Turk J Agric For
31 (2007) 319-325

The Effects of Biological and Chemical Treatment on Gray Mold Disease in Tomatoes Grown under Greenhouse Conditions

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Received: 02.11.2006

Abstract: A total of 163 bacterial strains isolated from tomato leaves were evaluated for their ability to suppress gray mold (*Botrytis cinerea* Pers. ex Fr.) on tomatoes, and 4 strains consistently reduced the incidence of *B. cinerea*. These antagonist strains were identified as *Pseudomonas fluorescens*. The bacterial strains with in vitro resistance to fungicides were combined with low-dose fenhexamid in vivo. One strain of *P. fluorescens* (Pf163) reduced the disease incidence by an average of 78% on tomato plants inoculated with *B. cinerea* in greenhouse trials. In comparison to the control treatment, other *P. fluorescens* strains applied with low-dose fenhexamid reduced the incidence of decay by 74.17%, 70.52%, and 65.74%, respectively. The antagonists significantly reduced gray mold decay when combined with low-dose (12.5 ml a.i. hl^{-1}) fenhexamid more than the control did. It was shown that antagonistic bacterial strains inhibited *B. cinerea* and that they have a potential use in combination with the fungicide in greenhouse experiments.

Key Words: Tomato, *Botrytis cinerea*, *Pseudomonas fluorescens*, biocontrol, chemical control

Sera Domates Yetiştiriciliğinde Görülen Kurşuni Küf Hastalığı Üzerinde Kimyasal ve Biyolojik Uygulamaların Etkileri

Özet: Sera bölgelerinden alınan domates yapraklarından izole edilen 163 bakterinin, kurşuni küf hastalığını (*Botrytis cinerea*) baskılayıcı etkisi bir seri testle ortaya konmuştur. Domates bitkilerinde kurşuni küf hastalığına karşı bu antagonist bakterilerinden özellikle dört adedi hastalığı baskılamada etkili olmuş ve bunlar *Pseudomonas fluorescens* olarak tanılanmıştır. Domates seralarında kullanılan fungisitlerin bu bakterileri etkilemedikleri testlerle saptanmıştır. Bunun sonucunda, 4 bakterinin sera koşullarında, fenhexamidin düşük dozuyla, hastalığa etkisi araştırılmıştır. Bu bakterilerden bir tanesi (Pf163) serada yapılan testlerde kurşuni küf hastalığını %78 oranında azaltmıştır. Kontrollerle karşılaştırıldığında, *Pseudomonas fluorescens*'in diğer izolatlarının fenhexamidin düşük dozu (12.5 ml a.i. hl^{-1}) ile birlikte hastalığı sırasıyla %74.17, %70.52 ve %65.74 oranında baskıladığı ortaya konmuştur. Bakteriyel antagonistlerin serada yetiştirilen bitkilerdeki hastalığı engellemede ve fungisitlerle birlikte kullanımlarında potansiyel bir etkileri olduğu ortaya konmuştur.

Anahtar Sözcükler: Domates, *Botrytis cinerea*, *Pseudomonas fluorescens*, biyolojik savaş, kimyasal savaş

Introduction

The tomato is Turkey's primary crop, and is primarily grown along the coasts of the Aegean and Mediterranean regions. Tomato cultivation in Turkey took place on almost 30,000 ha in 2000.

Botrytis cinerea (Pers. ex Fr.), the causal agent of gray mold, is a significant and potentially serious fungal pathogen affecting greenhouse tomatoes. In 1985, 672 greenhouses growing tomatoes that were surveyed in Antalya, Muğla, and İzmir were infested with *B. cinerea* (Yıldız and Delen, 1985). The pathogen has a very broad host range (Jarvis, 1977) and survives saprophytically for extended periods in plant debris or as sclerotia. The pathogen attacks the leaves, stems, flowers, and fruits of tomatoes growing in greenhouses.

Control of *B. cinerea* on tomatoes can be achieved with the frequent application of fungicide; however, resistance of the pathogen to common fungicides is

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widespread. The decreasing sensitivity to dicarboximide, benzimidazole, and even some classic fungicides has been studied in Turkey (Delen et al., 1984, 2000; Delen and Özbek, 1992, 1994; Delen and Tosun, 1996). The difficulty in controlling *B. cinerea* has led to alternative methods, which include biological control. Biological control is an alternative to reducing *Botrytis* infections and has been shown to be successful in many crops (Blakeman and Fokkema, 1982; Dubos, 1992; Elad and Freeman, 2002). Many organisms (bacteria, yeast, and fungi) living on the phylloplane can inhibit the pathogen by competing for nutrition (Blakeman and Fokkema, 1982; Nicot et al., 1996). Biological control of *B. cinerea* with *Trichoderma* species has been achieved in several studies (Dik et al., 1999; Elad., 2000). Some yeast species and bacteria, such as *Bacilli* and *Pseudomonads*, have been reported to be effective in suppressing gray mold on some crops (Redmond et al., 1987; Elad et al., 1994a).

