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# Biological control of apple gray mold by mixtures of *Bacillus Subtilis* and yeast isolates

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**In this study the effect of biocontrol agents *Candida membraniciens* (isolates A4, A5), *Pichia guilliermondii* (isolate A6) and *Bacillus subtilis* (isolates B2, B6) were evaluated individually and in combination on gray mold of apple. Our results show that the antagonists were compatible when they were tested *in vitro*. Also, results show the spore germination of *Botrytis cinerea* decreased significantly by the combination of B2+A5 more than the other treatments. In the dual culture test, the combinations of B2+A5 and B6+A6 prevented mycelial growth of pathogen and that the combined application of the two agents was more effective in control of gray mold of apple *in vivo* than the application of each one alone. In different proportions antagonist test, when the inoculum size favored A5 was 70 in the mixed, the biocontrol could be improved. This study suggested that antagonists are more effective in biological control of apple gray mold when used in proper combinations than when each one is used alone.**

**Key words:** Gray mold, biocontrol, mixtures antagonist, *Botrytis cinnerea*.

## INTRODUCTION

Fruits and vegetables suffer significant losses from fungal diseases after harvest (Filonow, 1998). Postharvest losses of fruits and vegetables are high, ranging from 10 to 40% depending on the species. Among them gray mold of apple is created by wound-invading necrotrophic *Botrytis. cinerea Pers.:Fr* (Romano et al., 1983). Currently, Thiabendazole, is the main fungicide globally used for the control of postharvest fungal fruit decays of apple (Pusey, 1989). The fungicide is applied as the drench treatment before or after the cold storage. The development of resistance in fungal pathogens to fungi-cides and the growing public concern over the health and environmental hazards associated with high levels of fungicides have resulted in a significant interest in the development of alternative nonchemical methods for control of post harvest diseases (Gullino et al., 1994). Biological control using some antagonistic microorganisms is the safe method for control of postharvest pathogens (Yu and Zheng, 2006). Puesey, (1984) and Lima et al. (1997) claimed that considerable success was achived by utilizing antagonistic microorganisms for controlling postharvest diseases. In recent years many attempts are made to develop the efficacy of existing biological agents. There are many aproaches to achieve this goal through: Enhancing efficacy by addition of fungicides (Chand-Goyal and Spotts, 1996); salt (Gholamnejad and Etebarian, 2009); nutrients (El-Ghaouth et al., 2000a); chitosan (El-Ghaouth et al., 2000b);integration with physical means (Stevens et al., 1997); and finally using mixture of antagonists (Janisiewicz, 1988). The mixture of

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**Abbreviations: OD,** Optical densities; **NYDB,** nutrient yeast dextrose broth; **NYDA**, nutrient yeast dextrose Agar; **PDB,** potato-dextrose broth; **Ee,** expected effect; **LR**, lesion reduction.

biological control agents have many benefits: it can increase the constancy of biological control of apples in the field and later in storage (Leibinger et al., 1997), and also it can be effective to control many post hasvest diseases at the same time (Janisiewicz, 1996). Also suggested, the employment of more than one biocontrol agent caused to survive biological control in unsteady position (Guetsky et al., 2001). And, the combination of antagonists with different mechanisms of disease suppression might simultaneously suppress many pathogens (Guetsky et al., 2002). Several studies have tested different biocontrol strains in combination, for example Leibinger et al. (1997) showed that mixture of two yeast isolates and one bacterium controlled the postharvest pathogens *Pencillium expansum*, *B. cinerea* and *Pezicula malicorticis* on apple. Calvo et al. (2003) showed that mixing different isolates of yeast *Rhabdotorula* and *Cryptococus* were considerably more effective in controlling *P. expansum* and *Botrytis cinerea* on Red Delicious apple fruits. Guetsky et al. (2001) combined *Pichia guilliermondii* with *Bacillus mycoides* to improve control of gray mold of strawberries by *B. cinerea* in storage. In this study, we applied different mixture of antagonists for controlling postharvest gray mold of apples.

## MATERIALS AND METHODS

### Atagonists

The antagonistic yeasts *Candida membranifuciens* (A4 & A5) and *Pichia guilliermondii* (A6) were obtained from plant pathology laboratory of Abureihan Campus, University of Tehran. The isolates were identified previously in Centralbureau voor Schimmelcultures (CBS). For biocontrol experiments, yeasts were grown on PDA media for 24 to 48 h at 25°C before use, then cultures were harvested by a bacteriological loop and resuspended in sterile distilled water and cell concentrations were adjusted with sterile distilled water to $1 \times 10^7$ cell/ml.

The *Bacillus subtilus* isolates B2 and B6 were also obtained from plant pathology laboratory of Abureihan campus, university of Tehran. *B. subtilus* isolates were grown in 250 ml erlenmeyer flasks containing 50 ml of NYDB (*Nutrient Yeast Dextrose Broth)* on a rotary shaker (150 rpm) at 25°C. Then the cultures were harvested by centrifugation and resuspended in sterile distilled water and the population of the bacterial cells was adjusted to $10^8$ cells/ml (optical densities (OD) 0/05 transmittance at 590 nm). For biocontrol experiments suspensions of the yeast and bacterium at each concentration were mixed in proportions of 50: 50 (v/v).

