# BIOCONTROL ACTIVITY OF ANTAGONISTIC YEASTS AGAINST *PENICILLIUM EXPANSUM* ON APPLE

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## SUMMARY

*Penicillium expansum* causes severe rots on apple fruit during storage and shelf life. Aiming at the development of new antagonistic yeast active in controlling postharvest pathogens of fruit, several isolates were obtained from fig (*Ficus carica*) and cactus pear (*Opuntia ficus-indica*) grown in untreated orchards in Northern Sardinia (Italy). Two yeast strains of *Candida guillermondii* were selected for their remarkable antagonistic properties against *P. expansum* on apple. A film-forming strain of *Saccharomyces cerevisiae* isolated from wine was also included in the experiments. In trials carried out on the cv. Golden Delicious and Fuji, the yeasts applied alone or in the presence of various additives reduced apple rot with up to 100% efficacy. Killed yeast cells and culture filtrates had no biocontrol activity. Addition of different sugars in the apple wound had no detrimental effect on the biocontrol potential of the tested yeasts. Conversely, several nitrates significantly inhibited the antagonistic capability of *C. guillermondii*, thus suggesting that competition for nitrogen should play a major role in the biocontrol activity of the antagonistic yeast.

*Key words*: postharvest diseases, *Penicillium expansum,* blue mould, *Candida guillermondii, Saccharomyces cerevisiae.*

## INTRODUCTION

*Penicillium expansum* Link causes severe rots on apple and pear fruit during storage and shelf life. This pathogen infects fruit through epicarpic wounds caused during harvesting and handling in the packing-house processing lines. Patulin, a mycotoxin produced by *Penicillium* spp. during fruit spoilage, is a major concern, since exposure can result in severe acute and chronic toxicity, including carcinogenic, mutagenic, and teratogenic effects (Beretta *et al*., 2000; Hasan, 2000; McCallum *et al*., 2002). Although *P. expansum* is usually controlled by chemicals such as thiabendazole, imazalil, or cyprodinil (Eckert and Ogawa, 1990; Viñas *et al*., 1991, 1993; Chen *et al*., 1995; Zhou *et al*., 2002), the development of fungicide resistance (Gullino *et al*., 1985; Viñas *et al*., 1991, 1993) and an increasing environmental concern over pesticide residues in food, has prompted an urgent need for alternative control measures. Under such circumstances, biological control of post-harvest diseases of fruit may be an effective alternative to chemical control (Wilson and Wisniewski, 1989, 1994; Janisiewicz and Korsten, 2002).

Yeasts are particularly suitable for post-harvest use, proving highly effective on several fruit commodities (Wilson and Wisniewski, 1989; 1994; Roberts, 1994; Janisiewicz and Korsten, 2002). Several biocontrol yeasts were developed to control postharvest decay of apple and pear fruit caused by *Penicillium* spp. (Janisiewicz, 1987; Jijakli *et al*., 1993; Chand-Goyal and Spotts, 1997; Teixidó *et al*., 1998; Benbow and Sugar 1999; Usall *et al*., 2000; Janisiewicz *et al*., 2001; Nunes *et al*., 2001, 2002; de Capdeville *et al*., 2002).

Several putative mechanisms have been proposed to play a role in biocontrol effectiveness of antagonistic yeasts: competition for space and nutrients (Droby *et al*., 1989; McLaughlin *et al*., 1990; Chand-Goyal and Spotts, 1996; Piano *et al*., 1997; Nunes *et al*., 2001; Spadaro *et al*., 2002), production of extracellular hydrolases (Wisniewski *et al*., 1991; Wilson and Wisniewski, 1994), ability to maintain normal metabolism at high osmotic potential (Wisniewski *et al*., 1995; Abadias *et al*., 2001b), resistance to oxidative stress (Castoria *et al*., 2003) and the induction of resistance responses at the wound site (Wilson and Wisniewski, 1994; Wilson *et al*., 1994).

