IN VITRO DIFFERENTIAL SENSITIVITY OF *PENCILLIUM ITALICUM* CAUSING POSTHARVEST ROT TO CITRUS FRUITS IN JORDAN TO CHEMICAL FUNGICIDES AND THEIR COMBINATIONS

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Abstract: In this study we evaluated "*in vitro*" the efficacy of six chemical fungicides and their mixtures as a strategy for the control of *Penicillium italicum*. The antifungal efficacy against four *P. italicum* isolates of thirty-one concentrations (0.01–3000 μg/ml) of each of the tested fungicides (Vydan, Blin exa, Canvil, Ranvil, Benomyl and Topsin M), in addition to six combined concentrations from each of ten fungicide mixtures were tested using agar well diffusion method. Regression analysis, one way ANOVA, and Post Hoc Multiple comparisons were carried out to test the significance of these treatments. Our results showed that benomyl completely inhibited the growth of tested isolates (Pi.1; Pi.3; Pi.5; and Pi.6) with MIC values of: 1000; 300; 150 and 40 μg/ml respectively. Canvil as compared to Blin exa, Ranvil and Vydan (no complete inhibition) showed high efficacy against isolates Pi.1 and Pi.5 (MIC values of 5 and 25 μg/ml respectively). The mixtures of Blin exa/Vydan and Topsin M/Canvil were the only mixtures that generated synergistic effects against tested isolates at all tested concentrations. The above mentioned mixtures showed at the first four tested combined concentrations (50:50, 100:100, 100:500 and 500:1000 μg/ml) either the largest inhibition zones (in the range of 47±1.40 mm to 51±1.49 mm) or complete inhibition of fungal growth at the last two (1000:1000 and 1000:2000 μg/ml) combined concentrations. The mixtures of Blin exa/Canvil and Blin exa/Ranvil as compared to the effect of each as singles showed additive effects against tested isolates. The mixtures of Benomyl/Vydan; Benomyl/Ranvil; Benomyl/Blin exa; Topsin/Vydan and Topsin/Blin exa (i.e. mixture of benzimidazole and DMI members) all generated antagonistic effects against tested isolates.

Key words: *Penicillium italicum*. citrus postharvest disease. chemical fungicides

INTRODUCTION

Citrus production has reached over 102 million tones and probably stands first largest among the fruit crop (Ismail and Zhang 2004). Citrus fruits are susceptible to a number of decay causing organisms (Bouzerda *et al*. 2003). Green and blue mould rots caused by *Penicillium digitatum* (Pers: Fr.) Sacc and *P. italicum* Wehmer respectively, are probably the most common post-harvest diseases affecting citrus fruits (Plaza *et al*. 2003; Samson *et al*. 2004). Blue mould is most prevalent in cold storage and it is able to germinate at cold temperatures even at 0°C (Palou *et al*. 2001; Plaza *et al*. 2004). The control of postharvest fungal decay of citrus fruits relies heavily on the massive use of chemical fungicides in order to maintain healthy crops and reliable yields of high quality product (Barkai-Golan 2001; Pramila and Dubey 2004). Benzimidazole [Thiabendazole (TBZ) and Benomyl] and Imadazole [Imazalil (IMZ)] groups of fungicides are applied to control post-harvest citrus rots in which their world-wide application has reached an approximate of 26% of the plant protection market in Europe and Asia and 6% in USA (Lopez-Garcia *et al*. 2003; Valiuskaite *et al*. 2006). In most of California packing houses, citrus fruits are treated with imazalil [a member of the sterol demethylation inhibitors (DMI) group of fungicides**]**, sodium *O*-phenyl phenate (*O*-phenyl phenol), and thiabendazole to control *Penicillium* decay (Plaza *et al*. 2003; Pramila and Dubey 2004**).** Most of the DMI group of fungicides including the systemic broad spectrum foliar fungicide Bayfidan Turf (Vydan) are derivatives of imadazoles or triazoles (Gopi *et al*. 2005; Ma *et al*. 2006). Hexaconazole (a new member of triazole fungicides) is a broad-spectrum DMI systemic fungicide, with eradicant and protectant activity against a wide range of plant pathogens, including members of Ascomycota and Basidiomycota (Sijaona and Mansfield 2001; Savocchia *et al*. 2004; Sugiura *et al*. 2006). Overseas, postharvest application of Benomyl (Benlate) and thiabendazole (benzimidazole derivatives) fungicides indicated that such fungicides are of the most effective ones against *Penicillium* mould that affect citrus fruits (Tsuda *et al*. 2004; Dalgie 2005; Valiuskaite *et al*. 2006). The benzimidazole fungicide Topsin M, (Thiophanate-methyl) is considered as an ultimate broad-spectrum systemic fungicide that controls several fungal diseases, such as powdery mildew, downy mildew, brown rust, smut and rust of wheat (Zamin *et al*. 1999; Siddiqui 1999).

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The recurrent multiplicity of the same active ingredient and the prolonged repeated storage of treated fruits in a facility would lead to serious commercial problems of fungal resistance to such fungicides and this reflects serious difficulties in disease control (McGrath 2001; Surviliene and Dambrauskiene 2006). The current investigation has aimed to overcome these threats through evaluating six fungicides (four of which are produced by national companies and two international ones) Also, to evaluate the efficacy of fungicides combination strategies in controlling the growth of the blue mold caused by *P. italicum* hoping in reaching new decay control options.

