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Control of Alternaria Spot Disease on Loquat (*Eriobotrya japonica* Lindl.) Using Detached Fruits and Leaf-disk Assay (*Eriobotrya japonica* Lindl.)

مكافحة مرض التبقع الالترناري على الأسكدنيا باستعمال طريقة العدوى على الثمار والأقراص الورقية

Yacoub Batta
Department of Plant Production and Protection, Faculty of Agriculture.
An-Najah National University. Nablus. Palestine.
E-mail: yabatta@najah.edu
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Abstract

Treatments with four types of new, slightly toxic and non-residual fungicides and two preparations of *Trichoderma harzianum* were applied against *Alternaria alternata*, causal organism of Alternaria leaf and fruit spot disease of loquat. Preventive and curative effects of the above-mentioned treatments were bioassessed by measuring lesion diameter of *A. alternata* and its sporulation density on detached loquat fruits and leaf discs. Results indicated that preventive and curative effects of the treatment with difenoconazole (Score®) and cyprodinil+flodioxonil (Switch®) applied at a rate of 0.35% (V/V) and 0.20% (W/V), respectively, significantly reduced the lesion diameter of *A. alternata* on treated fruits and leaf discs compared to the untreated control fruits and leaf discs. Also, application of the above-mentioned fungicides at the same rates completely inhibited the sporulation of *A. alternata* at preventive and curative treatment with Switch® and at curative treatment with Score® on treated leaf discs compared to the untreated control organs. Conidia of *T. harzianum* (strain Th_2) formulated in invert emulsion and applied at a concentration of $1.3X10^8$ conidia/ml of the preparation, significantly suppressed lesion diameter of *A. alternata* and its sporulation density on treated fruits compared to treatment with conidial suspension of *T. harzianum* in sterile distilled water, and the control (blank formulation of invert emulsion or sterile distilled water only). These results should be confirmed under field conditions before large scale application.

***Keywords*:** *Alternaria alternata*, *Trichoderma harzianum*, Difenoconazole, Cyprodinil + Flodioxonil, Metalaxyl + Mancozeb, Captan

ملخص

تم اجراء معاملات سيطرة على المسبب المرضي Alternaria alternata الذي يصيب أوراق وثمار الأسكدنيا باستخدام أربعة أنواع جديدة من المبيدات الفطرية ذات السمية القليلة والأثر المتبقي الضئيل بالاضافة إلى استخدام تحضيرين من الفطر المضاد Trichoderma harzianum . تمّ اختبار المعاملة الوقائية والعلاجية للسيطرة على المرض وبدلالة قياس قطر بقعة النسيج المتأثر بالمرض (شدة المرض) واعداد كونيديا الفطر المسبب للمرض على أقراص أوراق وثمار الأسكدنيا. أظهرت النتائج التي تم الحصول عليها ان التأثير الوقائي لكل من المبيدين الفطريين difenoconazole (سكور) و cyprodinil+flodioxonil (سويتش) المستعملين بتركيز مقداره 0.35% V/V) و0.20% W/V) على التوالي قد خفضت شدة المرض بشكل معنوي على أقراص الأوراق والثمار التي تمت معاملتها مقارنة مع الشاهد ، وكذلك فإن المبيدات الفطرية سالفة الذكر والمستعملة بنفس التراكيز السابقة قد منعت وبشكل كامل ظهور كونيديا الفطر المسبب للمرض على الثمار وأقراص الأوارق التي تمت معاملتها مقارنة مع الشاهد. أظهرت النتائج كذلك ان كونيديا الفطر المضاد T. harzianum والمستعملة بشكل مستحضر في محلول "انفرت" وبتركيز مقداره $1.3X10^{8}$ conidia/ml قد خفضت وبشكل معنوي شدة المرض واعداد كونيديا الفطر المسبب للمرض على الثمار التي تمت معاملتها مقارنة مع كونيديا الفطر المضاد والمستعملة في معلق مائي ومع الشاهد (محلول "انفرت" لوحده) أو مع الثمار غير المعاملة (مضاف إليها ماء مقطر معقم فقط). إن تأكيد النتائج التي تم الحصول عليها تحت ظروف الحقل هو أمر ضروري قبل أن يتم تعميم النتائج على نطاق واسع.

