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In vitro Evaluation of *Penicillium digitatum Sacc* Strains Sensitivity to Various Fungicides from Jordan

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Abstract: This investigation is the first report in Jordan, aimed for *in vitro* evaluation of six fungicides and their combinations, to control the post harvest green mold (*Penicillium digitatum*) of citrus fruits. Thirty one different concentrations (0.01-3000 μg mL^{-1}) of each fungicide, in addition to 6 combined concentrations from each of 7 fungicide mixtures, were tested using Agar well diffusion method against four fungal strains. Regression analysis, one way ANOVA and Post Hoc Multiple comparisons were carried out to test the significance of these treatments. Results of regression analysis indicated significant correlation ($p<0.01$) between fungicide concentration (μg mL^{-1}) and inhibition zone (mm) of tested strains. All applied fungicides have resulted in complete inhibition of fungal growth in the four tested strains with MIC values ranging from 5 to 2700 μg mL^{-1}. Canvil and Ranvil of the DMI family were the most effective against tested strains (except strain dg6) where an MIC in the range of 5 to 150 μg mL^{-1} was required. Benomyl has worked effectively with the least MIC values against the four tested strains. The obtained Benomyl's MIC values were: 20, 40, 300 and 40 μg mL^{-1} against strains dg2, dg4, dg5 and dg6, respectively. One way analysis of variance indicated that the following fungicide mixtures: Benomyl/Canvil; Topsin/Vydan; Blin/Canvil; Topsin/Blin and Topsin/Canvil had significantly ($p<0.001$) affected the sizes of inhibition zones, in strains dg2, dg2 and dg5, dg2 and dg4, dg2 and dg4, respectively. Scheffe multiple comparisons analysis showed that there were significant differences ($p<0.001$) between the combined concentrations of 50:50 μg mL^{-1} or 100:100 μg mL^{-1} of Benomyl/Canvil mixture and the rest of tested concentrations where complete inhibition of growth was achieved at a combination of 100:500 μg mL^{-1}. The mixtures of Benomyl/Canvil and Blin/Canvil were the most effective against strains where, wither complete inhibition or the largest inhibition zones were obtained at the least (50:50 μg mL^{-1}) combined concentrations.

Key words: *Penicillium digitatum*, citrus post harvest disease, chemical fungicides

INTRODUCTION

Citrus fruits are susceptible to a number of decay causing diseases that affect the quality and shelf-life of the crop (Bouzerda *et al*., 2003). The type and severity of decay depend on the disease causes organism, climate, variety of the fruit, agricultural practices and the pre- and post-harvest handling practices (Holmes and Eckertm, 1999). In absence of efficient disease control strategies, overseas citrus trade would significantly be reduced since the transport of citrus fruits from producers to consumers may take several weeks or months (Bouzerda *et al*., 2003). The post harvest losses of fresh citrus fruits are estimated to be between 10 to 30% worldwide and may reach a much higher percentage in less developed countries (Janisiewiez and Korsten, 2002; Spadaro and Gullino, 2004). Green and blue mold rots caused by *Penicillium digitatum* (Pers: Fr.) Sacc and *P. italicum* Wehmer respectively, are probably the most common post-harvest diseases, affecting citrus fruits worldwide (Holmes and Eckertm, 1999; Barkai-Golan, 2001; Plaza *et al*., 2003). These fungal species are strongly associated with citrus fruits as particular habitats (Samson *et al*., 2004). The green mold invades the fruit much more rapidly at room temperature and predominates in mixed infections, causing approximately 60-80% of decay (Palou *et al*., 2001; Skaria *et al*., 2003; Plaza *et al*., 2004). Citrus industry relies on the extensive use of chemical fungicides as a standard practice for the control of post harvest rots (Barkai-Golan, 2001; McGrath, 2001; Pramila and Dubey, 2004). These fungal diseases are commonly controlled worldwide (Lopez-Garcia *et al*., 2003; Valiuskaite *et al*., 2006) and particularly in California Citrus packinghouses (Holmes and Eckertm, 1999; Plaza *et al*., 2003), by the application of the sterol demethylation inhibitor (DMI) group of fungicides, such as Imazalil, O-phenyl phenol and thiabedazole fungicides. Furthermore, the DMI members of fungicides have shown protective activity against Ascomycota and basidiomycota (Sijaona and Mansfield, 2001; Savocchia *et al*., 2004; Gopi *et al*., 2005;

Sugiura *et al*., 2006). The systemic benzimidazole Benomyl (Benlate) fungicide which has a single site mode of action has shown high activity in upsetting mitotic cell division in *Penicillium* species, by inhibiting β-tubulin assembly (Tsuda *et al*., 2004; Dalgie, 2005). Topsin M (Methyl-thiophanate) is a broad-spectrum systemic benzimidazole fungicide, which shows great adhesion properties has provided excellent control for several fungal diseases including powdery and downy mildews, smut, wheat and brown rusts (Siddiqui *et al*., 1999). However, with the constant threats of having fungicide resistance in decay causing organisms, due to the recurrent use of the same active ingredients there is a need to expand and diversify decay control options (Irtwange, 2006; Surviliene and Dambrauskiene, 2006). This particular work has aimed to overcome these threats, through the discovery of new reduced risk fungicides by evaluating *in vitro* six fungicides including the ones that share the same active component but sold under different commercial names. Secondly, to evaluate the effectiveness of fungicide mixtures against tested strains of *P. digitatum* hoping in reaching new decay control options.

