Effect of Fungicides and Antagonistic Microorganisms on the Black Fruit Spot Disease on Persimmon

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ABSTRACT

Alternaria alternata is the causative agent of black fruit spot disease on Persimmon. This disease infects fruits in the field near the harvesting time, but develops during the postharvest period causing fruit rot. In this study, suspensions of different strains of antagonistic microorganisms such as *Trichoderma harzianum* and *Bacillus amyloliquefaciens* were tested against the disease, in addition to applying fungicidal treatments with new, non-residual fungicides against the disease. Artificial infections with the disease were made on harvested fruits and subsequently fungicides or antagonistic microorganisms were applied in the different experiments. Preventive and curative effects of tested fungicides or antagonistic strains were evaluated according to the extent of supression of disease lesion diameter. Results showed that significant differences between tested fungicides or antagonistic strains were obtained. Consequently, Difenoconazole, and Cyprodinil + Flodioxonil proved to be the most effective preventive fungicides against the disease. This significant prevention was also provided by the antagonistic strain Trichoderma-Th_1 of *Trichoderma harzianum*. In addition, the tested formulations of Metalaxyl + Mancozeb, Captan, and Cyprodinil + Flodioxonil had a significant curative effect against the disease. This curative effect was also shown by the strains Trichoderma-Th_1 and Bacillus-GA_1. A control program of the disease based on the above results comprising chemical and biological means is discussed. Such program should replace the current traditional fungicidal treatment since it is more effective and has no side-effects on the consumers or the local environment.

Key Words: Persimmon, *Alternaria alternata*, Fungicides, antagonistic microorganisms, *Trichoderma harzianum*, *Bacillus amyloliquefaciens*, Difenoconazole, Captan, Metalaxyl + Mancozeb, Cyprodinil + Flodioxonil.

INTRODUCTION

Persimmon (*Diospyros Kaki* L.) has been introduced to the West Bank (Palestine) since 1998 where newly established Persimmon orchards are found in Qalqilia district. Under the local warm-humid growing conditions especially during spring and summer seasons, persimmon production is suffering from several serious foliar diseases (Prusky and Ben-Arie, 1981). Infection with Alternaria black spot appears firstly as dark-brown to black necrotic spots on both upper and lower leaf surfaces with variable size and shape, then the infection appears during harvesting time as dark-brown to black lesions on fruits and fruit pedicels (Fig. 1-A, and B). These lesions, then, develop causing fruit rot during the postharvest stage (Fig. 1-C and D). The economic importance of the disease is represented by the leaf necrosis and leaf drop under field conditions, in addition to fruit spotting and rotting during harvest and postharvest periods which render the infected fruits unmarketable (Snowdon, 1990).

The isolated causative agent of the disease is the fungus *Alternaria alternata* (Fr.:Fr.) Keissl. Its morphological characteristics are identical to those described by Barnett and Hunter, 1998; and Rotem, 1994. Infections with this pathogen were reported on other crops such as tomato (Grogan et al., 1975), pepper (Halfon-Meiri and Rylski, 1983), potato tubers (Droby et al., 1984), brussels sprouts (Siemer et al., 1971), and on some weeds as black night-shade (Abbas et al., 1998). Also, *A. alternata* may appear along with other Alternaria species such as A. macrospora on cotton (Rotem et al., 1988), *A. carthami* on safflower (Mortensen et al., 1983), and

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with *A. cucumerina* on cucumber (Vakalounakis, 1990).

Alternaria diseases, especially those caused by *A. Alternata* are usually controlled by spraying chemical fungicides such as Difenoconazole and Tebuconazole to control *A. macrospora* on cotton (Shteinberg and Dreishpoun, 1991; Padaganur and Basavaraj, 1987), Chlorothalonil against *A. alternata* on tomato (Davis et al., 1997), Iprodione and Metiram against *A. alternata* on Minneola tangelo (Solel et al., 1996 and 1997), Prochloraz against *A. alternata* on eggplant (Temkin-Gorodeiski et al., 1993). However, the intensive and repeated use of these fungicides especially those with residual effect caused many problems such as environmental pollution and resistance to fungicides. To reduce these problems, non-residual fungicides and non-pollutant bioagents were tested, in this study, for controlling the disease. The objectives of the present study were: i) to test the efficacy of some new, non-residual chemical fungicides against the disease, and ii) to test the efficacy of some strains of the antagonistic fungus *Trichoderma harzianum* and the antagonistic bacterium *Bacillus amyloliquefaciens*.