### Pathogen

*B. cinerea* was isolated from decayed apple. The fresh cultures were grown on PDA plate at 25°C before use then spore suspension were prepared from the spourulating edges of 10 day old culture with a bacteriological loop, then suspending them in sterile distilled water containing 0.05% (v/v) Tween 80. Spore concentration was determined with hemacytometer and adjusted with sterile-distilled water to $1 \times 10^5$ spore/ml.

### Plant

Golden Delicious apples were obtained from organically grown position in orchard in Damavand, Tehran.

### Compatibility of biocontrol agents with each other

The compatibility of the biocontrol agents (yeast and bacteria isolates) with each other was tested following the methods of antibiosis and mixed culture tests, *in vitro*.

### Antibiosis test

15 mm diameter disks from 5 days old NYDA cultures of the three yeast isolates (A4 , A5 , A6) were cut and then they were spotted onto center of one surface dry plate PDA (9 cm plate). The plates were incubated for 72 h at 25°C. Following incubation, the plates of the yeast isolates were oversprayed with a suspension ($10^8$ cell/ml) of *Bacillus subtilis* isolates (B2, B6). The plates were incubated for 48 h at 25°C. Then, the plates were examined for the presence of inhibition zones. All treatments consisted of four replicates (Wilson and Lindow, 1994).

### Mixed culture test

To study interaction of the antagonists *in vitro*, the cultures of yeast and bacteria were resuspended in water (adjusted to $10^8$, $10^7$ cell/ml bacteria and yeast isolates respectively) and 100 micro liter of these suspensions were added individually to 10 ml Erlenmeyer flasks containing 5 ml of NYDB. Another set of flasks was inoculated with a combination of both organisms in the proportion of 50:50. The flasks were incubated on the shaker at 150 rpm and 24°C for 30 h. Samples from the flasks were taken at 15 and 30 h intervals and were plated on PDA or PDA supplemented with 25 mg of streptomycin per liter for recovering bacterial and yeast cells respectively. This samples were inoculated with mixture of the antagonists were plated in duplicate, once on regular PDA to recovery of the bacterial antagonist and the second on PDA supplemented with streptomycin sulfate at 25 mg/lL, which inhibited the bacterium but allowed the yeast to grow. Finally, for evaluate the yeasts population, the colonies were counted with a colony counter after incubation for 48 h. All treatments consisted of three replicates (Janisiewicz and Bors, 1995).

### *In vitro* antagonism

The biocontrol agents were tested alone and in different combinations for their effectiveness against the mycelial growth of *B. cinerea* by the dual culture technique (Etebarian et al., 2005). Half of the agar surface were streaked with 100 µl suspension of $10^7$ Cell/ml of the yeast isolates (A4, A5 and A6), $10^8$ cell/ml of *B. subtilis* isolates (B2 and B6) and an equal their mixtures (50:50). After 2 days of incubation, a mycelial disc (9 mm) of *B. cinerea* of ten-day old culture was placed on the other side of each plates. For control, the medium was incubated with the pathogen alone. Four replications were carried out for each treatment; the dishes were incubated at 25°C for 7 days and the percent 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 *B. cinerea* for the control and *b* is the colony area of *B. cinerea* treated with different combinations of the yeasts or the bacteria.

Effects of alone or the combination treatments of antagonists on spore germination of pathogen were carried out in PDB culture. The 100-µl of $1 \times 10^8$ Cell/ml of the washed cell suspension of bacteria

isolates (B2, B6) and 1 × $10^7$ Cell/ml of the yeast isolates (A4, A5, A6) and the sterile distilled water as the control were added into 10 ml glass tube containing 5 ml PDB respectively. Another set of glass was inoculated with same quantity of a combination of both antagonists in the proportion 50:50. At the same time, aliquots 100 µl of spore suspensions 1 × $10^5$ spores/ml of *B. cinerea* were added into each tube. After 20 h incubation at 25°C, at least 100 spores per replicate were observed microscopically to determine germination rate on a rotary shaker (150 rpm). All treatments consisted of four replicates (Droby et al., 1997).

### *In vivo* biological control studies

Fruits were surface-disinfected with sodium hypochlorite (0.1%) for 2 min and washed two times by immersion in distilled water,Then allowed to air-dry and wounded 3 × 3 × 3 mm in three places on the equator of the fruit with a sterilized needle , then 40 µl of suspension $10^8$ and $10^7$ Cell/ml of bacteria and yeast isolates respectively (singly or their mixtures 50:50) was pipeted in to apple wounds. after 24 h , the treated wounds were inoculated with 20 µl of *B. cinerea* spore (1×$10^5$ spore / ml ). The lesion diameters were determined 15 and 30 days after storage at 20 and 4°C in 95% relative humidity. Each apple constituted a single replicate and each treatment was replicated four times. Wounded apples were distributed into five sets: (1) Non-treated apples (control), (2) Apples treated with isolates yeast or bacteria alone (antagonist), (3) Apples inoculated with *B. cinerea* alone (pathogen), (4) Apples treated with isolates yeast or bacteria individual and then inoculated with *B.cinerea* (antagonist+ pathogen) (5) Apples treated with mixture of antagonists and inoculated with pathogen (mixture of antagonists + pathogen). The percentage of lesion reduction was estimated according to the formula:

$$LR = (Dc - Dt) / Dc \times 100$$

Where, Dc and Dt are, the lesion diameters of the control and treated apples respectively (Etebarian et al., 2005). Limpel's formula also was used to determine the presence of synergistic or antagonistic interactions between the antagonists in mixtures:

$$Ee = X + Y - X.Y/100$$

Where, *Ee* is the expected effect from additive responses of two inhibitory agents and *X* and *Y* are the pecentages of lesion reduction for each agent used alone. If the combination of the two agents produces any value of inhibition greater than Ee, then synergism exists, on the same basis, if the observed effect (pecentage of lesion reduction) would be less than the expected effect, then the mixture exhibits antagonism (Limpel et al., 1962).

### Biocontrol test *in vivo* in the various proportions of mixture antagonists

*In vivo* biological control studies when two treatments as B2+A5 and B6+A6 were combined at approximately equal biomass (50:50), they were consistently compaierd to the other combinations. The apple fruits were disinfected and wounded as described above, then aliquots 40 µl suspensions at $10^7$ and $10^8$ cell/ml yeast and bacterium were mixed in proportions from 0:100, 30:70, 70:30, 50:50, 60:40, 40:60, 100:0. This proprtions were pipetted into wounds after 24 h, 20 ml of $10^5$ spores/ml suspension of *B. cinerea* were inoculated into each wound. Treated fruits were stored at 20°C for 15 days or 4°C for 30 days. The lesion diameter was recorded afterwards. There were three replicate trials of 5 fruits per treatment with complete randomization (Janisiewicz and Bors, 1995).

### Recovery of the antagonists

Two combinations B2+A5 and B2+A6 was conducted to recover the antagonists. Three wounds per fruit were made as described above, and then 40 µl of each antagonist alone and an equal mixture (50:50) was pipetted to each wound. After 24 h, the tested wounds were inculated with 20 µl of *B. cinerea* spore (1 × $10^5$ spore/ml).The fruit apples were stored for 30 day at 4؛C, then the fruit samples were taken to determine antagonist populations after 15 and 30 days of storage. The wounded area was removed with a cork borer (no. 5) and placed in a sterile mortar, and ground with pestle. For each apple, recovered cell suspensions from the three wounds were diluted $10^3$. $10^4$ and $10^5$ Serial tenfold dilution and were plated on PDA or PDA supplemented with 25 mg of streptomycin per liter for recovering bacterial and yeast cells, respectively. Samples from flasks inoculated with the mixture of the antagonists were plated in duplicate on PDA and PDA plus streptomycin. The colonies were counted with a colony counter after incubation for 24 and 48 h for the bacteria and yeast, respectively (Teixido et al., 2000).

### Data 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). All assays were carried out with three/four_replicates. Statistical significance was judged at the level $p < 0.05$. 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 (Little and Hills, 1978).

## RESULTS

### Compatibility of biocontrol agents with each other

In the test antibiosis, the isolates which grew togother were compatible, but isolates that were separated by an inhibition zone were incompatible. From these results none of the antagonists showed antibiosis effect on each other. Both *B. subtilis* isolates (B2 and B6) and the yeast isolates grew together and it indicated that these isolates were compatible.

Interaction of the antagonists *in vitro* showed the mixtures of B2+A4, B2+A5, and B2+A6 the growth of yeast isolates wasn't effected by the presence of *B.subtilis* isolate B2 (P≤0.05). In NYDB inoculated with yeast isolate alone, the yeast population increased in all flasks after 15 h, and the cells increased until end of the experiment. However, the increases of the mixtures yeast isolate (A4, A5 and A6) with the bacteria, almost were equal with alone antagonists after 30 h *(*Figure 1 A). Also, in the mixtures of B6+A4, B6+A5 and B6+A6, the growth of yeast isolates was not affected by the presence of *B. subtilis* isolate B6 (P≤ 0.05). Results showed that the populations of the yeast isolates inoculated in mixture of *B.subtilis* increased in all flasks after 30 h. In the other hand, the application of the yeast population alone increased in all flasks after 15 h, and then it increased until end of the experiment (Figure 1 B).