Fungicides, biocontrol agents, modification of the greenhouse atmosphere, and cultural treatment are major control methods for gray mold disease. Biocontrol agents and fungicides are consistently applied together to increase their effectiveness (Malathrakis et al., 1996). Integration of biological and chemical control in disease suppression has been investigated in some studies (Elad et al., 1994a; Elad et al., 1994b; Shtienberg and Elad., 1997; Utkhede et al., 2001)

In the present study we aimed to assess the efficacy of the integrated control of gray mold on tomatoes by combining the application of some bacterial strains with low-dose fungicide and fungicide (at full dose and low dose) applied alone, in both pot and greenhouse conditions.

Materials and Methods

Bacterial strains

Tomato leaves used for the isolation of bacteria were obtained from commercial greenhouses in the Aegean and Mediterranean regions. All leaves were free of disease symptoms. We rinsed 20 g of leaves in 200 ml of distilled water after cutting into small pieces. The samples were shaken at 90 rpm on a rotary shaker for 30 min at room temperature. The dilutions of the suspension, at the rate of 0.01 ml, were added to 100 ml of King's B agar and plated onto the plates. After 48 h, selected colonies were isolated under UV light at 366 nm and streaked on King's B agar to obtain pure cultures.

Bacterial inoculum was prepared by growing bacteria strains in King's B broth for 48 h at 25 °C. Bacterial suspensions were centrifuged at 2795 × g for 10 min, the supernatant was poured down, and the pellet was resuspended to a concentration of 10^8 CFU of bacteria ml^{-1} with spectrophotometry.

Pathogen

During the winter of 2000, isolates of *B. cinerea* were obtained from 106 tomato greenhouses by harvesting from diseased leaves and putting in tubes on potato dextrose agar (PDA).

B. cinerea was grown for 10-14 days on PDA at 22 °C. Conidia was washed from the agar with sterile distilled water containing 1% carrot juice and gelatin, and was then filtered through 3-layered cheese cloth to prepare the inoculum suspension. The conidia concentration was adjusted to 1×10^5 conidia ml^{-1}.

Fungicides

The following fungicides, registered against *B. cinerea* on tomatoes in Turkey, were used in the experiments: imazalil (Magnete 50 EC, Hektaş, 50%); iprodione (Rovral 50 WP, Bayer, 50%); captan (Captan 50 WP, Syngenta, 50%); thiram (Pomarsol forte 80 WP, Bayer, 80%); fenhexamid (Teldor SC 500 Bayer, 500 g l^{-1}); pyrimethanil (Mythos SC, Bayer, 300 g l^{-1}); cyprodinil + fludioxonil (Switch 65.5 WG, Syngenta, 37.5% + 25%); prochloraz + folpet (Mirage F Plus, Flogaz, 150 g l^{-1} + 600 g l^{-1}).

To determine the sensitivity of *B. cinerea* isolates to the fungicides, petri dishes containing 20 ml of MM (minimal medium) (Maraite et al., 1980) amended with 0, 0.03, 0.1, 1.0, 3.0, 10.0, 30.0, and 100 µg ml^{-1} of fungicide were centrally inoculated with 5 mm plugs from the periphery of 3-day-old cultures and inoculated at 22 °C. The radial growth of the colony was measured in 2 orthogonal directions 4 and 5 days later, and 50% inhibition of mycelial growth (ED_{50}) values were calculated and recorded for each isolate.

Sensitivity of antagonists to fungicides

Biocontrol bacteria were tested in vitro for their sensitivity to the fungicides. The bacterial suspensions, at the concentration of 10^8 cfu ml^{-1}, were pipetted onto petri dishes containing King's B agar amended with the fungicides at 150, 300, and 600 µg ml^{-1} for cyprodinil + fludioxonil; at 250, 500, and 1000 µg ml^{-1} for fenhexamide; at 187, 375, and 750 µg ml^{-1} for iprodione; and at 75, 150, and 300 µg ml^{-1} for imazalil. The suspensions were dispersed over the medium. The dishes were then incubated at 25 °C for 48 h. The growing colonies were counted and the treatments were repeated 3 times.