Aims of the present investigation were: firstly, to evaluate the efficacy of antagonistic yeasts isolated from undisturbed ecosystems in Sardinia, Italy, in controlling *P. expansum* on apple fruit; secondly, to provide some preliminary data on the possible mechanism of action of two selected isolates of *Candida guillermondii*, and of a film-forming strain of *Saccharomyces cerevisiae*.

The first, second and third author have equally contributed to the experimental work; the fourth and fifth author contributed to the experimental design and to the critical revision of the manuscript; the sixth author coordinated the experimental design and wrote the manuscript.

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## MATERIALS AND METHODS

**Isolation of microorganisms.** Yeast isolates were obtained from the surface of fig (*Ficus carica* L.) and cactus pear [*Opuntia ficus-indica* (L.) Miller] fruits grown in unmanaged orchards in Northern Sardinia (Italy), by following a selection strategy developed by Wilson *et al.* (1993). Each fruit was dipped in 250 ml sterile Ringer's solution (containing 8.6 g NaCl, 0.3 g KCl, 0,33 g $CaCl_2$ per litre of distilled $H_2O$) contained in a 500-ml glass becker and incubated at 25°C for 30 min with gentle shaking (50 rpm). Apples (*Malus domestica* Borkh cv Golden Delicious), disinfected in sodium hypochlorite (0.8% as chlorine) and rinsed under tap water, were air dried and punctured with a sterile micropipette tip at the equatorial region (3-4 mm depth, 3 wounds per fruit). Twenty µl of fruit washing solution were pipetted into each wound and after 1 h incubation at 25°C, each wound was inoculated with *P. expansum* by pipetting 10 µl of a conidial suspension ($1{\cdot}10^6$ conidia $ml^{-1}$) prepared as follows: the pathogen was grown in Petri plates (90 mm diam.) containing 15 ml of potato dextrose agar (PDA; Merck & Co., Whitehouse Station, NY, USA) under constant fluorescent light; after 2 weeks incubation at 25°C, spores were collected by scraping the colony surface with a sterile scalpel, resuspended in sterile Ringer's solution, filtered through 2 layers of sterile cheese-cloth, and counted with a haemacytometer.

Inoculated apples were placed in polyethylene boxes (60x40x15 cm) and stored at 25°C and 85±5% relative humidity. The wounded areas remaining healthy after one week incubation were removed with a sterile scalpel, ground with mortar and pestle in sterile Ringer's solution and dilution plated on nutrient yeast dextrose agar (NYDA; B-D, Franklin Lakes, NJ, USA) medium for isolation of the candidate antagonists. After incubation of the plates at 25°C for 48 h, colonies were purified by following standard procedures.

**Yeast and pathogen isolates.** Two isolates, coded 3C-1b from fig and F1 from cactus pear, of *Candida guillermondii* (Castellani) Langeron & Guerra (non-ascospore forming state of *Pichia guillermondii* Wickerham), were used in this study in comparison with *Pichia guillermondii* Wickerham strain 5A (Arras *et al.*, 1998, 2002), which proved antagonistic against *Penicillium* spp. on citrus. In addition, a film-forming strain of *Saccharomyces cerevisiae* Meyen *ex* Hansen, isolated from wine (Budroni *et al.*, 2000) and coded M25, was tested for its biocontrol potential against *P. expansum* on apple. Yeast strains are stored in NYDA at 4°C and in 50% glycerol at -80°C.

Several isolates of *P. expansum* Link were obtained from decayed apple in storage and kept in the collection of the Dipartimento di Protezione delle Piante, University of Sassari. These pathogenic strains, which are stored as cultures in PDA at 4°C, were routinely reisolated from artificially inoculated apples and a mixture of 5 aggressive isolates was always used in biocontrol experiments.

