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In vivo SENSITIVITY OF *Penicillium digitatum* STRAINS INFECTING CITRUS FRUITS TO SIX FUNGICIDES AND THEIR COMBINATIONS

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By
Gh. J. M. Kanan

Department of Biological Sciences, Mu'tah University, Karak, Jordan

ABSTRACT

The effects of six fungicides and their mixtures on *in vivo* growth of four *Penicillium digitatum* strains and the development of post-harvest green mould on artificially inoculated orange and lemon citrus fruits were evaluated. Regression analysis, one way ANOVA, and Post Hoc multiple comparisons were carried out to test the significance of these treatments on fungal growth. All fungicides (except Blin exa) completely inhibited the growth of strain dg6 (the most sensitive) invading orange fruits. Benomyl has completely inhibited the growth of strains dg6, dg5 and dg4 infecting lemon fruits. Ranvil has generated complete inhibition of strains dg6 and dg5 infecting lemon fruits. The Benomyl/Ranvil mixture showed synergistic effect against strains: dg2 (the least sensitive) infected lemon and orange fruits, dg4 and dg5, infected orange fruits, where complete inhibition to fungal growth was observed. A combined concentration of 100:500 $\mu g.mL^{-1}$ of Topsin/Blin or Blin /Canvil mixture generated complete inhibition to the fungal growth as a result of synergistic effect against all strains infected both citrus fruit types.

Key words: *chemical control, fungicide mixtures, fungicides, green mould, in vivo, lemon, orange, Penicillium digitatum.*

1. INTRODUCTION

Citrus post-harvest green mould caused by *Penicillium digitatum* (Pers.) Sacc. is considered one of the most economically important universal diseases, which lead to spoilage of almost all kinds of mature citrus fruits (Plaza *et al.*, 2004). The fungus invades the fruit more rapidly at room temperature and predominates in mixed infections causing approximately 60 to 80% of decay (Palou *et al.*, 2001). Citrus industry relies heavily on the extensive use of chemical fungicides as standard practice for the control of post-harvest fungal decay of citrus fruits (Mc Grath, 2001and Pramila and Dubey, 2004). Members of the Sterol demethylation inhibitor (DMI) group of fungicides such as Imazalil and *O*-phenyl phenol have remained the most routinely used ones in California citrus packing houses in particular (Holmes and Eckert, 1999) and worldwide (Savocchia *et al.*, 2004 and Sugiura *et al.*, 2006). Furthermore, the DMI members have greatly inhibited the growth of Ascomycota and basidiomycota members (Savocchia *et al.*, 2004; Ma *et al.*, 2006). The benzimidazole precursor fungicides (azoles) such as Benomyl and Topsin M are ultimate broad-spectrum systemic fungicides (Tsuda *et al.*, 2004) which showed selective toxicity to several fungal diseases, and considered of the most effective against penicillium moulds (Zamin *et al.*, 1999 and Valiuskaite *et al.*, 2006). However, the widespread and recurrent use of chemicals having the same active gradients in commercial packing houses had lead to loss of their effectiveness, resulting in proliferation of resistant strains to these fungicides (Bus *et al.*, 1991; McGrath, 2001and Surviliene and Dambrauskiene, 2006). The occurrence of serious resistance problems had enhanced searching for alternative decay control options (Palou *et al.*, 2002 and Irtwange, 2006).

The current investigation aimed at participation in overcoming these threats through *in vivo* evaluation of four combined concentrations from each of seven fungicide mixtures. The hope in such strategy is to improve decay resistance at reduced risks and achieving cost saving benefits through reducing fruit spoilage and extending shelf life.

2. MATERIALS AND METHODS

2.1. *Penicillium digitatum* strains and medium

Conidiospores of four *P. digitatum* strains (dg2,

dg4, dg5, and dg6) were obtained from infected orange (*Citrus sinensis* Osbeck) and lemon (*Citrus limon* Burm .f.) fruits, collected from distributors in Irbid and Al-Karak cities in Jordan. Conidiospores from purified single colonies were used as a source of pure cultures. Routine Aspergillus growth medium and handling techniques were used as described by Cove (1966) with slight modifications, *i.e.* pH 5.5; supplemented with 10 mM glutamic acid and 10 gL^{-1} fructose as C- source.

2.2. Tested fungicides

Six fungicides were tested these are: (i) Bayfidan Turf - 25% EC (Vydan), containing 25% (w/v) triadimenol ($C_{14}H_{18}ClN_3O_3$) as an active gradient and produced by Vapco. Company – Jordan Blin exa - 5% Sc containing 5% (w/v) hexaconazole ($C_{14}H_{17}Cl_2N_3O$) as an active gradient and produced by IQV - Spain. Canvil - 5% contains 5% (w/v) hexaconazole and produced by Vapco. Company - Jordan. Ranvil - 5% contains also hexaconazole 5% (w/v) and produced by Chem.Vet - Jordan Benomyl - 50% W.P contains Benlate 50% (w/w) with the formula $C_{14}H_{18}N_4O_3$ and produced by Vapco. Company - Jordan. (vi) Topsin M - 70% W.P contains thiophanate-methyl 70% (w/w) with the formula $C_{12}H_{14}N_4O_4S_2$ and produced by Nippon Soda - Japan.

2. 3. Citrus fruit types

Two citrus fruit species at ripening maturity were used in this study and these are orange (*Citrus sinensis* Osbeck) and lemon fruits (*Citrus limon* Burm .f.).

2.4. Surface sterilization of fruits and inoculation with conidiospores and fungicides

Fruits were washed under running tap water for 5 min. This was followed by surface sterilization with 0.6% sodium hypochlorite solution for 10 min. After that fruits were washed three times (5 min / each) with sterilized water inside a UV sterilized culture room. Surface sterilized fruits were immediately inoculated in a laminar flow cabinet placed in a UV sterilized culture room. Each fruit was wounded two wounds [each of 5 x 5 mm. for orange fruits (thick coat) and approximately half that for lemon fruits without causing leakage of juice] at the equatorial side with sterilized stainless steel scalpel. 15 µL of conidiospores suspension (10^8 spore mL^{-1}) from any of the tested strains was inoculated into each wound under aseptic conditions. Two hours later eight different concentrations (10, 25, 50, 100, 200, 300, 500, and 1000 µg. mL^{-1}) from any of the tested fungicides were tested by inoculating 15 µL from each concentration into one wound (Palou *et al.*, 2001). Control fruits were inoculated with sterilized distilled water. Three replicates were used per each treatment for each fruit type and each test was repeated at least twice. The treated fruits were sealed in sterilized transparent nylon bags and incubated at room temperature (22-25 °C; optimal range for fungal growth) for two weeks then assessed thereafter for decay or infection symptoms.