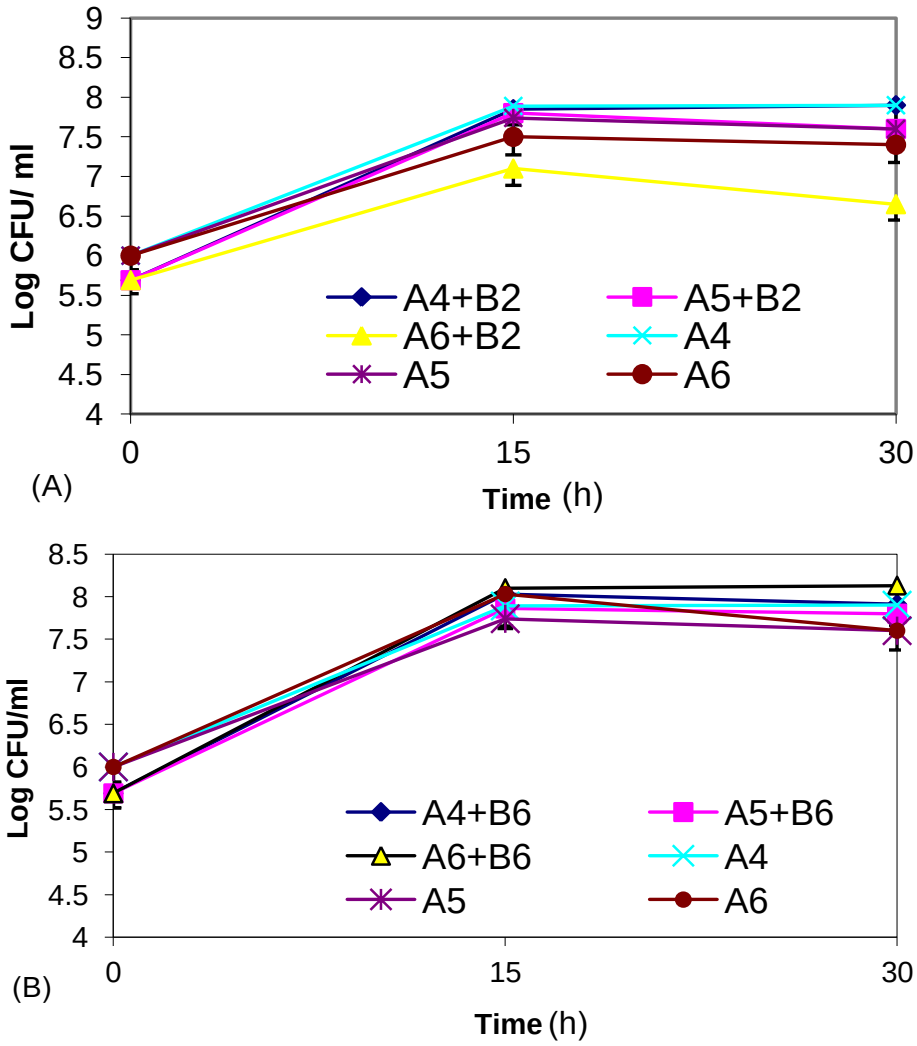


**Figure 1.** Population dynamics of yeast isolate (A4, A5, A6) by the presence of *B.subtilis* B2 (A), B6 (B) alone or in mixtures in NYDB. The samples were collected at 15 h intervals. Bars represent standard error.

### *In vitro* biological control studies

Results show that all treatments reduced significantly the mycelial growth of *B. cinerea* (P≤0.05). The tretment B2+A5 had the highest percent of inhibition at 53.26%, if the single isolates inhibition A4, A5, A6, B2, B6 were 20.5, 21.28, 32.21, 40.54 and 39.73 %,respectively. The percent inhibition of *B. cinerea* of other treatments such as B2+A4, B2+A6, B6+A5 and B6+A4 was equal to, or further than the inhibition achieved by the isolates applied alone (Table 1).

Spore germination of *B. cinerea* in PDB culture was inhibited strongly in the presence of active cells of antagonists and their mixtures (P≤0.05). At the end of 20 h incubation at 25°C, the spore germination of *B. cinerea* incubated with the combination B2+A5 was 10% while the spore germination of the other combinations were equal to, or further than the isolates applied alone (Table 2).

### *In vivo* biological control studies

The results show that wound diameter of all treated fruits were significantly lower than the untreated controls (P≤0.05). The lesion diameters of the biocontrol combinations B2+A5, B6+A6, B6+A4 on treated fruits were 5/23, 7/43, 11/66 mm, respectively, after storage at 20°C for 15 day, that provided better control than the antagonist applied alone B2, B6, A5 and A4 that had a lesion diameter of 15/06, 15/22 13/57 and 12/99 mm respectively.

On the other hand the lesion diameter, in the apple fruits treated with the combinations of yeast and bacteria, the lesion diameter of fruits with the best combination such as B2+A5, B6+A6 and B6+A5 was 3/48,3/87 and

**Table 1.** Effectiveness of mixture *Bacillus subtilis* and yeast isolates in inhibiting mycelial growth of *Botrytis cinerea.*

| Treatment isolateds[1] | Mycelial growth of the pathogen (mm) | Inhibition over control (%) |
|---|---|---|
| A4 | 31.96[b 2] | 20.5 |
| A5 | 31.65[bc] | 21.28 |
| A6 | 27.25[dc] | 32.21 |
| B2 | 23.9[de] | 40.54 |
| B6 | 24.23[de] | 39.73 |
| B2+A4 | 22.94[def] | 42.64 |
| B2+A5 | 18.79[f] | 53.26 |
| B2+A6 | 31.48[bc] | 21.7 |
| B6+A4 | 22.65[dfe] | 43.66 |
| B6+A5 | 23.53[de] | 41.47 |
| B6+A6 | 22.07[fe] | 45.09 |
| Control | 40.21[a] | 0.0 |

[1]*Bacilus subtilis* isolate B2 and B6 , *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6). [2]Values are the means of four replications. Means followed by a common letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

