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# Interaction Between *Pseudomonas fluorescens* Isolates and Thiabendazole in the Control of Gray Mold of Apple

A. Mikani, H.R. Etebarian, H. Aminian and A. Alizadeh
Department of Plant Protection, Abourayhan Campus,
University of Tehran, P.O. Box 11365/4117, Tehran, Iran

**Abstract:** Five isolates KW, 16P, 8, 11 and 7 of *Pseudomonas fluorescens and* fungicide thiabendazole were evaluated for control of gray mold of apple caused by *Botrytis mali.* Dual culture, cell free metabolite and volatile test showed that all five isolates of *Pseudomonas fluorescens* tested inhibited growth of the pathogen. Inhibition varied from 28.9-85.7% in dual culture, from 62-94.6% in cell free metabolite and from 45.9-87.9% in volatile test. Apples were wounded and inoculated with a 20 μL *Botrytis mali* ($10^5$ conidia $mL^{-1}$) combined with a range of concentration of fungicide. Thiabendazole controlled gray mold by over 83% at 5 μg $mL^{-1}$. *Pseudomonas fluorescens* isolates prevented *B. mali* decay from expanding to more than 49.4% at 20°C. Thiabendazole was combined with bacteria to control gray mold of apple. Mixing of thiabendazole and bacterial isolates provide more than 90.2% control at concentrations 5 μL $mL^{-1}$ and $10^7$ CFU $mL^{-1}$, respectively, on Golden delicious apples. A combination of thiabendazole and *Pseudomonas fluorescens* isolate 11 at concentration 10 μg $mL^{-1}$ and $1\times10^7$ CFU $mL^{-1}$, respectively controlled gray mold by 100%. Thiabendazole was not bactericidal and had no effect on five isloates of *Pseudomonas fluorescens.*

**Key word:** *Botrytis mali*, postharvest disease, *Malus*, *Pseudomonas fluorescens*

## INTRODUCTION

Some of the most important postharvest diseases of apples are caused by *Penicillium expansum* link ex Thom, *Botrytis cinerea* Pers. Ex Fr. and *Botrytis mali.* Ruehle *Botrytis* can cause significant economic losses on grape, strawberry, tomato, lettuce and kiwi fruit (Card *et al*., 2002).

Control of postharvest still relies mainly on the use of synthetic fungicides (Wilson *et al*., 1993). Thiabendazole is a systemic benzimidazole and used to control fruit and vegetable diseases. Blue mold of apple is mainly controlled by application of thiabendazole (450 ppm) as spray over the fruit on a packingline or incorporated in an anti-scaled bin drench. The use of fungicides has been becoming increasing more restricted because of health concerns due to of the development of resistance in many postharvest pathogens (Lennox and Spott, 2003).

Several bacteria and yeasts were reported as being effective in laboratory and pilot tests for controlling blue mold and gray mold (El Ghaouth *et al*., 2003; Etebarian *et al*., 2005; Yu *et al*., 2006). Currently, 2 biofungicides, BioSave 100 and 110 (*Pseudomons syringae*, Van Hall, EcoScience Longwood, Fl.) (Janisiewicz and Koresten, 2002) and Aspire (*Candida oleophila* Montrocher, Ecogen, Inc. Langhorne, Pa.) have been registered in the USA for postharvest use on apples (Droby *et al*., 1988).

The required level of fungal disease control with fungicides often approaches 95-98%, which is high compared with that obtained with biocontrol agents. (Chalutz and Droby , 1997). Extensive attempts have been made to increase the efficacy of biocontrol systems for the control of post harvest diseases by either improving the fitness and antagonistic traits of biocontrol agents (Chalutz and Droby, 1997) or by combining other disease-control practices with the biological-control approaches (Zhou *et al*., 1999). Addition of chemical fungicides at low rates can enhance biocontrol activity and reduce the population of antagonist required to achieve effective control (Mari *et al*., 2003).

Strain of *Bacillus amyloliquefaciens* mixed with iprodione fungicide at 50 ppm gave greater control of gray mold than either treatment alone (Mari *et al*., 1996). Similar results were obtained by combining yeasts with low dose of thiabendazole; the mixture gave significantly better control of blue mold than the low dose of TBZ alone and was comparable with the disease control achieved using a high dose of TBZ (Chand-Goyal and Spott, 1997).