2.5. Inoculation of fruits with conidiospores and fungicides mixture

The above mentioned procedure was carried out using four combined concentrations (50:50, 100:100, 100:500, and 500:1000 µg. mL^{-1}) from each fungicide mixture against fungal strains, where these mixtures showed homogenous solution without precipitation. Seven fungicide mixtures were used these are: Benomyl/Ranvil; Topsin M/Vydan; Vydan/Canvil; Blin exa/Canvil; Topsin M/Blin exa; Blin exa/Vydan; Topsin M/Canvil. Each combined concentration of fungicides mixture was loaded to the same wound, where three replicates from each fruit type were used for each combination and each treatment was repeated at least twice then fruits decay was assessed as mentioned before.

2.6. Estimation of fruit surface area

Since the fruit has an ellipsoid shape, which has greater polar radius (a) than the equatorial (b) one (a>b) then the quantity (e) = $\sqrt{(1-b^2/a^2)}$ is the eccentricity of the ellipse (Anton, 1995) and the surface area (S) of the prolate ellipsoid is given by the following equation:

$S = 2\pi b^2 [1+ (a/b) \arcsin(e) /e)]$

$\text{Arcsine}(e) = \tan^{-1}\{e/\sqrt{1-e^2}\}$

so, the polar as well as the equatorial radius of each fruit were measured, then both measurements were applied to a mathematical equation which was introduced into a visual basic computer program, in order to calculate the fruit surface area, and the percentage of cleared surface area.

2.7. Statistical analysis

The IC_{50} values were calculated by regression analysis for the relationship between the size of inhibition zone (mm.) and the fungicide concentration (Log value) using Microsoft Excel 2003 and the SPSS program version 10. One way ANOVA was carried out to determine the significant effect of each of the seven fungicide mixtures on sizes of inhibition zones of the studied strains. This was followed by Post Hoc mul330tiple comparisons to determine the significance level of combined concentrations of

fungicide mixtures and their interactions on sizes of inhibition zones of *P. digitatum* strains.

3. RESULTS

3.1. *In vivo* sensitivity of *P. digitatum* strains to some fungicides

Results of regression analysis indicate that there was a significant correlation (at the 0.01 level-2 tailed) between fungicide concentration (µg. mL^{-1}) and the size of inhibition zone (mm.) for the tested strains invading lemon and orange fruits (Table 1). All fungicides completely inhibited the growth of strain dg6 invading orange fruits where, the obtained IC_{50} values have ranged from 35 µg. mL^{-1} (with Benomyl Topsin) to 230 µg. mL^{-1} with Canvil (Table 1). However, Benomyl was the only fungicide which showed complete inhibition (IC_{50} = 375 µg. mL^{-1}) of growth to strain dg4 on orange fruits (Table 1). In addition, Benomyl fungicide completely inhibited the growth of strains dg6, dg5 and dg4 infecting lemon fruits (Fig. 1b) and the obtained IC_{50} values of the three strains were 30, 237 and 132.5 µg. mL^{-1}, respectively. Furthermore, Ranvil (a hexaconazole member) has also generated complete inhibition of strains dg6 and dg5 (Fig. 1b) invading lemon fruits and the obtained IC_{50} values were 138 and 144 µg. mL^{-1} respectively. On the other hand, none of the tested fungicides caused completely inhibit the growth of strain dg2 (The least sensitive strain) on lemon fruits where, the obtained zones of inhibition at the concentration of 1000 µg. mL^{-1} have ranged from 7.0 ± 2.30 µg. mL^{-1} with Vydan (The least effective fungicide) to 42.5 ± 3.57 µg. mL^{-1} with Benomyl (Table 1).

3.2. *In vivo* sensitivity of *P. digitatum* strains to various combined concentrations of fungicides mixtures

One way analysis of variance (ANOVA) indicated that the fungicide mixtures (except Benomyl/Ranvil and Topsin/Blin exa) have significantly (P=0.000) affected the sizes of inhibition zones of the four tested fungal strains infecting both lemon and orange fruits.

3.3. Schaffe multiple comparisons

3.3.1. Effect of benomyl/ranvil mixture on growth of *P. digitatum* strains

The Benomyl/Ranvil mixture showed synergistic effect at all tested concentrations against strain dg2 infecting both lemon and orange fruits, where complete inhibition (100% cleared fruit surface area – CSA) of fungal growth was obtained (Table 2). The same mixture (Benomyl/Ranvil) showed also, synergistic effect against strains dg4 and dg5 infecting orange fruits (complete inhibition was achieved at a concentration of 100:500 µg. mL^{-1}). In contrast, the same mixture of fungicides resulted in antagonistic effects against strains dg4 and dg6 infecting lemon fruits where the obtained maximum zones of inhibition were 15 and 50 mm., respectively, and this resulted in 5.83 and 88.97% (CSA), respectively (Table 2). In addition, obtained results indicate that there was a significant difference (P=0.000) between the combined concentration 50:50 µg. mL^{-1} of Benomyl/Ranvil mixture and the rest of combinations (100:100;100:500 and 500:1000 µg. mL^{-1}) on size of inhibition zones of strains dg4 and dg6 infecting lemon, but not orange (P=1.000) fruits. Furthermore, there was significant difference (P=0.000) between the combined concentration of 100:100 µg. mL^{-1} and each of the following combinations: 100:500 and 500:1000 µg. mL^{-1} on zone size of strain dg5 which infects both lemon (Fig. 1b) and orange fruits.

3.3.2. Effect of topsin M/Canvil mixture

Results indicate that Topsin/Canvil mixture has generated synergistic effects against strains dg4 and dg5 infecting both fruit types, whereas, antagonistic effect was obtained against strain dg2 infecting both fruit types (Table 2). However, additive effects were obtained against zones of strain dg6, whether invading orange or lemon fruits where, all treatments have generated complete inhibition of fungal growth on orange fruits but not on lemon. Furthermore, results indicate that there was a significant difference between combined concentrations of 50:50 and 100:500 µg. mL^{-1} on zones of strain dg4 infecting both citrus fruit types (P=0.000 for both fruit types). Also, there was a significant difference between the combination of 100:100 µg. mL^{-1} and each of the following combinations: 100:500 (P=0.002 for lemon; P=0.001 for orange) and 500:1000 µg mL^{-1} (P=0.000 for both fruit types) on zones of strain dg5 infecting lemon and orange fruits, and on zones of strain dg6 infecting lemon (p=0.000) but not orange (p=1.000) fruits. Moreover, there was a significant difference between the combined concentrations of 100:100 and 500:1000 µg. mL^{-1} on zones of strain dg2 infecting lemon (P=0.000) and orange (P=0.039) fruits, respectively.