MATERIALS AND METHODS

***Penicillium italicum* isolates**

Conidiospores of four (Pi.1; Pi.3; Pi.5; and Pi.6) *P. italicum* isolates were obtained from three types of spoiled citrus fruits and these include: orange fruits *(Citrus sinensis* L.), lemon (*Citrus limon* L.), and calamondin (*Citrus mitis* L.). The spoiled fruits were collected from distributors in three Jordanian cities: Amman the capital, Irbid in the northern part and Al-Karak in the southern part. This study was conducted during the year 2006 in laboratories of Biological Sciences Department, at Mu'tah University – Jordan.

Media used

The standard *Aspergillus nidulans* complete (CM) medium described previously by Cove (1966) was used (gave maximum zone of growth as compared to potato dextrose agar (PDA) media) with slight modification (i.e. pH 6 and supplemented with 10 mM ammonium, and 10 g/l glucose as C-source).

Purification of fungal isolates

Single inoculum from each tested isolate was grown for 7 days at 25ºC on CM plates to confirm their purity and identity. Conidiospores were suspended in 5 ml sterile normal Saline/Tween 80 (0.05%) solution and used at a concentration of $1x10^8$ spores per milliliter. Aliquot of 100 µl from a dilution of 10^{-6} or 10^{-7} were plated again on complete media (CM) in order to achieve single pure colony as a source of pure culture for further studies (Zhang *et al*. 2004).

Optimal growth conditions of tested fungal isolates

Nine replications (for each tested condition) of conidiospores' suspension (20 µl) from each tested isolate were inoculated into complete media, having different pH regimes (i.e. 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9) for optimal pH testing. The following *N*-sources were used at a concentration of 10 mM each: Urea; *L*-proline, *L*-lysine, *L*-arginine, *L*-adenine, *L*-glutamine, NH_4^+, NO_3^-, and *L*-histidine) for best serving nitrogen source at optimal pH of 6.0. In order to determine the optimal temperature of growth plates of complete media adjusted to optimal pH of 6.0, and supplemented with 10 mM proline as best serving *N*-source were incubated at five temperature regimes (i.e. 10°C, 20°C, 25°C, 30°C, 37°C). Also, various *C*-sources (glucose, sucrose, sorbitol, fructose, and maltose) were tested at a final concentration of 10 g/l to determine the best serving *C*-source. Each group of nine replications was incubated for 5 days, at 20°C or at the tested temperature, then the radius of each growing colony was measured in two directions at right angles to each other.

Tested fungicides

Six chemical fungicides were tested and these are: (i) Bayfidan Turf – 25% EC (Vydan), containing 25% (w/v) triadimenol as an active gradient with the chemical formula $C_{14}H_{18}ClN_3O_3$ and produced by Vapco company – Jordan (II) Blin exa – 5% Sc, containing hexaconazole 5% (w/v) as an active gradient and produced by IQV–Spain (III) Canvil – 5% which contains hexaconazole 5% (w/v) as an active gradient and produced by Vapco – Jordan. (IV) Ranvil – 5 Sc, which also contains hexaconazole 5% (w/v) as an active gradient and produced by Chem.Vet – Jordan, where the latter three fungicides have the same chemical formula $C_{14}H_{17}Cl_2N_3O$ (V) Benlate – 50% W.P, containing Benomyl 50 (w/w) with the formula $C_{14}H_{18}N_4O_3$ and produced by Vapco – Jordan. (VI) Topsin M – 70% WP, containing methyl thiophenate 70% (w/w), having the formula $C_{12}H_{14}N_4O_4S_2$ and produced by Nippon soda – Japan.

Application of fungicides– non–amended medium

Aliquot of 100 µl spores suspension (approx: 10^8 spores/ml) of each tested isolate was cultured by streaking in radial patterns on the surface of 9 cm in diameter sterile plastic petridishes containing the above mentioned complete medium. The minimum inhibitory concentration (MIC) of each tested fungicide was determined using the agar well diffusion method (Nostro *et al*. 2000; Bhattacharjee *et al*. 2006; Ogundare *et al*. 2006; Ndukwe *et al*. 2006). Wells of 6 mm in diameter were performed (3 wells/plate/concentration) then a range of concentrations from 0.01 to 3000 µg/ml (0.01; 0.05; 0.1; 0.5; 1; 2; 5; 10; 15; 20; 25; 30; 35; 40; 50; 100; 150; 200; 250; 300; 350; 400; 450; 500; 750; 1000; 1500; 2000; 2400; 2700; 3000 µg/ml) from each of the six tested fungicides (3 plates and each with 3 wells were used for each fungicide concentration) were used against each tested isolate**.** The plates were incubated at 20°C for 5 days before measuring the radius for the zone of inhibition around each well. Each experiment was repeated three times for conformation of results. Control experiments were carried out along with each treatment where sterile distilled water was loaded into wells instead of fungicide.

Application of fungicides' combinations

The procedure described above for *in vitro* application of single fungicide treatment was followed here using 6 different combined concentrations (50:50; 100:100; 100:500; 500:1000; 1000:1000; 1000:2000 µg/ml) with each pair-wise mixture of fungicides. Ten different fungicide mixtures were used against four tested fungal isolates, and these mixtures are: Benomyl/Ranvil; TopsinM/Canvil; Blin exa/Vydan; TopsinM/Blin exa; Vydan/Canvil; TopsinM/Vydan; Blin exa/Canvil; Benomyl/Blin exa; Ranvil/Blin exa and Benomyl/Vydan. However, each combined concentration of a particular fungicide mixture was loaded into the same well, where 3 plates and each with 3 wells were used for each combination.