MATERIALS AND METHODS

This study was conducted during the year 2007 in laboratories of biological sciences department at Mu'tah University-Jordan.

***Penicillium digitatum* tested strains:** Conidiospores of four (dg2, dg4, dg5 and dg6) *P. digitatum* strains were obtained from spoiled citrus (orange (*Citrus sinensis* L.) and lemon (*Citrus limon* L.) fruits, collected from two Jordanian cities: Irbid and Al-Karak.

Media used: The *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 5.5, supplemented with 10 mM glutamic acid and 10 g L^{-1} fructose as C-source).

Purification of strains: Conidiospores from each tested strain were grown for 7 days at 25° C on CM plates (Cove, 1966) to confirm their purity and identity. Purified conidiospores suspensions in 5 mL physiological Saline/Tween 80 solution were used at a concentration of approximately 1×10^{8} spores mm^{-1}. Aliquot of 100 μL from a serial dilution of 10^{-6} or 10^{-7} were plated again on complete media in order to achieve single pure colony as a source of pure culture (Zhang *et al*., 2004).

Optimal growth conditions of tested fungal strains: Nine replicates (for each tested condition) of conidiospores suspension (20 μL) from each tested strain 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 5.5. In order to determine the optimal temperature of growth plates of complete media adjusted to optimal pH of 5.5 and supplemented with 10 mM glutamic acid as best serving N-source were incubated at four temperature regimes (i.e., 10, 20, 25, 30 and 37°C). Also, various C-sources (glucose, sucrose, sorbitol, fructose and maltose) were tested at a final concentration of 10 g L^{-1} to determine the best serving C-source. Each group of nine replicates were incubated for 5 days, at 25°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: (1)- Bayfidan Turf $_{25\%}$ EC (Vydan) containing Triadimenol as an active gradient: $C_{14}H_{18}CIN_3O_3$ Vapco. Com. Jordan. (2)- Blin exa $_{5\%}$SC, IQV Spain. (3)- Canvil $_{5\%}$ Vapco.Com. Jordan. (4)- Ranvil $_{5\%}$ Chem.Vet. Jordan, where all of the latter three fungicides contain hexaconazole 5% as an active ingredient and have the same formula: $C_{14}H_{17}CI_2N_3O$. (5)- Benlate (Benomyl) $_{50\%}$ W.P: $C_{14}H_{18}N_4O_3$, Vapco. Com. Jordan. (6)- Topsin M $_{70\%}$ W.P: $C_{12}H_{14}N_4O_4S_2$, Nippon soda Japan.

Application of fungicides-non-amended medium *in vitro*: Aliquot of 100 μL spores suspension (approx: 10^8 spores/mL) of each tested strain 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 (Ogundare *et al*., 2006; Ndukwe *et al*., 2006). Wells of 6 mm in diameter were performed (3 wells/plate/concentration) then a range of 31 concentrations (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 and 3000 μg mL^{-1}) from each of the six tested fungicides were used against each tested strain. The plates were incubated at 25°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 *in vitro*: 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 and 1000:2000 μg mL^{-1}) with each pair-wise mixture of fungicides. Seven different fungicide mixtures were used against the four tested fungal strain and these mixtures are: Benomyl/Canvil; TopsinM/Canvil; Blin exa/Vydan; TopsinM/Blin exa; Vydan/Canvil; TopsinM/Vydan and Blin exa/Canvil. 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 were 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 seven fungicide's mixtures on sizes of inhibition zones of studied *P. digitatum* strains. This was followed by Post Hoc multiple comparisons to determine the significance level of applied combined concentrations of fungicide's mixtures and their interactions on sizes of inhibition zones for the studied strains of *P. digitatum*.

RESULTS

Optimal growth conditions for tested fungal strains: The obtained results indicated that fructose was serving as the best sole source of carbon for the growth of the four (dg2, dg4, dg5 and dg6) tested isolates of *P. digitatum* under two temperature regimes: 25 and 20°C. The obtained zones of growth at these temperatures were 30.5±1.7 and 35.5±1.8 mm, respectively. Furthermore, glutamic acid and ammonium were serving as the best sole sources of nitrogen when cultures were incubated at 25 and 20°C, respectively. The obtained maximum zones of growth have reached 30.4±3.2 and 31.6±2.3 mm, respectively. The obtained optimal pH (within a range of pH from 5 to 9) for growth of isolates at either 25 or 20°C was pH 5.5, where the maximum zone of fungal growth has reached 24.55±5.6 mm at 25°C and 22.32±3.3 mm at 20°C.

Sensitivity of four wild-type strains of *P. digitatum* to six chemical fungicides (*in vitro*): Results of regression analysis *in vitro* indicated that there was significant correlation (at the 0.01 level- 2-tailed) between fungicide's concentration (μg mL^{-1}) and the size of inhibition zone (mm) for the tested strains (dg2, dg4, dg5 and dg6) of *P. digitatum* (Table 1). The fungicide Canvil was serving as the most effective against strains dg4 and dg5 where, MIC values of 5 μg mL^{-1} (IC_{50} = 1.9 μg mL^{-1}) and 25 μg mL^{-1} (IC_{50} = 21.7 μg mL^{-1}) were obtained, respectively. In contrast, the same fungicide (Canvil) had shown antagonistic effect against strains dg6 and dg2 (i.e., being the least effective) where the obtained MIC values against the strains were 2700 μg mL^{-1} (IC_{50} = 2470 μg mL^{-1}) and 50 μg mL^{-1} (IC_{50} = 10 μg mL^{-1}), respectively. However, results indicated that Ranvil was the most effective against strain dg2 (Fig. 1) where, an MIC value of 10 μg mL^{-1} and an IC_{50} value of 6 μg mL^{-1} were obtained. Whereas, Benomyl was the most effective against strain dg6 in which an MIC of 40 μg mL^{-1} and IC_{50} of 36 μg mL^{-1} were obtained (Table 1).