3.3.3. Effect of Topsin/Vydan mixture

There was a significant difference between all combinations of applied concentrations (50:50; 100:100; 100:500 and 500:1000 µg. mL^{-1}) of

Table (1): *In vivo* **sensitivity of four** *Penicillium digitatum* **strains to six fungicides, 14 days after incubation at 22-25°C.**

Fungicide/ Fruit type	Fungicide/ Conc/range (µg mL $^{-1}$)†	Fungal strain	Mean inhibition zone (range) (mm) ± SD	IC_{50}	Coeff (r-value)	Sig (2-tailed)
Benomyl/ Orange	10-25 50-1000	dg6	14 ± 4.24 – 17 ± 4.46 Ci‡	35	0.797*	0.018
Benomyl/ Lemon	10 – 25 50 – 1000	dg6	34.5 ± 2.33 – 39.5±4.74 Ci	30	0.801*	0.017
Topsin M/ Orange	10 – 25 50 - 1000	dg6	15 ± 1.41 – 18.5 ± 2.12 Ci	35	0.892**	0.003
Topsin M/ Lemon	10 - 1000	dg6	0.0 – 22.5 ± 2.11		0.906**	0.002
Ranvil/ Orange	10 – 100 200 - 1000	dg6	4 ± 2.82 – 9 ± 2.88 Ci	145	0.874**	0.005
Ranvil/ Lemon	10 – 100 200 - 1000	dg6	5 ± 7.07 – 18.5 ± 2.12 Ci	138	0.892**	0.003
Vydan/ Orange	10 – 100 200 - 1000	dg6	10 ± 2.32 – 30 ± 1.65 Ci	128	0.913**	0.002
Vydan/ Lemon	10 - 1000	dg6	0.0			
Canvil/ Orange	10 – 200 300 - 1000	dg6	2.0 ± 2.82 – 28 ± 4.48 Ci	230	0.886**	0.003
Canvil/ Lemon	10 - 1000	dg6	0.0 – 15 ± 3.12		0.902**	0.002
Blin exa/ Orange	10 - 1000	dg6	6.5 ± 0.70 – 44.5 ± 2.12		0.893**	0.003
Blin exa/ Lemon	10 - 1000	dg6	0.0 – 23 ± 2.82		0.982**	0.000
Benomyl/ Orange	10 - 1000	dg5	11 ± 1.65 – 21.5 ± 2.21		0.982**	0.000
Benomyl/ Lemon	10 – 200 300 - 1000	dg5	4 ± 1.44 – 21.5 ± 2.20 Ci	237	0.862**	0.006
Topsin M/ Orange	10 - 1000	dg5	0.0 – 13.5 ± 4.99		0.905**	0.002
Topsin M/ Lemon	10 - 1000	dg5	0.0 – 15 ± 8.48		0.955**	0.000
Ranvil/ Orange	10 - 1000	dg5	4.5 ± 0.77 – 13.5 ± 3.56		0.980**	0.000
Ranvil/ Lemon	10 – 100 200 - 1000	dg5	4 ± 1.42 – 11 ± 1.43 Ci	144	0.879**	0.004
Vydan/ Orange	10 - 1000	dg5	4.0 ± 2.23 – 13.0 ± 3.32		0.875**	0.004
Vydan/ Lemon	10 - 1000	dg5	0.0 – 0.0			
Canvil/ Orange	10 - 1000	dg5	1.0 ± 1.41 - 8.5 ± 0.77		0.959**	0.000
Canvil/ Lemon	10 - 1000	dg5	3.0 ± 1.11 – 8.5 ± 2.87		0.980**	0.000
Blin exa/ Orange	10 - 1000	dg5	5.0 ± 1.41 – 16.5 ± 2.13		0.952**	0.000
Blin exa/ Lemon	10 - 1000	dg5	1.5 ± 2.12 – 11.0 ± 3.11		0.991**	0.000
Benomyl/ Orange	10 - 1000	dg2	5.0 ± 1.43 – 37.0 ± 1.71		0.953**	0.000
Benomyl/ Lemon	10 - 1000	dg2	15.0 ± 1.44 – 42.5 ± 3.57		0.986**	0.000
Topsin M/ Orange	10 - 1000	dg2	0.0 ± 15.0 ± 2.83		0.984**	0.000

(Continued)

Table (1): Continued.

Fungicide/ Fruit type	Fungicide/ Conc/range (μg mL $^{-1}$)†	Fungal strain	Mean inhibition zone (range) (mm) ± SD	IC_{50}	Coeff (r-value)	Sig (2-tailed)
Topsin M/ Lemon	10 - 1000	dg2	3.5 ± 4.95 – 35 ± 1.88		0.972**	0.000
Ranvil/ Orange	10 - 1000	dg2	4.5 ± 0.71 – 20.5 ± 3.55		0.972**	0.000
Ranvil/ Lemon	10 - 1000	dg2	3.5 ± 2.21 – 15.5 ± 3.53		0.981**	0.000
Vydan/ Orange	10 - 1000	dg2	0.0– 6.0 ± 1.36		0.791*	0.019
Vydan/ Lemon	10 - 1000	dg2	0.0 – 7.0 ± 2.30		0.553	0.155
Canvil/ Orange	10 - 1000	dg2	4.0 ± 1.61 – 36.0 ± 2.36		0.944**	0.000
Canvil/ Lemon	10 - 1000	dg2	14.0 ± 2.61 – 40.0 ± 3.34		0.986**	0.000
Blin exa/ Orange	10 - 1000	dg2	3.0 ± 1.41 – 16.0 ± 5.65		0.958**	0.000
Blin exa/ Lemon	10 – 100 200 - 1000	dg2	4.0 ± 1.44 – 9.5 ± 1.77 Ci	143	0.875**	0.004
Benomyl/ Orange	10 – 300 500 - 1000	dg4	8.5 ± 0.77 – 19.5 ± 2.02 Ci	375	0.765*	0.027
Benomyl/ Lemon	10 – 100 200 - 1000	dg4	15.5 ± 0.71 – 25.5 ± 2.12 Ci	132.5	0.886**	0.003
Topsin M/ Orange	10 -1000	dg4	0.0 – 14.5 ± 4.95		0.908**	0.002
Topsin M/ Lemon	10 - 1000	dg4	0.0 – 19.0 ± 1.44		0.959**	0.000
Ranvil/ Orange	10 - 1000	dg4	4.0 ± 1.41 – 9.5 ± 2.61		0.962**	0.000
Ranvil/ Lemon	10 - 1000	dg4	0.0 – 14 ± 4.24		0.969**	0.000
Vydan/ Orange	10 - 1000	dg4	0.0 – 15 ± 1.42		0.950**	0.000
Vydan/ Lemon	10 -1000	dg4	0.0 – 20.5 ± 2.70		0.987**	0.000
Canvil/ Orange	10 - 1000	dg4	4.5 ± 1.76 – 14.0 ± 2.83		0.964**	0.000
Canvil/ Lemon	10 - 1000	dg4	0.0 – 19.5 ± 2.12		0.959**	0.000
Blin exa/ Orange	10 - 1000	dg4	4.0 ± 1.41 – 27.5 ± 3.53		0.820*	0.013
Blin exa/ Lemon	10 - 1000	dg4	0.0 -57.0 ± 6.81		0.827*	0.011

