µl of *R. fluviale* viable cell plus 20 µl of *B. cinerea* BNM 0527. Thereafter, we incubated the apples at 15°C for 24 and 48 h, respectively and the samples taken from the wound and healthy tissue. Samples were fixed and dehydrated by the technique as described by Chan and Tian (2005) and dried using an oven at a temperature of 55°C for approximately 12 h.

The dried samples mounted in "stubs" of aluminum, covered with a gold layer of 30 nm in a "sputter" standard under argon, and finally observed in a scanning electron microscope (SEM, LEO 1450 VP.) in the Laboratory of Electron Microscopy and Microanalysis (LABMEM) of the Universidad Nacional de San Luis (UNSL).

Study the host response to the presence of the antagonist, the pathogen, and antagonist-pathogen mixture

To assess the induction of β-1,3-glucanase, apples were wounded using the tip of a disinfected 10-penny nail (3 mm diameter and 3 mm deep) on the three opposite sides of

each fruit. The fruits were inoculated with the following treatments: 1) 40 µl of sterile distilled water; 2) 40 µl of viable *R. fluviale* cells; 3) 40 µl of non-viable *R. fluviale* cells; 4) 40 µl *B. cinerea*; 5) 20 µl of viable *R. fluviale* 20 µl of *B. cinerea* and 6) 20 µl of non-viable *R. fluviale* and after 2 h, 20 µl of *B. cinerea*. We carried out the treatments using both strains of *Botrytis* (BNM 0527 and BNM 0528) and the apples incubated at 15°C during 5 and 10 days. According to the procedure of El Ghaouth (2003) with some modifications, the tissue samples were extracted and treated.

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. We fractioned the homogenate into Eppendorf tubes which were centrifuged at 12000 rpm for 15 min using a refrigerated centrifuge at 4°C, (Presvac EPF-12). The supernatant obtained was used for the assay of β-1,3-glucanase activity.

Assay of β-1,3-glucanase activity

Assay of glucanase activity was based on the ability of this enzyme to hydrolyze laminarin and release glucose (Kulminskaya et al., 2001); 50 µl of 4% Laminarin (substrate) and 50 µl of supernatant (enzyme) were put into Eppendorf tubes. Tubes were incubated for two hours in BM at 40°C; and then glucose released quantified by the technique of glucose oxidase/peroxidase according to the protocol provided by the Wiener Laboratory equipment. Determination of glucose in supernatants before incubation (t=0) was the control value.

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.) and three replicates of each treatment analyzed.

Assessment of the production of β-1,3-glucanase by *Rhodospiridium fluviale*

To assess if *R. fluviale* could be glucanase producer, it was cultured in modified Lilly-Barnett minimal salt medium. This medium contains 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). We prepared cell wall of *B. cinerea* (BNM 0527 or BNM 0528) according to Chan and Tian method (2005).

For the assay, we inoculated 50 ml of culture medium contained in 250 ml Erlenmeyer flasks with 5 ml of *R. fluviale* (1×10^6 cells ml⁻¹) and the cultures maintained at 15°C on a rotary shaker (140 rpm) for 120 h. After this time, the cultures centrifuged for 15 min at 12000 rpm using a Sorvall SS-3 centrifuge and supernatants used to

Table 1: Effect of *R. fluviale* (viable or nonviable cells) on conidial germination of *B. cinerea* BNM 0527 and *B. cinerea* BNM 0528 at 15°C.

| Conidial germination (%) | 7 h | 23 h |
|--------------------------------------|-------------------------|------------------------|
| <i>B. cinerea</i> 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 ± 6.33 ^c | 23 ± 5.3 ^b |
| Preventive effect - non-viable cells | 11 ± 4.33 ^{bc} | 13 ± 3.12 ^a |
| Curative effect - non-viable cells | 10 ± 3.65 ^b | 12 ± 3.3 ^a |
| <i>B. cinerea</i> 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 cells | 39 ± 5.3 ^{ef} | 37 ± 5.3 ^c |
| Preventive effect - non-viable cells | 28 ± 2.9 ^d | 42 ± 3.9 ^{cd} |
| Curative effect - non-viable cells | 35 ± 4.2 ^e | 48 ± 6.3 ^{de} |

Assays were performed in Apple Dextrose Broth (ADB). To assess the preventive effect *R. fluviale* was incubated for two hours and then conidial suspension of *B. cinerea* was added. To simulate the curative effect conidial suspension of *B. cinerea* was incubated for two hours before the addition of *R. fluviale* suspension. In the control tubes, yeast suspension was replaced by sterile distilled water. Means in the same column followed by the same letters are not significantly different at p=0.05.

determine the extracellular glucanase.