**Biocontrol tests.** Apples (cv. Golden Delicious or Fuji), were disinfected in sodium hypochlorite, rinsed under tap water, air dried and punctured with a sterile micropipette tip at the equatorial region as described. Yeast cultures were routinely prepared as described by Droby *et al.* (1989) by growing yeast in 250-ml Erlenmeyer flasks containing 75 ml of nutrient yeast dextrose broth (NYDB; B-D) during 24 h at 25°C on a rotary shaker (150 rpm). Yeast cells were then recovered by centrifuging at 150 *g* for 10 min, washed twice and resuspended in sterile Ringer's solution, and brought to a final concentration of $1{\cdot}10^8$ cells $ml^{-1}$ by direct counting with a haemocytometer. Ten µl of a cell suspension of each yeast strain were pipetted into each wound and after 2 h incubation at 25°C, each wound was inoculated with *P. expansum* as described previously. A healthy control was included in some experiments (Table 1) by mock-inoculating 10+10 µl of sterile Ringer's solution within each wound. When dry, apples were placed in polyethylene boxes and stored at 25°C and 85±5% relative humidity for 1 week. The incidence of *Penicillium* rot was determined by measuring the diameter of decayed tissue around lesions every 48 h. Results are expressed as lesion diameter (in mm or as percent of the inoculated control) at the end of the experiment. There were 5-10 fruit per treatment, the treatments were arranged in a completely randomised block design and each experiment was repeated at least two times.

**Effect of culture filtrates or autoclaved cells.** Yeast culture filtrates were prepared by centrifuging cultures of the antagonists at 150 *g* for 10 min and by filtering the supernatant through a 0.2 µm nitro-cellulose filter (Sartorius AG, Göttingen, Germany). Centrifuged yeast cells were resuspended in sterile Ringer's solution and autoclaved at 120°C for 10 min. Autoclaved yeast cells and culture filtrates were used in some experiments to evaluate their efficacy in reducing *Penicillium* decay on apple (cv. Golden Delicious or Fuji). Ten µl of culture filtrate or Ringer's solution containing $1{\cdot}10^8$ autoclaved cells $ml^{-1}$ were pipetted into each artificial wound and after 2 h incubation at 25°C, each wound was inoculated with *P. expansum* as described.

There were 5 fruit per treatment arranged in a completely randomised block design and the experiment was repeated two times.

**Effect of different substances on biocontrol activity.** The following GRAS (generally regarded as safe) substances were tested for their effect on biocontrol activity of the antagonistic yeasts: calcium chloride ($CaCl_2$, 11 g $l^{-1}$), ammonium molibdate [$(NH_4)_6\,Mo_7O_{24}{\cdot}4H_2O$, 6.17 g $l^{-1}$], sodium carbonate ($Na_2CO_3$, 20 g $l^{-1}$), 2-deoxy-D-glucose (2 g $l^{-1}$). An aliquot (1 ml) of each of these substances (all provided by Merck & Co., Whitehouse Station, NY, USA), previously diluted in sterile $H_2O$ at double concentration, was mixed with 1 ml of yeast cell

**Table 1.** Biological control activity of different yeasts against *Penicillium* decay on apple (cv. Golden Delicious, experiments 1 and 3; cv. Fuji, experiment 2).

| Treatment | Lesion diameter (%) [a] | | |
|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 |
| Inoculated control | 100 ± 2.7 d [b] | 100 ± 5.9 c | 100 ± 3.2 d |
| Healthy control | 0 a | 0 a | 0 a |
| *Candida guillermondii* 3C-1b | 16.6 ± 4.4 ab | 27.4 ± 10.2 b | 13.9 ± 6.3 ab |
| *Candida guillermondii* F1 | 38.3 ± 7.0 c | 16.9 ± 6.6 ab | 19.9 ± 4.7 b |
| *Pichia guillermondii* 5A | N.T. [c] | N.T. | 53.5 ± 5.4 c |
| *Saccharomyces cerevisiae* M25 | 29.5 ± 7.7 bc | 25.9 ± 10.7 b | 21.6 ± 5.2 b |

[a] Results of three separate experiments are expressed as percent lesion diameter of the inoculated control (32.1, 30.8, and 22.3 mm, respectively).
[b] Values in each column followed by a same letter are not statistically different by Duncan's multiple range test ($P < 0.01$).
[c] N.T. = not tested.

suspension in double strength Ringer's solution; 10 ml aliquots of the resulting suspension were pipetted into each apple wound and 1 h later the pathogen was inoculated as described.