Introduction

Alternaria spot disease caused by the fungus *Alternaria alternata* (Fr.:Fr.) Keissl is one of the most important diseases on leaves and fruits of loquat (*Eriobotrya japonica* Lindl.). It appears as dark-brown necrotic lesions (almost circular) on leaves and fruits (Fig.1-A) that may coalesce to form large necrotic areas. The importance of the disease is attributed to causing leaf necrosis and leaf drop under field conditions, also causing fruit spotting and rotting during harvest and postharvest stages. The latter type of damage renders the fruits unsuitable for marketing.

Infections with *A. alternata* were reported by various investigators on other types of fruit trees and vegetable crops such as tomatoes (*Lycopersicon esculentum*) (15,23), peppers (*Capsicum annum*) (9,16), potato tubers (*Solanum tuberosum*) (14), brussel sprouts (*Brassica oleracae*) (30), figs (*Ficus carica*) (4), persimmons (*Diospyros virginiana*) (5, 24), and on certain weeds such as black night-shade (*Solanum nigrum*) (1). Moreover, *A. alternata* may appear along with other species of *Alternaria* in form of mixed infections such as *A. macrospora* on cotton (*Gossypium hirsutum*) (26, 27), *A. carthami* on safflower (*Carthamus tinctorius*) (21), and *A. cucumerina* on cucumber (*Cucumis sativus*) (35).

Alternaria diseases on different crops are usually controlled by spraying with chemical fungicides such as tebuconazole and difenoconazole to control *A. macrospora* on cotton (22, 28, 29), difenoconazole and metalaxyl + mancozeb to control *A. alternata* on fig trees (4), difenoconazole and cyprodinil+flodioxonil to control *A. alternata* on persimmon fruits (5), chlorothalonil against *A. alternata* on tomato (13), iprodione and metiram against *A. alternata* on minneola tangelo (*Citrus tangelo*) (32, 33), prochloraz against *A. alternata* on eggplant (*Solanum melongena*) (34). In addition to fungicides, few antagonistic microorganisms such as *Pseudomonas putida*, *P. cepacia*, *Bacillus subtilis*, *Trichoderma harzianum*, and yeasts (*Pichia* and *Candida* species) were investigated as biocontrol agents against rot and decay diseases of apple (7, 8, 17, 18), plum and apricot (25), citrus (10, 11, 31), pepper (10), potato (12), pear (19), tomato (10), peach and nectarine (25), persimmon (5), fig (4), and cucumber (6). The intensive and repeated application of highly toxic and residual fungicides caused many problems such as environmental pollution and resistance of disease-causative organisms to fungicides (20). To reduce these problems, slightly toxic and non-residual fungicides, in addition to non-pollutant bioagents could be used. The objective of the present research were: i) to test the efficacy of some new, slightly toxic and non-residual fungicides against the disease on loquat leaf discs and fruits; ii) to test the efficacy of formulated and non-formulated conidia of *T. harzianum* against the disease on loquat leaf discs and fruits.

Materials and Methods

Plant material

The loquat cultivar used in this study was "Akka 13". Healthy mature loquat fruits picked at harvesting stage, and leaf-discs (20mm diameter) cut from healthy young loquat leaves were used in the tests.

Fungal inocula

The causative fungus of the disease *Alternaria alternata* (strain Alt.3) was isolated from infected loquat fruits (cv: Balady) and cultured on potato dextroze agar (PDA) medium to obtain the fungus conidia to be used in subsequent inoculations (Fig.1-B and E). The antagonistic fungus *Trichoderma harzianum* (strain Th_2) was obtained from the Faculty of Agriculture, University of Gembloux (Belgium) and subcultured on plates with oat meal agar medium to be used against *A. alternata*. Young cultures

(14-day-old) of the above-mentioned fungi were used to carry out the different tests. The concentration of the conidial suspension prepared from these cultures was $5.5X10^5$ conidia/ml for the strain Alt.3 and $1.3X10^8$ conidia/ml for the strain Th_2.

