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Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple

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Abstract

In the present study, two yeast antagonists, *Cryptococcus laurentii* (strain 317) and *Candida ciferrii* (strain 283) isolated from the surface of healthy apples, controlled blue mold of apple caused by *Penicillium expansum*. Both antagonists reduced the incidence of blue mold by 80% at 25 °C. At 5 °C *C. ciferrii* (strain 283) maintained the efficacy of disease control, but *C. laurentii* (strain 317) only reduced disease incidence by 50%. Moreover *C. ciferrii* (strain 283) exhibited significant protection at lower concentrations than *C. laurentii* (strain 317). The population of both strains increased in wounds of apples at 25 and 5 °C, and both strains maintained viable over a period of 35 days at 5 °C. Nutrient competition into wounds appeared to be the principal mode of action of these antagonists. Nitrogen rather than carbon appeared to be the limiting factor to both the antagonists and the pathogen. Further research will explore commercial potential of these antagonists and the possibility of enhancing biocontrol efficacy by using mixtures of antagonists or additives such as calcium chloride or deoxyglucose. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Postharvest losses of fruits and vegetables are high, ranging from 10 and 40% depending on the species and technologies used in the packing-houses (Arras and Arru, 1999; Wilson and Wisniewski, 1994). Such losses are mainly due to pathogenic fungi (Wilson and Wisniewski, 1989) which usually infect the host through wounds

made during harvest, handling and processing. In the case of apples, postharvest losses are mainly caused by *Penicillium expansum* Link (blue mold) and *Botrytis cinerea* Pers.: Fr. (grey mould). Synthetic chemical fungicides, such as benomyl and iprodione, have been traditionally used to control these pathogens. Fungicide efficacy, however, is frequently decreased by the development of resistant strains of pathogens. In addition, public concern and regulatory restrictions about the presence of fungicide residues on crops have em-

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phasized the need to find alternative methods for disease control (Smilanick, 1994).

In the recent years, biological control has been explored as an alternative to the use of synthetic fungicides for managing postharvest decay (Wilson and Wisniewski, 1989). Several species of bacteria and yeast have been reported to reduce postharvest fungal decay of pome fruits (Janisiewicz, 1985; Mari et al., 1996; Mercier and Wilson, 1994; Chand-Goyal and Spotts, 1997). At least two, yeast-based products, are now commercially available (Aspire based on *Candida oleophila*, and Yield Plus based on *Cryptococcus albidus*). The products Bio-save-100 and Biosave-110, based on the bacterium *Pseudomonas syringae*, are also available for postharvest disease control. In previous work (Vero, 1998) the microflora from surfaces of organically-grown apple fruits was isolated and yeast and bacterial strains were tested as biocontrol agents of blue mold on apples. Two yeast strains were selected for their potential as biocontrol agents due to their strong inhibitory activity against *P. expansum* rot on apple wounds. The present work was undertaken to further characterize these yeast strains and to study their possible mode of action.

2. Materials and methods

2.1. Fruit

Apples (*Malus pumila* 'Red Delicious') of uniform size and maturity without wounds or rot, were used in this study.

2.2. Pathogen

P. expansum DSM 1994 was obtained from the German Collection of Microorganisms and Cell Cultures (DSM). Cultures were grown on Malt Agar at 25 °C for 4 days and maintained on the same medium at 5 °C.

2.3. Biocontrol agents

Biocontrol agents used in this study were yeast strains isolated from the surface of healthy apple

fruits collected from organic production orchards in the south of Uruguay. Identification of selected strains was carried out according to Kurtzman and Fell (1998) and using the API system. Optimum growth temperature in apple juice was determined for each strain. Temperatures assayed were 5, 25, 30, 37 and 45 °C.

2.4. Biocontrol assays

Biocontrol assays were performed at 25 and 5 °C.

Fruits were surface-disinfected with sodium hypochlorite (0.1%) for 2 min and then rinsed with running tap water. Four wounds (5 mm deep × 7 mm wide) were cut at the equator of each apple with a cork borer. Two of the wounds were inoculated with 40 µl of a yeast suspension (10^7 cfu ml⁻¹) and the other two with 40 µl of sterile saline (0.9%) as a control. Fruit were then placed in covered plastic containers at 25 or 5 °C. High humidity was maintained by adding water to the bottom of the tray. After 24 h wounds were inoculated with 40 µl of conidial suspension of the pathogen (10^4 conidia ml⁻¹). This pathogen concentration had previously proved to produce 100% infection of wounds (Vero, 1998).