Bioassays in Growth Chamber

The effect of the bacterial strains on *B. cinerea* was tested on 4 full sets of the leaves of tomato cv. Rio Grande plants. In all, 3 of these plants for each strain to be tested were sprayed to runoff with 4 ml of bacteria at a concentration of 10^8 cfu ml^{-1} using a hand sprayer. Then, 2 days later they were sprayed to runoff with a spore suspension produced as previously described, which contained 1×10^5 spores ml^{-1} (Delen et al., 1984), and were put into a growth chamber at 23 °C for 10 days after covering with plastic bags.

Greenhouse experiments with tomato

Tomato cv. M19 plants were planted in a simple heated experimental greenhouse (10^8 m^2) for 3 vegetation periods. Experiments were performed with 4-month-old tomato plants carrying fully expanded leaves. The following treatments were selected based on their performance in the pot experiments: Bacterial strains (Pf9/11, Pf52/16, Pf141, and Pf163) and low- (12.5 ml a.i. hl^{-1}) and full-dose (50 ml a.i. hl^{-1}) fenhexamid, alone or combined, were applied to the tomato plants. The experiments were arranged in randomized blocks with 4 replicates of each treatment. Each plot consisted of 7 plants in 1 row. Thrips and other insect pests were regularly controlled. Using a knapsack sprayer in the greenhouse, tomato plants were sprayed to runoff with a suspension of bacteria at a concentration of 10^8 CFU ml^{-1} in 0.05% Tween 20 in December. The control plants were sprayed only with 0.05% Tween 20 and all plants were allowed to dry overnight in the greenhouse.

The following day, fungicide was applied to the plants. Finally, the plants were inoculated with *B. cinerea*. The spraying was repeated 2 times within 10 days.

Statistical analysis

The severity of infection was rated on a 0-4 visual scale (0 = no infection, 1 = 5%, 2 = 25%, 3 = 50%, and 4 = infected leaf area about 75%-100%) (Yıldız, 2000). The disease indices were calculated based on the following formula:

$$\text{Disease severity} = \frac{\Sigma(\text{scale} \times \text{number of infected leaves}) \times 100}{\text{total number of leaves} \times \text{highest scale}}$$

Observations were submitted to one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests to separate means ($P \leq 0.01$).

Results

Mycelial growth tests

For the 47 *B. cinerea* isolates from 106 greenhouses that were tested with 8 fungicides, ED_{50} values were selected to characterize the sensitivity of each *B. cinerea* isolate (Table 1).

Table 1. Mean ED_{50} values of several concentrations of 8 fungicides for the inhibition of mycelial growth of *B. cinerea* isolates.

	Distribution of ED_{50} values of the *B. cinerea* isolates						
Fungicides	< 0.01	0.01 0.09	0.10 0.99	1.0 2.9	3.0 9.9	10.0 29.9	30-100
cyprodinil + fludioxonil	36	11	-	-	-	-	-
Fenhexamid	21	22	4	-	-	-	-
pyrimethanil	2	24	5	1	-	-	1
Imazalil	6	22	19	-	1	-	-
prochloraz + folpet	1	19	25	1	-	1	-
Iprodione	1	5	25	10	3	2	2
Thiram	-	2	10	14	16	4	-
Captan	2	-	2	1	8	24	10

All isolates were sensitive to fenhexamid and cyprodinil + fludioxonil, according to the ED_{50} values. Additionally, 2 isolates were resistant to pyrimethanil, 2 isolates were resistant to prochloraz + folpet, and 1 isolate was resistant to imazalil. A significant number of the isolates were moderately sensitive to iprodione.

Efficacy of the bacterial strains on potted plants

In total, 163 bacterial strains isolated from the leaves of tomato plants were evaluated for their ability to suppress gray mold. In the first experiments, 51 strains of the 163 bacteria screened suppressed the development of *B. cinerea* by 70% in potted tomatoes. Bacterial strains that were shown to inhibit *B. cinerea* in vivo were tested for their ability to reduce gray mold on tomatoes inoculated with *B. cinerea*. These further tests revealed that 5 of the 51 bacterial strains (Pf9/8, Pf9/11, Pf40/6, Pf52/16, and Pf98/6) significantly reduced the level of subsequent gray mold disease in tomatoes artificially inoculated with *B. cinerea*. The percentage of inhibition varied between 73% and 94% (Figure).