Statistical analysis

The concentration of fungicide producing 50% growth inhibition (IC_{50}) and the minimum inhibitory concentration (MIC) of fungicide, or fungicides' combination was calculated by regression analysis for the relationship between the size of inhibition zone (mm) and the fungicide concentration (Log value). The Microsoft Excel 2003 and the SPSS program version 10 were used in such analysis. One way ANOVA was carried out to determine the significant effect of ten fungicides mixtures on sizes of inhibition zones of studied *P. italicum* isolates. This was followed by Post Hoc multiple comparisons to determine the significance level of applied combined concentrations of fungicides' mixtures and their interactions on sizes of inhibition zones for the studied isolates of *P. italicum*.

RESULTS

1. Optimal growth conditions for tested fungal isolates

The slight modification for the used complete media (gave larger zones of fungal growth as compared to PDA media) has required optimization for all growth condition including: pH, temperature, N-source and C-source. The obtained results indicated that glucose, was served as the best sole source of carbon, for the four (Pi.1; Pi.3; Pi.5; and Pi.6) tested *P. italicum* isolates under two temperature regimes (i.e. 25°C and 20°C). The generated growth zones at the indicated temperatures were 18.8±0.43 mm and 22.8±0.19 mm respectively. Furthermore, lysine and ammonium were served as the best sole sources of nitrogen when cultures were incubated at 25°C and 20°C respectively. The obtained zones of growth at these temperatures reached 24.6±3.6 mm and 32.1±1.7 mm respectively. The optimal pH (within the range of pH 5 to pH 9) for growth of isolates at either 25°C or 20°C was pH 6, where the zones of fungal growth have reached a mean value of 21.7±2.8 mm at 25°C and 20.5±2.4 mm at 20°C.

2. Sensitivity of *P. italicum* isolates to six chemical fungicides

Results of regression analysis showed that there was a significant correlation (at the 0.01 level – 2-tailed) between the fungicide's concentration (μg/ml) and the size of inhibition zone (mm) for all tested isolates of *P. italicum* (Table 1). The fungicide Benomyl as compared with the rest of tested fungicides has shown the highest efficacy, where complete inhibition was achieved for the four (Pi.1; Pi.3; Pi.5; and Pi.6) tested isolates (Table 1, Fig. 1). The obtained MIC values of Benomyl against the above mentioned isolates were: 1000, 300, 150, and 40 μg/ml respectively. In contrast, the fungicides: Ranvil, Blin exa (except for isolate Pi1; IC_{50} = 205), Vydan (except for isolate Pi6; IC_{50} = 418) and Topsin M (benzimidazole member) did not lead to complete inhibition of hyphal growth at a range of concentrations from 0.01 to 3000 μg/ml with all tested isolates of *P. italicum* (Table 1). However, isolates Pi.1 and PI.5 were the most sensitive to the fungicide Canvil in particular and to all tested fungicides in general. The obtained IC_{50} values for the above mentioned isolates were 2.75 μg/ml and 21.5 μg/ml respectively.

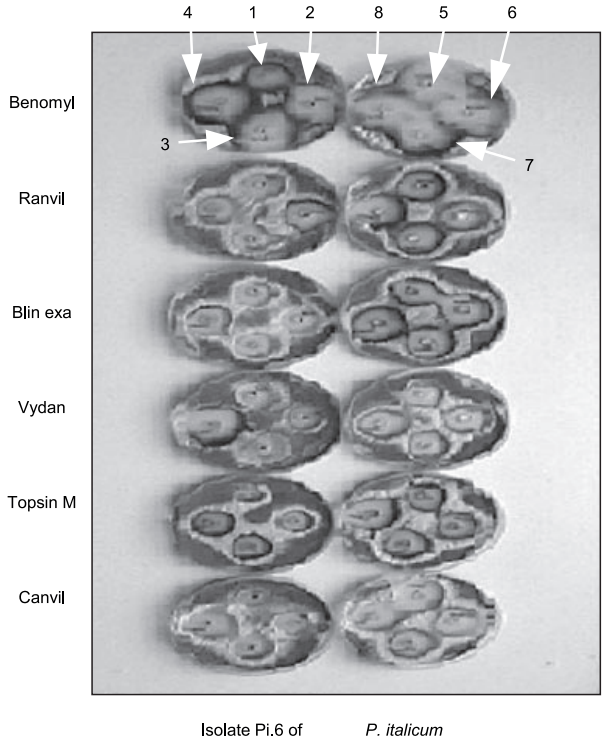

Fig. 1. Sensitivity of Pi.6 isolate of *P. italicum* to six chemical fungicides

3. Sensitivity of *P. italicum* isolates to various combined concentrations of fungicides mixtures

3.1. One way ANOVA

One way analysis of variance indicated that two (Vydan/Canvil (p = 0.005) and Topsin/Vydan (p = 0.001)) fungicide mixtures out of ten tested combinations (Table 2) have significantly affected the sizes of inhibition zones in the four tested *P. italicum* isolates.

3.2. Shaffe multiple comparison

3.2.1. Effect of Vydan/Canvil mixture of fungicides on growth of tested fungal isolates

None of the combined concentrations of Vydan/Canvil mixture generated complete inhibition of tested isolates of *P. italicum*. The obtained zones of inhibition with this mixture reached a value of 16±1.20 against isolate Pi.3 at a combined concentration of 50:50 μg/ml and a value of 25±1.27 against isolate Pi.6 at a combined concentration of 1000:2000 μg/ml (Table 2). Results indicated that there was a significant difference between the combined concentrations of 50:50 and 1000:2000 μg/ml of Vydan/Canvil mixture of fungicides and on the sizes of inhibition zones of the four tested *P. italicum* isolates (p = 0.015). In addition, the combined concentrations of Vydan/Canvil and Ranvil/Blin exa mixtures showed a significant correlation between them (r = 0.981*, p = 0.001) in terms of affecting the growth of isolate Pi.3 (Table 4).