The fruit were then incubated again in the same conditions as above. After the incubation period (7 days at 25 °C or 28 days at 5 °C), wounds were examined and the lesion diameters were measured. Two parameters were recorded: percentage of incidence reduction and percentage of severity reduction.

Incidence was defined as:

$$\%Inc = \frac{\text{Number of rotten wounds}}{\text{Number of total wounds}} \times 100$$

Severity was defined as:

$$\%Severity = \frac{LdA}{LdC} \times 100$$

LdC = Average lesion diameter in control inoculated wounds; LdA = Average lesion diameter in wounds treated with antagonists, prior to inoculation with a pathogen. Lesion diameter = Total lesion diameter-wound diameter. Ten fruit were used in each biocontrol assay and assays were repeated at least twice.

At 5 °C, biocontrol assays on wounded fruits were performed as described above, varying the concentration of antagonists suspensions in order to apply 10^3 – 10^6 cfu per wound. The colony forming units of conidial suspension of the pathogen was maintained (10^4 conidia per ml) and 40 μ l were applied to each wound. Concentrations of antagonists and pathogen suspensions were confirmed by plating appropriate dilutions on malt agar.

2.5. Mechanisms of biocontrol

2.5.1. Antifungal metabolite production.

Each strain was tested in dual cultures against pathogens in apple juice agar (Wisniewski et al., 1991).

2.5.2. Production of chitinolytic enzymes

It was assayed on plates, as described by Frändberg and Schnürer (1994).

2.5.3. Colonization of wound site

Growth curves were done in fruit wounds at 25 and 5 °C. Wounds (5 mm deep \times 7 mm wide) were made in surface-disinfected apple fruit with a cork borer. Pieces of apple (approximately 0.2 g) bearing a wound were cut and placed in 1.5 ml cotton capped Eppendorf tubes. The wounds were inoculated with 40 μ l of yeast suspension of known concentration (10^7 cfu ml⁻¹) and incubated for 7 days at 25 °C and for 35 days at 5 °C. Controls were inoculated with saline (0.9%). At different times, three tubes per treatment and three controls, were weighed and 1 ml sterile saline (0.9%) was added to them. Samples were then homogenized in vortex for 2 min. Quantification of viable yeast cells in the resulting abstract was performed by plate count on malt agar. Residual sugars, as glucose, sucrose and fructose, were determined in filtered saline extract by HPLC at room temperature using a Shimadzu HPLC fitted with a Chromapack OA 1000 column and a refraction index detector. The mobile phase was sulphuric acid 0.05 M.

To study the influence of nitrogen and glucose concentration on yeast growth, growth curves in fruit wounds were done, as described above, but

inoculating with 40 μ l of cells suspended in a solution of amino acids (0.05% total nitrogen concentration) or in a glucose solution (3%). The composition of the amino acid solution was similar to the one reported for apple juices: aspartic acid (21%), asparagine (17%), glutamic acid (15%), serine (10%), alanine (7%) (Casas Canamiñana, 1979). To determine the effect of additional nitrogen supply on antagonism, biocontrol assays were performed at 25 °C, as described before, but antagonists were applied suspended in the amino acid solution. Controls without amino acid amendment were performed. Fifteen fruit were used for each treatment.

2.5.4. Nutrient competition

Biocontrol assays on fruit were performed at 25 °C as described above, but the pathogen applied was suspended in different sterile substrate solutions. Substrates used were apple juice, sugars (sucrose 2.5%, glucose 2.4%, fructose 5.0%), and sodium nitrate (0.3%).

2.6. Statistical analysis

Severity (difference in diameter growth) was subjected to analysis of variance; the model included strain effect and temperature nested on strain. Differences between means were tested using least significant difference.

Incidence was analyzed with the same model but a binomial distribution and logit transformation was used (maximum likelihood). Contrasts between means were performed when significant effects were found (Toutenburg, 1995) All analysis were performed in Statistical Analysis System, Release 6.12 (SAS/STAT®, 1996, SAS Institute, Cary, NC, USA) PROC MIXED and PROC GENMOD.

3. Results

Both yeast strains, identified as *C. laurentii* (strain 317) and *C. ciferrii* (strain 283), appeared to be good antagonists of blue mold on apples at 25 and 5 °C ($\alpha < 0.05$; Fig. 1). Incidence of blue mold was lowered to 21% when both antagonists

were applied at 25 °C, and to 46 and 25%, respectively, when applied at 5 °C. Neither showed antifungal activity in dual cultures with the pathogen, and no chitinolytic activity was detected (data not shown). Optimum growth temperature was 25 °C for *C. laurentii* (strain 317) and 30 °C for *C. ciferrii* (strain 283).