† Range of used concentrations of fungicides was: 10, 25, 50, 100, 200, 300, 500 and 1000 μg mL $^{-1}$; ‡ Ci denotes for complete inhibition of fungal growth. * Correlation is significant at the 0.01 level of significance (2-tailed);
**: Correlation is significant at the 0.05 level of significance (2-tailed).

Topsin/Vydan mixture (P values were within the range of P=0.000 to P=0.039) on size of inhibition zones of the four strains (except strain dg5 infecting lemon fruits; P values were within the range of P=0.193 to P=0.693) infecting both lemon (Fig. 1a) and orange fruits. Synergistic effects against zones of strain dg2 infecting orange fruits (inhibition zone = 32 mm. equivalent to 19% CSA) and strain dg4 infecting both orange (inhibition zone = 53 mm. equivalent to 56% CSA) and lemon (56 mm; equivalent to 85% CSA) fruits were obtained. However, additive effects against zones of strain dg2 infecting lemon fruits (Maximum zones of 46 mm; equivalent to 84% CSA) were obtained. In contrast, antagonistic effects were seen against zones of strain dg6 infecting orange fruits, (maximum zone was 30 mm as compared to complete inhibition with the singly tested fungicides (Table 1).

3.3.4. Effect of Vydan/Canvil mixture

Table (2). Indicates that no significant difference between the combination of 100:500 μg. mL^{-1} and 500:1000 μg. mL^{-1} on inhibition zone of strains dg4; dg5 and dg6 infecting orange fruits (Fig. 2b and d). The obtained P-values were 1.000; 0.693; and 1.0, respectively. Furthermore, synergistic effects were obtained against zone of strains dg4 infecting orange fruits (complete inhibition was obtained at a combined concentration of 100:500 μg. mL^{-1}) and strain dg5 infecting both fruit types (47.4% CSA on lemon as compared to inhibition zones in the range of 0.0 – 8.5 mm. with the singly used fungicides). Also, synergistic effects were obtained against zones of strain dg6 infecting lemon (46.48% CSA as compared to 0.0 mm. inhibition zone with Vydan fruits). However, additive effects against zones of strain dg6 infecting orange fruits were obtained, where complete inhibition was achieved with the combined concentration of 100:500 μg. mL^{-1} (Table 2).

3.3.5. Effect of Topsin/Blin exa mixture

Topsin/Blin mixture recorded synergistic effect against zones of strains dg2, dg4, dg5 and dg6, (Table 2) where complete inhibition at a combined concentration of 100:500 μg. mL^{-1} was obtained against strains dg2 (infecting orange and lemon fruits), dg4 (infecting lemon) and dg6 (infecting both fruit types). However, such mixture has caused complete inhibition to strain dg4 (infecting orange), at all tested concentrations (Table 2). There was no significant difference Between the combination of 100:500 and 500: 1000 μg. mL^{-1} against strains dg2; dg4 and dg6 infecting both fruit types (Fig. 2a).

3.3.6. Effect of blin exa/vydan mixture

This mixture of fungicides resulted in synergistic effect against zones of strains dg2, dg4; and dg6 infecting both orange and lemon fruits, where, complete inhibition of fungal growth was generated with the combination of 100:500 μg. mL^{-1} on orange and lemon fruits, except strain dg2, where an inhibition zone of 16 mm. (9% CSA) was obtained (Table 2). However, there were significant differences among the combined concentrations (P values within the range of P=0.000 to P=0.039) on zones of the four strains, infecting both fruit types (Fig. 2c) with exception of the combinations of 100:500 μg. mL^{-1} and 500:1000 (P values have ranged from 0.693 to 1.000).

3.3.7. Effect of Blin exa/Canvil mixture

Results indicate that synergistic effects were obtained against zones of the four strains, where the growth of strains dg2 and dg6 on both fruit types was completely inhibited at the combined concentration of 100:500 μg. mL^{-1} (Table 2). However, the combination of 500:1000 μg. mL^{-1} resulted in complete inhibition to strain dg4 infecting both fruit types (Table 2), and strain dg5 infecting orange fruits, whereas, the growth of the same strain on lemon fruits was completely inhibited at a concentration of 100:500 μg. mL^{-1} (Table 2). Moreover, results indicate that there was no significant difference between combined concentration of 100:500 μg. mL^{-1} and 500:1000 μg. mL^{-1} on zones of strains: dg2 (P=1.000) infecting both fruit types; dg5 infecting lemon (P=1.000) and dg6.

3.3.8. Correlation between fungicides mixture and size of inhibition zones of *P. digitatum* strains

Results of regression analysis indicate significant correlation (at the 0.01 level: 2-tailed) between applied combined concentrations of: Topsin/Vydan; Vydan/Canvil; Blin exa/Vydan; and Blin exa/Canvil mixtures of fungicides and inhibition zones of the four tested strains, whether such strains grown on lemon or orange fruits. Furthermore, the applied combined concentrations of Benomyl/Ranvil mixture showed significant correlation (at the 0.01 level: 2-tailed) with zones of strains: dg4 infecting lemon fruits; dg5 infecting both citrus fruit types; and dg6 infecting lemon fruits.

Table (2) :Percentage of cleared (spore-free) citrus fruit (orange and lemon) surface area as an effect of different combined concentrations of fungicides mixtures.

