



## Characterization of Biocontrol Activity of Two Yeast Strains from Iran against Blue Mould of Apple in Order to Reduce the Environmental Pollution

Jalal Gholamnejad\*, H.R. Etebarian, N.A. Sahebani, A. Roustaei

Department of plant protection, Aboraihan Campus, University of Tehran, Iran

Received December 12, 2008; Accepted April 01, 2009

**Abstract:** In this present research, two yeast antagonists *Rhodotorula mucilaginosa* (strain A7) and *Pichia guilliermondii* (strain A6) isolated from the surface of healthy apples, controlled blue mould of apple caused by *Penicillium expansum*. This yeast applied instead of fungicide, whatever hazard health and environment. Both antagonists were evaluated as a potential biological control agent for apple blue mould caused by *P. expansum*. Dual culture, cell free metabolite and volatile test were used *in vitro* assay. Colony area was recorded compared with controls and percentage of growth inhibition was calculated. Both yeast strains of two genus inhibited growth of *P. expansum*, the inhibition varied among isolates of two genuses and ranged from 34.51 to 57.62, in dual culture, from 71.86 to 82.6 in volatile metabolite and from 86.03 to 88.77 in cell free metabolite test. Both antagonists reduced the incidence of blue mould by 70% at 20 °C. At 5 °C *P. guilliermondii* (strain A6) maintained the efficacy of disease control, but *R. mucilaginosa* (strain A7) only reduced disease incidence by 60%. Moreover *P. guilliermondii* (strain A6) exhibited significant protection at lower concentrations than *R. mucilaginosa* (strain A7). The population of both strains increased in wounds of apples at 20 and 5 °C, and both strains maintained viable over a period of 32 days at 5 °C. Separately, effect of CaCl<sub>2</sub> was mixed yeast was evaluated in 25°C. The different of concentration CaCl<sub>2</sub> reduced decay area from 185.07 to 1738.037 mm<sup>2</sup> compared to 2452.84mm<sup>2</sup> in control after incubation for 15 days.

**Keywords:** Biocontrol, Blue mould, Postharvest disease, Dual capture, Volatile and cell free metabolite test, environment

### Introduction

The fungal decay of fruits and vegetables in postharvest storage greatly limits their economic value. Postharvest losses of fruits and vegetables are high, ranging from 10 and 40% depending on the species and technologies used in the packinghouses (Arras & Arru, 1999; Wilson & Wisniewski, 1989). In the case of apples, postharvest losses are mainly caused by *Penicillium expansum* Link (blue mold) and *Botrytis cinerea* Pers. Fr. (grey mould).

Although fungicide treatments, such as benomyl, iprodione and thiabendazol, have been the main method for controlling postharvest diseases, public concern about fungicide residues in food and environment and also the development of fungicide resistance by pathogens has increased the search for alternative means of controlling disease. Biological control of postharvest decays of fruits and vegetables has emerged recently as a promising alternative to the use of synthetic fungicides (Wilson & Wisniewski, 1989; Wisniewski & Wilson, 1992).

Treatment of fruit with microbial agents has been demonstrated to be an efficient method for control of several postharvest decays (Janisiewicz & Roitman, 1988; Chalutz & Wilson, 1990; Wilson *et al.*, 1993; Fan & Tian, 2000). Some yeasts and bacteria are reported to reduce effectively various postharvest decays of fruits (Janisiewicz & Roitman, 1988; Chalutz & Wilson, 1990; McLaughlin *et al.*, 1990; Piano *et al.*, 1997; Fan & Tian, 2001).

\*Corresponding: E-mail: jalalgholamnejad2006@gmail.com; Tel: 00983515252254; Fax: 00982923025292

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 order to enhance biocontrol activity of antagonists against fungal pathogens, certain strategies, such as adding calcium salts, carbohydrates, amino acids and other nitrogen compounds to biocontrol treatments, are proposed (Conway, 1982; Conway et al., 1987; Janisiewicz & Marchi, 1992). Many researchers have shown that calcium plays an important role in the inhibition of postharvest decay of fruits (Conway & Sams, 1985; Conway et al., 1992) and in enhancing the efficacy of postharvest biocontrol agents (Conway et al., 1991).

The present work was carry out to characterize two antagonistic yeast strains, *Rhodotorula mucilaginosa* (strain A7) and *Pichia guilliermondii* (strain A6) and to study their possible mode of action as well as to evaluate the effect of calcium treatment on the biocotrol activity of the yeast antagonists.