Sensitivity of *P. digitatum* strains to combined concentrations of fungicides mixtures

One way ANOVA: One way analysis of variance had indicated that the combination of Benomyl/Canvil had significantly affected the sizes of inhibition zones in strains dg2 (p = 0.000) and dg5 (p = 0.000) of *P. digitatum*, but not dg4 (p = 0.527) and dg6 (p = 0.556) strains. However, the combinations of Topsin M/Canvil and Blin exa/Vydan had shown significant effect (p = 0.000) on sizes of inhibition zones of the tested strains, except strain dg2 where, the obtained p-values have reached 0.859 and 0.558, respectively. Furthermore, the combination of Topsin M/Blin exa had significantly (p = 0.000) affected the zones of all tested strains (Fig. 2), except strain dg4 (p = 0.081) whereas, the combination of Blin exa/Canvil had significantly (p = 0.000) affected the size of zones in tested strains except strain dg6 (p = 0.334).

Scheffe multiple comparison: Effect of Benomyl/Canvil Mixture of Fungicides on Growth of Tested Fungal Strains Result presented in Table 2 indicated that the mixture of Benomyl/Canvil had caused complete inhibition of strain dg2 growth at a combined concentration of 100:500 and over. Whereas, the remaining tested strains have shown minimum zones of inhibition in the range of 40.5±8.5 to 49±6.9 and maximum zones in the range of 51.5±10.6 to 56.5±6.6 at combined concentration of 50:50 and 1000:2000 μg mL^{-1} respectively (Table 2). Scheffe multiple comparison indicated that there was significant difference (p = 0.000) between the 50:50 and 100:100 μg mL^{-1} combined concentrations of Benomyl/Canvil and the rest of the tested combinations (i.e., 100:500; 500:1000; 1000:1000 and 1000:2000) on zones of strain dg2. Also, there was significant difference (at the

Table 1: Sensitivity of four wild-type strains of *Penicillium digitatum* to six chemical fungicides (*in vitro*)

Fungicide/concentration range (μg mL^{-1})	Fungal strain	Mean inhibition zone (mm)±SD (Range)	IC_{50}	MIC (μg)	Equation	Coeff (r)	Sig (2-tailed)
Benomyl	dg4				Y = 23.29x + 17.30	0.837**	0.000
0.01-35		0.0- 13.5±1.42	37	40			
40-3000		CI†					
Canvil	dg4				Y = 14.87x+53.114	0.855**	0.000
0.01-2		14±5.70-45.5±7.78	1.9	5			
5-3000		CI					
Vydan	dg4				Y = 20.87x+17.05	0.851**	0.000
0.01-150		0.0-36.5±9.19	157	200			
200-3000		CI					
Ranvil	dg4				Y = 19.563x+35.84	0.837**	0.000
0.01-15		11±1.33-20±4.65	16.8	20			
20-3000		CI					
Blin exa	dg4				Y = 23.68x+11.79	0.838**	0.000
0.01-50		1.0-37±2.26	58	100			
100-3000		CI					
Topsin M	dg4				Y = 22.04x+23.59	0.887**	0.000
0.01-35		0.0-34.5±2.71	36	40			
40-3000		CI					
Benomyl	dg6				Y = 21.88x+23.8	0.884**	0.000
0.01-35		0.0-30.5±3.41	36	40			
40-3000		CI					
Canvil	dg6				Y = 6.65x+12.85	0.521**	0.003
0.01-2400		0.0-30±2.83	2470	2700			
2700-3000		CI					
Vydan	dg6				Y=19.43x+0.881	0.754**	0.000
0.01-400		0.0-20.5±1.75	420	450			
450-3000		CI					
Ranvil	dg6				Y = 11.96x+4.45	0.550**	0.001
0.01-1500		0.0-20±3.52	1720	2000			
2000-3000		CI					
Blin exa	dg6				Y = 10.64x+3.99	0.644**	0.000
0.01-2000		0.0-20±11.2	2140	2400			
2400-3000		CI					
Topsin M	dg6				Y = 11.87x+0.008	0.681**	0.000
0.01-2000		0.0-28.5±1.42	2110	2400			
2400-3000		CI					
Benomyl	dg2				Y = 19.86x+34.10	0.875**	0.000
0.01-20		10±1.42- 29.5±0.71	20.7	25			
25-3000		CI					
Canvil	dg2				Y = 17.52x+37.03	0.921**	0.000
0.01-40		17.5±4.95-51.5±7.78	10	50			
50-3000		CI					
Vydan	dg2				Y = 22.004x+22.80	0.870**	0.000
0.01-34		4.5±6.4-27±1.41	36	40			
40-3000		CI					
Ranvil	dg2				Y = 15.66x+49.69	0.856**	0.000
0.01-5		15.5±2.12-33.5±7.8	6	10			
10-3000		CI					
Blin exa	dg2				Y = 20.15x+37.73	0.888**	0.000
0.01-10		0.0-43.5±4.95	10.2	15			
15-3000		CI					
Topsin M	dg2				Y = 22.21x+22.84	0.883**	0.000
0.01-35		0.0-27.5±1.74	36.5	40			
40-3000		CI					
Benomyl	dg5				Y = 18.90x+16.01	0.796**	0.000
0.01-250		10.5±1.53-27±1.77	262	300			
300-3000		CI					
Canvil	dg5				Y = 21.024x+30.81	0.796**	0.000
0.01-20		3.5±1.36- 26±1.44	21.7	25			
25-3000		CI					
Vydan	dg5				Y = 12.81x+13.39	0.668**	0.000
0.01-750		10.5±0.71-25.5±0.71	825	1000			
1000-3000		CI					
Ranvil	dg5				Y = 22.115x+21.33	0.941**	0.000
0.01-100		0.0-52±1.46	29	150			
150-3000		CI					