The following nutrients were also tested: galactose, maltose, glucose, melibiose, fructose, raffinose, lactose, mannose, $NH_4NO_3$, $NaNO_3$, $Ca(NO_3)_2 \cdot 4H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $KNO_3$, $Fe(NO_3)_3 \cdot 9H_2O$ (Merck & Co., Whitehouse Station, NY, USA). All sugars were tested at the concentration of 50 g $l^{-1}$, while nitrates were tested at 1 g $l^{-1}$. An aliquot (10 ml) of each nutrient solution was pipetted into the wound 30 min before inoculation of the antagonist. Two hours later, the pathogen was inoculated, apples were placed in polyethylene boxes and stored at 25°C and 85±5% relative humidity for 1 week. The incidence of *Penicillium* rot was determined as described previously.

There were 10 fruits per treatment arranged in a completely randomised block design and each experiment was repeated once.

**Statistical analysis.** The incidence and severity of *Penicillium* decay was analysed by an analysis of variance and Duncan's multiple range test with Statgraphics version 5 software (Manugistic Inc., Rockville, MD, USA). Statistical significance was applied at the level P<0.01. When the analysis was statistically significant, the least significant difference test was applied to separate means.

## RESULTS

**Biocontrol activity of selected yeast.** In preliminary experiments, the biocontrol potential of selected antagonistic yeast isolates against *P. expansum* on apple cv. Golden Delicious and Fuji was evaluated. Results of three separate tests are shown in Table 1. All yeasts were effective in reducing significantly *Penicillium* decay after one-week storage at 25°C. *C. guillermondii* 3C-1b allowed the highest reduction of decay to 13.9-27.4% of the inoculated control (Table 1).

In order to test the role of toxic metabolites in the biocontrol potential of *C. guillermondii* isolates 3C-1b and F1 and of *S. cerevisiae* strain M25, killed cells and cell-free culture filtrates of the antagonistic yeasts were tested for their efficacy in controlling *Penicillium* decay on apple cv Golden Delicious and Fuji.

While the living antagonists were able to reduce the lesion diameter significantly, both autoclaved cells and culture filtrates had no biocontrol effect and generally induced an increase in the severity of disease, probably due to the presence of nutrients at the wound site (Table 2).

**Enhancement of biocontrol efficacy by GRAS substances.** To test the feasibility of enhancing the effectiveness of biocontrol treatments against *Penicillium* decay on apple, different substances were added to artificial wounds alone or in combination with *C. guillermondii* isolates 3C-1b and F1 or *S. cerevisiae* M25. All yeasts confirmed their antagonistic potential against *P. expansum* when applied alone, and their effectiveness could be improved (although not always significantly) when combined with calcium chloride, ammonium molibdate, sodium carbonate, or 2-deoxy-D-glucose (Tables 3-5). Calcium chloride, ammonium molibdate and 2-deoxy-D-glucose were particularly effective when coupled to *C. guillermondii* 3C-1b or F1, leading to complete inhibition of the pathogen (Tables 3-4). It is noteworthy that calcium chloride and ammonium molibdate were effective in some experiments also when applied alone (Tables 4-5).