Table 1. Sensitivity of four *Penicillium italicum* isolates to six chemical fungicides

Fungicide/ concentration range [μg/ml]	Fungal isolate	Mean inhibition zone [mm]±SD (range)[a]	IC_{50} [μg]	MIC [μg]	Regression equation[c]	Correlation coefficient (r)[d]
Benomyl 0.01–750 1000–3000	Pi.1	0.0–27±1.41 CI[b]	830	1000	Y=3.538X+15.226	0.875**
Benomyl 0.01–250 300–3000	Pi.3	2.5±3.54–24.5±0.71 CI	270	300	Y=5.5136X+11.694	0.965**
Benomyl 0.01–100 150–3000	Pi.5	0.0–26.66±2.88 CI	117	150	Y=7.475X+16.475	0.945**
Benomyl 0.01–35 40–3000	Pi.6	3.5±0.71–33.0±7.07 CI	36	40	Y=8.9383X+18.663	0.989**
Ranvil 0.01–3000	Pi.1	0.0–47.5±0.71			Y=8.971X+12.459	0.979**
Ranvil 0.01–3000	Pi.3	0.0–24±11.32			Y=11.468X+2.5573	0.862**
Ranvil 0.01–3000	Pi.5	0.0–32±1.41			Y=4.651X+17.185	0.930**
Ranvil 0.01–3000	Pi.6	0.0–24±5.66			Y=5.26X+2.7263	0.886**
Canvil 0.01–2 5–3000	Pi.1	0.0–33.5±3.52 CI	2.75	5	Y=13.55X+32.052	0.945**
Canvil 0.01–3000	Pi.3	0.0–19±1.42			Y=1.8152X+10.934	0.766**
Canvil 0.01–20 25–3000	Pi.5	0.0–28.5±.1 CI	21.5	25	Y=6.266X+22.928	0.829**
Canvil 0.01–3000	Pi.6	5 ± 7.07–22.67±2.89			Y=4.1218X+6.6605	0.883**
Blin exa 0.01–200 250–3000	Pi.1	0.0–38.5±0.71 CI	205	250	Y=11.461X+13.428	0.942**
Blin exa 0.01–3000	Pi.3	0.0–24.5±3.54			Y=4.889X+1.979	0.861**
Blin exa 0.01–3000	Pi.5	0.0–30.5±0.71			Y=5.716X+12.719	0.882**
Blin exa 0.01–3000	Pi.6	0.0–24±0.0			Y=4.7331X+0.37	0.823**
Vydan 0.01–3000	Pi.1	0.0–27.67±7.37			Y=5.794X+3.587	0.929**
Vydan 0.01–3000	Pi.3	0.0–24.5±4.94			Y=5.8779X+2.753	0.896**
Vydan 0.01–3000	Pi.5	0.0–25±1.4			Y=4.668X+7.673	0.945**
Vydan 0.01–400 450–3000	Pi.6	0.0–22.333±3.06 CI	418	450	Y=5.555X+2.105	0.790**
Topsin M 0.01–3000	Pi.1	4.0 ± 5.66–28.3±1.53			Y=3.948X+11.479	0.875**
Topsin M 0.01–3000	Pi.3	4.0 ± 5.7–25.5±4.89			Y=4.122X+9.5793	0.938**
Topsin M 0.01–3000	Pi.5	0.0–28.67±1.15			Y=5.652X+9.1861	0.973**
Topsin M 0.01–3000	Pi.6	4.0±5.66–30±3.61			Y=5.1298X+6.5475	0.913**

[a]values are means ± SD of three independent experiments
[b]CI – denotes Complete Inhibition of fungal growth
[c]Y – is the radius of inhibition zone (mm)
X – is the log concentration of fungicide ((μg/ml)
[d]**correlation is significant at the 0.01 level (2-tailed). Results indicated significant correlation between fungicide concentration and the size of inhibition zone for tested isolates. Benomyl was the most effective fungicide

Table 2. Effect of different combined concentrations of fungicide mixtures on the size of inhibition zone of *P. italicum* isolates