At 5 °C, *C. laurentii* (strain 317) was effective only when applied at 10⁶ cells per wound whereas *C. ciferrii* (strain 283) exhibited significant protection at 10⁴ cells per wound (Figs. 2 and 3). When applied to apple wounds, both yeast strains grew at both temperatures and remained viable over a period of 35 days at 5 °C (Fig. 4A and B).

Analysis of sugars in inoculated wounds showed that the concentration of sucrose and glucose decreased whereas the concentration of fructose slightly increased during the studied period of time. Glucose concentration after 7 days of yeast growth was half of the initial concentration (Table 1). Yeast populations in wounds in the presence of added glucose were the same as obtained without addition of sugars (data not shown).

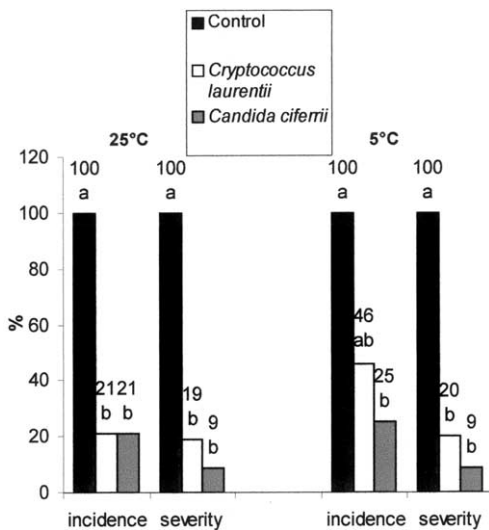


Fig. 1. Incidence and severity of blue mold on apples inoculated with *C. laurentii* 317 or *C. ciferrii* strain 283 and *P. expansum* DSM 1994. Controls were not treated with antagonists. Fruit were held for 28 days at 5 °C. Treatments with the same letter are not significantly different ($P = 0.05$).

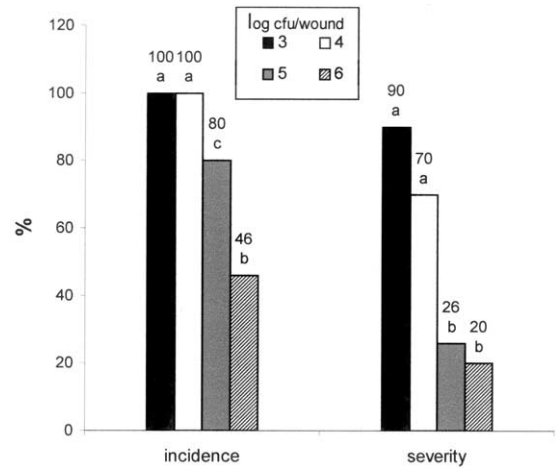


Fig. 2. Incidence and severity of blue mold on apples after applying different concentrations of *C. laurentii* strain 317. Fruit were held for 28 days at 5 °C. Treatments with the same letter are not significantly different ($P = 0.05$).

Growth curves for both antagonists in apple wounds, with and without the addition of amino acids, indicated that the populations of both strains were greater in the presence of amino acids (Fig. 5A and B). Surprisingly, biocontrol was not significantly enhanced by the addition of amino acids along with the antagonists. There was only a

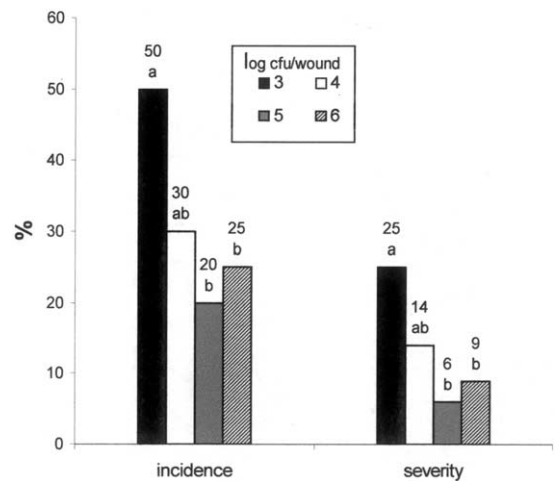


Fig. 3. Incidence and severity of blue mold on apples after applying different concentrations of *C. ciferrii* strain 283. Fruit were held for 7 days at 25 °C or 28 days at 5 °C. Treatments with the same letter are not significantly different ($P = 0.05$).