**Table 2.** Effect of living cells, autoclaved cells and cell-free culture filtrates of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Golden Delicious and Fuji).

| Treatment | Lesion diameter (%) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| *Experiment 1 (Golden Delicious)* | | | |
| Inoculated control | 100 ± 8.1 b [b] | 100 ± 8.1 b | 100 ± 8.1 ab |
| Cell suspension | 37.8 ± 7.0 a | 21.8 ± 3.5 a | 71.9 ± 11.4 a |
| Autoclaved cells | 115.3 ± 3.2 bc | 139.8 ± 3.0 c | 128.9 ± 3.5 ab |
| Culture filtrate | 153.7 ± 1.4 c | 148.9 ± 2.1 c | 142.0 ± 2.1 b |
| *Experiment 2 (Fuji)* | | | |
| Inoculated control | 100 ± 5.7 b | 100 ± 5.7 b | 100 ± 5.7 b |
| Cell suspension | 14.2 ± 6.1 a | 2.6 ± 1.0 a | 54.6 ± 2.3 a |
| Autoclaved cells | 86.9 ± 7.3 b | 91.1 ± 2.4 b | 95.7 ± 6.3 b |
| Culture filtrate | 73.0 ± 8.1 b | 123.1 ± 5.0 b | 89.9 ± 10.8 b |

[a] Results of two separate experiments are expressed as percent lesion diameter of the inoculated control (18.3 and 17.3 mm, respectively).
[b] In each experiment, values in the same column followed by a same letter are not statistically different by Duncan's multiple range test ($P < 0.01$).

**Table 3.** Effect of calcium chloride, ammonium molibdate and sodium carbonate on the biological control activity of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Fuji).

| Treatment | Lesion diameter (mm) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| Inoculated control | 30.3 ± 2.2 c [b] | 30.3 ± 2.2 c | 30.3 ± 2.2 c |
| $CaCl_2$ | 28.8 ± 2.1 c | 28.8 ± 2.1 c | 28.8 ± 2.1 b |
| $(NH_4)_6 Mo_7 O_{24} \cdot 4 H_2O$ | 17.8 ± 7.2 b | 17.8 ± 7.2 b | 17.8 ± 7.2 a |
| $Na_2CO_3$ | 28.1 ± 2.4 c | 28.1 ± 2.4 c | 28.1 ± 2.4 b |
| Yeast | 4.4 ± 2.3 a | 5.8 ± 5.3 a | 10.9 ± 7.6 a |
| Yeast + $CaCl_2$ | 0 a | 3.1 ± 0 a | 11.4 ± 9.4 a |
| Yeast + $(NH_4)_6 Mo_7 O_{24} \cdot 4 H_2O$ | 9.5 ± 4.1 ab | 11.0 ± 1.8 ab | 9.4 ± 5.2 a |
| Yeast + $Na_2CO_3$ | 7.0 ± 0 a | 8.0 ± 5.2 a | 9.2 ± 3.1 a |

[a] Results are expressed as lesion diameter (mm).
[b] See Table 1.

**Table 4.** Effect of calcium chloride, ammonium molibdate, sodium carbonate and 2-deoxy-D-glucose on the biological control activity of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Fuji).

| Treatment | Lesion diameter (mm) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| Inoculated control | 24.7 ± 6.1 d [b] | 24.7 ± 6.1 f | 24.7 ± 6.1 c |
| $CaCl_2$ | 7.0 ± 5.7 ab | 7.0 ± 5.7 abc | 7.0 ± 5.7 ab |
| $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ | 11.0 ± 8.4 abc | 11.0 ± 8.4 bcd | 11.0 ± 8.4 ab |
| $Na_2CO_3$ | 20.0 ± 3.3 cd | 20.0 ± 3.3 def | 20.0 ± 3.3 bc |
| 2-deoxy-D-glucose | 12.4 ± 3.7 bc | 12.4 ± 3.7 cde | 12.4 ± 3.7 abc |
| Yeast | 7.6 ± 5.4 ab | 4.6 ± 2.7 abc | 2.8 ± 4.8 a |
| Yeast + $CaCl_2$ | 12.0 ± 10.7 bc | 11.0 ± 11.0 bcd | 6.6 ± 9.9 ab |
| Yeast + $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ | 0 a | 1.7 ± 2.9 ab | 2.2 ± 3.8 a |
| Yeast + $Na_2CO_3$ | 11.2 ± 7.5 abc | 21.4 ± 2.2 ef | 9.0 ± 12.6 ab |
| Yeast + 2-deoxy-D-glucose | 0 a | 0 a | 8.0 ± 8.0 ab |

[a] See Table 3.
[b] See Table 1.