**Corresponding Author:** H.R. Etebarian, Department of Plant Protection, Abourayhan Campus, University of Tehran, P.O. Box 11365/4117, Tehran, Iran

The combination of TBZ with *C. laurentii* HRA5 controlled *P. expansum* (Chand-Goyal and Spott, 1997). Zhou *et al.* (2002) reported that a combination of cyprodinil and *Pseudomonas syringae* MA-4 at concentrations of 2.5 μg $mL^{-1}$ and $1\times10^{8}$ CFU $mL^{-1}$, respectively controlled gray mold on apple by 100%.

The objective of the research presented here were, firstly, to test the efficacy of TBZ for the control of gray mold; secondly, to study the interactions between the biocontrol isolates of *Psedomonas fluorescens* and TBZ on the control of gray mold and thirdly to determine the potential use of previously identified isolates of *Pseudomonas fluorescens* in postharvest control of gray mold on apple.

## MATERIALS AND METHODS

**Biocontrol agents:** Fourty *Pseudomonas* isolates were obtained from rhizosphere of melon and rice with serial dilution methods on King, B medium. These isolates were screened *in vitro* against *B. mali*. Five promising isolates, KW, 16P, 8, 11 and 7 were selected and evaluated as potential biological agent for gray mold of apple. *Pseudomonas fluorescens* identified by conventional biochemical bacterial tests (Schaad *et al.*, 2001).

**Pathogen:** *Botrytis mali* M14 , from *Malus domestica* Golden delicious from Ghazvin, 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.

***In vitro* biological control studies:** Dual culture (Dennis and Webster, 1971; Etebarian *et al.*, 2003), cell free culture (Weller, 1988) and volatile metabolites tests (Fiddaman and Rossall, 1993) were used to observe the effect of 5 *Pseudomonas* isolates on mycelial growth of *B. mali*. All anatagonist-pathogen combinations were examined on 10-15 mL of potato dextrose agar in 9 cm petri plate with three replications The plates were incubated 20 days for dual culture and 18 days for volatile metabolites and cell free culture at 25°C. All *vivo* and *vitro* experiments were carried out in the Department of Plant Protection Abourayhan Campus, University of Tehran, Pakdasht, Iran during 2005 and 2006.

The percent growth inhibition was calculated using the formula n= (a- b)/a ×100, where n is the percent growth inhibition; a is the colony area of uninhibited *Botrytis* sp. and b is the colony area of treated *Botrytis mali* (Etebarian *et al.*, 2005).

**Efficacy of fungicide thiabendazole against gray mold pathogen:** Isolates M14 was grown on PDA plates (90 mm diameter) for 8 days. Conidia were harvested by pouring a few ML of steride distilled water (SDW) containing 0.05% tween 20 on the plates. The conidia suspensions were adjusted to $1\times10^{5}$ conidia $mL^{-1}$ with hemacytometer.

Thiabendazole was suspended in water giving a series concentrations required for the treatments. Final concentrations of fungicide were 0.5, 25, 50 and 100 μg $mL^{-1}$.

*Malus domestica* Golden delicious apples that have been harvested at commercial maturity and kept at l±0.5°C in cold storage were used in this study. The apples were washed in 70% ethanol for 30 sec. followed by dipping in. 0.1% sodium hypocholorite solution and rinsed with sterile distilled water. The suspension of fungicide and pathogen were mixed 1:1 individually, resulting in final concentration 0. 5, 10, 25, 50 and 100 for TBZ and $10^{5}$ conidia $mL^{-1}$ for *B. mali*, respectively.

The Golden delicious apple were wounded in triplicate with a 2.5 mm-diameter nail to a depth of 3 mm and inoculated with 20μL drop of the appropriate pathogen-fungicide combination. The treated apples were incubated at 20°C in humid condition for 13 days.

**Gray mold control with combination of five isolates of *Pseudomonas fluorescens* and thiabendazole:** TBZ was suspended in sterile distilled water at final concentration 5 and 10 μg $mL^{-1}$. The bacterial inoculum was prepared in potato dextrose broth on rotary shaker at 150 r $min^{-1}$ for two days at room temperature. The produced bacterial cells were collected by centrifugation at 6500×g for 5 min and resuspended in water to obtain the desired concentrations. Concentrations were determined by dilution plating on potato dextrose agar (Zhou *et al.*, 2001).

The inoculum of *B. mali* was prepared as described above. The fruit were wounded in triplicate with 2.5 mm diameter nail to a depth of 3 mm, 20 μL aliquot of each bacteria ($10^{7}$ CFU $mL^{-1}$) and 20 μL aliquot of each concentration of fungicide were dispensed in each wound.