Identification of bacterial strains

The 10 bacterial strains used in the pot tests were identified as *Pseudomonas fluorescens* by several analyses, such as Gram staining (-), fluorescent pigments (+) levan production (-), oxidase (-), arginine dihydrolase (-), gelatin hydrolase (+), and potato tests (-), and some carbohydrate tests, such as sucrose (-), sorbitol (-), tartrate (+), and trehalose (+) (Buchanan et al., 1974). The bacterial antagonists were also identified as *P. fluorescens* according to fatty acid analysis with the microbial identification system (MIS). Under growth chamber conditions 5 *P. fluorescens* reduced fungal infection of tomatoes inoculated with *B. cinerea*.

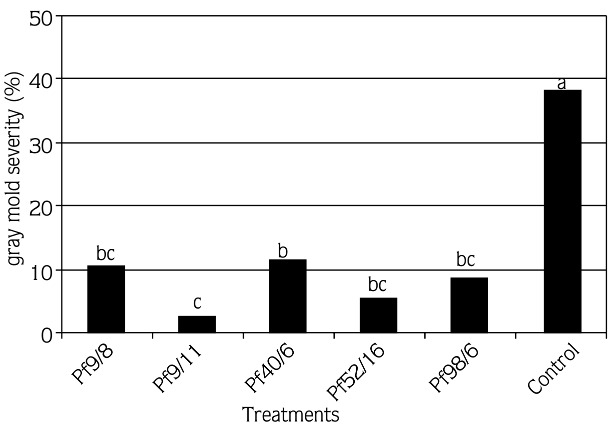

Figure. Percentage of gray mold severity on tomato plants treated with different *P. fluorescens* strains. In the pot tests, tomato plants were treated by spraying with a suspension containing 10^8 cfu ml^{-1} of strains Pf9/8, Pf9/11, Pf40/6, Pf52/16, and Pf98/6. Treatments marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Sensitivity of the bacterial strains against some fungicides

Some bacterial strains found to be effective in this study were tested in vitro. The medium was amended with 3 concentrations of 4 fungicides. No decline was observed in the colonial growth of *P. fluorescens* with low doses of fungicide (Table 2).

The combination treatments carried out to control *B. cinerea* in greenhouse experiments

The ED_{50} values of fenhexamid for mycelial growth of *B. cinerea* ranged from 0.1 µg ml^{-1} to < 0.001 µg ml^{-1} throughout the tests. Fenhexamid and the 2 most effective bacterial strains (Pf9/11 and Pf52/16) were selected based on their performance in the tests.

In the greenhouse experiments, all antagonists applied in combination with fenhexamid reduced gray mold decay compared to the control, which did not (Table 3). One antagonist, *P. fluorescens* (Pf163), applied alone reduced the disease incidence by 78.33%.

In comparison to the control, *P. fluorescens* strains (Pf52/16, Pf 163, and Pf9/11) applied with fenhexamid reduced the incidence of decay by 74.17%, 70.52%, and 65.74%, respectively. The fungicide applied alone at full-dose reduced the incidence of decay by 75.12%.

Discussion

In the experiments, *P. fluorescens* strains (Pf163, Pf52/16, and Pf9/11) showed a high level of antagonistic activity against *B. cinerea* on tomato plants. Two *P. fluorescens* strains (Pf141 and Pf163), which were previously shown to control gray mold on tomatoes, were added to the greenhouse tests (Yıldız, 2000).

The performance of 2 *P. fluorescens* (Pf9/11 and Pf52/16) and 1 *P. fluorescens* (Pf163) from the previous studies, which were shown to control gray mold in tomatoes (Yıldız, 2000), were used alone and in combination with the fungicide in the greenhouse experiments.

Table 2. Sensitivity of selected *P. fluorescens* strains to fungicides.