**Table 5.** Effect of calcium chloride, ammonium molibdate, sodium carbonate and 2-deoxy-D-glucose on the biological control activity of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Golden Delicious).

| Treatment | Lesion diameter (mm) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| Inoculated control | 23.6 ± 3.5 e [b] | 23.6 ± 3.5 c | 23.6 ± 3.5 d |
| $CaCl_2$ | 20.8 ± 4.6 e | 20.8 ± 4.6 c | 20.8 ± 4.6 cd |
| $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ | 13.3 ± 5.9 d | 13.3 ± 5.9 b | 13.3 ± 5.9 ab |
| $Na_2CO_3$ | 20.3 ± 4.2 e | 20.3 ± 4.2 c | 20.3 ± 4.2 cd |
| 2-deoxy-D-glucose | 20.4 ± 5.2 e | 20.4 ± 5.2 c | 20.4 ± 5.2 cd |
| Yeast | 0.9 ± 1.4 a | 13.8 ± 4.2 b | 11.8 ± 10.8 ab |
| Yeast + $CaCl_2$ | 0.6 ± 1.8 a | 7.2 ± 5.4 a | 19.8 ± 6.5 cd |
| Yeast + $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ | 5.9 ± 3.4 bc | 9.0 ± 7.2 ab | 8.2 ± 3.9 a |
| Yeast + $Na_2CO_3$ | 7.5 ± 6.1 c | 9.8 ± 6.9 ab | 12.5 ± 6.8 ab |
| Yeast + 2-deoxy-D-glucose | 2.7 ± 3.1 ab | 6.5 ± 8.8 a | 15.5 ± 8.3 bc |

[a] See Table 3.
[b] See Table 1.

**Table 6.** Effect of different nitrates on the biological control activity of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Golden Delicious).

| Treatment | Lesion diameter (mm) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| Inoculated control | 17.6 ± 2.6 d [b] | 17.6 ± 2.6 c | 17.6 ± 2.6 b |
| Yeast | 5.0 ± 3.4 ab | 0 a | 9.9 ± 3.4 ab |
| Yeast + $NH_4NO_3$ | 13.3 ± 8.5 bcd | 13.8 ± 0.2 bc | 9.9 ± 6.0 ab |
| Yeast + $NaNO_3$ | 16.4 ± 4.6 cd | 12.4 ± 4.0 bc | 12.5 ± 5.3 ab |
| Yeast + $Ca(NO_3)_2 \cdot 4H_2O$ | 7.4 ± 6.4 abc | 11.4 ± 4.3 b | 8.2 ± 7.3 ab |
| Yeast + $Mg(NO_3)_2 \cdot 6H_2O$ | 8.5 ± 1.7 abcd | 18.4 ± 1.9 c | 8.8 ± 1.9 ab |
| Yeast + $KNO_3$ | 10.4 ± 4.1 abcd | 13.7 ± 6.0 bc | 8.5 ± 5.9 ab |
| Yeast + $Fe(NO_3)_3 \cdot 9H_2O$ | 2.4 ± 4.2 a | 0.5 ± 0.9 a | 3.7 ± 3.8 a |

[a] See Table 3.
[b] See Table 1.