Table 1: Continued

Fungicide/concentration range (μg mL^{-1})	Fungal strain	Mean inhibition zone (mm)±SD (Range)	IC_{50}	MIC (μg)	Equation	Coeff (r)	Sig (2-tailed)
Blin exa	dg5				Y = 22.48x+25.54	0.909**	0.000
0.01-30		0.0-46.5±9.19	21.2	35			
35-3000		CI					
Topsin M	dg5				Y = 9.36x+8.92	0.599**	0.000
0.01-2000		0.0-23±2.65	2113	2400			
2400-3000		CI					

Values are means±SD of three independent experiments. †: Denotes for Complete Inhibition of fungal growth, **Correlation is significant at the 0.01 level (2-tailed) , *Correlation is significant at the 0.05 level (2-tailed)

Table 2: Effect of different combined concentrations of fungicides mixtures on the size of inhibition zone of four (dg2, dg4, dg5 and dg6) wild-type strains of *P. digitatum*

	Zone of Inhibition (mm) = Mean±SD						
	Combined concentrations						
Fungicides combinations	50:50	100:100	100:500	500:1000	1000:1000	1000:2000	Strain
Beno/Can‡	42±9.9	46.5±7.8	47.5±9.2	50±11.3	50±8.5	52.5±10.6	dg4
	42.5±6.9	41.5±6.8	CI †	CI	CI	CI	dg2
	49±6.9	48.5±6.9	52±6.89	52.5±6.83	53.5±6.7	56.5±6.6	dg5
	40.5±8.5	43±8.6	45.5±8.7	48±8.2	47±8.3	51.5±10.6	dg6
Top/Vyd	34±2.6	38±3.9	42±4.5	46±6.6	52±7.3	58±5.2	dg4
	32±5.8	36±4.6	39±3.6	42±5.2	CI	CI	dg2
	31±4.5	33±3.8	39±2.9	39±2.4	CI	CI	dg5
	28±1.6	33±2.2	38±6.2	42±3.3	48±1.5	53±4.1	dg6
Vyd/Can	41±3.6	42.5±3.6	43±3.3	47.5±2.6	47.5±3.7	49±3.4	dg4
	42±2.6	47±2.2	47±1.8	51±1.1	53±3.4	56±4.5	dg2
	28±5.6	32±5.9	34±3.6	37±2.5	41±1.7	43±3.1	dg5
	27±2.8	31±5.3	34±3.3	39±4.2	43±2.5	46±2.2	dg6
Blin/Can	41±1.2	52±1.8	CI	CI	CI	CI	dg4
	43±5.3	53±3.6	56±2.1	CI	CI	CI	dg2
	41±4.6	41±6.2	41.5±1.5	41.5±3.4	41.5±2.4	42.5±6.2	dg5
	32±5.6	36±5.5	38±7.2	43±4.6	46±3.2	48±5.6	dg6
Top/Blin	40.5±5.2	42±3.2	42±2.6	45±3.3	46.5±2.2	48±4.1	dg4
	41±2.1	46±3.7	53±4.6	58±7.5	CI	CI	dg2
	38±1.5	41±4.6	44±2.3	49±3.4	52±1.8	56±5.4	dg5
	26±2.5	31±4.8	34±7.1	37±2.5	39±4.5	39±5.6	dg6
Top/Can	42±1.5	46±4.5	52±4.6	CI	CI	CI	dg4
	39±2.8	43±5.7	43.5±3.2	43.5±3.8	44.5±3.6	47±5.5	dg2
	37±4.7	39±5.7	41±2.5	43±3.4	43±6.1	47±3.5	dg5
	36±3.4	36±2.6	37±3.5	39±2.3	40±4.1	40.5±2.5	dg6
Blin/Vyd	29.5±1.5	33±2.5	38±4.3	41.5±3.6	45.5±3.5	49.5±7.2	dg4
	41±2.6	41.5±3.3	42.5±3.3	42.5±4.2	42.5±5.1	49±2.6	dg2
	29.5±1.4	32.5±1.3	38.5±2.2	41.5±4.3	43.5±7.1	45.5±3.6	dg5
	33.5±2.4	34±3.2	35±2.6	36.5±3.3	37±4.2	38.5±2.6	dg6

Values are means±SD of three independent experiments. ‡Beno, denotes for: Benomyl; Ran, denotes for Ranvil; Top, Topsin M; Vyd, Vydan; Can, Canvil; Blin, Blin exa fungicide. †Denotes for complete inhibition

0.05 level of significance) between the former combinations and the 1000:1000 and 1000:2000 μg mL^{-1} combinations on zones of strain dg5.