## Materials and Methods

### Fruit

*Malus domestica*, apples that had been harvested at commercial maturity and kept at  $1\pm0.5$  in cold storage was used to evaluate the antagonistic potential of yeast on fruit.

### Pathogen

*P. expansum* (P<sub>11</sub>), from *Malus domestica* Golden delicious from Damavand, Iran was used in this study. The culture were derived from single spore isolate and maintained on Potato Dextrose Agar (PDA) at 4 °C in darkness until needed.

### Biocontrol agents

Biocontrol agents used in this study were yeast strains isolated from the surface of healthy apple fruits collected from organic production orchards. Identification of selected strains was carried out by "Identification Service CBS". Optimum growth temperature in PDA was determined for each strain. Temperatures assayed were 5, 25, 30, 37 °C.

### In vitro biological control studies

Dual culture (Etebarian et al., 2003), cell free culture (Weller, 1988) and volatile metabolite tests (Lillbro, 2005) were used to observe effect of yeast strains on mycelia growth of *P. expansum*. All antagonist- pathogen combinations were examined on potato dextrose agar in 9 cm Petri plate with four replications. The plates were incubated 18 days for both dual culture and cell free culture as well as 25 days for volatile metabolite at 25°C. The percentage growth inhibition was calculated using the formula  $n=(a-b)/a \times 100$ , where  $n$  is the percent growth inhibition;  $a$  is the colony area of uninhibited *P. expansum* and  $b$  is the colony area of treated *Penicillium expansum* (Etebarian et al., 2005).

### Biocontrol assays

Biocontrol assays were performed at 20 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×3 mm wide) were cut at the equator of each apple with a cork borer. Two of the wounds were inoculated with 40µl of a yeast suspension ( $10^7$  cfu ml<sup>-1</sup>) and the other two with 40 µl of sterile saline (0.9%) as a control. Fruit were then placed in covered plastic containers at 20 or 5 °C. High humidity was maintained by adding water to the bottom of the tray. After 24 h wounds were inoculated with 20µl of conidial suspension of the pathogen ( $10^5$  conidia ml<sup>-1</sup>). This pathogen concentration had previously proved to produce 100% infection of wounds (Vero et al., 2002).

The fruit were then incubated again in the same conditions as above. After the incubation period (15 days at 20 °C or 32 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:

$$\%Inc = \frac{\text{Number of rotten wounds}}{\text{Number of rotten wounds}} \times 100 \quad (1)$$

Severity was defined as:

$$\% \text{Severity} = \frac{\text{LdA}}{\text{LdC}} \times 100 \quad (2)$$

Where:

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  $10^4$ – $10^7$  cfu per wound. The colony forming units of conidial suspension of the pathogen was maintained ( $10^5$  conidia per ml) and 20 µl were applied to each wound. Concentrations of antagonists and pathogen suspensions were confirmed by plating appropriate dilutions on malt agar.

### ***Antagonistic effect of $\text{CaCl}_2$ concentration on biocontrol activity of yeast***

Aliquots of 50 mL NYDB with different concentration of  $\text{CaCl}_2$  of 0 mmol  $\text{l}^{-1}$ , 173 mmol  $\text{l}^{-1}$  (2%), 347 mmol  $\text{l}^{-1}$  (4%) , and 521 mmol  $\text{l}^{-1}$  (6%)  $\text{CaCl}_2$ , in 250 ml conical flasks were autoclaved (121°C, 15 min) prior to adding suspension of antagonistic yeast to some flask. The cell suspensions of the antagonists (A7) and  $\text{CaCl}_2$  were adjusted to concentrations of  $1 \times 10^7$  CFU  $\text{ml}^{-1}$ . The same percentages of  $\text{CaCl}_2$  without antagonistic yeast were prepared as described above.

Wounds were inoculated with a 40 µl of this solution. After 24-h, a 20 µl *P.expansum* suspension of P<sub>11</sub> isolate in a concentration of  $1 \times 10^5$  spores  $\text{ml}^{-1}$  was added to each wound, and the apples was stored for 15 days at 25 °C (Tian *et al.*, 2002).

### ***Colonization of wound site***

Growth curves were done in fruit wounds at 20 and 5 °C. Wounds (5 mm deep×3 mm wide) were made in surface-disinfected apple fruit with a sterile needle. Pieces of apple (approximately 1 g) bearing a wound were cut and placed in 9 ml cotton tubes. The wounds were inoculated with 40 µl of yeast suspension of known concentration ( $10^7$  cfu  $\text{ml}^{-1}$ ) and incubated for 15 days at 20 °C and for 32 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 (Vero, 1998).