**Table 7.** Effect of different sugars on the biological control activity of *Candida guillermondii* isolates 3C-1b and F1, and *Saccharomyces cerevisiae* strain M25 against *Penicillium* decay on apple (cv. Golden Delicious).

| Treatment | Lesion diameter (mm) [a] | | |
|---|---|---|---|
| | 3C-1b | F1 | M25 |
| Inoculated control | 23.8 ± 2.1 c [b] | 23.8 ± 2.1 d | 23.8 ± 2.1 b |
| Yeast | 9.6 ± 2.0 b | 7.3 ± 1.0 bc | 17.2 ± 2.0 a |
| Yeast + galactose | 0.7 ± 1.3 a | 1.5 ± 2.7 ab | 17.0 ± 5.2 a |
| Yeast + glucose | 8.3 ± 2.6 b | 0 a | 18.9 ± 3.8 ab |
| Yeast + fructose | 4.6 ± 8.1 ab | 1.1 ± 1.9 ab | 21.0 ± 1.0 ab |
| Yeast + melibiose | 1.3 ± 2.3 a | 1.8 ± 3.2 ab | 21.2 ± 4.2 ab |
| Yeast + lactose | 0 a | 1.0 ± 1.7 ab | 22.7 ± 4.0 ab |
| Yeast + mannose | 9.0 ± 4.7 b | 9.9 ± 8.3 c | 23.0 ± 3.6 ab |
| Yeast + maltose | 1.1 ± 1.9 a | 4.4 ± 4.5 abc | 23.9 ± 3.4 b |
| Yeast + raffinose | 6.0 ± 0.7 ab | 2.0 ± 1.9 ab | 23.9 ± 2.5 b |

[a] See Table 3.
[b] See Table 1.

**Nutrient competition.** Several nitrates were tested for their capacity to suppress the antagonistic potential of the yeast strains. The addition in each wound of either $NH_4NO_3$, $NaNO_3$, $Ca(NO_3)_2 \cdot 4H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, or $KNO_3$ before inoculation of the antagonist, generally decreased, although not always significantly, the biocontrol effect of *C. guillermondii* 3C-1b and F1, while *S. cerevisiae* M25 was not effective under the tested conditions (Table 6). The addition of $Fe(NO_3)_3 \cdot 9H_2O$ had no significant influence on the biocontrol effect of *C. guillermondii*, enhancing the suppression of *Penicillium* decay when combined with *S. cerevisiae* M25. This result is likely due to fungistatic effect of the compound: indeed, a significant inhibitory effect was observed when iron nitrate was added in the absence of any biocontrol yeast (data not shown).

The addition of sugars (except mannose) generally enhanced the biocontrol effect of *C. guillermondii*: such effect was significant for galactose, melibiose, lactose and maltose applied in combination with 3C-1b, and for glucose applied with F1. Maltose and raffinose decreased significantly the efficacy of *S. cerevisiae* M25 against *Penicillium* decay (Table 7).

## DISCUSSION

Control of *Penicillium* decay of apple fruit is known as very difficult to achieve by biological means, due to the high competitiveness of the pathogen in the wound niche (Wilson and Wisniewski, 1989, 1994; Spotts *et al*., 1999; Janisiewicz and Korsten, 2002). This impels development of an integrated approach to post harvest disease management, where the role and mechanism of action of selected yeast isolates should be evaluated under variable environmental condition.

The selection strategy adopted in our study (Wilson *et al*., 1993) allowed the isolation of two antagonistic *C. guillermondii* isolates with remarkable antagonistic properties against *P. expansum* on apple fruit. The biocontrol activity was not influenced by the apple cultivar, the decay being inhibited on apple cv. Golden Delicious, Fuji (this study), Red Delicious and Starking Delicious (data not shown). Also, the use of a mixture of *P. expansum* isolates in all the experiments should provide a high level of reliability of the data obtained.

In agreement with previous reports (Droby *et al*., 1989; Piano *et al*., 1997; Spadaro *et al.,* 2002), living cells of all the tested yeasts were necessary to achieve consistent biocontrol activity, as neither autoclaved cells or culture filtrates had any antagonistic effect and generally induced an increase in the severity of disease. Most probably, this effect was due to the presence of nutrients at the wound site.