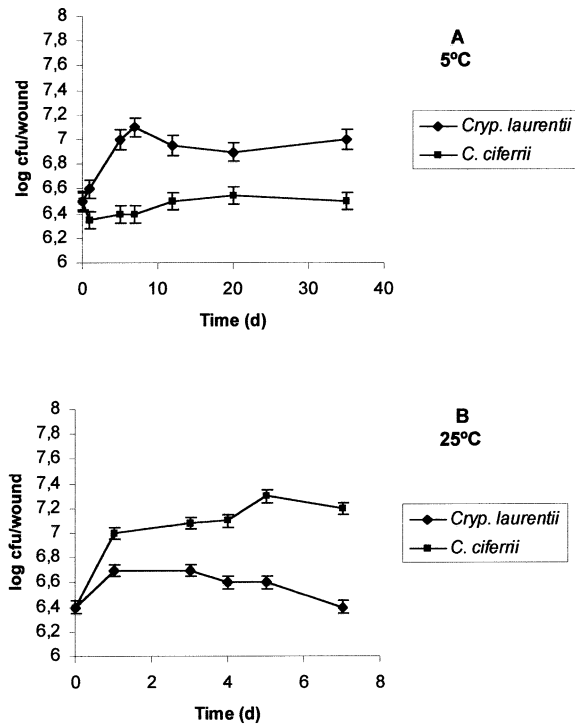


Fig. 4. Population sizes of *C. laurentii* strain 317 and *C. ciferrii* strain 283 in apple wounds at 5 °C (A) or 25 °C (B). Bars indicate S.E.M.

statistically significant ($P = 0.05$) reduction in severity when *C. laurentii* (strain 317) was applied together with amino acids (Fig. 6). Fig. 7 shows the effect of applying the pathogen, suspended in different nutrient solutions and saline, to wounds previously inoculated with the yeast antagonists. Complete loss of biocontrol activity was observed when apple juice or nitrate solution was used. In the process of identifying the yeast strains it was determined by auxanogram that nitrate could not be used as a nitrogen source by the yeast strains

Table 1

Sucrose, glucose and fructose concentrations in apple wounds before and 7 days after applying *C. laurentii* strain 317 or *C. ciferrii* strain 283

Time (days)	Antagonist	Sucrose (g kg ⁻¹)	Glucose (g kg ⁻¹)	Fructose (g kg ⁻¹)
0	None	2.3	2.4	7.2
7	<i>C. laurentii</i> (317)	1.1	1.4	7.0
7	<i>C. ciferrii</i> (283)	0.1	1.5	8.0

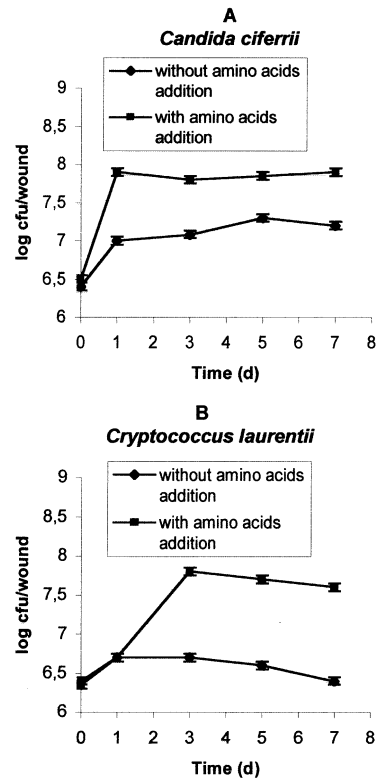


Fig. 5. Population sizes of *C. laurentii* strain 317 (A) and *C. ciferrii* strain 283 (B) in apple wounds with and without amino acids addition at 25 °C. Bars indicate S.E.M.

but could be assimilated by the pathogen, *P. expansum*.

4. Discussion

The use of biocontrol agents to manage postharvest decay of fruit has been explored as an alternative to the use of synthetic fungicides

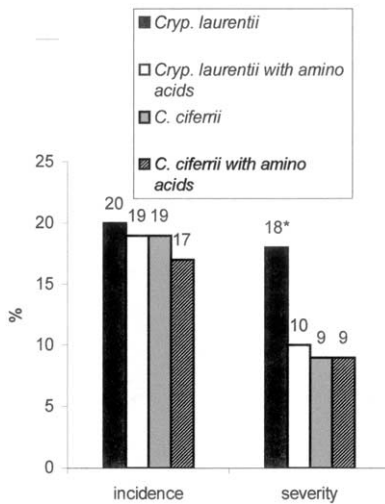


Fig. 6. Incidence and severity of blue mold on apples inoculated with antagonists in presence and absence of added amino acids, and *P. expansum* DSM 1994. Fruit were held 7 days at 25 °C. *, Indicates a significantly different result ($P = 0.05$).