Treatments and data analyses

Four treatments with fungicides were used against the disease at the recommended dosages: 0.30% (W/V) for metalaxyl+mancozeb (sold as Ridomil® MZ 63.5 WP, produced by Ciba Geigy Ltd. Co., Bazil-Switzerland, concentration of a.i.=7.5% metalaxyl + 56% mancozeb); 0.35% (V/V) for difenoconazole (sold as Score® 250 EC, produced by Novartis Ltd. CO., Bazil-Switzerland, concentration of a.i.=250 g/l); 0.35% (W/V) for captan (sold as Merpan® 50 WP, produced by Macktichim Chemical Factories, Israel, concentration of a.i=50%); and 0.20% (W/V) for cyprodinil+flodioxonil (sold as Switch® 62.5 WG, produced by Novartis Ltd. Co., Bazil-Switzerland, concentration of a.i.=375 g/Kg cyprodinil + 250 g/Kg flodioxonil).

Two treatments with *Trichoderma harzianum* (strain Th2) were used against the disease: conidial suspension in sterile distilled water, and formulated conidia in invert emulsion (water-in-oil type). Concentration of the fungal conidia in the two treatments was $1.3X10^8$ conidia/ml. Ingredients of the invert emulsion used in the experiment were identical to those of invert emulsion used by Batta, 2004a (7). They are: sterile distilled water (45.25%), glycerine (4.00%), water-soluble wax or Dehymuls K® (0.75%), Tween 20 (2.50%), and a mixture of 19.00% coconut oil + 28.50% soybean oil. Conidia of *T. harzianum* (strain Th2) harvested from 14-day-old culture of the fungus on plates with oat meal agar medium were introduced into the invert emulsion according to the technique developed by Batta, 2004a (7).
Two additional control treatments were included; one with sterile distilled water only, and the other with blank formulation of invert emulsion. The experimental treatments were distributed according to completely randomized design (CRD) with 4 to 5 replicates representing 4 fruits or 5 leaf discs per treatment. Data obtained were subjected to statistical analysis using ANOVA and DMRT.

Inoculation and assessment of treatment effect

Prior to inoculation, all discs and fruits were disinfected with 0.025% sodium hypochlorite and then rinsed with sterile distilled water. Inoculation of *A. alternata* was accomplished by depositing a 25-µl droplet of conidial suspension containing 14,750 conidia (original suspension contained $5.5X10^5$ conidia/ml) on the leaf-disc center or on the fruit surface after being wounded. Treatment effect with fungicides and *T. harzianum* on *A. alternata* as preventive treatment (application at time of *A. alternata* inoculation), and curative treatment (application 24 h after *A. alternata* inoculation) was assessed. Twenty five-µl droplet of fungicide solutions (as indicated earlier) or formulated *T. harzianum* or its conidial suspension containing $3.25X10^6$ conidia (original suspension contained $1.3X10^8$ conidia/ml) was used. The droplet was deposited at the same site of *A. alternata* inoculation on leaf disc or fruit surface immediately after the inoculation or 24 h later. Inoculated and treated leaf discs and fruits were then incubated either in Petri-dishes (5 discs per dish) or in closed plastic cans (9.5cm diameter by 6.5cm deep) with one fruit per can. Incubation periods were 5 and 7 days at 22±2^{o}C for measuring the disease-lesion diameter on fruits and leaf discs, respectively, and 8 and 10 days for measuring *A. alternata*-sporulation density on the respective organs.

Evaluation of treatment effect on disease control

Evaluation of the treatment with each fungicide or *T. harzianum*-form was done according to their effectiveness in reducing the lesion development of *A. alternata* or its sporulation density on leaf discs or fruits compared to the untreated control. The lesion diameter of *A. alternata* and its sporulation density on fruits were then measured 5 and 8 days after the treatment, respectively, and on leaf-discs 7 and 10 days after the treatment, respectively. Mean lesion-diameter and sporulation-density of *A. alternata* per lesion on each organ in each treatment were calculated. Sporulation density of *A. alternata* was evaluated on the basis of a scale consisting of 4 levels: level 1=no sporulation; level 2= light density (1-49 conidia); level 3= moderate density (50-199 conidia); and level 4= high density (>200 conidia).