GRAS substances, such as calcium chloride, ammonium molibdate, sodium carbonate, or 2-deoxy-D-glucose, were tested for their influence on the antagonistic potential of *C. guillermondii* and *S. cerevisiae* against *P. expansum*. Calcium chloride, ammonium molibdate and 2-deoxy-D-glucose, when coupled to *C. guillermondii* 3C-1b or F1, led to complete inhibition of the pathogen. It should be mentioned that ammonium molibdate showed phytotoxic effect (leading to the appearance of necrotic tissue) at the wound level when applied to apple fruit of the cv Fuji at the tested dosage. This effect was not reported by Nunes *et al*. (2002) who first proposed the use of this compound to inhibit postharvest decay of pear. Conversely, the addition of a nutrient analogue such as 2-deoxy-D-glucose, which cannot be metabolised by the pathogen, inhibited *Penicillium* decay by giving advantage to the antagonist.

Based on previous studies on the nutrient competition of *Metschnikowia pulcherrima* (Piano *et al*., 1997), several nitrates and sugars were tested for their capacity to suppress the antagonistic potential of the yeast strains. As suggested by Janisiewicz and Korsten (2002), nitrogen is likely to represent a limiting factor in the carbon-rich environment of apple wound and our results support the hypothesis that, once again, nutrient competition for nitrates plays a major role in the biocontrol effect of *C. guillermondii* 3C-1b and F1. The inhibitory effect of $FeNO_3$ *per se* towards *P. expansum* is responsible for the reduction of decay in the presence of both *C. guillermondii* and *S. cerevisiae* and deserves further investigation.

The addition of sugars, such as galactose, glucose, melibiose, lactose and maltose, generally enhanced the biocontrol effect of *C. guillermondii* against *Penicillium* decay. The effect of galactose and lactose is particularly promising because of the role of these sugars as cryoprotectants during freeze drying and lyophilization (Abadias *et al*., 2001a).

The reported experiments were all carried at 25°C, in order to test the biocontrol potential of selected yeast isolates during shelf life and in the presence of a high concentration of pathogen inoculum. More studies are now needed to evaluate the effectiveness of *C. guillermondii* during prolonged storage in controlled atmosphere.

A major drawback hampering the practical use of isolates of this species in commercial biocontrol formulations resides in the significant, although not frequently observed, cases of onychomycosis and fungaemia in humans, and bovine mastitis caused by *C. guillermondii* (Moretti *et al*., 1998; Krcmery and Barnes, 2002; Ellabib *et al*., 2002). The pathogenic behaviour and the genetic structure of *C. guillermondii* isolates selected as antagonists should be therefore carefully examined in comparison to clinical specimens before starting any registration process as biocontrol products.

Noteworthy, the film-forming *S. cerevisiae* strain M25 showed some effect in reducing postharvest decay on apple caused by *P. expansum*. It is well known that during the biological aging of wines such as the Fino sherry or the Malvasia, the formation of aroma compounds takes place as a result of the oxidative metabolism of the *S. cerevisiae flor* yeasts, which form a film on

the surface of the wine. These microrganisms utilise compounds such as ethanol, glycerol, organic acids (acetic, lactic, citric and succinic), and amino acids, including proline. At the same time, *flor* yeasts produce other compounds, namely higher alcohols (isobutanol and isoamilic), acetaldehyde and acetoin (Martinez *et al*., 1998; Budroni *et al*., 2000; Dos Santos *et al*., 2000). The reported biocontrol effect of this particular strain may be therefore due to the accumulation of higher alcohols, acetaldehyde or acetoin within the wound site. Additional work is now needed to shed light on the role of these substances in the inhibition of the pathogen. Although the practical possibility to develop *S. cerevisiae* as a biocontrol agent is probably weak, the results presented here open new avenues for a genome-based approach to the study of the mechanism of action of antagonistic yeasts (Berman and Sudbery, 2002; Brazhnik *et al*., 2002; Kumar and Snyder, 2002; Kessler *et al*., 2003).

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