**Table 2.** Effectiveness of mixture *Bacillus subtilis* and yeast isolates in inhibiting Spore germination of *Botrytis cinerea.*

| Treatment isolateds[3] | Spore germination[1] (%) |
|---|---|
| A4 | 59[ab 2] |
| A5 | 45.33[cb] |
| A6 | 13[de] |
| B2 | 14.66[de] |
| B6 | 19.66[de] |
| B2+A4 | 28[dc] |
| B2+A5 | 10[e] |
| B2+A6 | 55.33[ab] |
| B6+A4 | 30[cd] |
| B6+A5 | 21.66[de] |
| B6+A6 | 19.33[de] |
| Control | 82.33[a] |

[1]Germination rate were measured microscopically after 20 h incubation at 25°C in PDB. [2]Each value is the mean of four experiments. Values in each column followed by different letters are statistically different according to Duncan's multiple range test at( P≤0.05). [3]*Bacilus subtilis* isolate B2 and B6, *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6) .

5/16 mm, respectively, wheras the biocontrol isolates applied alone such as B2, B6, A6, A5 and A4, it was only 13.29, 10/83, 8/97,11/67 and 16/01 respectively, after storage for 30 day at 4°C (Figure 2 A and B).

The expected effect of Ee, accordance to the formula Limpele, showed that B2+A5 and B6+A6 compounds at 4°C and B2+A5 at 20°C had a synergistic effect in controlling gray mold. Its reason is that the calculated LR was more than Ee.In other compounds, the number of calculated Ee was more than the number of suppresions percent LR, it showed that these compounds had the antagonistic effects against gray mold (Table 3).

### Biocontrol test *in vivo* in the various proportions of mixture antagonists

The results showed that the different proportion of the antagonists affected the lesion diameter, significantly (P≤0.05). Also, the results show, the mixture of B2+A5 (proportion of 30% of bacterium and the proportion of 70% of yeast), reduced the lesion diameter to 5 mm, this compounds didn't have significant difference with other mixtures (with different proportions antagonists) expect of B2(70)+A5(30) at 20°C. Also, the results showed that there is a significant difference between treatments at 4°C (P≤0.05). The treatment of B2+A5 with 50:50 ratio for yeast and bacteria, and other mode with 30:70 ratio for bacreria and yeast was more effective than other proportions and the antagonists applied individually on lesion development, respectively (Figure 3 A and B).

### Recovery antagonists

The results showed that both *B. subtilis* (B2) and yeast isolates (B6) multiplied rapidly in apple wounds in the presence of *B*. *cinerea*. At the start of the experiment (time 0), the number of per yeast and bacterium strain was $2 \times 10^5$ CFU/ml for mixture of B2+A5, also the population of *C. Membranifuciens* (A5) and *B. subtilis* (B2) increased from $1.1 \times 10^7$ and $6.31 \times 10^6$ CFU/wounds, respectively at 15 d. But after 30 days incubation at 4°C, the yeast and bacteria population began to decline. The comparison between logarithm of mixed antagonists and their individual applications showed that the population of B2 and yeast A5 in mixture is more than they applied alone. Indeed the results showed that a positive interaction probably occurred between these antagonists in mixture and in this position, the quantity of both antagonist's cell is more than when they applied alone. The study of antagonist's population in mixture of B2+A6 have been showed that the population logarithm of alone antagonists as more than their mixture in all test period, this can be due to an antagonism effect among them (Figure 4 B).