Effect of topsin/canvil mixture of fungicides on growth of tested fungal strains: The combination of TopsinM/Canvil had generated complete inhibition in strain dg4, at a combined concentration of 500:1000 μg mL^{-1} and higher (Table 2). In addition, the obtained results indicated significant difference ($p = 0.000$) between the combined concentrations of 50:50 μg mL^{-1} of TopsinM/Canvil fungicides and the rest of combined concentrations (excluding the combination of 100:100; $p = 0.210$) on the sizes of inhibition zones of strain dg4. There was significant difference between the combination of 50:50 and 1000:1000 ($p = 0.031$) or 1000:2000 ($p = 0.003$) μg mL^{-1} on sizes of strain dg5 zones. In contrast, the latter combinations had reflected no significant difference on sizes of inhibition zones in strains dg2 and dg6.

Effect of blin exa/vydan mixture of fungicides on growth of tested fungal strains: None of the combined concentrations of Blin exa/Vydan mixture of fungicides had generated complete inhibition, in any of the tested strains of *P. digitatum* (Table 2). However, all combined concentrations of Blin exa/Vydan had significantly affected the sizes of inhibition zones in strains dg4 and

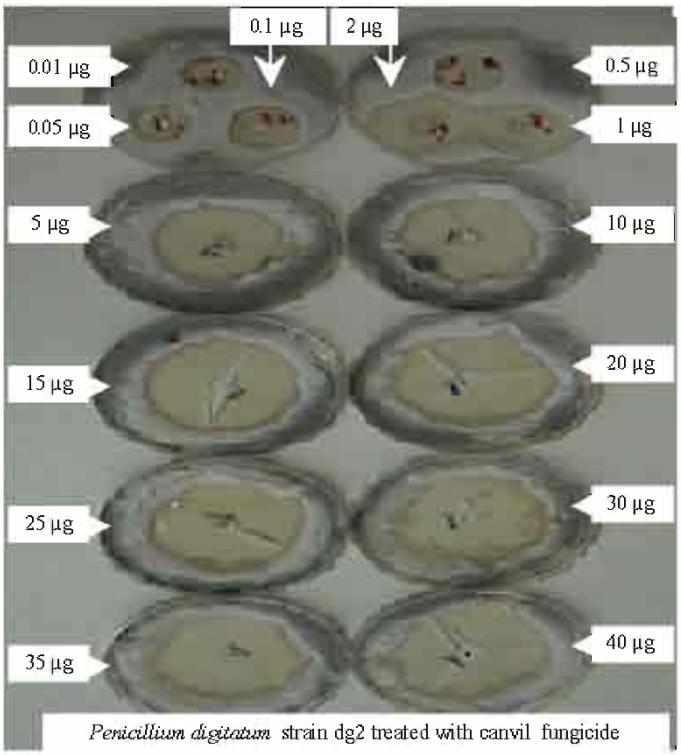

Fig. 1: *In vitro* study of the effect of 14 different concentrations (0.01; 0.05; 0.1; 0.5; 1.0; 2.0; 5.0; 10; 15; 20; 25; 30; 35 and 40 µg mL^{-1}) of the chemical fungicide canvil, on the size of inhibition zone of *Penicillium digitatum* strain dg2 using agar well diffusion method

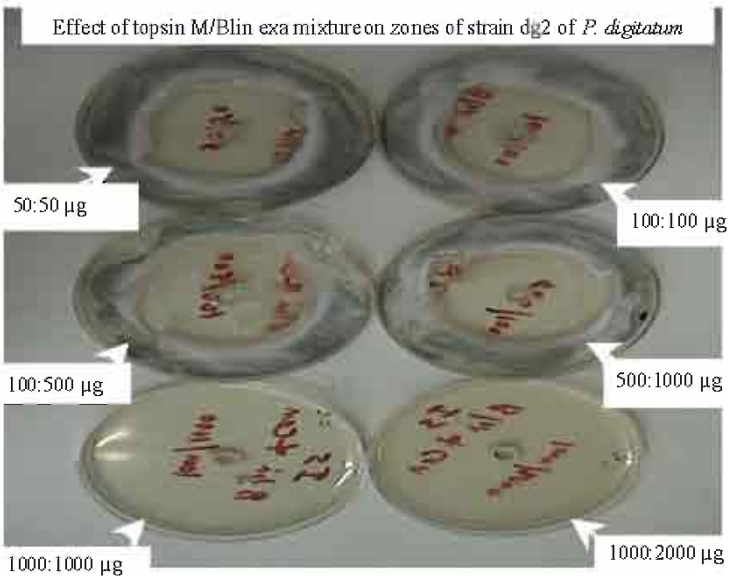

Fig. 2: The effect of different combined concentrations (50:50; 100:100; 100:500; 500:1000; 1000:1000 and 1000:2000 µg mL^{-1}) of topsin m and blin exa mixture of fungicides, on the size of inhibition zone of *Penicillium digitatum* strain dg2 using agar well diffusion method (*in vitro*). Complete inhibition of fungal growth was obtained at the highest combined concentrations

dg5. In contrast, there was no significant difference between the tested combined concentrations on zones of strain dg6 except between the combinations of 50:50 or 100:100 μg mL^{-1} and the combination of 1000:2000 μg mL^{-1}.