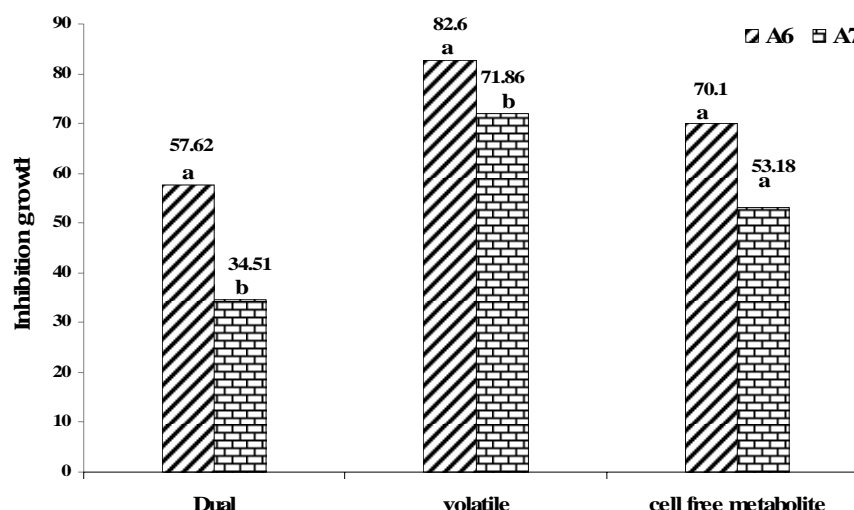
### ***Statistical analysis***

The in vitro and in vivo assays were analyzed by an analysis of variance (ANOVA) with SAS Software (SAS Institute, version 9.0, Cary, NC). Statistical significance was judged at the level of  $P < 0.05$ . When the analysis was statistically significant, Duncan's Multiple-Range Test (SSR Test) was used to test mean separations among mean values of each treatment.

## **Results**

### ***Effects of the Antagonist in vitro***

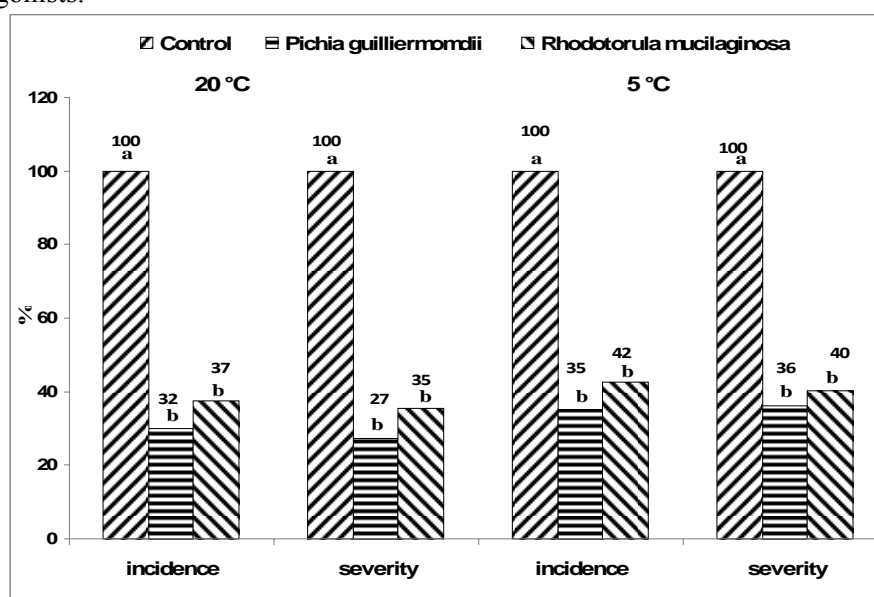
Two yeast antagonist tested inhibited mycelia growth of *P.expansum* in dual culture. The percent average of growth inhibition of *P.expansum* was 57.62% by strain A6 of *P. guilliermondii*, and 34.51% by strain A7 of *R.mucilaginosa* in dual culture (Figure 1). Volatile metabolite was produced by both yeast strains. Strain A6 of *P. guilliermondii* reduced the colony area of pathogen to 82.6%, and strain A7 of *R.mucilaginosa* reduced it to 71.86(Figure 1). Cell free metabolite of strain A6 of *P. guilliermondii* reduced growth of *P.expansum* to 88.77, and also strain A6 of *R.mucilaginosa* reduced the colony area of *P.expansum* to 86.03 (Figure 1).