MATERIALS AND METHODS

For testing the efficacy of certain fungicides against the disease, inoculated persimmon fruits (variety: Triumph) were picked near the harvesting stage and treated with the fungicide, either at the time of inoculation with the fungus (preventive effect) or 24 hours after the inoculation (curative effect). This was accomplished, in both cases, by depositing 25 µl of conidial suspension of Pr_1 strain of *A. alternata* on the fruit surface, which had been superficially disinfected, and then wounded superficially near the blossom end of each fruit. A similar drop (25 µl) of fungicide solution was also deposited at the same site immediately after inoculation or 24 hours after inoculation. The concentration of conidial suspension of *A. alternata* used in the inoculation was 3×10^5 conidia/ ml. The recommended dosages of fungicides used in the experiments were 0.30% (w/v) for Metalaxyl + Mancozeb (sold as Ridomil® MZ 63.5 WP, produced by Ciba Geigy Ltd., Bazil-Switzerland, concent. of a.i. = 7.5% Metalaxyl + 56% Mancozeb),0.35% (v/v) for Difenoconazole (sold as Score® 250 EC, produced by Novartis Ltd., Bazil-Switzerland, concent. of a.i. = 250 g/l), 0.35% (w/v) for Captan (sold as Merpan® 50WP, produced by Macktichim Chemical Factories, Israel, concent. of a.i. = 50%), and 0.20% (w/v) for Cyprodinil + Flodioxonil (sold as Switch® 62.5WG, produced by Novartis Ltd., Bazil-Switzerland, concent. of a.i. = 375 g/kg Cyprodinil + 250 g/kg Flodioxonil). The fruits, after inoculation and treatment with fungicides, were incubated at 20 ± 1°C for 7 days in sterile closed plastic boxes (95 mmϕ and 65 mm deep) and distributed at a rate of one fruit per box. The experimental treatments were distributed and then analyzed according to the Completely Randomized Design (CRD) with 5 replicates representing 5 fruits in each treatment. The experiment was repeated 3 times.

A similar experimental method was followed in testing the efficacy of four strains of the antagonistic fungus *Trichoderma harzianum* and two strains of the antagonistic bacterium Bacillus amyloliquefaciens against the disease. These strains were provided by the faculty of Agriculture - Gembloux (Belgium). The persimmon test variety was the same as in the case of fungicides. The preventive and curative effects of Trichoderma and Bacillus strains against the disease were tested in the same manner as in the fungicide test. The experiment was repeated three times. The concentration of conidial suspension of *A. alternata* (strain Pr1), method of inoculation on fruits, incubation of fruits after inoculation and treatment with antagonistic strains, in addition to the experimental design were also similar to fungicide efficacy test. The concentration of conidial suspension of *Trichoderma* strains was 2.4×10^7 conidia per ml for Trichoderma-Th_1 Trichoderma-Th_2, Trichoderma-Th_3, and Trichoderma - CI306, whereas the concentration of bacterial suspension of *Bacillus* strains was 1.2×10^7 cfu per ml for Bacillus-GA_1 and Bacillus-GA_2 ($Y_1 = 0.17620 X_1 + 0.13624$, where Y1 = optical density and X_1 = cfu/ml for GA_1; $Y_2 = 0.14320 X_2 + 0.10213$, where Y_2 = optical density and X_2 = cfu/ml for GA_2). Culturing and subculturing of *Trichoderma* and *Bacillus* strains were carried out on Oat Meal Agar (OMA) and Potato Dextroze Agar (PDA) culture media, successively.

Evaluation of the efficacy test was carried out according to the capacity of each fungicide or strain of antagonistic microorganisms to reduce the disease lesion development. Therefore, the lesion diameter was measured in mm in all replicates 7 days after disease inoculation and treatment. The mean lesion diameter for each treatment with fungicide or antagonistic strain was calculated and used in the efficacy comparisons. In these comparisons, differences between the treatments were based on statistical analysis using ANOVA table and mean separation according to Duncan's Multiple Range Test.