Results

Effect of fungicides and *T. harzianum* on *A. alternata*-lesion development On detached fruits

In both preventive and curative treatment effects, significant differences were found between means of *A. alternata*-lesion diameter on detached fruits treated with the tested fungicides and *T. harzianum* as compared with the control treatment (Table 1). Means of lesion diameter in the preventive and curative functions indicated significant reduction by 86.1, 84.1, and 84.1 and 83.1% using Score® and Switch®, respectively, compared to the control with sterile distilled water only (Table 1). Also, Merpan® and Ridomil® significantly reduced lesion diameter but by only 61.5, 44.6, and 46.1 and 43.1%, respectively (Table 1). Treatments with the formulated conidia of *T. harzianum* in invert emulsion and conidial suspension of *T. harzianum* in sterile distilled water also significantly reduced lesion diameter compared to sterile distilled water control but only by 53.8, 36.9, and 35.4 and 29.2% in the preventive and curative functions, respectively (Table 1).

The general classification of preventive-treatment efficacy in a descending order was as follows: Score® or Switch®, Merpan® or formulated conidia of *T. harzianum* in invert emulsion, Ridomil®, conidial suspension of *T. harzianum* in sterile distilled water, blank formulation of invert emulsion (control treatment) or sterile distilled water only (control treatment), respectively. For the curative-treatment efficacy, the descending order was as follows: Score® or Switch®, Merpan® or Ridomil®, formulated conidia of *T. harzianum* in invert emulsion, conidial suspension of *T. harzianum* in sterile distilled water, blank formulation of invert emulsion or sterile distilled water only, respectively (Table 1; Fig.1-D). The above-mentioned classification was based on the significance of reduction level in disease-lesion diameter resulted from the treatments compared to the control.

On leaf discs

The preventive and curative effects of treatment with fungicides and *T. harzianum* on *A. alternata* -lesion development were almost similar to those obtained on fruits. Significant differences were found between means of lesion diameter on leaf-discs as a result of treatment with fungicides and *T. harzianum* in comparison with the control (Table 1). Means of lesion diameter in the preventive and curative functions indicated significant reduction by 89.5, 100, and 100 and 100% using Score® and Switch®, respectively, compared to the control with sterile distilled water only (Table 1). Also, Merpan® and Ridomil® significantly reduced lesion diameter but by only 84.9, 55.8, and 57.0 and 77.9%, respectively (Table 1). Treatments with the formulated conidia of *T. harzianum* in invert emulsion and conidial suspension of *T. harzianum* in sterile distilled water also significantly reduced lesion diameter compared to sterile distilled water control but only by 67.4, 55.8, and 59.3 and 46.5% in the preventive and curative functions, respectively (Table 1).

The general classification of preventive-treatment efficacy in a descending order was therefore as follows: Switch®, Score® or Merpan®, formulated conidia of *T. harzianum* in invert emulsion or Ridomil®, conidial suspension of *T. harzianum* in sterile distilled water, blank formulation of invert emulsion (control treatment), and sterile distilled water only (control treatment), respectively. For curative-treatment efficacy, the descending order was as follows: Score® or Switch®, Ridomil®, Merpan® or formulated *T. harzianum*-conidia in invert emulsion, conidial suspension of *T. harzianum* in sterile distilled water, blank formulation of invert emulsion, and sterile distilled water only, respectively (Table 1, Fig.1C).

Effect of fungicides and *T. harzianum* on *A. alternata*-sporulation density on detached fruits and leaf discs

Treatment with fungicides and *T. harzianum* also caused significant reduction in *A. alternata*-sporulation density on detached fruits and leaf discs of loquat compared to the untreated control (Table 2). Preventive and curative treatment with Score® or Switch® significantly reduced fungus sporulation density from density level 4(>200 conidia) on untreated control fruits and leaf discs to density level 1 (0 conidia) on treated leaf discs and to density level 2 (1-49 conidia) on treated fruits. (Table 2). Also, preventive and curative treatment with formulated conidia of *T. harzianum* in invert emulsion significantly reduced the sporulation density from density level 4 (>200 conidia) on the control to density level 2 (1-49 conidia) (Table2).