## DISCUSSION

In earlier studies the multiple strain mixtures of bio-

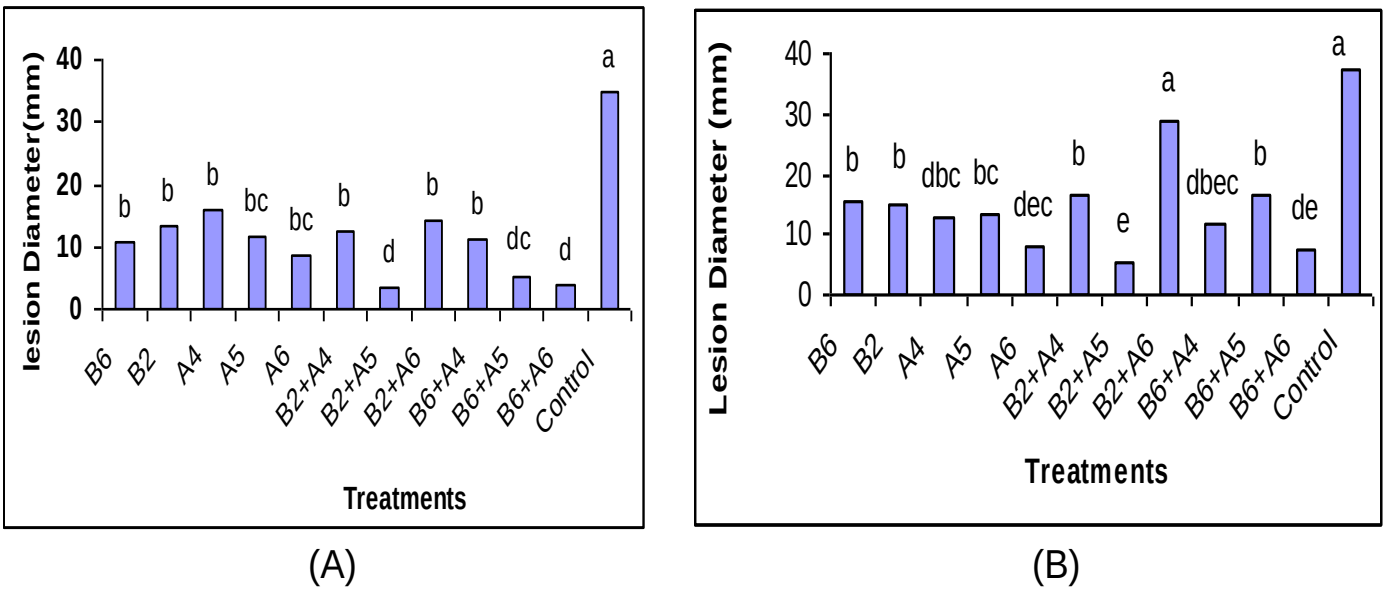


**Figure 2.** Inhibition of gray mold in apple fruits .the lesion diameters were measured after 30 days incubation at 4°C (A), and 15 days incubation at 20°C (B). Different letters in the assay indicate significant differences between means according to Duncan's multiple range test ($p \leq 0.05$).

**Table 3.** percentage of lesion reduction of mixture *B.subtilis* isolate (B2,B6) and yeast isolates of (A4,A5 and A6) and expected effect (Ee) of mixtures according to Limpel's formula.

| | 20°C | | 4°C | |
|---|---|---|---|---|
| **Treatment isolateds[2]** | **lesion reduction (LR) %** | **Ee** | **lesion reduction (LR) %** | **Ee** |
| A4 | 65.14$^{bc1}$ | | 54.24$^{c}$ | |
| A5 | 60.56$^{bc}$ | | 66.64$^{bc}$ | |
| A6 | 77.93$^{a}$ | | 72.22$^{abc}$ | |
| B2 | 59.63$^{bc}$ | | 62$^{c}$ | |
| B6 | 55.55$^{dc}$ | | 69.09$^{cb}$ | |
| B2+A4 | 66.59$^{b}$ | 91.09 | 64.38$^{c}$ | 90.58 |
| B2+A5 | 86.94$^{a}$ | 85.07 | 90.02$^{a}$ | 87.32 |
| B2+A6 | 28.41$^{e}$ | 85.92 | 58.96$^{c}$ | 82.61 |
| B6+A4 | 59.32$^{dbc}$ | 90.18 | 68.32$^{cb}$ | 92.32 |
| B6+A5 | 50.21$^{d}$ | 82.46 | 85.23$^{ab}$ | 89.66 |
| B6+A6 | 80.01$^{a}$ | 84.50 | 88.93$^{a}$ | 85.83 |

[1]Each value is the mean of three experiments. Values in each column followed by different letters are statistically different according to Duncan's multiple range test at (P≤%05). [2]*Bacilus subtilis* isolate B2 and B6, *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6) .

control agents have been employed successfully against plant pathogens (Janisiewicz, 1996). Several authors have suggested that combinations of bio-control agents have to be compatible with each other for more consistent results of biological control (Raaijmakers et al., 1995). According to *in vitro* antibiosis test the selected antagonist strains do not have any antibiotic element against each other. Interaction of the antagonists *in vitro*, also showed that the growth of yeast isolates were not affected by the presence of *B.subtilis* B2 and B6 isolates in NYDB culture. It indicated that the *B.subtilis* isolates B2 and B6 were compatible with yeast strains A4, A5 and A6. Tillugavati et al. (2007) so as to affirm the compatibility between two bacterium (*Bacillus* and *Psudomonase*) and one fungus (*Trichdema*) in mixture, used the dual culture test of these antagonists with pathogen. They concluded that these three biocontrol agents create more inhibition of growth of mycelia of *B. cinerea* than single antagonists used alone, it was suggested its reason is the compatibility between bio-