Fungicides mixture	Combined. conc ($\mu g\ mL^{-1}$)[a]	orange				Lemon				
		IZ[b] (cm)	IZA[c] (cm^2)	SA[d] (cm^2)	CSA[e] (%)	IZ (cm)	IZA (cm^2)	SA (cm^2)	CSA (%)	Fungal strain
Benomyl/ Ranvil	1; 2; 3; 4	Ci[f]		216.4	100	Ci		119.43	100	dg2
	1	Ci		178.29	100	1	3.14	121.23	2.6	dg4
	2	Ci			100	1.4	6.15		5.08	
	3	Ci			100	1.4	6.15		5.08	
	4	Ci			100	1.5	7.07		5.83	
	1	0.5	0.79	187.86	0.42	1.4	6.15	89.2	6.9	dg5
	2	0.9	2.54		1.35	2.5	19.63		22.00	
	3; 4	Ci			100	Ci			100	
	1	Ci		184.62	100	2.4	18.09	88.23	20.50	dg6
	2	Ci			100	2.5	19.63		22.24	
	3	Ci			100	3	28.26		32.03	
	4	Ci			100	5	78.50		89.97	
Topsin/ Canvil	1	1.1	0.95	161.32	0.59	1.5	7.07	84.71	8.34	dg2
	2	1.5	1.77		1.10	2	12.56		14.83	
	3	1.5	1.77		1.10	2	12.56		14.83	
	4	1.8	2.55		1.58	3	28.26		33.36	
	1	1.8	2.54	55.59	4.58	0.9	2.54	53.47	4.76	dg4
	2	2.1	3.46		6.23	1.7	9.08		16.97	
	3	2.7	5.73		10.29	2.3	16.61		31.07	
	4	3.6	10.18		18.30	3.1	30.18		56.43	
	1	0.5	0.20	53.59	0.37	1.5	7.07	25.22	28.01	dg5
	2	1.3	1.33		2.48	1.8	10.17		40.34	
	3	1.9	2.83		5.29	2.3	16.61		65.86	
	4	2.3	4.15		7.75	2.6	21.23		84.17	
	1	Ci		204.42	100	1.3	5.31	100.84	5.26	dg6
	2	Ci			100	1.6	8.04		7.97	
	3	Ci			100	2.2	15.20		15.07	
	4	Ci			100	2.5	19.63		19.46	
Blin/ Vydan	1	0.9	0.64	207.8	0.31	0.8	2.01	88.12	2.28	dg2
	2	1.3	1.33		0.64	1.2	4.52		5.12	
	3	Ci			100	1.5	7.07		8.01	
	4	Ci			100	1.6	8.04		9.11	
	1	1.8	2.54	42.23	6.03	2.8	24.62	63.01	39.07	dg4
	2	2.4	4.52		10.71	3.3	34.19		54.27	
	3; 4	Ci			100	Ci			100	
	1	1.8	2.54	62.67	4.06	0.4	0.503	25.22	1.99	dg5
	2	2.1	3.46		5.53	1.1	3.80		15.07	
	3; 4	Ci			100	Ci			100	
	1	0.6	0.28	158.35	0.18	1.0	3.14	98.39	3.19	dg6
	2	0.8	0.50		0.32	1.1	3.80		3.86	
	3	1.0	0.79		0.50	1.4	6.15		6.26	
	4	1.1	0.95		0.60	1.5	7.07		7.18	
Topsin/ Blin	1	1.3	1.33	39.20	3.38	2.8	24.62	77.39	31.81	dg2
	2	1.9	2.83		7.23	3.7	42.99		55.55	
	3; 4	Ci			100	Ci			100	
	1	Ci		204.44	100	1.8	10.18	156.57	6.50	dg4
	2	Ci			100	2.0	12.56		8.02	
	3; 4	Ci			100	Ci			100	
	1	1.5	1.77	99.20	1.78	1.9	11.34	88.26	12.84	dg5
	2	2.1	3.46		3.49	2.6	21.23		24.05	
	3	3.3	8.55		8.62	3.6	40.69		46.11	
	4	5.2	21.23		21.40	4.7	69.36		78.59	
	1	3.2	8.04	42.58	18.89	1.3	5.31	22.33	23.76	dg6
	2	4.1	13.20		30.99	2.4	18.09		81.0	
	3; 4	Ci			100	Ci			100	

(Continued)

Table (2): Continued.

Fungicides mixture	Combined. conc (μg mL $^{-1}$)[a]	orange				Lemon				
		IZ[b] (cm)	IZA[c] (cm^2)	SA[d] (cm^2)	CSA[e] (%)	IZ (cm)	IZA (cm^2)	SA (cm^2)	CSA (%)	Fungal strain
Vydan/ Canvil	1	1.1	0.95	36.67	2.59	0.7	1.54	24.04	6.40	dg2
	2	1.9	2.84		7.73	1.3	5.31		22.07	
	3	2.1	3.46		9.45	1.8	10.17		42.32	
	4	2.6	5.31		14.47	2.1	13.85		57.60	
	1	0.3	0.071	191.12	0.037	0.7	1.54	68.38	2.25	dg4
Vydan/ Canvil	2	0.4	0.13		0.066	0.8	2.01		2.94	
	3	Ci			100	1.0	3.14		4.59	
	4	Ci			100	1.3	5.31		7.76	
	1	1.4	1.54	55.59	2.77	0.7	1.54	55.75	2.76	dg5
	2	1.8	2.54		4.58	1.2	4.52		8.11	
	3	3.3	8.55		15.38	2.3	16.61		29.79	
	4	3.4	9.07		16.32	2.9	26.41		47.37	
	1	2.3	4.15	51.08	8.13	0.3	0.28	21.89	1.29	dg6
	2	3.4	9.07		17.77	0.9	2.54		11.62	
	3	Ci			100	1.3	5.31		24.24	
	4	Ci			100	1.8	10.17		46.48	
Topsin/ Vydan	1	0.3	0.071	41.45	0.17	1.2	4.52	79.57	5.68	dg2
	2	0.8	0.50		1.21	1.9	11.34		14.25	
	3	1.7	2.27		5.47	3.7	42.99		54.02	
	4	3.2	8.04		19.39	4.6	66.44		83.50	
	1	0.7	0.38	39.19	0.98	1.4	6.15	119.57	5.15	dg4
	2	1.9	2.83		7.23	3.1	30.18		25.24	
	3	3.2	8.04		20.51	4.2	55.39		46.32	
	4	5.3	22.05		56.27	5.6	100		85.32	
	1	0.9	0.64	48.58	1.31	1.6	8.04	58.07	13.84	dg5
	2	1.2	1.13		2.33	1.7	9.07		15.63	
	3	1.8	2.54		5.24	1.7	9.07		15.63	
	4	2.1	3.46		7.13	1.8	10.17		17.52	
	1	1.5	1.77	178.27	0.99	1.1	3.8	58.08	6.54	dg6
	2	1.8	2.54		1.43	1.6	8.04		13.84	
	3	2.0	3.14		1.76	2.1	13.85		23.84	
	4	3.0	7.07		3.96	3.2	32.15		55.36	
Blin/ Canvil	1	2.3	4.15	48.53	8.56	2.4	18.09	57.39	31.51	dg2
	2	2.9	6.60		13.60	3.7	42.99		74.90	
	3; 4	Ci			100	Ci			100	
	1	1.5	1.77	39.19	4.51	2.1	13.85	93.52	14.81	dg4
	2	1.9	2.83		7.23	3.2	32.15		34.38	
	3	3.8	11.34		28.92	5.3	88.20		94.31	
	4	Ci			100	Ci			100	
	1	0.9	0.64	88.23	0.72	1.0	3.14	39.66	7.92	dg5
	2	1.3	1.33		1.50	1.1	3.8		9.58	
	3	2.8	6.15		6.98	Ci			100	
	4	Ci			100	Ci			100	
	1	0.4	0.13	184.62	0.07	0.6	1.13	57.64	1.96	dg6
	2	0.5	0.20		0.11	1.4	6.15		10.68	
	3	Ci			100	3.3	34.19		59.32	
	4	Ci			100	Ci			100	