***Table 1**: Preventive and curative effect of fungicides and Trichoderma harzianum treatment on lesion development of Alternaria alternata on detached loquat fruits and leaf discs 5 and 7 days after inoculation and treatment, respectively*

	Means of *A. alternata*-lesion diameter (in mm)			
	on detached fruits		on leaf discs	
Treatments	Preventive effect [1)]	Curative effect [1)]	Preventive effect [1)]	Curative effect [1)]
Switch® (cyprodinil+flodioxonil)	2.50 a [2)]	2.75 a [2)]	0 a [2)]	0 a [2)]
Score® (difenoconazole)	2.25 a	2.50 a	1.80 ab	0 a
Merpan® (captan)	6.25 b	9.00 b	2.60 b	7.60 c
Formulated conidia of *T. harzianum* in invert emulsion	7.50 bc	10.25 c	5.60 c	7.60 c
Ridomil® (metalaxyl+mancozeb)	8.75 c	9.25 b	7.40 c	3.80 b
Conidial suspension of *T. harzianum* in sterile distilled water	10.50 d	11.50 d	7.00 c	9.20 c
Blank formulation of invert emulsion (control treatment)	15.50 e	15.50 e	14.20 d	14.20 d
Sterile distilled water only (control treatment)	16.25 e	16.25 e	17.20 e	17.20 e

[1)] The preventive treatment against the disease was done immediately after inoculation with the disease-causative organism (*A. alternata*). Five leaf-discs (20mm diameter) or 4 fruits which represent 5 and 4 replicates of the above organs, respectively, per treatment were used. The curative treatment against the disease was done 24h after disease-causative organism inoculation. Five leaf-discs (20mm diameter) or 4 fruits which represent 5 and 4 replicates of these organs, respectively, per treatment were used.

2) Means of disease-lesion diameter within the column (in each type of effect and in each organ) followed by different letters are significantly different at $p<0.05$ according to ANOVA table and Duncan's multiple range test (DMRT).

***Table 2**: Preventive and curative effect of fungicides and Trichoderma harzianum treatments on sporulation density of Alternaria alternata on detached loquat fruits and leaf discs 8 and 10 days after inoculation and treatment, respectively*

	Means of *A. alternata*-sporulation density on the lesion surface [1]			
	on detached fruits		on leaf discs	
Treatments	Preventive effect [2]	Curative effect [2]	Preventive effect [2]	Curative effect [2]
Switch® (cyprodinil+flodioxonil)	2	2	1	1
Score® (difenoconazole)	2	2	2	1
Merpan® (captan)	2	2	2	2
Formulated conidia of *T. harzianum* in invert emulsion	2	2	2	2
Ridomil® (metalaxyl+mancozeb)	3	3	2	2
Conidial suspension of *T. harzianum* in sterile distilled water	2	2	3	3
Blank formulation of invert emulsion (control treatment)	4	4	4	4
Sterile distilled water only (control treatment)	4	4	4	4

1) Sporulation density was measured according to the following scale: 4= high density (>200 conidia); 3= moderate density (50-199 conidia); 2= light density (1-49 conidia); and 1= no sporulation (0 conidia).

2) The preventive treatment against the disease was done immediately after inoculation with the disease-causative organism (*A. alternata*). Five leaf-discs (20mm diameter) or 4 fruits which represent 5 and 4 replicates of these organs, respectively, per treatment were used. The curative treatment against the disease was done 24h after the disease inoculation. Five leaf-discs (20mm diameter) or 4 fruits which represent 5 and 4 replicates of these organs, respectively, per treatment were used.