(Wilson and Wisniewski, 1989) and several commercial products are now available (Bull et al., 1997; Droby et al., 1998). Further identification of new antagonists is desirable because antagonists identified in specific geographic areas may be more effective against the pathogen strains present in that locale. Furthermore, the financial

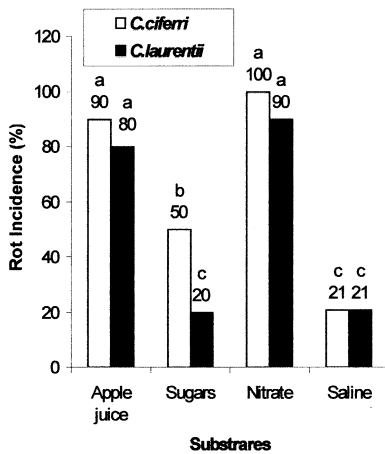


Fig. 7. Incidence and severity of blue mold on apples inoculated with antagonists and pathogen suspended in different solutions. Treatments with the same letter are not significantly different ($P = 0.05$).

costs involved in the registration of a product may inhibit its widespread availability in several countries. In the present research, we have identified two yeast antagonists that exhibit biocontrol efficacy against blue mold of apples caused by *P. expansum*. This is the first report of *C. ciferrii* as antagonist of blue mold of apples. Strains of this species have been isolated from soil and also from animals (Kurtzman and Fell, 1998). *C. laurentii* has been previously described as a biocontrol agent of postharvest diseases (Roberts, 1990; Castoria et al., 1997).

Growth curves of the antagonists demonstrated that both could colonize and grow in apple wounds. Even after a period of 35 days at 5 °C, the number of viable microorganisms was similar to or greater than that originally introduced into the wound. Our studies demonstrated that 10^5 – 10^6 cfu per wound of viable yeast cells of both strains were enough to prevent rot in laboratory assays (Figs. 2 and 3). These data indicate that only one application of the antagonists may be enough to prevent blue mold rot for at least a period of 35 days.

Cessation of exponential growth in apple wounds after 24 h at 25 °C indicates that nutrients may have become limited for antagonists and, most likely for the pathogen. Availability of carbon in the form of sugars did not appear to be a limiting factor since the population of antagonists in apple wounds did not increase when extra glucose was supplied. Rather, our studies showed that microbial growth in apple wounds was limited by nitrogen depletion, since populations of both yeast strains were one order greater when amino acids were supplied (Figs. 5 and 6). Nitrogen seems to be depleted in apple wounds after 24 h of yeast growth at 25 °C. The addition of yeasts suspended in the amino acid solution, did not result in an enhancement of the biocontrol most likely because they were also available to the pathogen (Fig. 6). Screening of specific amino acids may identify ones that specifically enhance the growth of the yeast antagonist but not the pathogen. Janisiewicz et al. (1992) have already demonstrated that L-asparagine and L-proline enhanced the biocontrol of blue mold on apples by a saprophytic strain *P. syringae* (strain L-59-66).

They have also shown that both amino acids were utilized readily by the antagonist but poorly by the pathogen in in vitro assays (Janisiewicz and Marchi, 1992).

The loss of biocontrol activity with the addition of potassium nitrate indicates that if a nitrogen source is supplied in wounds after the application and growth of antagonists, *P. expansum* could grow, despite the presence of viable cells of the biocontrol agents. Collectively, these results indicate that nitrogen competition in wounds would be one of the main mechanisms involved in blue mold control by *C. ciferrii* and *C. laurentii*.

5. Conclusions

In summary, this research identified two yeast antagonists that exhibited biocontrol efficacy against blue mold of apples. The antagonists, *C. laurentii* (strain 317) and *C. ciferrii* (strain 283) were isolated from the surface of locally grown fruit. Both species of yeast effectively colonized wounds of apple at 5 and 25 °C. Nutrient competition appeared to be the principal mode of action as the production of lytic enzymes or antimicrobial peptides was not detected. Furthermore, antagonism could be overcome by the addition of nutrients to the wound. Nitrogen, rather than carbon, appeared to be limiting to both the antagonists and the pathogen. Further research will explore the possibility of biocontrol enhancement using mixtures of antagonists or additives such as calcium chloride or deoxyglucose.

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