[a]- combined conc, 1: denotes combined concentrations of 50:50 μg mL $^{-1}$; 2: denotes 100:100; 3: denotes 100:500; 4: denotes 500:1000 μg mL $^{-1}$. [b] IZ –denotes inhibition zone (cm). [c] IZA-denotes inhibition zone area (cm2). [d] SA-denotes fruit's surface area (cm2). [e] %CSA-denotes % of cleared fruit's surface area (spores-free). [f] Ci-denotes complete inhibition of fungal growth.

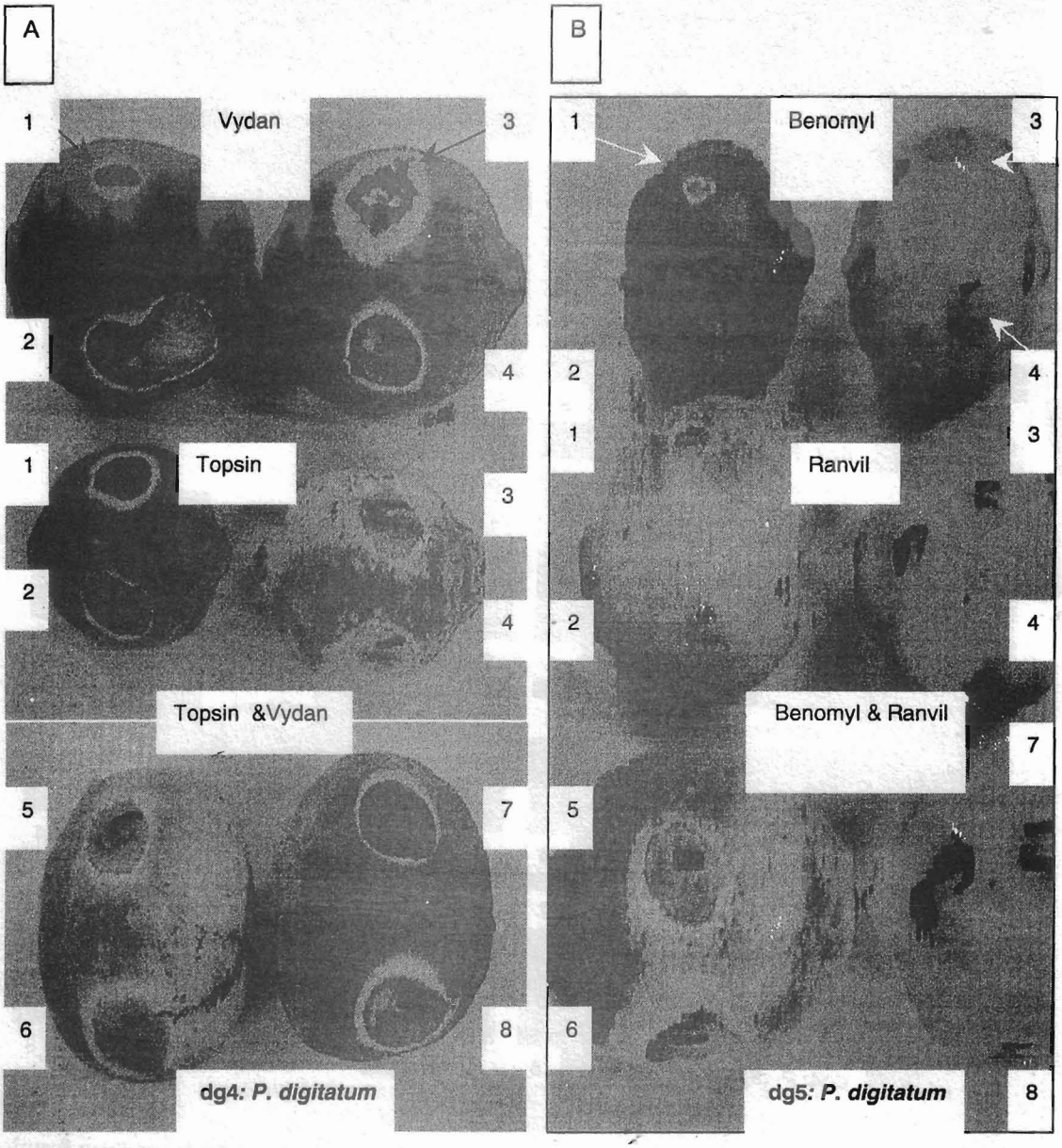

Fig. (1): *In vivo* sensitivity of two *P. digitatum* strains infecting lemon fruits to two fungicides and their combinations. Panel a: Inhibition zones generated by strain dg4 as the effect of treatment by Vydan; Topsin and a combination of both. Panel b: Effect of Benomyl; Ranvil; and a combination of both on growth of strain dg5 of *P. digitatum*.1: denotes for 50 µg mL $^{-1}$; 2: 100 µg mL $^{-1}$; 3: 500 µg mL $^{-1}$; 4: 1000 µg mL $^{-1}$; 5; 6; 7; 8: denote for combined concentrations of 50:50; 100:100; 100:500; and 500:1000 µg mL $^{-1}$from both fungicides respectively. Note complete inhibition to strain dg5 growth was obtained at concentrations of 500 and 1000 µg mL $^{-1}$of Benomyl; and Ranvil. Also, a mixture of both generated complete inhibition for the same strain at combined concentrations of 100:500 and 500:1000 µg mL $^{-1}$.