We also performed biocontrol assays using cells of *R. fluviale* cultured in modified Lilly-Barnett minimal salt medium. For this, we inoculated apple wounds with 20 µl of *R. fluviale* (1×10^6 cell ml⁻¹) and after two hours, we inoculated the treated wounds with 20 µl of *B. cinerea* (BNM 0527 or BNM 0528), 1×10^5 conidia ml⁻¹. After ten days at 15°C, we examined the wounds for rot. We repeated this experiment twice and expressed the results as the percent of the reduction in decay severity.

Statistical analysis

Data were processed by an analysis of variance (ANOVA). The statistically significant differences were analyzed (p=0.05) using Test of Multiple Range of Duncan, Statistic Software InfoStat, 2008.

RESULTS AND DISCUSSION

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

In the preliminary assays, we evaluated the ability of yeast *R. fluviale* to prevent the installation of the pathogen (preventive effect) or to control the pathogen already installed (curative effect) 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 characteristics of pathogenicity and resistance to fungicide iprodione (Sansone et al., 2011).

Table 1 shows the results. 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 at seven hours shows a typical mechanism of competition for nutrient (Spadaro and Droby, 2016). The results of the inhibition of germination in the case of the curative effect would indicate a reasonable control of the pre-existing infections through competition, but made difficult by the previous presence of the pathogen. In respect to non-viable yeast cells, their preventive effect was higher against *B. cinerea* BNM 0527 than *B. cinerea* BNM 0528 but there was no a significant difference for the curative effect.

In general, the BNM 0527 strain was the most inhibited by *R. fluviale*. According to Sansone et al. (2011), of the two strains, the BNM 0527 strain is more pathogenic but also more sensitive to iprodione.

Effectiveness of *Rhodospiridium fluviale* for controlling *Botrytis cinerea* on apples

Viable and non-viable 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.

Figure 1 shows the results of simulation of preventive effect using viable and non-viable yeast cells. 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 50% at 10 days. On the other hand, the