The treated apples were placed on cardboard trays that were then enclosed in plastic bags. The inside the bags were sprayed with SDW to maintain high relative humidity in the bags. A 20 μL aliquot of conidial suspension was applied to each wound 24 h after inoculation with *Pseudomonas fluorescens* and TBZ. The apples were incubated at 20°C and lesion diameter was measured after 13 days, using calipers and lesion area was calculated. Each apple constituted a single and each treatment was replicated 4 times. This experiment was repeated at 5°C. All *vivo* and *vitro* experiments were carried out in the Department of Plant Protection Abourayhan Campus, University of Tehran, Tehran, Iran during 2005 and 2006.

**Statistical analysis:** Data on the percent growth inhibition were subjected to arcsin square root transformation before analysis, and data no decay area were subjected to plus 0.5 square root transformation before analysis.

The completely randomized design was used for all experiments. Analysis of variance was performed on the data and means were separated using Duncan's Multiple Range test at $p<0.005$ (Little and Hills, 1978).

## RESULTS

**Effect of *Pseudomonas fluorescens* on *B. mali in vitro*:** Five isolates of *P. fluorescens* tested inhibited mycelial growth of *B. mali* in dual culture. However there were significant differences among bacterial isolates. Growth inhibition of *B. mali* by *P. fluorescens* isolate 11 was significantly greater than that of the other isolates, but *P. fluorescens* isolate KW had less effect on the growth of the pathogen (Table 1).

Cell free metabolites of five fluorescent Pseudomonas reduced growth of *B. mali* by 62-89.5% (Table 1).

The results shown in Table 1 indicated that the antifungal activity of volatile metabolites of the *P. fluorescens* isolate KW was less than those of the other isolates tested ($p<0.05$).

**Effect of thiabendazole against gray mold:** The percent reduction of gray mold was determined after 7 day incubation at 20°C. TBZ controlled gray mold by over at concentration of 5 μg $mL^{-1}$, thus TBZ was used in subsequent experiment because of it's effectiveness against pathogen (Table 2).

**Effect of *P. fluorescens* on gray mold lesion development:** Five isolates of *P. fluorescens* prevented *B. mali* decay from 1.7-6.2 $cm^2$ compared with 12.34 $cm^2$ in control after incubation for 13 days at 20°C.

At 20°C the lesion size was from 0.5-2.54 $cm^2$ for antagonistic treatments and 6.5 $cm^2$ for control after 25 days.

**Effects of thiabendazole on colonization of *Pseudomonas fluorescens*: *In vitro* and *vivo*:** TBZ could not prevent the growth of *Pseudomonas fluorescens in vitro*. At 20°C, there were no significant difference between treatments with and without the addition of TBZ at 5 μg $mL^{-1}$ in population of bacterial cell 2, 7 and 13 days after inoculation. The log number of bacterial cells for each wound (20 μL) increased and closely followed for 16P a linear function. The equation $y = 0.1263x + 7.1141$ where y is the log number of bacterium and x is the number of days after inoculation (Fig. 1).

Table 1: Percentage of growth inhibition of *B. mali* (M14) by different isolates of *Pseudomonas fluorescens in vitro*

| Treatments | Dual culture 20 days after inoculation | Cell free metabolite 18 days after inoculation | Volatile metabolite 18 days after inoculation |
|---|---|---|---|
| 11 | 85.6a | 79.1c | 76.3a |
| 7 | 69.4b | 97.6a | 74.2a |
| 16P | 48.9c | 89.5b | 87.8a |
| 8 | 41.5cd | 62.0d | 84.7a |
| KW | 28.8d | 68.9d | 45.8b |

Each treatment was replicated 3 times. Means within columns followed by the same letter do not differ significantly at $p<0.05$ according to Duncan,s Multiple Range Test

Table 2: Percent reduction of gray mold by thiabendazole(TBZ) on Golden delicious apples

| Thiabendazole (μg $mL^{-1}$) | Gray mold reduction (%) |
|---|---|
| 0 | 0.0b |
| 5 | 86.1a |
| 10 | 97.3a |
| 25 | 100.0a |
| 50 | 100.0a |
| 100 | 100.0a |

The apples were inoculated with *B. mali* (M14) and treated with or without (0 μg $mL^{-1}$) TBZ. The percentage of inoculation sites with diseases was assessed after inocubation for 7days at 20°C. Means within columns followed by the same letter do not differ significantly at $p<0.05$ according to Duncan,s Multiple Range Test