Effect of topsin/blin exa mixture of fungicides on growth of tested fungal strains: The combination of Topsin M/Blin exa had lead to complete inhibition of strain dg2 growth (Fig. 2) at combined concentrations of 1000:1000 and 1000:2000 μg mL^{-1} (Table 2). In addition, there was significant difference between the combined concentrations of 50:50 μg mL^{-1} and the rest of tested concentrations (except the combination of 100:100 μg mL^{-1}; p = 0.178) on the size of inhibition zones of strain dg2 (Fig. 2). Moreover, results indicated that there was significant difference between the mixture of 50:50 μg mL^{-1} and any of the following combinations: 500:1000 (p = 0.016); 1000:1000 (p = 0.001); 1000:2000 μg mL^{-1} (p = 0.000) on the sizes of strain dg5 zones of inhibition. There was significant difference between the combination of 50:50 μg mL^{-1} and any of the following combinations: 500:1000 (p = 0.016); 1000:1000 (p = 0.002); or 1000:2000 μg mL^{-1} (p = 0.002) on zones of strain dg6. In contrast, the tested combined concentrations had shown no significant difference on zones of strain dg4.

Effect of vydan/canvil mixture of fungicides on growth of tested fungal strains: None of the combined mixtures of Vydan/Canvil fungicides had generated complete inhibition, in any of the tested strains of *P. digitatum*. The maximum zones of inhibition obtained with this mixture have reached a value in the range of 43±3.1 to 56±4.5 mm against the tested strains at a combined concentration of 1000:2000 μg mL^{-1} (Table 2). However, there was significant difference between the mixture of 50:50 μg mL^{-1} and any of the following mixtures: 500:1000 μg mL^{-1} (p = 0.019); 1000:1000 (p = 0.005); or 1000:2000 μg mL^{-1} (p = 0.001) on zones of strain dg2. Furthermore, there was significant difference between the combination of 1000:2000 μg mL^{-1} and any of the 50:50 μg mL^{-1} (p = 0.002) or 100:100 μg mL^{-1} combinations (p = 0.021) on zones of strain dg5. Moreover, there was significant difference between the mixture of 50:50 μg mL^{-1} and the rest of tested combinations (excluding the combination of 100:100; p = 0.316) on zones of strain dg6. In contrast, significant difference was noticed between the combination of 100:500 μg mL^{-1} and the rest of tested combinations (except the combination of 500:1000 μg mL^{-1}; (p = 0.076)) on zones of strain dg6.

Effect of topsin/vydan mixture of fungicides on growth of tested fungal strains: This mixture had generated complete inhibition of growth in strains dg2 and dg5 at combined concentrations of 1000:1000 and 1000:2000 μg mL^{-1} (Table 2). In addition, the obtained results indicated that there was significant difference between the combined concentration of 50:50 μg mL^{-1} and any of the following combinations: 500:1000 (p = 0.004); 1000:1000 (p = 0.000); 1000:2000 (p = 0.000) on zones of strain dg4. Moreover, there was significant difference between the mixture of 100:500 μg mL^{-1} and the mixture of 1000:1000 μg mL^{-1} (p = 0.014) or 1000:2000 μg mL^{-1} (p = 0.000) on zones of strain dg4. There was significant difference between the mixture of 50:50 of Topsin M/Vydan and the rest of tested combinations (except the combination of 100:100 μg mL^{-1} (p = 0.241)) on zones of strain dg2 (Fig. 3). Also, there was significant difference between the combination of 100:500 μg mL^{-1} and the rest of combinations (excluding the combination of 500:1000 μg mL^{-1}; (p = 0.747)) on zones of dg2 strain (Fig. 3). Concerning the effect of Topsin M/Vydan mixture on zones of strain dg5, results indicated that there was significant difference between the mixture of 50:50 μg mL^{-1} and the combination of 1000:1000 (p = 0.000) or 1000:2000 μg mL^{-1} (p = 0.000). Also, there was significant difference between the combination of 50:50 μg mL^{-1} and the rest of combinations (except the combination of 100:100 μg mL^{-1}; (p = 0.289)) on zones of strain dg6 (Fig. 3). Furthermore, there was significant difference between the combination of 500:1000 μg mL^{-1} and the rest of combinations (except the combination of 1000:1000 μg mL^{-1}; (p = 0.236)) or between the combination of 1000:1000 μg mL^{-1} and the rest of combinations (except the combination of 1000:2000 μg mL^{-1}; (p = 0.350)) on zones of dg6 strain.

Effect of blin exa/canvil mixture of fungicides on growth of tested fungal strains: The combination of Blin exa/Canvil had generated complete inhibition, of fungal growth in strains dg4 and dg2 at combined concentrations of 100:500 and 500:1000 μg mL^{-1}, respectively (Table 2). There was significant difference between the combined concentrations of 50:50; or 100:100 μg mL^{-1} and the rest of tested concentrations on sizes of strain dg4 zones of inhibition. Concerning strain dg2 zones of inhibition, there was significant difference between the combination of 50:50 μg mL^{-1} and the rest of combinations. Also, there was significant difference between the combined concentration of 50:50 μg mL^{-1} and the combination of 1000:1000 μg mL^{-1}; (p = 0.038) or the combination of 1000:2000 μg mL^{-1} (p = 0.009) on zones of strain dg6. In contrast, there was no significant difference between all of the tested combinations on sizes of strain dg5 zones of inhibition. Results presented in Table 3 indicated that