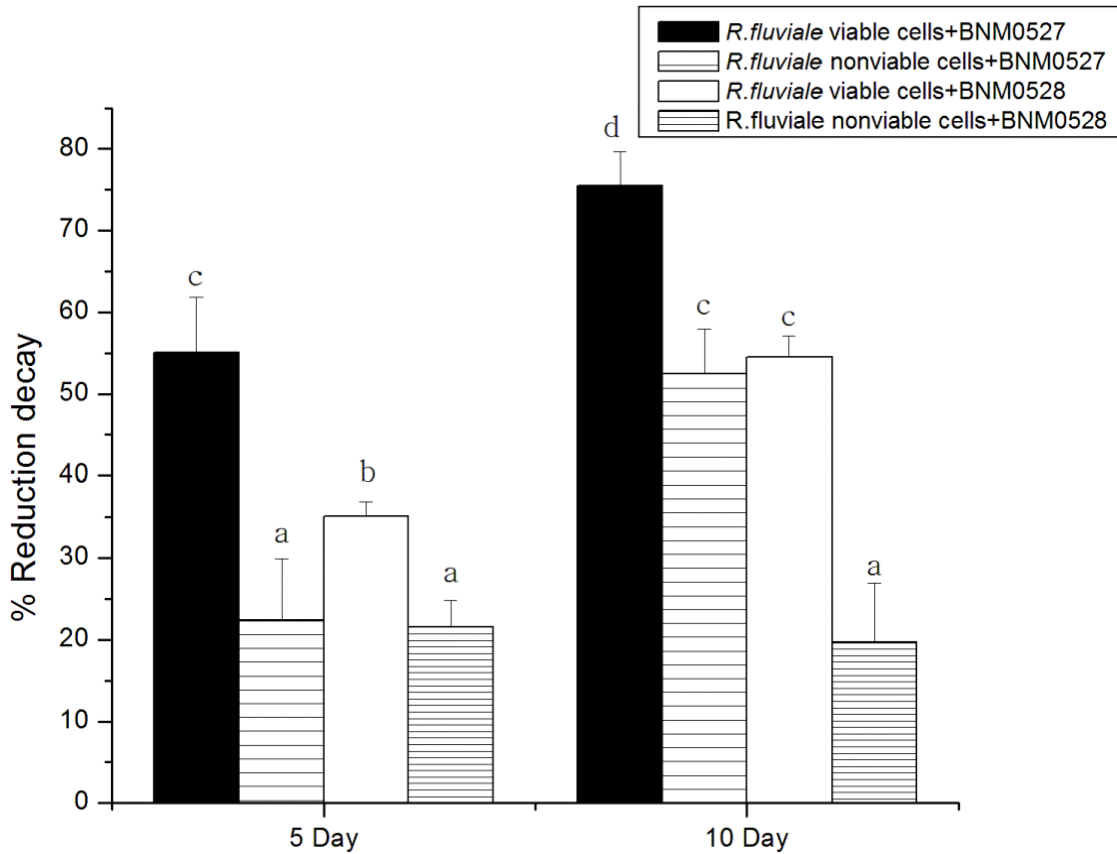


Figure 1: Preventive effect using viable and non-viable yeast cells of *Rhodosporidium fluviale* against *Botrytis cinerea* BNM 0527 and BNM 0528. An aliquot (20 μ l) of *R. fluviale* suspension at 1×10^6 cell ml^{-1} (viable or nonviable cells) was put into each wound site, and after two hours, 20 μ l of a suspension of *B. cinerea* BNM 0527 or *B. cinerea* BNM 0528 (1×10^5 conidia ml^{-1}) was inoculated. Apples were stored at 15°C for 5 days (5d) and 10 days (10d). Each value is the mean of three experiments. Bars represent the standard error of the mean. Means with the same letters within the same set of columns are not statistically different at $p=0.05$. Lesion Diameters of *B.cinerea* control: BNM 0527: 14.32 mm (5 d), 53 mm (10 d); BNM 0528: 9.8 mm(5d) , 44.26 mm (10 d).

reduction in the severity of the lesion by non-viable cells at 10 days was 50% for strain BNM 0527 and 20% for the other strain.

Figure 2 shows results of the curative effect. There, it can be seen that for BNM 0527 strain, the reduction of decay was 50% at 10 days using viable cells and there was no significant difference with non-viable cells treatments. Reduction of decay was minor for BNM 0528 strain and reached 35 and 25% with viable cells and non-viable cells respectively.

In general, *Botrytis* control was more effective when the yeast was present before infection (preventive effect) than when the infection was already in place (curative effect). For viable cells, in the case of the curative effect, the significant difference between 5 and 10 days of incubation demonstrated the existence of competition for nutrients and space as one mechanism of biocontrol of *R. fluviale*. Tian (2007), who used the yeast *Cryptococcus laurentii* as a control agent against *Penicillium expansum* and *Alternaria alternata*, reported similar results.

The effectiveness of non-viable cells of *R. fluviale* was higher against *B. cinerea* BNM 0527 than *B. cinerea* BNM 0528 and was independent of the time of application. Non-viable cells had similar efficacy for prevention and cure. 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 non-viable cell suspensions showed the presence of chitin (data not shown) and various authors demonstrated that this compound may be useful in the control of post-harvest fungal diseases (Lu et al., 2014; Fu et al., 2016).

Interaction assays between antagonist, pathogen and host

The interaction between antagonist, pathogen and host was evaluated by microscopic observation of wounds of apples inoculated with the microorganisms by determining the