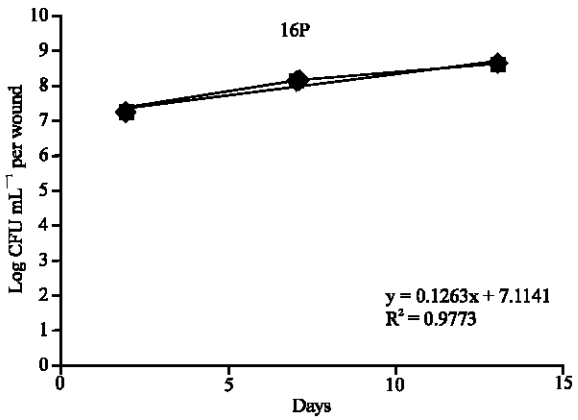


Fig. 1: Growth curve of *P. fluorescens* 16P in apple wounds containing *B. mali* over 13days at 20°C

**Effect of combination of *P. fluorescens* and TBZ on controlling of gray mold:** *P. fluorescens* and thiabendazole were effective in controlling gray mold (Table 3 and 4). Isolate 11 that was the most effective in reducing decay and controlled gray mold by 85.6% at 20°C after 13days. TBZ alone at 5 and 10 μg $mL^{-1}$ controlled gray mold by 83.7 and 95%, respectively.

At 20°C a combination of TBZ at 5 μg $mL^{-1}$ with isolate 11 of bacteria at $10^7$ CFU $mL^{-1}$ significantly increased gray mold control to 98.8% compared with the individual treatments of 83.7 and 85.6%, respectively.

At 5°C the addition of TBZ at 10 μg $mL^{-1}$ to 16P at $10^7$ CFU $mL^{-1}$ resulted in higher control of gray mold than 16P alone.

Table 3: Decay area on Golden delicious apples with *B. mali* (M14) *challenged* with *P. fluorescens* and Thiabendazole and incubated for 25 days at 5°C

| Treatments | Decay area ($cm^2$) after 15 days | Decay area ($cm^2$) after 25 days |
|---|---|---|
| *B. mali* (M14) | 1.930a | 6.540a |
| KW | 0.987b | 1.890c |
| 8 | 0.796bc | 2.700b |
| 11 | 0.597c | 1.760c |
| 7 | 0.597c | 0.516e |
| 5 μg $mL^{-1}$ | 0.226d | 0.780d |
| 16P | 0.180d | 0.977d |
| KW+5 μg $mL^{-1}$ | 0.026d | 0.042fg |
| 7+5 μg $mL^{-1}$ | 0.025d | 0.043fg |
| 8+5 μg $mL^{-1}$ | 0.021d | 0.057fg |
| 8+10 μg $mL^{-1}$ | 0.012d | 0.050fg |
| KW+10 μg $mL^{-1}$ | 0.011d | 0.028fg |
| 11+5 μg $mL^{-1}$ | 0.009d | 0.025fg |
| 10 μg $mL^{-1}$ | 0.005d | 0.226f |
| 11+10 μg $mL^{-1}$ | 0.001d | 0.035fg |
| 16P+5 μg $mL^{-1}$ | 0.000d | 0.000g |
| 16P+10 μg $mL^{-1}$ | 0.000d | 0.000g |
| 7+10 μg $mL^{-1}$ | 0.000d | 0.000g |

Each treatment was replicated 4 times.Means followed by the same letter within a column differ significantly at $p<0.05$ according to Duncan's Multiple Range Test, 7, 8, 11, 16P and KW are isolates of *Pseudomonas fluorescens*

Table 4: Decay area on Golden delicious apples with *B. mali* (M14) *challenged* with *P.fluorescens* and Thiabendazole and incubated for 13 days at 20°C

| Treatments | Decay area ($cm^2$) after 8 days | Decay area ($cm^2$) after 13 days |
|---|---|---|
| *B. mali* (M14) | 3.625a | 13.085a |
| KW | 1.902b | 7.000b |
| 8 | 1.03cd | 6.897b |
| 7 | 1.355c | 4.34c |
| 16P | 0.282e | 3.055d |
| 5 μg $mL^{-1}$ | 0.85d | 2.000e |
| 11 | 0.797d | 1.745e |
| 8+5 μg $mL^{-1}$ | 0.016f | 1.395ef |
| KW+5 μg $mL^{-1}$ | 0.021f | 1.09fg |
| 7+5 μg $mL^{-1}$ | 0.084ef | 0.92g |
| 10 μg $mL^{-1}$ | 0.165ef | 0.437h |
| 16P+5 μg $mL^{-1}$ | 0.009f | 0.286hi |
| 8+10 μg $mL^{-1}$ | 0f | 0.156hi |
| KW+10 μg $mL^{-1}$ | 0.003f | 0.054i |
| 11+5 μg $mL^{-1}$ | 0f | 0.035i |
| 7+10 μg $mL^{-1}$ | 0.001f | 0.024i |
| 16P+10 μg $mL^{-1}$ | 0f | 0.003i |
| 11+10 μg $mL^{-1}$ | 0f | 0.001i |