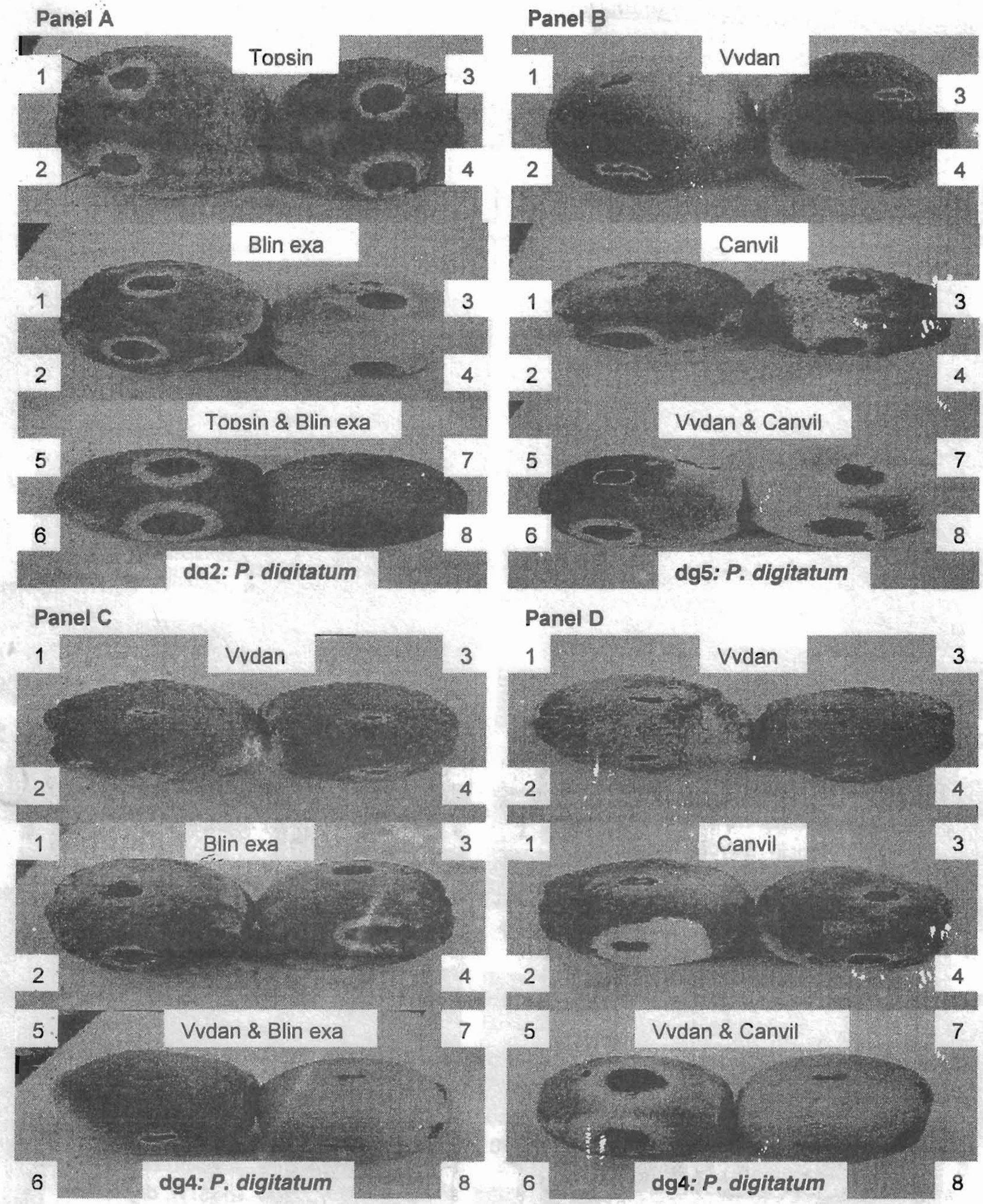

Fig. (2): ***In vivo*** **sensitivity of three** ***P. digitatum*** **strains infecting orange fruits to four fungicides and their combinations. Panel a: Inhibition zones generated by strain dg2 as an effect of treatment by Topsin; Blin exa and a combination of both. Panel b: Effect of Vydan; Canvil; and a combination of both on growth of strain dg5. Panel c: Effect of Vydan; Blin exa and a combination of both on growth of strain dg4. Panel d: Effect of Vydan; Canvil and a combination of both on growth of strain dg4. Number 1: denotes for 50 µg mL $^{-1}$; 2: 100 µg mL $^{-1}$; 3: 500 µg mL $^{-1}$; 4: 1000 µg mL $^{-1}$; 5; 6; 7; 8: denote for combined concentrations of 50:50; 100:100; 100:500; and 500:1000 µg mL $^{-1}$from both fungicides respectively. Complete inhibition of strains dg2 and dg4 growth was obtained at combined concentrations of 100:500 and 500:1000 µg mL $^{-1}$of Topsin & Blin; Vydan & Blin; and Vydan & Canvil.**