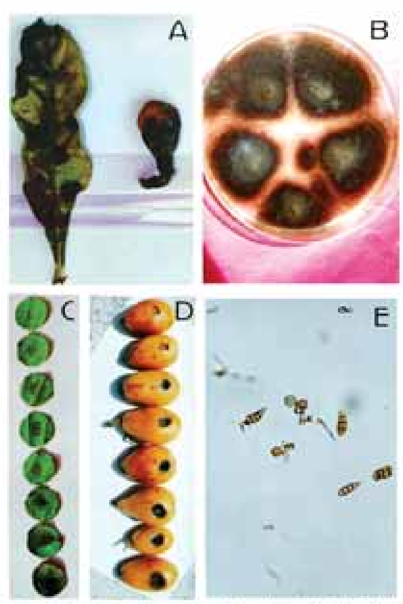

Figure 1: Alternaria spot disease (*Alternaria alternata*) on loquat leaves and fruits. A. typical lesions of the disease on infected loquat leaves and fruits as dark-brown necrotic lesions (almost circular) which may coalesce to form large necrotic areas. Lesions are covered with dense dark fungal growth (chains of fungus conidia), B. typical culture of *A. alternata* isolated from loquat fruits and grown on potato dextrose agar medium. It was characterized by dark-brown colonies with some concentric fungal growth, C. comparison of the disease-lesion development on loquat leaf-discs after disease-inoculation and treatment, the descending order of treatment efficacy was Switch®, Score®, Merpan®, Formulated conidia of *T. harzianum* in invert emulsion, Ridomil®, conidial suspension of *T. harzianum* in sterile distilled water, formulation blank of invert emulsion as check treatment, and sterile distilled water only as check treatment (order from top to bottom, respectively), D. comparison of the disease-lesion development on loquat fruits after disease inoculation and treatment, the descending order of treatment efficacy was almost similar to that of leaf-discs but, with higher values of disease-lesion diameter on fruits. E. young and mature typical conidia of *A. alternata* (grown on PDA-medium) characterized by brown color with very short peak and formed in long chains of 10 conidia or more and have 4-8 transverse septs per mature conidium.

Discussion

Under the local conditions, loquat growers usually apply traditional fungicides that are known for their residual effects, when the disease appears in their fields on loquat trees. In addition to their inefficacy, no specific spraying program for traditional fungicides was followed by the growers during application of these fungicides (personal communication). Previous studies have indicated that systemic fungicides with no residual effect such as difenoconazole, cyprodinil+flodioxonil, and metalaxyl+mancozeb were effectively applied against *A. alternata* on fig leaves and persimmon fruits (4, 5) and against *A. cucumerina* on cucumber (6). In the present study, application of the same non-residual systemic fungicides especially difenoconazole, and cyprodinil + flodioxonil, also, significantly reduced the disease infection or disease-lesion diameter on loquat leaves and fruits. Score® (at a rate of 0.35% V/V) and Switch® (at a rate of 0.20% W/V) were recommended to be used in the disease control. To avoid development of resistance to these fungicides, alternation of treatment with *T. harzianum*-conidia formulated in invert emulsion (concentration $1.3X10^{8}$ conidia/ml) was suggested in this study. The development of resistance to systemic fungicides applied against *A. alternata* on other crops was reported in other countries (20). For example, *A. alternata* f.sp. Kikuchiana on pears and *A. alternata* f.sp. mali on apple were found resistant to Iprodione and polyoxin (12). The same type of resistance was also developed to fenitin hydroxide, fenitin acetate, and tebuconazole when applied, respectively, against *A. macrospora* on cotton in India and Israel (2, 3, 22, 28, 29).

No previous study was found dealing with the effect of treatment with fungicides or antagonistic bioagents on the sporulation density of *A. alternata*. In the present study, a direct relationship between the treatment effect with fungicides or *T. harzianum* on *A. alternata*-lesion diameter or its sporulation density could be deduced since all tested fungicides and *T. harzianum*-preparations which significantly reduced the disease-lesion diameter reduced also the sporulation density of the fungus. Consequently, the tested fungicides: difenoconazole and cyprodinil+flodioxonil completely inhibited the fungus sporulation on leaf discs when applied against the disease preventively or curatively. Also, formulated *T. harzianum* in invert emulsion significantly reduced the fungus sporulation. Finally, the above-mentioned results obtained on the fungus sporulation are important from epidemiological viewpoint since inhibition of the fungus sporulation greatly affects the disease overwintering and development in the next season.

Finally, the results obtained on efficacy of the tested fungicides and *T. harzianum* conidia formulated in invert emulsion against the disease-lesion development and its sporulation using detached fruits and leaf-disk assay should be tested under field conditions on loquat trees. Recommended dosages of the effective fungicides and *T. harzianum* used in the study should be confirmed under field conditions since the disease attacks the leaves and fruits at harvest and postharvest stages. Alternation of fungicidal sprays and formulated *T. harzianum* treatments is recommended to avoid development of disease resistance to fungicides.

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