RESULTS

The chemical fungicides Captan, Difenoconazole, Metalaxyl + Mancozeb, and Cyprodinil + Flodioxonil, had significant preventive effect against the black fruit spot disease on persimmon (Table 1). The disease lesion diameter was significantly smaller on fruits treated with fungicides than on the control. Specifically, the fruits treated with Difenoconazole, and Cyprodinil + Flodioxonil had the least mean diameter of disease lesion in all treatments (Table 1, Fig. 1-E). Similarly, the curative effect of the four fungicidal treatments on disease lesion development was also significant in comparison with the control (Table 1). The most significant reduction of disease lesion diameter was obtained after treatment with Metalaxyl + Mancozeb or Captan or Cyprodinil + Flodioxonil compared with the control in all treatments (Table 1, Fig. 1-F).

All of the four strains of *Trichoderma harzianum* and the two strains of *Bacillus amyloliquefaciens* tested against the disease showed a significant preventive effect against the disease as compared with the control in all treatments (Table 2). These results showed that the lesion diameter of the disease was significantly less after treatment with the strain Trichoderma-Th$_1$ compared with other strains or with the control (Table 2). This treatment gave the least mean diameter of disease lesion in all treatments (Table 2, Fig. 1-E). Also, significant differences were obtained in mean lesion diameter of the disease after curative treatment with all strains compared with the control (Table 2). In this case, the strains Trichoderma-Th$_1$ and Bacillus-GA$_1$ gave the least mean diameter of disease lesion (Table 2, Fig. 1-F).

DISCUSSION

For controlling the disease, local farmers usually treat Persimmon trees with traditional and residual fungicides endangering both local environment and health of Persimmon consumers (Personal Communication with Growers). The new fungicides Difenoconazole (Score® and Cyprodinil + Flodioxonil (Switch®) which proved to be the best during our tests with significant preventive effect against *A. alternata*, could be used in the control program as alternative to the traditional and residual fungicides. Difenoconazole (1 - 2 - [4 - (4-chlorophenoxy)- 2-chlorophenyl] -4- methyl-1, 3-dioxolan-2, 2yl-methy 1) -1H-1, 2, 4 triazole) is a new triazole fungicide with a slight toxicity (Oral LD_{50} to rats = 2125 mg/kg), and acts through inhibition of ergosterol biosynthesis in treated fungi (Ware, 1994, and Technical sheet of the fungicide). This explains the preventive effect of this fungicide by preventing the conidial germination and/or subsequent penetration in plant tissues if germination takes place. The ergosterol inhibition was reported by Ragsdal and Sisler, 1972 in the sporidia of Ustilago maydis after treating the fungus with other triazole fungicide: Triarimol (one of the pyrimidine derivatives). Switch® consisting of Cyprodinil [(4-cyclopropyl-6-methyl-N-phenyl-2yl) aniline] and Flodioxonil [4-(2,2-difluro-1, 3-benzodioxol-4-yl) pyrrole-3-carbonitrile] combines systemic and contact action where cyprodinil affects methionine synthesis and hydrolytic enzyme secretion in treated fungi, but Flodioxonil prevents the addition of energy to protein through the action of protein Kinase which interferes with signal transmission in treated fungi [Technical sheet of the fungicide]. This double action of Switch® explains its significant preventive and curative effect against *A. alternata* obtained in our tests. Other tested fungicide Ridomil® consisting of metalaxyl [N-(2,6-dimethylphenyl)- N- (methoxyacetyl)-alanine methyl ester] and Mancozeb [coordination product of zinc ion and manganese ethylene bisdithiocarbamate] has also both systemic and contact action (Table 1). This explains its significant curative effect against *A. alternata* by inactivating the -SH group of amino acids contained within the individual fungal cell and inhibiting elongation and ability to penetration of germ tube after conidial germination.