**Figure 1.** Percentage of growth inhibition of *P. expansum* by *P. guilliermondii* (A6) and *R. mucilaginosa* (A7) in vitro. Values are the mean inhibition growth percentage from four replication. Values with the same letter within each test do not differ significantly ( $P \leq 0.05$ ) according to Duncan's multiple range Test.

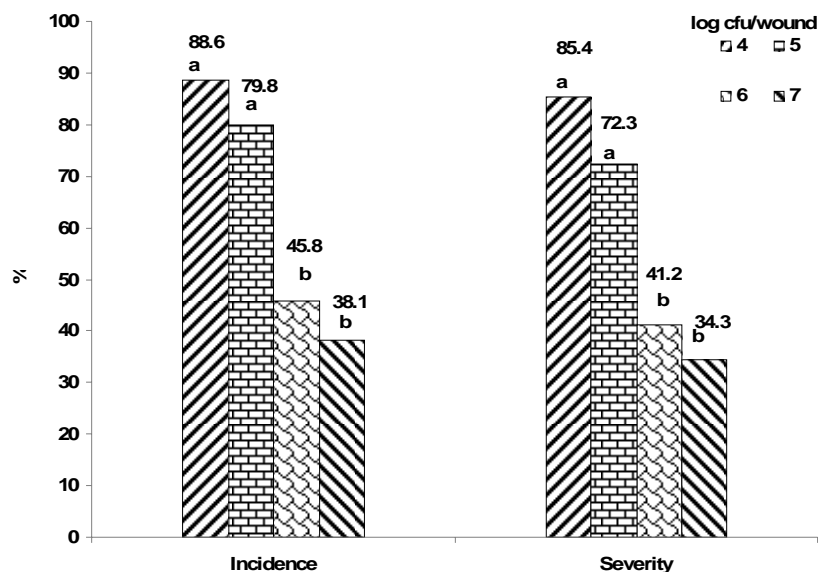
### Effects of the Antagonist in vivo

Both yeast strains, identified as *Rhodotorula mucilaginosa* (A7) and *Pichia guilliermondii* (strain A6), appeared to be good antagonists of blue mold on apples at 20 and 5 °C ( $p \leq 0.05$ ; Figure 2). Incidence of blue mould was lowered to 30.02 and 37.47% when both antagonists were applied at 20 °C, and to 35 and 42.5 %, respectively, when applied at 5 °C. Optimum growth temperature was 25 °C for both antagonists.

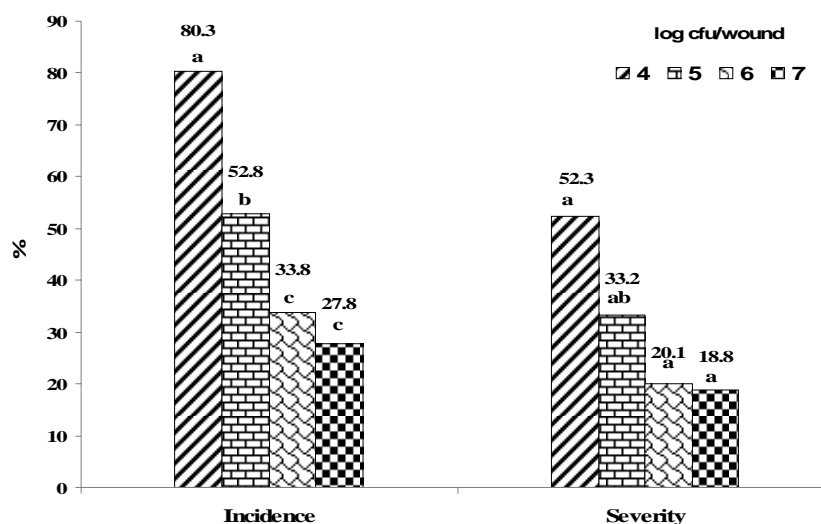


**Figure 2.** Incidence and severity of blue mould on apples inoculated with *P. guilliermondii* (A6) or *R. mucilaginosa* (A7) and *P. expansum*. Controls were not treated with antagonists. Fruit were held for 32 days at 5 °C. Values with the same letter within each test do not differ significantly ( $P \leq 0.05$ ) according to Duncan's multiple range Test

At 5°C, *R. mucilaginosa* (A7) was effective only when applied at  $10^6$  cells per wound whereas *P. guilliermondii* (A6) exhibited significant protection at  $10^5$  cells per wound (Figures. 3 and 4). When applied to apple wounds, both yeast strains grew at both temperatures and remained viable over a period of 35 days at 5 °C (Figure 5 A and B).