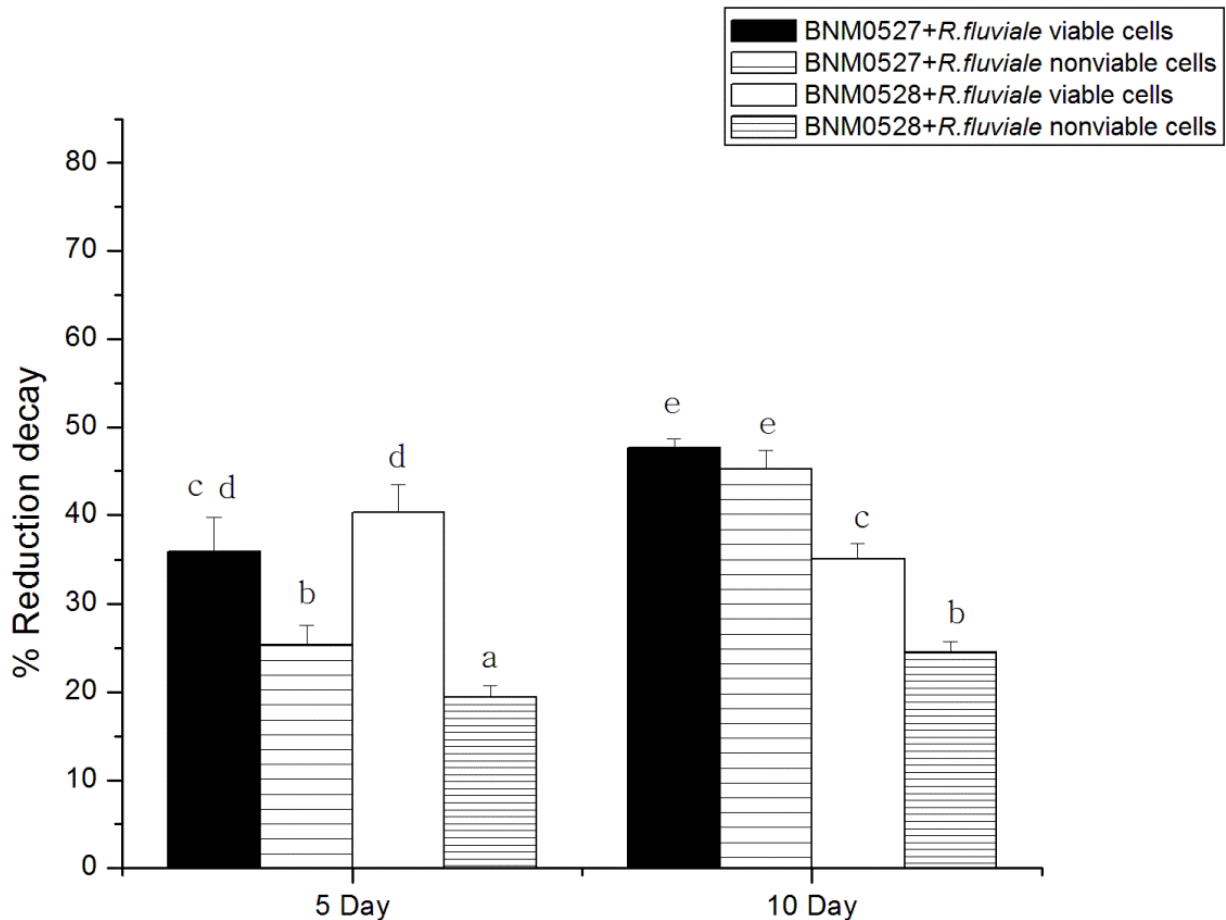


Figure 2: Curative effect using viable and nonviable yeast cells of *Rhodosporidium fluviale* against *Botrytis cinerea* BNM 0527 and BNM 0528. A conidial suspension (20 μ l) of *B. cinerea* BNM 0527 or *B. cinerea* BNM 0528 (1×10^5 conidia ml^{-1}) was inoculated into the apple wounds. After two hours, an aliquot (20 μ l) of *R. fluviale* suspension at 1×10^6 cell ml^{-1} (viable or non-viable cells) was put into each wound site. Apples were stored at 15°C for 5 days (5 d) and 10 days (10 d). Each value is the mean of three experiments. Bars represent the standard error of the mean. Means with the same letters within the same set of columns are not statistically different at $p=0.05$. Lesion Diameters of *B. cinerea* control: BNM 0527: 14.32 mm (5 d), 53 mm (10 d); BNM 0528: 9.8 mm (5 d), 44.26 mm (10 d).

β -1,3-glucanase activity in apples.

Observation of the interactions by Electron Microscopy

Figure 3 shows electron microscopy images of apple tissue samples taken after 48 h of the inoculation with *R. fluviale* and *B. cinerea* BNM0527. 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). Both micrographs evidence a predominance of the yeast grouped and fixed to the apple tissue.