Each treatment was replicated 4 times. Means within columns followed by the same letter differ significantly at $p<0.05$ according to Duncan`s Multiple Test, 7, 8, 11, 16P and KW are isolates of *Pseudomonas fluorescens*

## DISCUSSION

Thiabendazole is very active against apple gray mold confirming its previously described activity in cold storage (Sharom and Edgington, 1985).

The biocontrol activity of bacterial antagonists such as *Pseudomonas syringae* (Zhou *et al*., 2001) and *Pseudomonas fluorescens* (Etebarian *et al*., 2005) against several important plant diseases have been attributed to the production of antifungul metabolite. For example many *P. fluorescens* produce pyrrolnitrin that has strong antifungal activity (Kirner *et al*., 1998).

Delany *et al.* (2000) reported that *P. fluorescens* produce 2, 4- diacetyl pholoroglucinol that plays a major role in its biocontrol capabilities. All the *Pseudomonas fluorescens* isolates reduce mycelial growth of pathogen by means of dual culture, cell free and volatile metabolite test. Zone of inhibition were observed between the colonies of pathogen and bacteria. Benizri *et al.* (1995) demonstrated that antagonist activity of *Pseudomonas* sp was due to the production antibiotic, volatile compounds and siderophore.

In cell free culture test *P. fluorescens* 7 produces large inhibition zone on PDA indicating that they synthesize compounds that are highly active against pathogen. Etebarian *et al.* (2005) demonstrated that *P. fluorescens* population on wounded apple inoculated with *P. expansum* increased with 10-100 fold at wound site over 20 days at 20°C, in our case, the wound carrying capacity was approx. $1\times10^9$ CFU $mL^{-1}$ per wound after only 13 days. This may indicate that isolate 11 has a high capacity for colonizing apple wounds and could be potentially a better biocontrol agent.

In this study the result for apples stored at 5°C for 25 days and 20°C for 13 days showed that these five isolates reduced growth of *B. mali* and were effective material for control of pathogen. These isolates have also ability for suppressing other pathogens such as charcoal rot of melon (*Macrophomina phaseolina*) (Kheiri, 2004), common smut of wheat (*Tilletia foetida*) (Khodaygan, 2003). The adaptation of these five isolates 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.

The activity of both bacterial isolates and TBZ against pathogen provided an opportunity to combine these agents at lower concentration while maintaining a high level of effectiveness and consistency of performance are two major factors limiting the use of biocontrol agents in plant disease control (Chalutz and Droby, 1997). The strategy of combining a biocontrol agent with a fungicide has been used previously. Zhou *et al.* (2002) showed that combinations of *Pseudomonas syringae* strain MA-4 at $10^7$ to $3\times10^7$ CFU $mL^{-1}$ with cyprodinil at 5-10 μg $mL^{-1}$ gave a satisfactory control of both blue and gray mold on Northen spy and Jonagold apples.

Present results in this study showed that the control of apple gray mold by five isolates of *P. fluorescens* at

$10^7$ CFU $mL^{-1}$ was increased by combination of 5 isolates of *P. fluorescens* with TBZ at a low concentration could improve the efficacy of postharvest disease control.

Bacteria and TBZ had a significant synergistic effect on the reduction of gray mold on golden delicious apple. TBZ has not effect on bacteria *in vitro* and *vivo.*

A combination of a biocontrol agent with a fungicide both significantly reduced at concentrations from what would be needed if used alone may greatly reduce their residues on fruit (Zhou *et al*., 2002). Studies have shown that residue level of chemicals were proportional to the concentrations used in many cases (Papadopulou-Mourkidou, 1991) and the amount of biocontrol agent recovered from the surface of fruits was correlated with the concentration applied (Usall *et al*., 2001).

In conclusion, five isolate of *P. fluorescens* tested here reduced disease severity of gray mold of apple at 5 and 20°C. and the combination of these isolates and TBZ were most effective on controlling of gray mold of apple. These isolates warrant further investigation for their ability to control gray mold in commercial situations.

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