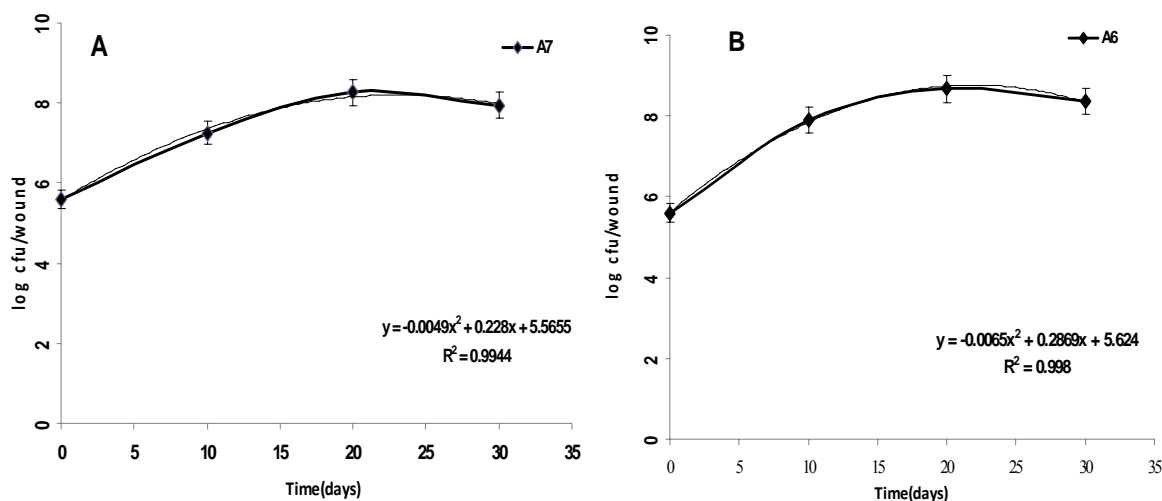
**Figure 3.** Incidence and severity of blue mould on apples after applying different concentrations of *R. mucilaginosa* strain A7. Fruit were held for 32 days at 5 °C. Values with the same letter within each test do not differ significantly ( $P \leq 0.05$ ) according to Duncan's multiple range Test



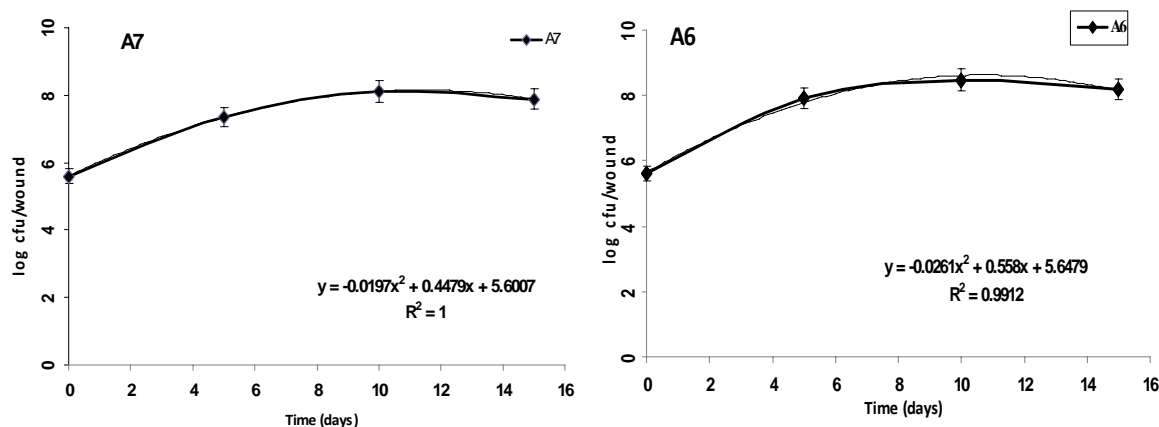
**Figure 4.** Incidence and severity of blue mould on apples after applying different concentrations of *P. guilliermondii* strain A6. Fruit were held for 32 days at 5 °C. Treatments with the same letter are not significantly different ( $P < 0.05$ ).