The yeast cells colonized efficiently and quickly the wound. Electron micrographs that show the physical interaction between the antagonist and the pathogen in the apple wound also confirmed the competition by space as a mechanism of action.

Induction of β -1,3-glucanase activity in apple

Figure 4 shows the results of the induction of glucanase enzyme 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 fw. 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 fw 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 activity values reached 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. The activity values obtained with the mixture of non-viable yeast plus pathogen cells were not significantly different from those achieved with either

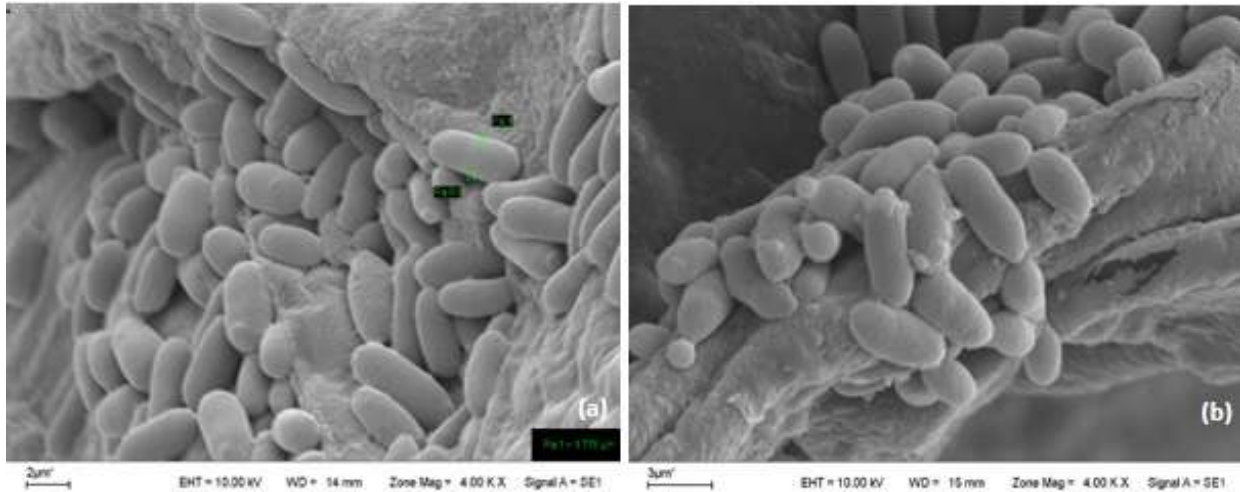


Figure 3: SEM micrographs: (a) Apple wound inoculated with *R. fluviale* after 48 h of incubation (b) Apple wound inoculated with *R. fluviale* and *B. cinerea* BNM 0527 after 48 h of incubation (SEM, LEO 1450 VP).