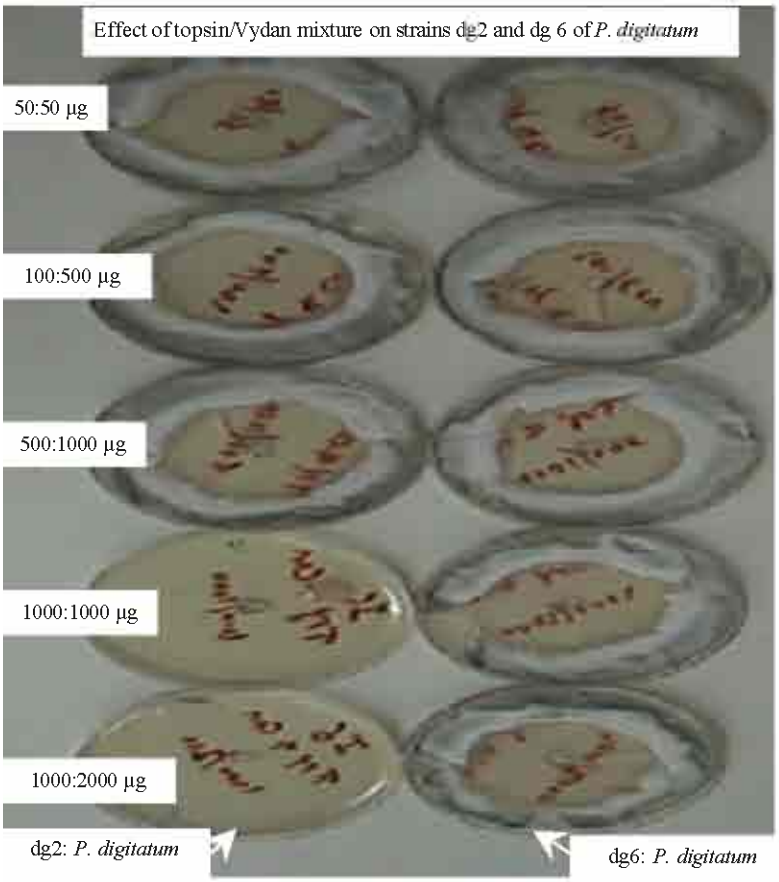

Fig. 3: *In vitro* study of the relationship between the combined concentrations (50:50; 100:500; 500:1000; 1000:1000; 1000:2000 µg mL^{-1}) of topsin m and vydan mixture of fungicides and the size of inhibition zone (mm) for two wild-type strains (dg2 and dg6) of *P. digitatum* using agar well diffusion method. Complete inhibition of growth was obtained in strain dg2 at the highest two combined concentrations

Table 3: Regression analysis for the relationship between the size of inhibition zone (mm) and the concentration (µg mL^{-1}) of combined fungicides on four wild-type strains of *P. digitatum* (*in vitro*)

Combined‡ Fungicides	Beno/Can (r) sig	Top/Can (r) sig	Vyd/Can (r) sig	Blin/Vyd (r) sig	Top/Blin (r) sig	Top/Vyd (r) sig	Conc (r) sig	Strain
Beno/Can							0.826*	dg2
							0.043	
Top/Can	0.715						0.916*	dg2
	0.110						0.010	
Vyd/Can	0.744	0.941**					0.982**	dg2
	0.090	0.005					0.000	
Blin/Vyd	0.480	0.776	0.741				0.750	dg2
	0.336	0.070	0.092				0.086	
Top/Blin	0.695	0.813*	0.911*	0.661			0.941**	dg2
	0.125	0.049	0.012	0.153			0.005	
Top/Vyd	0.587	0.759	0.859*	0.651	0.989**		0.886*	dg2
	0.220	0.080	0.028	0.161	0.000		0.019	
Blin/Can	0.786	0.772	0.928**	0.543	0.840*	0.766	0.925**	dg2
	0.064	0.072	0.008	0.266	0.036	0.076	0.008	
Combined Fungicides	Beno/Can (r) sig	Top/Can (r) sig	Vyd/Can (r) sig	Blin/Vyd (r) sig	Top/Blin (r) sig	Top/Vyd (r) sig	Conc (r) sig	Strain
Beno/Can							0.959**	dg4
							0.003	
Top/Can	0.878*						0.915*	dg4
	0.022						0.010	