### Colonization of wound site

The population dynamics of two yeast were evaluated after 5, 10 and 15 days at 20 °C, and 10, 20 and 30 at 5 °C. The maximum population was recovered 10 and 20 days after the yeast application at 20 °C and 5 °C, respectively. Initially,  $4 \times 10^5$  cfu g<sup>-1</sup> inoculated on the wounds, 10 days after applications of *R. mucilaginosa* (strain A<sub>7</sub>) at 20 °C, population were maximum, about  $1.2 \times 10^8$  cfu g<sup>-1</sup>, after that population was decreased, until end of the experiment (. At 5 °C, on the twentieth day population was maximum, about  $1.8 \times 10^8$  cfu g<sup>-1</sup>, after that decrease population, until end of the experiment. (Figures 5 and 6)



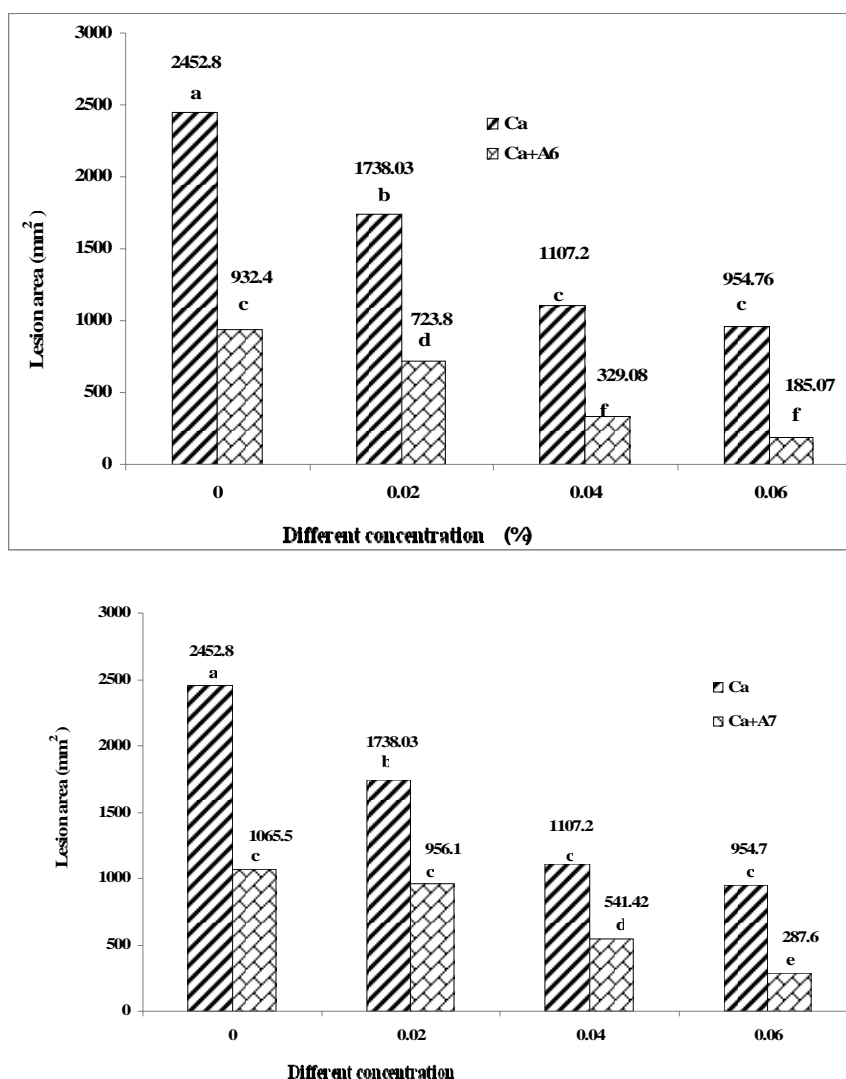
**Figure 5.** Population sizes of *P. guilliermondii* strain A6 and *R. mucilaginosa* strain A7 in apple wounds at 5°C.