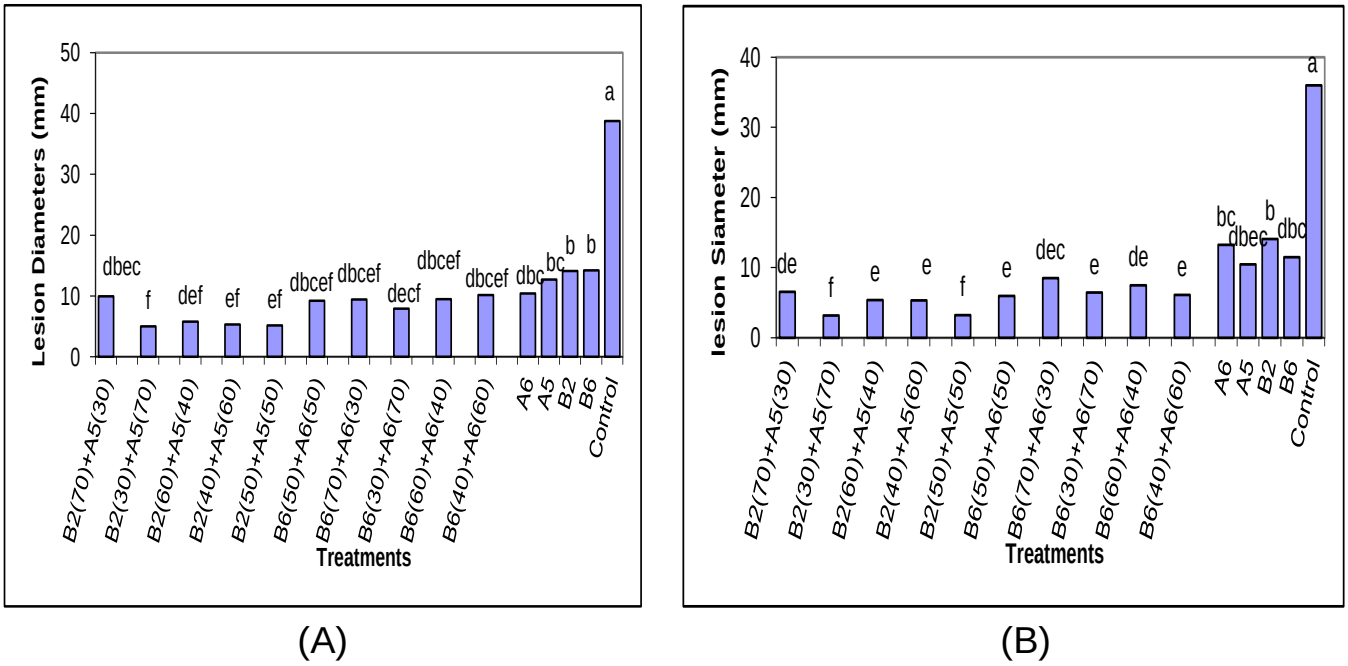


**Figure 3.** Inhibition of grey mold in apple fruits as affected by various proportions(0:100, 30:70,70:30,50:50,60:40,40:60,100:0) treatments of the antagonist. lesion diameters were measured after 15 days incubation at 20°C (A), and 30 days incubation at 4°C (B). Values followed by different letters are significantly different according to Duncan's multiple range test at $p \leq 0.05$.

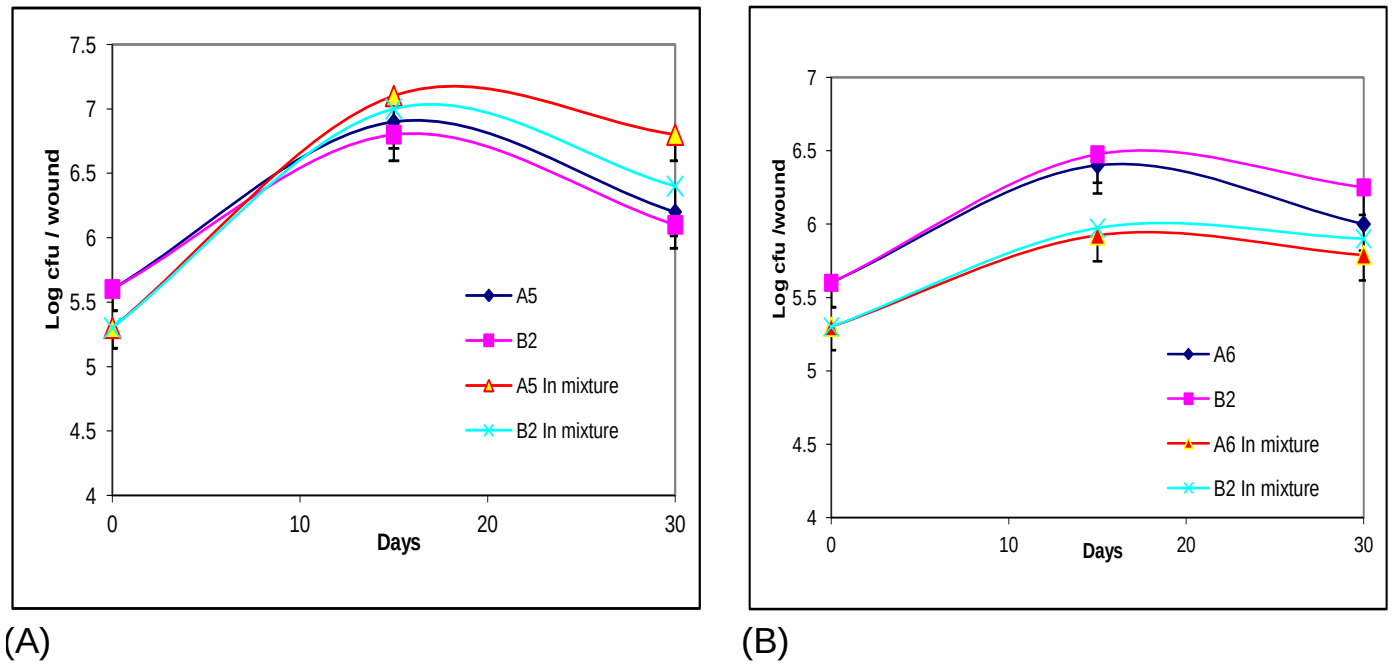


**Figure 4.** Population dynamics in wounds of Golden Delicious apples inoculated with *B.subtilis* B2 and *C.Membranifuciens* A5 isolate (A) and *B.subtilis* B2 and *Pichia guilliermondi* A6 isolate (B) the individually or in a 50:50 mixture and incubated at 4 °C. Bars represent standard error.