Fungicide mixture[a]	Mean zone of inhibition diameters [mm] within each combined concentration [μg/ml] of fungicides mixture						
	50:50[b]	100:100	100:500	500:1000	1000:1000	1000:2000	isolate
Beno/Ran	22±2.0[c]	22± 1.52	29± 3.27	31± 2.36	35± 2.37	41± 3.1	Pi.1
	41± 4.27	41± 1.44	41± 2.36	44± 2.46	44± 3.87	45± 2.37	Pi.5
	25± 2.16	25± 1.40	26± 1.37	29± 2.41	30± 3.17	30± 3.21	Pi.6
Top/Vyd	21± 2.46	25± 2.30	25± 1.46	25± 1.26	26± 2.06	28± 2.46	Pi.1
	20± 1.36	20± 2.27	25± 2.37	25± 3.36	25± 1.25	25± 2.77	Pi.5
Beno/Vyd	25± 2.21	30± 1.63	30± 1.25	30± 2.44	32± 3.56	39± 1.26	Pi.1
Vyd/Can	16± 1.20	16± 1.16	16± 1.44	22± 1.26	22± 2.35	25± 1.27	Pi.3
	17± 2.35	21±2.30	21± 1.56	21± 1.20	21± 1.25	21± 1.17	Pi.6
Blin/Can	21± 1.53	21±1.21	26± 2.25	30± 2.21	30± 2.41	32± 1.41	Pi.3
	44± 1.31	45± 1.26	46± 2.35	49± 3.20	CI[d]	CI	Pi.5
Ran/Blin	26± 1.55	26± 2.20	27± 2.36	30± 2.32	31± 1.30	32± 1.51	Pi.3
Top/Blin	24± 1.35	26± 1.44	30± 1.32	30± 2.27	30± 1.26	34± 1.66	Pi.5
	20± 2.27	20± 1.46	20± 1.47	21± 1.26	21±1.17	25± 1.35	Pi.6
Beno/Blin	27± 1.25	28± 2.35	28± 2.46	28± 1.44	30± 2.26	30± 1.10	Pi.5
Top/Can	45± 1.16	47± 1.41	51± 2.26	52± 1.29	CI	CI	Pi.6
Blin/Vyd	47± 1.40	49± 2.06	51± 1.32	51± 1.49	CI	CI	Pi.6

[a]denotes fungicides mixture where: Beno, denotes Benomyl; Ran, denotes Ranvil; Top, Topsin M; Vyd, Vydan; Can, Canvil; Blin, Blin exa fungicide

[b]denotes combined concentrations of fungicides mixtures i.e. 50:50 indicates combination of 50 μg/ml from each fungicide within the mixture

[c]values are means ± SD of three independent experiments

[d]CI – denotes for complete inhibition. The largest zones were generated against isolates Pi.5 and Pi.6 with the combinations of Blin exa/Vydan, Topsin/Canvil, and Blin exa/Canvil. Also, the last two concentrations generated complete inhibition of fungal growth

3.2.2. Effect of Topsin/Vydan mixture of fungicides on growth of tested fungal isolates

There was a significant difference between 50:50 μg/ml combined concentrations of Topsin/Vydan mixture and the rest of combined concentrations on the sizes of inhibition zones in the four (Pi.1; Pi.3; Pi.5; and Pi.6) tested isolates, where, the obtained p values (at the 0.05 level of significance) were 0.024, 0.024, 0.015 and 0.002, respectively. The combined concentrations of Topsin M/Vydan mixture that were tested against isolate Pi.5 showed approximately the same effect as that produced by each single fungicide alone. Furthermore, the combinations of Topsin M/Vydan and Topsin M/Blin exa, were the only combinations of fungicides that showed significant correlation between them (r = 0.880*, p = 0.021) in term of affecting the inhibition zones of isolate Pi.5. Moreover, there was a significant correlation between Benomyl/Vydan and Topsin/Vydan mixtures (r = 0.942**, p = 0.005) in terms of affecting inhibition zones of isolate Pi.1 (Table 4). In addition, results of regression analysis (Table 3) indicated that the mixture of Topsin/Vydan showed a significant correlation (at either the 0.01 or the 0.05 level of significance) with all tested fungicide mixtures except for Blin exa/Canvil (r = 0.641; p = 0.170).

3.2.3. Effect of Topsin/Canvil or Blin exa/Vydan mixtures of fungicides on growth of tested fungal isolates

The combined concentrations of 1000:1000 and 1000:2000 μg/ml of Topsin/Canvil or Blin exa/Vydan mixtures lead to a complete inhibition of *P. italicum* (Fig. 2). In addition, results presented in Table 2 indicated that the

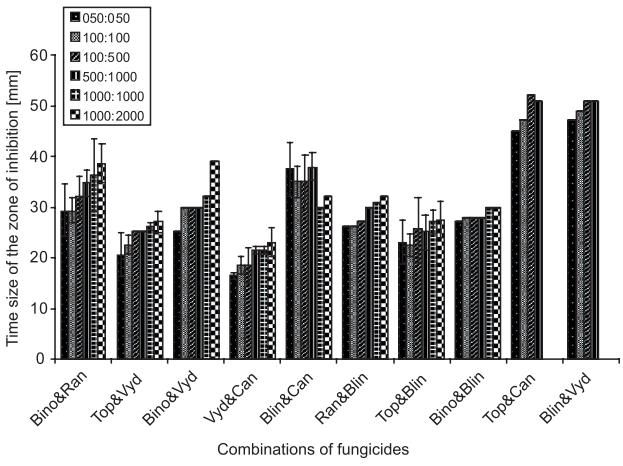

Fig. 2. Effect of combined concentration of fungicide mixtures on the size of inhibition zone of *P. italicum* isolates

largest zones of inhibition were generated with the mixture of Blin exa/Vydan at all used combined concentrations. Moreover, results of regression analysis presented in Table 4 indicated that there was a significant correlation between the combination of Topsin M/Canvil, and Blin exa/Vydan that were used against isolate Pi.6 of *P. italicum* (r = 0.975*; p = 0.025). All of the tested combined concentrations of Topsin M/Canvil and Blin exa/Vydan fungicides showed approximately the same sized zone of inhibition towards isolate Pi.6 as a result of synergistic effect.