On the other hand, for reducing the excessive application of Difenoconazole on fruits especially during the harvesting time, *Trichoderma harzianum* (strain Trichoderma-Th$_1$) could be included in the control program as a mycofungicide (preventive effect) against *A. alternata.* This antagonistic fungus acts through secretion of the antifungal antibiotic viridin or through production of gliotoxin (antifungal substance) where both of them inhibited germination of *A. alternata* conidia. Certain authors reported the Trichoderma antifungal effect against certain causative agents of fungal plant pathogens such as Pythium damping-off and wood rotting fungi (Ware, 1994). Also, recent studies conducted by Yucheng, 2000 indicated the antagonistic effect of a strain of *Bacillus subtilis* used in biocontrol of *Alternaria alternata.* This supports our results obtained on the significant curative effect of the strain Bacillus-GA_1 of *Bacillus amyloliquefaciens* against *A. alternata* (Table 2). This curative effect indicates the potential of using this strain to reduce the infection with the disease on Persimmon fruits and then reduce frequency and dose of applying chemical fungicides.

In conclusion, a combination of biological and chemical treatments based on the above results may be applied to control the disease. So far all infections with Alternaria species especially *A. alternata* and *A. macrospora* on different hosts were reduced through treatments with different types of fungicides including those of systemic-type (Shteinberge and Dreishpoun, 1991; Padaganur and Basavaraj, 1987; Solel et al., 1996 and 1997; and Temkin-Gorodeiski et al., 1993). This combination may serve to minimize the dose and frequency of using chemical fungicides, thus reducing environmental pollution. Therefore, the following control program of the disease may be applied under field conditions: i) conducting alternate sprays of Difenoconazole (recommended dose 0.35% v/v) and Cyprodinil + flodioxonil (recommended dose 0.20% w/v) two or three times at 2-week intervals in the beginning of the growing season or immediately after bud-break or leaf enlargement in order to prevent infection with the disease especially on leaves; ii) spraying conidial suspension of *Trichoderma harzianum* (strain Trichoderma-Th1) at a concentration of 2.4 x 10^7 conidia per ml at 2-week intervals during the harvesting period to prevent the disease incidence especially on fruits. This biological treatment substituting the chemical treatments with fungicides has neither toxic effect on treated persimmon fruits, nor residual effect in the local environment; iii) carrying out alternate sprays of Metalaxyl + Mancozeb (recommended dose 0.30% w/v) and Cyprodinil + Flodioxonil (0.20% w/v) and Captan (0.35% w/v) two or three times at 2-week intervals to reduce the infection with the disease especially on leaves at favorable conditions during the growing season; and iv) spraying conidial suspension of Trichoderma harzianum (strain Trichoderma-Th_1; recommended concentration 2.4 x 10^7 conidia per ml) or bacterial suspension of *Bacillus amyloliquefaciens* (strain Bacillus-GA_1; recommended concentration 1.2 x 10^7 cfu per ml) to decrease the disease development when infection occurs at the harvesting period or after that.

Table 1: Effect of four fungicides on black fruit spot infection on Persimmon fruits (temperature: 20 ± 1°C, evaluation of the effect 7 days after inoculation).

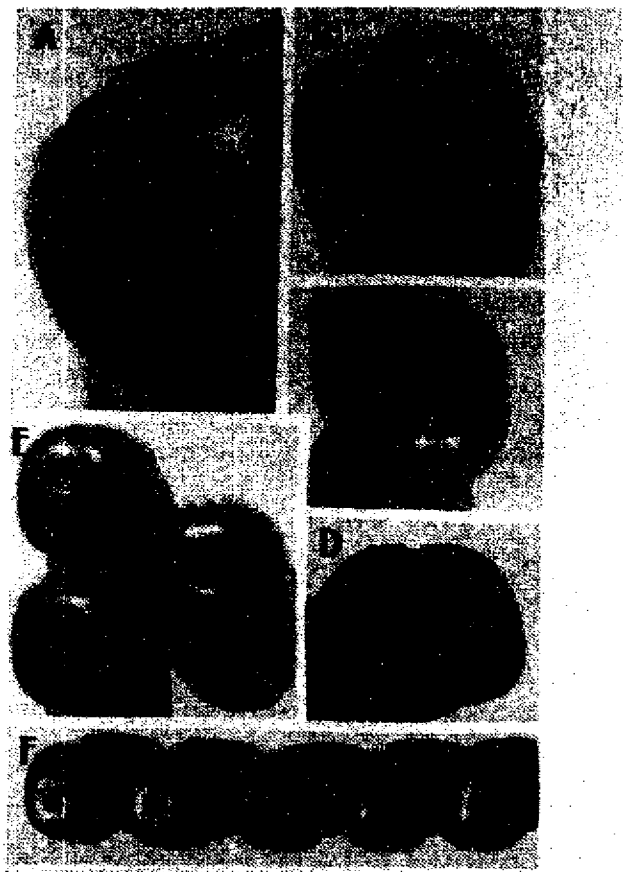
Treatments or types of fungicides used	Mean diameter (in mm) of lesions on Persimmon fruits	
	Preventive effect	Curative effect
Metalaxyl + Mancozeb	9.8[t] c	2.0[t] a
Difenoconazole	1.0 a	6.5 b
Captan	5.0 b	1.8 a
Cyprodinil + Flodioxonil	1.0 a	2.5 a
Control	16.2 d	16.2 c