**Figure 6.** Population sizes of *P. guilliermondii* strain A6 and *R. mucilaginosa* strain A7 in apple wounds at 20°C.

### ***Antagonistic effect of CaCl<sub>2</sub> concentration on biocontrol activity of yeast***

There was a significant reduction in diseases with the Ca<sup>2+</sup>- yeast treatments, as controlled to yeast treatments alone ( $P \leq 0.05$ ) (figure.7). The control of pathogen was correlated to concentrations of Ca<sup>2+</sup>; CaCl<sub>2</sub> at 521mmol/L provided better control than 347 mmol/L but wasn't significant. In result, 347 mmol/L was the best concentration Ca<sup>2+</sup>. The results shown all concentrations of Ca<sup>2+</sup> had significant effect on lesion diameter.



**Figure 7.** Effect of different concentrations of  $\text{Ca}^{2+}$  alone and with antagonistic yeast *R. mucilaginosa* strain A7 and *P.guilliermondii* strain A6 on lesion area in apple ( $p < 0.05$ )

## Discussion

The results of this study indicate that *Penicillium expansum* causes postharvest decay of apple and that *Rhodotorula mucilaginosa* and *Pichia guilliermondii* have antagonistic biocontrol efficacy. The biocontrol efficacy of *P. guilliermondii* was superior. Biocontrol of postharvest decay caused by different epiphytes may need different antagonists.

Yeast was the most common biological control agent, it can be used instead of chemical fungicide. The yeast is not harmful but useful for environment and human health. This research presents a valuable method to control epiphyte pathogen caused postharvest decay, by using antagonistic yeasts. However, the methods of yeast biocontrol have many aspects and need to further study. Better methods are needed to assist to the decay control and to combine microorganisms with storage of farm produce.

In the present study, we have identified two yeast antagonists that exhibit biocontrol efficacy against blue mould of apples caused by *P. expansum*. Strains of this species have been isolated from the surface of healthy apple.

The result for apples stored at 5 °C for 32 days and 20 °C for 15 days showed that these two strains reduced growth of *P. expansum* and were effective material for control of pathogen. These strains have also ability for suppressing other pathogen such as green mold of oranges (*Penicillium digitatum*; Zheng et al. 2004), Grey Mould on Tomato (*Botrytis cinerea*) (Bello et al. 2008). The

adaptation of these two strains to a wide range of temperature provides great market potential for this product for control of postharvest diseases on apples in storage and transportation, as well as under arbitrary temperature in the market places and consumers' home.

Growth curves of the antagonists demonstrated that both could colonize and grow in apple wounds. Even after a period of 32 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 (Figures 3 and 4). These data indicate that only one application of the antagonists may be enough to prevent blue mould rot for at least a period of 35 days.

The activity of both yeast strains and  $\text{CaCl}_2$  against pathogen provided an opportunity to combine these agents at lower concentration while maintain a high level of effectiveness and consistency of performance are two major factors limiting the use of biocontrol agent in plant disease control (Chalutz et al, 1988). Yeast and  $\text{CaCl}_2$  had a significant synergistic effect on reduction of blue mould on golden delicious apple.  $\text{CaCl}_2$  not effect on yeast until 6% (w/v) in vitro. A combination of a biocontrol agent with a salt both significantly reduced at concentration from what would be needed if used alone may greatly reduce their residue on fruit (Zhou et al., 2002). 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.

## Conclusions

In summary, this research identified two strains of antagonistic yeast that exhibited biocontrol efficacy against blue mould of apples. All two isolates of yeast effectively colonized wounds of apple at 5 and 20 °C. They have good effect in vivo assay. They can be applied in storage under commercial condition instead of chemical fungicide.

**Acknowledgements:** This research was financially supported by college of Aboraihan university of Tehran and Iran, National Science Foundation (INSF), Tehran, Iran. Particular thanks to M. Mohammadifar, who cooperated in part of the experiments, and to the anonymous reviewers for their useful suggestions.