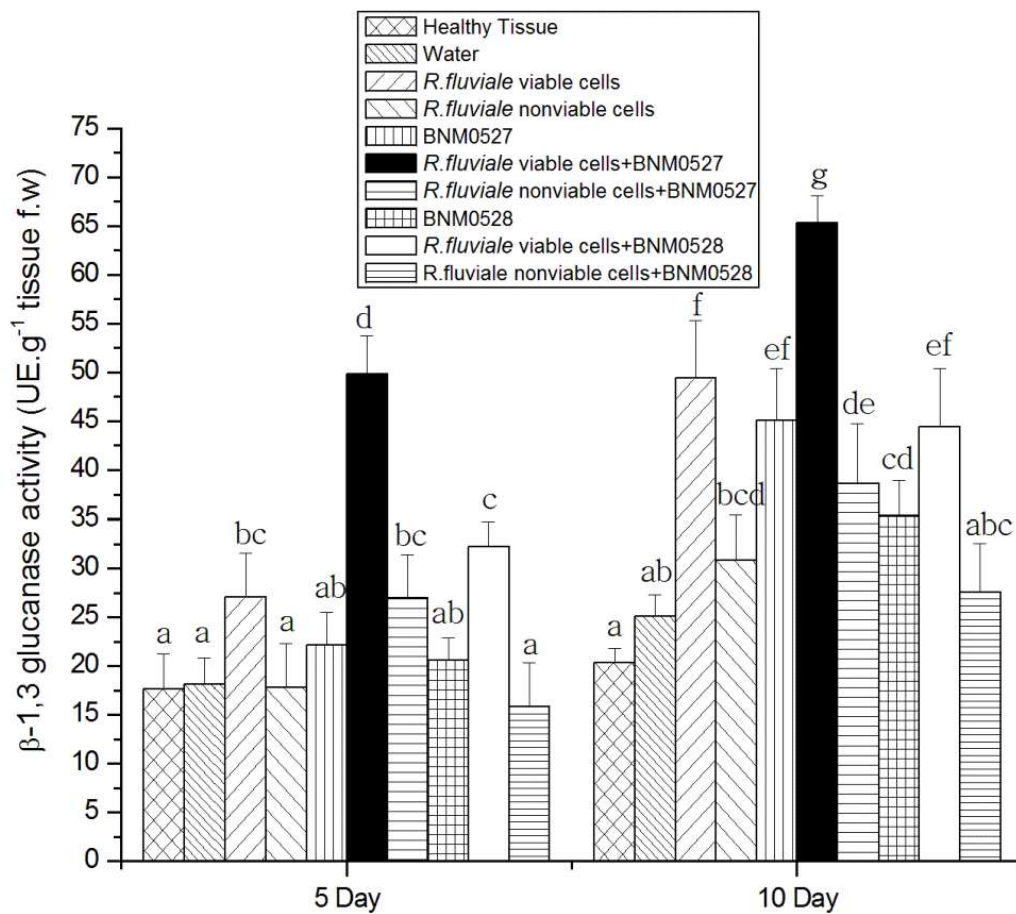


Figure 4: Stimulation of β -1,3-glucanase in apples by *Rhodosporidium fluviale* and *Botrytis cinerea*. The following treatments were applied to apple wounds: 1) 40 μ l of sterile distilled water; 2) 40 μ l of viable *R. fluviale* cells, 3) 40 μ l of non-viable *R. fluviale* cells, 4) 40 μ l of *B. cinerea*, 5) 20 μ l of viable *R. fluviale* and after 2 h, 20 μ l of *B. cinerea*, 6) 20 μ l of non-viable *R. fluviale* and after 2 h, 20 μ l of *B. cinerea*. Each value is the mean of three experiments. Bars represent the standard error of the mean. Means with the same letters within the same set of columns are not statistically different at $p=0.05$.

antagonist or pathogen alone. There was not a difference between treatments with non-viable cells or water. Taking into account that there was no difference between the inductions provoked by either of the strain of *B. cinerea*, the strong induction caused by the mixture of the viable cells of *Rhodosporidium* and the strain BNM 0527 at five days of storage was remarkable. Zhang et al. (2016) reported similar results with co-inoculated *Streptomyces rochei* A1 and the pathogen *Botryosphaeria dothidea* in apple fruit. According to Calvo et al. (2003), it could be a synergistic effect because the effect obtained was greater than the addition of individual effects. In addition, the production of glucanase by the antagonist could be an explanation for the phenomenon observed. However, the negative result obtained when *R. fluviale* grew in a culture medium with *B. cinerea* walls allowed us to discard that possibility and attribute the increase of glucanase only to the host defense mechanism. We also evaluated the ability of *R. fluviale*, cultured in the presence of *B. cinerea* wall to reduce the severity of lesion generated by this pathogen. Reduction of severity at 15°C after ten days of incubation, for both strains, *B. cinerea* BNM 0527 and BNM 0528, was 59 and 40% respectively. These results showed that the growth of the antagonist in a glucanase inductor medium did not enhance the ability of yeast for controlling *B. cinerea*.

Conclusions

We evaluated the yeast *R. fluviale* isolated from the epiphytic microbiota of the apple for controlling *B. cinerea* and demonstrated that this yeast has potential as a biological control agent. The results showed the existence of three possible mechanisms of action of *R. fluviale* to control *B. cinerea*:

- 1) Competence for nutrients and space that favored the preventive effect more than the curative effect;
- 2) The induction of a defense response in the host by stimulation of the β 1,3 glucanase enzyme and;
- 3) The action of cellular components, probably chitin, present in the yeast cell wall.

This last mechanism manifested by the effectiveness of non-viable cells inhibits germination of *Botrytis* conidia and reduces the severity of apple lesions.

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

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