Table 3. Regression analysis for the relationship between the size of inhibition zone ([mm] and the concentration [μg/ml] of combined fungicides on *P. italicum* isolates

Fungicides Mixture[a]	Beno/Ran (r)[b] (sig)	Top/Vyd (r) (sig)	Beno/ Vyd (r) (sig)	Vyd/Can (r) (sig)	Blin/Can (r) (sig)	Ran/Blin (r) (sig)	Top/Blin (r) (sig)	Beno/ Blin (r) (sig)	Top/Can (r) (sig)	Conc.[c] (r) (sig)
Beno/Ran										0.984** 0.000
Top/Vyd	0.914* 0.011									0.957** 0.003
Beno/Vyd	0.846* 0.034	0.849* 0.032								0.891* 0.017
Vyd/Can	0.949** 0.004	0.904* 0.014	0.856* 0.029							0.968** 0.002
Blin/Can	0.617 0.192	0.641 0.170	0.664 0.151	0.549 0.259						0.680 0.137
Ran/Blin	0.987** 0.000	0.867* 0.025	0.792 0.060	0.959** 0.002	0.590 0.218					0.965** 0.002
Top/Blin	0.942** 0.005	0.924** 0.008	0.776 0.070	0.821* 0.042	0.706 0.117	0.893* 0.016				0.932** 0.007
Beno/Blin	0.874* 0.023	0.850* 0.032	0.859* 0.028	0.847* 0.033	0.907* 0.013	0.860* 0.028	0.863* 0.027			0.917** 0.010
Top/Can	0.817 0.183	0.984* 0.016	0.757 0.243	0.697 0.303	0.165 0.835	0.653 0.347	0.925 0.075	0.757 0.243		0.899 0.101
Blin/Vyd	0.818 0.182	0.998** 0.002	0.870 0.130	0.802 0.198	0.196 0.810	0.690 0.310	0.828 0.172	0.870 0.130	0.975* 0.025	0.944 0.056

[a]Beno, denotes for Benomyl; Ran, for Ranvil; Top, for Topsin M; Vyd, for Vydan; Can, for Canvil; Blin, for Blin exa fungicide
[b](r) denotes correlation coefficient and (Sig) for significance level
**correlation is significant at the 0.01 level (2-tailed)
*correlation is significant at the 0.05 level (2-tailed)
[c]conc denotes concentration. Results indicated that the tested combined concentrations of Blin exa/Canvil, Topsin/Canvil, and Blin exa/Vydan mixtures have reflected no significant differences on inhibition zones

3.2.4. Effect of Blin exa/Canvil and Benomyl/Ranvil mixtures of fungicides on the growth of tested fungal isolates

Results presented in Table 2 indicated that all combined concentrations of Blin exa/Canvil and Benomyl/Ranvil fungicides showed the largest zones of inhibition against isolate Pi.5. The inhibition zones obtained with Benomyl/Ranvil mixture ranged from 41±4.27 at a combined concentration of 50:50 μg/ml to 45±2.37 at a combined concentration of 1000:2000 μg/ml. Furthermore, the combined concentrations of 1000:1000 and 1000:2000 μg/ml of Blin exa/Canvil were the only concentrations that caused complete inhibition of fungal growth, whereas, the rest of tested combinations i.e. from 50:50 to 500:1000 μg/ml showed inhibition zones in the range of 44±1.31 to 49±3.20 respectively. In contrast, the combined concentrations of either Blin exa/Canvil or Blin exa/Ranvil mixtures showed additive effect in terms of affecting the sizes of inhibition zones against isolate Pi.3. Moreover, the obtained results indicated that the combinations of Benomyl/Ranvil, Topsin/Vydan and Benomyl/Vydan that were tested against isolate Pi.1 showed antagonistic effect, in terms of affecting the sizes of inhibition zones as compared to each of the individually tested fungicides (Tables 1, 2). The combinations of Benomyl/Ranvil and Benomyl/Vydan were significantly correlated with each other ($r = 0.879^*$, $p = 0.021$). In addition, the mixture of Benomyl/Blin exa showed a significant correlation with all tested mixtures of fungicides except for Topsin/Canvil ($r = 0.757$; $p = 0.243$) and Blin exa/Vydan mixture ($r = 0.870$; $p = 0.130$) (Table 3).

Table 4. Regression analysis for the effect of fungicides mixtures on sizes of fungal inhibition zones [mm]

Fungicides Mixture[a]	Top/Vyd (r)[b] (sig)	Beno/Vyd (r) (sig)	Conc.[c] (r) (sig)	Fungal isolate		
Beno/Ran	0.826* 0.043	0.879* 0.021	0.978** 0.001	Pi.1		
Top/Vyd		0.942** 0.005	0.891** 0.017	Pi.1		
Beno/Vyd			0.891* 0.017	Pi.1		
Fungicides mixture	Blin/Can (r) (sig)	Ran/Blin (r) (sig)	Conc. (r) (sig)	fungal isolate		
Vyd/Can	0.705 0.118	0.981** 0.001	0.925** 0.008	Pi.3		
Blin/Can		0.605 0.203	0.421 0.417	Pi.3		
Ran/Blin			0.965** 0.002	Pi.3		
Fungicides mixture	Blin/Can (r) (sig)	Top/Blin (r) (sig)	Beno/Blin (r) (sig)	Beno/Ran (r) (sig)	Conc. (r) (sig)	fungal isolate
Top/Vyd	0.802 0.198	0.880* 0.021	0.632 0.178	0.693 0.127	0.828* 0.042	Pi.5
Blin/Can		0.823 0.177	0.309 0.691	0.00 1.000	0.478 0.522	Pi.5
Top/Blin			0.696 0.125	0.671 0.144	0.759 0.080	Pi.5
Beno/Blin				0.798 0.062	0.917* 0.010	Pi.5
Beno/Ran					0.919** 0.010	Pi.5
Fungicides mixture	Top/Blin (r) (sig)	Top/Can (r) (sig)	Blin/Vyd (r) (sig)	Beno/Ran (r) (sig)	Conc. (r) (sig)	fungal isolate
Vyd/Can	0.294 0.571	0.757 0.243	0.870 0.130	0.504 0.308	0.655 0.158	Pi.6
Top/Blin		0.262 0.738	0.302 0.698	0.361 0.483	0.578 0.229	Pi.6
Top/Can			0.975* 0.025	0.653 0.347	0.899 0.101	Pi.6
Blin/Vyd				0.690 0.310	0.944 0.056	Pi.6
Beno/Ran					0.946** 0.004	Pi.6