## References

- Arras G, Arru S, (1999) Integrated control of postharvest citrus decay and induction of phytoalexins by *Debaryomyces hansenii*. *Adv. Hort. Sci.* **13**, 76-81.
- Chalutz E, Droby S, Wilson CL, (1988) Microbial protection against postharvest diseases of citrus fruit. *Phytoparasitica* **16**, 195-196.
- Chalutz E, Wilson CL, (1990) Postharvest biocontrol of green and blue mold and sour rot of citrus fruits by *Debaryomyces hansenii*. *Plant Dis* **74**, 134.
- Conway WS, (1982) Effect of postharvest calcium treatment on decay of delicious apples. *Plant Dis.* **66**, 402.
- Conway WS, Abbott JA, Bruton BD, (1991) Postharvest calcium treatment of apple fruit to provide broad-spectrum protection against postharvest pathogens. *Plant Dis* **75**, 620.
- Conway WS, Greene GM, Hickey KD, (1987a) Effects of preharvest and postharvest calcium treatments of peaches on decay caused by *Monilinia fructicola*. *Plant Dis* **71**, 1084.
- Conway WS, Sams CE, (1985) Influence of fruit maturity on the effect of postharvest calcium treatment on decay of Golden Delicious apples. *Plant Dis* **69**, 42.
- Conway WS, Sams CE, McGuire RG, Kelman A, (1992) Calcium treatment of apples and potatoes to reduce postharvest decay. *Plant Dis* **76**, 329.
- Etebarian HR, Scott ES, T.J. Wicks, (2003) Evaluation of streptomyces strains as potential biological agents of phytophthora erythroseptica. *Iran J. Plant Pathol.*, **39**, 49.
- Etebarian HR, Sholberg PL, Eastwell KC, Sayler RJ, (2005) Biological control of apple blue mold with *Pseudomonas fluorescens*. *Microbiol.* **51**, 591-598.
- Fan Q, Tian SP, (2001) Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus* (Saito) Skinner. *Postharvest Biol and Technol* **21**, 341-50.
- Fan Q, Tian SP, (2000) Postharvest biological control of *Rhizopus* rot on nectarine fruits by *Pichia membranefaciens* Hansen. *Plant Disease* **84**, 1212.



- Janisiewicz, W.J., Marchi, A., (1992) Control of storage rots on various pear cultivars with a saprophytic strain of *Pseudomonas syringae*. *Plant Dis.* **76**, 555–560.
- Janisiewicz WJ, Roitman J, (1988) Biological control of blue mold on apple and pear with *Pseudomonas cepacia*. *Phytopathol* **78**, 1697.
- Lillbro, M. (2005) Biocontrol of *Penicillium roqueforti* on grain-a comparison of mode of action of several yeast species. Master thesis for the Agriculture Programme, animal science, performed at the Department of Microbiology. Swedish University of Agricultural Sciences **21**pp.
- McLaughlin, R.J., Wisniewski, M.E., Wilson, C.L., Chalutz, E. (1990) Effects of inoculum concentration and salt solution on biological control of Postharvest diseases of apple with *Candida* sp. *Phytopathol.* **80**, 456-461.
- Piano, S., Neyrotti, V., Migheli, Q., Gullino, M.L., (1997) Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. *Postharvest Biol. Technol.* **11**: 131–140.
- Tian SP, Fan Q, Xu Y, Jiang AL., (2002) Effects of calcium on biocontrol activity of yeast antagonists against the postharvest fungal pathogen *Rhizopus stolonifer*. *Plant Dis.* **51**, 352-358.
- Vero S, (1998) Metabolismo secundario de hongos filamentosos. Doctoral thesis, Universidad de la Repu'blica, Facultad de Qui'mica, Montevideo, Uruguay.
- Vero S, Mondino P, Burgaeno J, Soubes M, Wisniewski M, (2002) Characterization of biological activity of two yeast strains from Uruguay against blue mould of apple. *Postharvest biology and Technol.* **26**, 91-98.
- Wisniewski M, Droby S, Chalutz E, Eilam Y, (1995) Effects of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on *Botrytis cinerea* and *Penicillium expansum* in vitro and on the biocontrol activity of *Candida oleophila*. *Plant Pathol* **44**, 1016.
- Weller, D.M. (1988) Biological control of soil borne plant pathogens, in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26**:379-407.
- Wilson, C.L., Wisniewski, M.E., 1989. Biological control of postharvest diseases of fruits and vegetables. An emerging technology. *Annu. Rev. Phytopathol.* **27**, 425–441.
- Wilson CL, Wisniewski ME, Droby S, Chalutz E, (1993) A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. *Sci. Hort.* **53**, 183.
- Wisniewski M, Wilson CL, (1992) Biological control of postharvest diseases of fruits and vegetables: recent advances. *Hort. Sci.* **27**, 94.
- Zheng XD, Zhang HY, Sun P, (2004) Biological control of postharvest green mould decay of oranges by *Rhodotorula glutinis*. **12**, 34-42.
- Zhou T, Northover J, Schaneider K, Lu X, (2002) Interaction between *Pseudomonas syringae* MA-4 and cyprodinil in the control of the blue mould and gray mould of apples. *Can. J. Plant Pathol.*, **24**: 154-161.