Dose of the fungicides μg ml^{-1}		*P. fluorescens* strains and the numbers of the colonies (10^8 cfu ml^{-1})						
		Pf9/8	Pf9/11	Pf40/6	Pf52/16	Pf98/6	Pf141	Pf163
Control		2.4a[a]	2.3a	0.3bcd	0.3d	3.2a	0.6a	3.7ab
cyprodinil + fludioxonil	150	2.4a	2.4a	0.3bcd	0.8abcd	2.8a	0.9a	4.9ab
	300	2.3a	1.9a	0.2cd	1.0abcd	3.4a	1.1a	5.3a
	600	2.0a	0.2a	0.3bcd	0.7abcd	3.3a	0.6a	4.3ab
fenhexamid	250	2.3a	1.9a	0.7ab	1.3abc	3.2a	1.0a	4.4ab
	500	2.6a	2.4a	0.7a	1.5a	2.9a	0.9a	4.5ab
	1000	2.2a	2.3a	0.5abc	1.4ab	2.8a	1.0a	3.2b
imazalil	75	2.2a	1.9a	0.3bcd	0.9abcd	3.1a	0.7a	3.9ab
	150	2.0a	1.9a	0.2cd	0.9abcd	3.4a	1.0a	4.4ab
	300	2.4a	1.2a	0.03d	0.4cd	0.4a	0.9a	4.5ab
iprodione	187	2.2a	1.3a	0.07d	0.4cd	4.0a	0.9a	4.6ab
	375	2.1a	1.7a	0.2cd	0.2d	3.1a	0.8a	4.9ab
	750	2.0a	1.0a	0.2cd	0.1d	2.4a	1.2a	4.4ab

[a]Means were separated by using Duncan's multiple range test at $P < 0.01$.

Table 3. The effect of some *P. fluorescens* strains and low-dose fenhexamid on gray mold disease.

The effects of treatments on disease severity and effectiveness				
Treatments	Alone	Effect %	12.5 ml a.i. hl^{-1}	Effect %
P. fluorescens Pf9/11	13.56 ab[a]	63.37	12.68 a	65.74
P. fluorescens Pf52/16	16.70 ab	54.88	9.56 a	74.17
P. fluorescens Pf141	17.53 ab	52.64	14.55 ab	60.69
P. fluorescens Pf163	8.02 a	78.33	10.91 a	70.52
Fenhexamid[b]	9.21 a	75.12	-	-
Fenhexamid[c]	9.67 a	73.87	-	-
Control	37.02 b	-	-	-

[a]Means were separated by using Duncan's multiple range test at $P \leq 0.05$.
[b]50 ml a.i. hl^{-1} full dosage
[c]12.5 ml a.i. hl^{-1} quarter dosage

In the tests, *P. fluorescens* (Pf163) significantly decreased gray mold infections when applied alone. Bacteria plus low-dose fungicide (25% of the commercially recommended dose) showed an activity comparable to that of the fungicide applied alone at full dose (Table 3).

Fluorescent *Pseudomonads* have been shown to antagonize a wide range of fungal pathogens on various plants. The *P. fluorescens* PB92B10E strain reduced the average *B. cinerea* disease incidence by 77% on petunia plants (Gould et al., 1996). Swadling and Jeffries (1998) reported that 2 bacterial strains (*Bacillus pumilus* and *P. fluorescens*) successfully controlled *B. cinerea* in strawberries. In 2 experiments, 4 fluorescent *Pseudomonads* were shown to reduce gray mold lesions by an average of 79% in lettuce (Card et al., 2002).

Moreover, in the present study 47 isolates from 106 greenhouses were tested for their sensitivity to commercial fungicides and 1 isolate of *B. cinerea* obtained from a tomato greenhouse was applied in the experiments. This work showed that fenhexamid was effective against gray mold and resistant isolates were not detected (Study et al., 1999; Haensler et al., 2000).

Biocontrol bacteria tested in vitro for resistance to 3 doses of commercial fungicide showed good tolerance (Table 2). The integration of biological treatment with low-dose fungicide will help avoid the development of fungicide resistance. In greenhouse experiments 4 bacteria reduced gray mold when applied in combination with low-dose fenhexamid, reducing decay by 61%-74%.

The integration of biological and chemical controls has been investigated in some studies. The number of fungicide sprays was minimized with the combination of *Trichoderma harzianum* T39 (Shtienberg et al., 1996; Shtienberg and Elad., 1997). Trichodex controlled the disease effectively when applied in alternation (Elad et al., 1994).

In the present experiments *P. fluorescens* (Pf9/11, Pf52/16, Pf141, and Pf163) showed a high level of antagonistic activity against *B. cinerea* on tomatoes.

This study demonstrates that utilization of integrated control, combining bacterial strains with low-dose fungicide could be an effective strategy in reducing gray mold decay in tomatoes.

Acknowledgments

We thank the Scientific and Technological Research Council of Turkey, TÜBİTAK (TARP-2412) and The Ege Science and Technology Center, EBİLTEM (99-BIL-013) for their financial support of this study.

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