[t] For fungicidal treatments preventively or curatively, means within each column followed by the same letter are not significantly different ($P<0.05$) according to Duncan's multiple range test based on ANOVA table for the completely randomized design (CRD).

Table 2: Effect of six strains of antagonistic microorganisms on black fruit spot infection on Persimmon fruits (temperature: 20 ± 1°C, evaluation of the effect 7 days after inoculation).

Treatments or strains of antagonistic microorganisms used	Mean diameter (in mm) of lesions on Persimmon fruits	
	Preventive effect	Curative effect
Bacillus-GA_1	12.5[t] c	9.2[t] a
Bacillus-GA_2	10.2 bc	10.2 a
Trichoderma-Th_1	5.8 a	9.2 a
Trichoderma-Th_2	9.2 b	10.8 a
Trichoderma-Th_3	11.8 bc	11.5 a
Trichoderma-CI306	10.8 bc	11.0 a
Control	17.0 d	17.0 b

t For treatments with antagonistic strains preventively or curatively, means within each column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test based on ANOVA table for the completely randomized design (CRD).



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تاثير المبيدات الفطرية والكائنات الحية الدقيقة المضادة على مرض تبقع الثمار الاسود في الفرسمون

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ملخـــص

يتسبب مرض تبقع الثمار الأسود في الفرسمون عن فطر *Alternaria alternata* الذي يصيب الثمار في الحقل عند اقتراب نضـجها ثم يتطور في فترة ما بعد الحصاد ليؤدي الى تعفن كامل الثمار فيما بعد. تم في هذه الدراسة اختبار فعالية عدد من السلالات لكائنات حية دقيقة مضادة للمرض *(Bacillus amyloliquefaciens & Trichoderma harzianum)* بالاضافة الى اجراء معاملات ضد المرض باستعمال مبيدات فطرية جديدة وعديمة الاثر المتبقي. ولتحقيق ذلك، تمت عدوى الثمار بالمرض ثم معاملتها بالمبيدات او السلالات المضادة في كافة التجارب، وتم تقييم كل من التأثير الوقائي والعلاجي لهذه السلالات او المبيدات تبعا لمقدرتها على منع زيادة قطر بقعة المرض بعد العدوى.

أظهرت النتائج وجود فروقات معنوية بين المبيدات او السلالات المضادة التي تم اختبارها، حيث اثبتت كل من المبيدات دايفنو كونازول وسايبرودينيل + فلوديوكسونيل كفاءة عالية في منع حدوث المرض وكانت ذات تاثير وقائي قوي على المرض، كما انه ثبت وجود هذا التاثير الوقائي القوي لدى سلالة الفطر $Trichoderma\text{-}Th_1$ واظهرت النتائج كذلك وجود التاثير العلاجي لدى كل من المبيدات كابتان ومتالاكسيل + مانكوزب وسايبرودينيل + فلوديو كسونيل ولدى كل من السلالتين $Trichoderma\text{-}Th_1$ و $Bacillus\text{-}GA_1$. ونظرا لكون المكافحة الكيماوية باستعمال المبيدات الفطرية التقليدية هي الطريقة المتبعة حاليا لمكافحة المرض، فقد تم اقتراح برنامج يشتمل على وسائل كيماوية واخرى حيوية وهو لا يشكل ضررا على البيئة المحلية او على مستهلكي ثمار الفرسمون.

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