4. DISCUSSION

The recurrent multiplicity of the same active ingredient will lead to a major commercial problem of fungal resistance to such fungicides, which reflected serious difficulties in disease control (Cunningham, 2005 and Surviliene and Dambrauskiene, 2006). In this work, Ranvil (a DMI member) showed high efficacy in controlling the infection by *P. digitatum* strains (gives complete inhibition or the largest zones after Benomyl) than Canvil; Blin exa and Vydan although, all have the same active component (hexaconazole) but produced by different national and international companies under different trade marks. Furthermore, Vydan has shown to be the least effective, especially when strains infect lemon rather than orange fruits. These findings agreed with the results of *in vitro* study (Kanan, 2008) which revealed that Ranvil and Benomyl were the most effective against tested strains, whereas, Canvil was the least. However, these results disagreed with the findings of Lam and Lim (1993) and with that of Savocchia and his co-workers (2004) who stated that Vydan has shown an excellent control of white rust on Chrysanthemum and powdery mildew on roses and grapevines. The triazole fungicides are sterol demethylation inhibitors (DMI) that inhibit the enzyme, C14-demethylase, leading to depletion of ergosterol which serves as a bioregulators for membrane fluidity and integrity in fungal cells resulting in alteration in their cell walls (Ma *et al.*, 2006 and Sugiura *et al.*, 2006). Possible mechanisms leading to DMI resistance include mutations in the DMI target enzyme C14-alpha-demethylase (CYP51) which lead to decrease affinity of DMI to target protein (De'lye *et al.*, 1997). Resistance to demethylation inhibitors (DMI) in biotypes of *Penicillium* species is thought to be controlled by a polygenic system (Van Tuyl, 1977 and Kalamarakis *et al.*, 1987) where the development of resistance would appear due to cumulative or additive effects of mutations in several minor genes (De Waard *et al.*, 1982 and Georgopoulos and Skylakakis, 1986). In this kind of resistance (*i.e.* quantitative or continuous resistance) pathogens exhibit a range of sensitivity to the fungicide, depending on the type and number of altered genes where, variations in sensitivity within the population is continuous, and selection occurs in a directional manner. Resistance in this case is seen as erosion of disease control that can be regained by either applying higher concentrations of the fungicide, or by more frequent use of the fungicide. However, the strain could be revert back to be sensitive if the fungicide is no longer used. Other mechanisms include over-expression of ATP binding cassette (ABC) transporters encoding efflux pumps that effectively pump toxic chemicals out of the cell and here the strain may gain resistance to several fungicides using the same mechanism (Hayashi *et al.*, 2002 and Zwiers *et al.*, 2002). Furthermore, the deposition of fungicide in lipid droplets and change in pH leading to protonation of fungicide is possible mechanism of resistance (McGrath, 2001). Obtained results showed that the benzimidazole systemic fungicide Benomyl (Benlate) was the most effective (mostly generates complete inhibition or the largest zones) in terms of restraining fungal growth. In contrast, the fungicide Topsin (TBZ member as benomyl) did not show complete hyphal growth inhibition, at a range of concentrations from 10^{-1} to 1000 $\mu g.mL^{-1}$, with the four tested strains infecting both fruit types (except strain dg6 infecting orange fruits). These findings disagreed with the *in vitro* findings of Kanan (2008) also with that of Zamin and his co-workers (1999) who indicated that the systemic fungicide Topsin M has controlled several fungal diseases including powdery mildew, downy mildew which infect grapes and wheat leaf brown rust. Concerning Benomyl mode of action it is firstly transformed into methyl-2-benzimidazole carbamate metabolites, (carbendazim) that causes morphological distortion of germinating spores (Tsuda *et al.*, 2004). This fungicide binds to microtubules inhibiting β-tubulin assembly and interferes with cell division (Dalgie, 2005). Furthermore, Benomyl shows selective toxicity to several microorganisms including fungi and interferes with intracellular transportation causing loss of membrane transport ability (Amar and Reinhold, 1973). Resistance to benzimidazole fungicides is thought to be a kind of qualitative type since it is resulted from modification of a single major gene, where pathogens became either resistant or sensitive to the fungicide and here disruptive selection occurs (Mc Grath, 2001). Resistance in this case is seen as complete loss of disease control, which resulted in conformational changes at the target site that can not be regained by using higher concentrations or more frequent fungicides application. This type of mutations resulted in high selection pressure during fungicides application and there is low selection pressure to remove them in absence of fungicide (Van Tuyl, 1977and Mc Grath, 2001). Concerning the effect of fungicides mixture, when Benomyl and Topsin

M were mixed with another fungicide from the DMI group, more controllable effects were obtained. These findings agreed with the *in vitro* study (Kanan, 2008) which indicated that inhibition zones of tested strains were significantly affected by fungicides mixture and complete inhibition was mostly obtained, especially when either Benomyl or Topsin M was mixed with a DMI member. These results agreed also with the suggestions of Shaw (1993) who stated that studies of resistance development revealed that the combination of two selective fungicides to combat resistance is reasonable strategy, only when used against wild population of the pathogen with an extremely low frequency of resistance to both fungicides. In addition, the sequential use of two unrelated fungicides may be more effective strategy, because, the application of fungicides mixture showing the same mode of action would lead to resistance as a result of positive cross resistance. However, when resistance to one chemical class lead to increase sensitivity to the other, this result is negative cross resistance. The mechanisms that underlie the development of fungicide resistance, have mainly proved to be some kind of modification of the bio-chemical target site in the pathogen, which render the site more sensitive to damage by combined fungicides. This suggestion agreed with the findings of Mc Grath (2001) also with that of Surviliené and Dambrauskiené (2006).

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حساسية سلالات الفطر (*Penicillium digitatum*) الممرضة لثما ر الحمضيات لستة أنواع من المبيدات الفطريه ومزيجها الثنائي

غسان كنعان

قسم العلوم البيولوجية – جامعة مؤتة – الكرك – الاردن

ملخص

تهدف هذه الدراسة الى تقييم فاعلية ستة أنواع من المبيدات الفطرية بالإضافة إلى إختبار مزيج ثنائي من هذه المبيدات لتثبيط نمو أربع سلالات من فطر بنسيليوم ديجيتاتم المسبب لمرض العفن الأخضر بعد حقنها في ثمار البرتقال و الليمون. كما تم إستخدام عدة تحاليل إحصائية لبيان مدى الإرتباط والفروق المعنوية بين المعاملات. أظهرت النتائج أن جميع المبيدات (بإستثناء المبيد بلين) أدت إلى تثبيط نمو السلالة dg6 المحقونة في ثمار البرتقال بشكل كامل. أدى المبيد بينوميل الى تثبيط كامل لنمو السلالات dg6 و dg5 و dg4 المحقونة في ثمار الليمون. أدى المبيد رانفيل الى تثبيط نمو السلالات dg6 وdg5 المحقونة في ثمار الليمون (بلغت قيم IC_{50} ١٤٤ و ١٣٨ ميكروجرام/مل على التوالي). أظهر المزيج من بينوميل/رانفيل فاعلية تعاونية عالية ضد السلالة dg2 المحقونة في ثمار الليمون والبرتقال والسلالتين dg4, dg5 المحقونتان في ثمار البرتقال حيث أدى ذلك إلى تثبيط كامل للنمو. أدى التركيز المشترك ١٠٠:٥٠٠ ميكروجرام/مل من خليطي توبسين/بلين وكذلك بلين/كانفيل إلى فاعلية تعاونية مما سبب تثبيط كامل لنمو جميع السلالات المحقونة في الثمار قيد الدراسة.

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