[a]Beno, denotes Benomyl; Ran, denotes Ranvil; Top, for Topsin M; Vyd, for Vydan; Can, for Canvil; and Blin, for Blin exa fungicide
[b](r) denotes correlation coefficient; (Sig) for significance level
**correlation is significant at the 0.01 level (2-tailed)
*correlation is significant at the 0.05 level (2-tailed)
[c]conc. denotes concentration of fungicides in µg/ml. There was significant correlation between mixtures of: Benomyl/Vydan and Topsin/Vydan; Vydan/Canvil and Ranvil/Blin exa; Topsin/Blin exa and Topsin/Vydan, and between Blin exa/Vydan and Topsin/Canvil that were used against isolates Pi.1; Pi.3; Pi.5 and Pi.6 respectively

DISCUSSION

Since new growth media was used instead of the commonly used PDA medium it was essential to optimize the growth conditions, so that the efficacy of tested fungicides could be better evaluated, otherwise, weakened or delayed mould growth could be mistakenly attributed to fungicidal effect resulting in false evaluation of efficacy. In addition, the modified medium provided maximum zones of growth as compared to PDA medium. Obtained results indicated that there was a significant correlation between fungicide concentration and the size of inhibition zone for all tested isolates. These findings agreed with the results obtained by both *in vitro* (Kanan 2008) and *in vivo* (G. J. Kanan, Mutah University, unpublished data) tests of studied fungicides against *P. digitatum* (the green mould) isolates. The benzimidazole fungicide Benomyl worked effectively with the least MIC values against all tested isolates and this agreed with the results of both *in vitro* (Kanan 2008) and *in vivo* (tested on orange and lemon fruits) tests against *P. digitatum* isolates (G. J. Kanan, Mutah University, unpublished data). Ranvil (DMI member) and Topsin (TBZ member) did not lead to complete inhibition in all tested isolates within a range of concentrations from 0.01 to 3000 µg/ml. These results disagreed with the *in vitro* results of studied fungicides against *P. digitatum* isolates where, all tested fungicides lead to complete inhibition in all tested isolates (Kanan 2008). Benomyl, also showed selective toxicity to several microorganisms including fungi (Stringer and Wright 2006) and interferes with intracellular transportation i.e. loss of membrane transport ability (Spencer *et al.* 1998). The TBZ as well as DMI are systemic fungicides that act on specific targets in such a way that mutations in the corresponding gene (s) of the target pathogen may develop resistance due to modification of the bio-chemical target site in the pathogen (Lopez-Garcia *et al*. 2003; Survilienė and Dambrauskienė 2006). Concerning Benomyl's mode of action, it is first transformed into methyl-2-benzimidazole carbamate that causes morphological distortion of germinating spores (Dalgie 2005), and is thought to alter mitotic cell division where it binds to microtubules and inhibits ß-tubulin assembly (Dalgie 2005; Spencer *et al*. 1998). Resistance to benzimidazole is suggested to be a kind of qualitative resistance, since it is resulted from conformational change at the target site of the fungus. The resistance here leads to complete loss of disease control which can not be regained by increasing fungicide concentration or by the frequent application of the fungicide. This type of resistance leads to disruptive selection of biotypes, because such resistance resulted from modification of a single major gene, and this agreed with the suggestions of McGrath (2001). Canvil showed high efficacy against tested isolates as compared to Blin exa, Ranvil and Vydan although all are DMI members and contain the same active component, but produced by different national and international companies (Savocchia *et al*. 2004). Resistance to DMI fungicides in certain fungal species including *P. italicum* biotypes is controlled by a polygenic system (Van Tuyl 1977; Kalamarakis *et al*. 1987), where it is suggested that several minor genes control DMI fungicides resistance in wild-type strains of *Penicillium* spp. (De Waard and Van Nisterrooy 1990). These minor genes interact in an additive fashion to confer a significant level of resistance to fungicides (Georgopoulos and Skylakakis 1986). However, when resistance resulted from modification of several interacting genes, pathogens exhibit a range in sensitivity to the fungicide, depending on the type and number of altered genes. Variation 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. Such type of resistance is termed quantitative resistance, and is exemplified by resistance to demethylation inhibition (DMI) fungicides. Possible mechanisms of DMI resistance include: (I) mutations in the DMI target enzyme, C14 alpha-demethylase (CYP51), leading to a decreased affinity of DMIs to the target protein or tolerance of toxic sterols, detoxification of DMIs or even detoxification of sterols (Delye *et al*. 1998; Asai *et al*. 1999; McGrath 2001). Otherwise, the chemical will inhibit the enzyme leading to depletion of ergosterol which serves as a bioregulator for membrane fluidity, symmetry and integrity in fungal cells i.e. it is essential for the development of functional cell wall (Sugiura *et al*. 2006). (ii) Over-expression or increased copy number of the (CYP51) gene, leading to increased production of the target enzyme (Hamamoto *et al.* 2000; Ma *et al*. 2006). (III) Over-expression of ATP binding cassette (ABC) transporters encoding efflux pumps resulting in increased efflux (Hayashi *et al.* 2002; Zwiers *et al*. 2002). (iv) Failure in activation of the fungicide, deposition of fungicide in lipid droplets and change in pH leading to protonation of fungicide and this agreed with the suggestions of McGrath (2001). However, when pair-wise combinations of triazoles together or triazoles and benzimidazoles were applied complete inhibition of growth was achieved. The Blin/Vydan and Topsin/Canvil mixtures of fungicides either showed the largest zones of fungal inhibition at the least combined concentration or lead to complete inhibition at higher (1000:1000 µg/ml) combined concentrations. These findings disagreed with the results of *in vitro* study against *P. digitatum* isolates where, no complete inhibition was achieved (Kanan 2008). However, when Blin/Vydan mixture was tested *in vivo* against *P. digitatum* isolates infecting orange and lemon fruits mostly 100% cleared fruit surface area (CSA; i.e. complete inhibition) was obtained at a combined concentration of 100:500 µg/ml (G. J. Kanan, Mutah University, unpublished data). In contrast, no complete inhibition on both fruit types was obtained against *P. digitatum* isolates when Topsin/Canvil was used, where the maximum % CSA on orange fruits reached 8% , whereas that on lemon fruits reached 84% (G. J. Kanan, Mutah University, unpublished data). The combined concentrations of Benomyl/Blin exa and Benomyl/Ranvil showed antagonistic effects on the size of zone of inhibition where it seemed to be that either Blin exa or Ranvil negatively influenced the activity of Benomyl, in which the complete inhibition caused by Benomyl was cancelled by the effects of either Blin exa or Ranvil (both contain the same active component but produced by different companies). In contrast,