control agents. In our experiments, the compounds of B2+A5 and B6+A6 created the most inhibition of the growth of mycelia and spore germination of *B. cinerea*; this can be due to the compatibility between biocontrol

agents with each other. The combinations of B2+A5 and B6+A6 and a less extent B2+A4, B6+A4 and B6+A5, controlled apple gray mold more effectively when they were used alone. In addition, Limpel's formula (Limpel et al., 1962) and population size of yeasts allowed us to evaluate the interactions between yeast and bacteria isolates when they were used in mixtures. According to Limpel's formula the *B. subtilis* (B2) and *C. membranifaciens* (A5) mixture showed synergism against *B. cinerea*, because the LR% was calculated higher than Ee. Dynamic of populations, also explained that both *C. membranifaciens* A5 and *B. subtilis* B2 reached a higher cell concentration when they used in a mixture mode instead of using alone. These results agree with the findings of Janisiewicz and Bors who said carrying capacity of the wounds could be greater than the population of the single antagonists and they mentioned that another antagonist may caused to increase the population of the antagonists in the wounds to increase more improves of biocontrol (Janisiewicz and Bors, 1995). Also, they found that screening of specific amino acids may specifically enhance the growth of the antagonists but had no effect on pathogen. It was when the specific nutrient is available, the growth of the antagonist which metabolize it enhance, so both antagonists cannot compete for this nutrient and it creates desirable position for growth of both antagonists**.** Also, this caused even greater depletion of essential nutrients for development of *B.cinerea* and eventually better biocontrol. These results also agree with the findings of Calvo et al. (2003) and Janisiewicz (1996). In a more comprehensive study Gutsky et al. (2002) showed types of biocontrol mechanisms *B. cinerea* on strawberry by mixture of yeasts (*Pichia guilermondii*) and a bacterium (*Bacillus mycodes*) by scan of Electron Microscopy, they concluded that in the presence of yeast, some of *B. cinerea* spore cannot germinate, They indicated that *P. guilermondii* competed with *Botrytis cinerea* for glucose, sucrose, adenine, histidine, and folic acid. It was found that the half of spores of *B. cinerea* hydrolyzed fully in presence of *B. mycodes*. They suggested that these observations may be due to the presence of cell wall hydrolyzing enzymes. But when the mixture of two biocontrol agents was used, the spores were hydrolyzed with more intensity. Likewise, they expressed when both biocontrol agents were applied in a mixture, their activity reflected the sum of the biocontrol mechanisms. Most of researches on the mixtures of biocontrol agents showed that combinations of antagonists caused to improve biocontrol. But, studies show that some combinations of biocontrol agents could not improve suppression of disease compared with the separate antagonists (Thilagavathi et al., 2007; Leibinger et al., 1997). In this study, the mixture of B2+A6, had an equal or/and lower control than the separate antagonists. This may be due to an incompatibility between *B. subtilis* isolate B2 and *P.guilliermondi* isolate A6. Also, Limpel's formula showed that the interaction between them is antagonism. Morever, the dynamic of populations of B2 has a negative effect on the growth of A6 and application of isolate A6 in alone mode is more effective than in the mixture mode. Results of compatibility of biocontrol agents with each other *in vitro*, demonstrated that at least inhibitory substances (antibiosis) are not involved, probably. However many same studies showed that the basis of this incompatibility is unknown (Schisler et al., 1997).We can compare these results with Leibinger's test that saied a mixture of two yeast isolates and one bacterium controled the post-harvest pathogens *Penicillium expansum*, *B. cinerea* and *Pezicula malicorticis in vivo*. In their test the population size of yeast isolate *A. pullulans* reduced when it was applied in combination with *B. subtilis*, they reported that the basis of this incompatibility could result from the production of antibiotic compounds by *Bacillus subtilis* (Leibinger et al., 1997). From the test of different proportions of antagonist in B2+A5 mixture, we can understand that biocontrol would be improved when we use more inocoulom yeast A5 with 70%, its reason could be more colonization of yeasts in apple's wounds. It shows that yeasts, for control of the pathogens, have stronger mechanism than bacterium that include competition for nutrients and space (Leibinger et al., 1997). Calvo et al. (2003) showed that the gray mold in mixture of *Rhodotorula glutinis* SL1 and SL30 with inocoulom 1:2 for SL1, is controlled more better than proportion 2:1 for SL30. They concluded that the control of disease is depended on the size of inocoulom of antagonists in mixture. In this reserch, we showed that application of antagonists in combination mode is more effective than the application used alone, for a period of 30 days at 4°C and a period of 15 days at 20°C. The adaptation of these strains to a wide range of temperature (4 to 20 C°) provides great potential for this antagonist mixture for control of post-harvest diseases on apples in storage and transportation conditions. We hope that other biologists, physicians and agronomists work together for culturing and formulating of these agents and make commercial using of these antagonist microorganisms in the near future.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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