the *in vivo* results indicated that Benomyl/Ranvil mixture had lead to 100% cleared surface area (% CSA) of orange fruits and to 88–100% CSA of lemon fruits that were infected by *P. digitatum* isolates (G.J. Kanan, Mutah University, unpublished data). These findings would agree with the suggestion of Shaw (1993) who stated that theoretical studies of resistance development revealed that the combination of two selective fungicides to combat resistance was reasonable strategy only when used against wild population of the pathogen. These pathogens should have a low frequency of resistance to both fungicides, even the sequential use of two unrelated fungicides may be a more effective strategy.

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POLISH SUMMARY

BADANIA *„IN VITRO"* NAD WRAŻLIWOŚCIĄ GRZYBA *PENICILLIUM ITALICUM*, SPRAWCY ZGNILIZNY OWOCÓW CYTRUSOWYCH PO OKRESIE ZBIORÓW W JORDANII NA FUNGICYDY ORAZ ICH MIESZANINY

Oceniano *„in vitro"* skuteczność sześciu fungicydów oraz ich mieszanin w zwalczaniu grzyba *Penicillium italicum.* Zbadano przeciwgrzybowe działanie w stosunku do czterech izolatów *P. italicum* trzydziestu jeden koncentracji (0.01–3000 μg/ml) każdego fungicydu z osobna (Vydan, Blin exa, Canvil, Ranvil, Benomyl i Topsin M) oraz sześciu koncentracji tych samych fungicydów zastosowanych w formie mieszanin, stosując metodę dyfuzji w agarze. Wykorzystano analizę regresji, jednokierunkową metodę ANOVA oraz wielokrotne porównania Post Hoc do oceny istotności różnic zastosowanych kombinacji. Wyniki przeprowadzonych badań wykazały, że benomyl całkowicie hamował wzrost kolonii testowanych izolatów (Pi.1; Pi.3; Pi.5 oraz Pi.6) odpowiednio przy minimalnej inhibicyjnej koncentracji rzędu: 1000; 300; 150 i 40 μg/ml. Fungicyd Canvil w porównaniu do fungicydów Blin exa, Ranvil i Vydan (gdzie obserwowano brak całkowitej inhibicji wzrostu kolonii grzyba) wykazał wysoką skuteczność przeciwko izolatom Pi.1 oraz Pi.5, a wielkości minimalnej inhibicyjnej koncentracji wynosiły odpowiednio 5 i 25 ug/ml. Mieszaniny fungicydów Blin exa/Vydan oraz Topsin M/Canvil były jedynymi mieszaninami wykazującymi synergistyczne działanie we wszystkich zastosowanych koncentracjach przeciwko testowanym izolatom grzyba. Wyżej wymienione mieszaniny fungicydów zastosowane w przypadku czterech pierwszych koncentracji (50:50, 100:100, 100:500 oraz 500:1000 μg/ml) powodowały największą strefę zahamowania wzrostu kolonii patogena (w zakresie od 47±1.40 mm do 51±1.49 mm) lub całkowitą inhibicję dla dwóch ostatnich koncentracji (1000:1000 oraz 1000:2000 μg/ml) Porównując skuteczność mieszaniny fungicydów Blin exa/Canvil i Blin exa/Ranvil do skuteczności każdego z zastosowanych preparatów oddzielnie stwierdzono, że mieszaniny te wykazały addytywne działanie przeciwko badanym izolatom patogena. Mieszaniny fungicydów: Benonyl/Vydan; Benomyl/Ranvil; Benomyl/Blin exa; Topsin M/Vydan oraz Topsin M/Blin exa (jak na przykład mieszaniny benzymidazoli z prepararatami należącymi do grupy DMI) wykazywały antagonistyczne działanie w stosunku do testowanych izolatów *P. italicum.*