Table 3: Continued

Combined Fungicides	Beno/Can (r) sig	Top/Can (r) sig	Vyd/Can (r) sig	Blin/Vyd (r) sig	Top/Blin (r) sig	Top/Vyd (r) sig	Conc (r) sig	Strain
Vyd/Can	0.939**	0.982**					0.961**	dg4
	0.006	0.000					0.002	
Blin/Vyd	0.955**	0.911*	0.956**				0.999**	dg4
	0.003	0.011	0.003				0.000	
Top/Blin	0.928**	0.940**	0.979**	0.971**			0.979**	dg4
	0.008	0.005	0.001	0.001			0.001	
Top/Vyd	0.936**	0.889*	0.945**	0.993**	0.980**		0.995**	dg4
	0.006	0.018	0.004	0.000	0.001		0.000	
Blin/Can	0.863*	0.786	0.793	0.860*	0.738	0.794	0.844*	dg4
	0.027	0.064	0.060	0.028	0.094	0.059	0.035	
Combined Fungicides	Top/Can (r) sig	Beno/Can (r) sig	Top/Vyd (r) sig	Blin/Vyd (r) sig	Top/Blin (r) sig	Vyd/Can (r) sig	Conc (r) sig	Strain
Top/Can							0.977**	dg5
							0.001	
Beno/can	0.962**						0.961**	dg5
	0.002						0.002	
Top/Vyd	0.790	0.833*					0.877*	dg5
	0.061	0.039					0.022	
Blin/Vyd	0.957**	0.943**	0.801				0.980**	dg5
	0.003	0.005	0.056				0.001	
Top/Blin	0.977**	0.953**	0.873*	0.973**			0.997**	dg5
	0.001	0.003	0.023	0.001			0.000	
Vyd/can	0.959**	0.927**	0.893*	0.971**	0.991**		0.996**	dg5
	0.002	0.008	0.017	0.001	0.000		0.000	
Blin/can	0.888*	0.964**	0.724	0.833*	0.714	0.995**	0.845*	dg5
	0.018	0.002	0.104	0.040	0.111	0.000	0.034	
Combined Fungicides	Top/Can (r) sig	Beno/Can (r) sig	Top/Vyd (r) sig	Blin/Vyd (r) sig	Top/Blin (r) sig	Vyd/Can (r) sig	Conc (r) sig	Strain
Top/Can							0.961**	dg6
							0.002	
Beno/Can	0.786						0.891*	dg6
	0.064						0.017	
Top/Vyd	0.951**	0.917					0.998**	dg6
	0.004	0.010					0.000	
Blin/Vyd	0.968**	0.889	0.988**				0.990**	dg6
	0.001	0.018	0.000				0.000	
Top/Blin	0.925**	0.861	0.962**	0.926**			0.960**	dg6
	0.008	0.028	0.002	0.008			0.002	
Vyd/Can	0.954**	0.885*	0.996**	0.953**	0.986**		0.999**	dg6
	0.003	0.019	0.000	0.003	0.000		0.000	
Blin/Can	0.947**	0.928**	0.998**	0.971**	0.982**	0.989**	0.993**	dg6
	0.004	0.008	0.000	0.001	0.000	0.000	0.000	

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). ‡: Beno, denotes for: Benomyl; Ran, denotes for Ranvil; Top, Topsin M; Vyd, Vydan; Can, Canvil; Blin, Blin exa fungicide

there is significant correlation between the combined concentrations, of six out of seven, (excluding Blin exa/Vydan mixture (r = 0.750 and p = 0.086) fungicide mixtures and the resulting zones of inhibition in strain dg2 of *P. digitatum*. In addition, there is significant correlation between the combined concentrations of Topsin M/Canvil and Vydan/Canvil (r = 0.941**; p = 0.005) or between Topsin/Canvil and Topsin M/Blin exa (r = 0.813*; p = 0.049) mixtures and the sizes of inhibition zones in strain dg2. Also, there was significant correlation between the combined concentrations of Vydan/Canvil and that of Topsin M/Blin exa (r = 0.911*; p = 0.012) or Topsin M/Vydan (r = 0.859*; p = 0.028) or even Blin exa/Canvil (r = 0.928**; p = 0.008) and the zones sizes of strain dg2 (Table 3). Moreover, results presented in table 3 indicated that there is significant correlation between the combined concentrations of the seven tested mixtures of fungicides and the resulting sizes of inhibition zones in strain dg4 of *P. digitatum*. Also, there is significant correlation between the combined concentrations of Benomyl/Canvil mixture of fungicides and the combined concentrations of the rest of tested mixtures, in relation to sizes of inhibition zones of strain dg4 (Table 3). Furthermore, there is significant correlation between the combination of Topsin M/Canvil and the rest of combined mixtures, except with Blin exa/Canvil mixture (r = 0.786; p = 0.064) on zones of strain dg4 (Table 3). Furthermore, there is significant correlation between combined concentrations of Blin exa/Vydan and the remaining combined mixtures, also, between the combined concentration of Topsin M/Blin exa and Topsin M/Vydan (p = 0.001), but not with Blin exa/Canvil (p = 0.094) on zones of strain dg4 (Table 3).

Moreover, results indicated that there is significant correlation between the combined concentration of the seven tested fungicide's mixtures and the sizes of inhibition zones in strain dg5. There is significant correlation between combined concentrations of Benomyl/Canvil or Blin exa/Vydan or even Vydan/Canvil and the remaining of tested fungicides mixtures (Table 3) on zones of strain dg5. However, the combination of Topsin M/Canvil was found to be significantly correlated with the remaining mixtures, except with Topsin M/Vydan ($r = 0.962$; $p = 0.061$) in terms of increasing the sizes of inhibition zones in strain dg5 (Table 3). In addition, the mixture of Topsin M/Vydan was significantly correlated with the rest of combinations except with Blin exa/Vydan ($r = 0.801$; $p = 0.056$) or with Blin exa/Canvil ($r = 0.724$; $p = 0.104$). Concerning the effect of such combinations on zones of strain dg6 results indicated that all combined concentrations of tested mixtures of fungicides are significantly correlated with sizes of inhibition zones of strain dg6. The mixtures of Vydan/Canvil; Topsin M/Blin exa; Blin exa/Vydan and Topsin M/Vydan were found to be significantly correlated with the remaining mixtures of fungicides, in terms of influencing the sizes of inhibition zones of strain dg6.

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 can be better evaluated otherwise, weakened or delayed mold growth could be mistakenly attributed to fungicidal effect resulting in false evaluation of efficacy. In addition, the modified medium has provided maximum zones of growth as compared to PDA medium. It is concluded from the obtained results that Canvil and Ranvil of the DMI family were the most effective fungicides against all strains (except dg6; MIC in the range of 2000 to 2700 μg mL^{-1}) where an MIC in the range of 5 to 150 μg mL^{-1} was required. More specifically, Canvil was the most effective against strains dg4 and dg5 where an MIC of 5 and 25 μg mL^{-1}, respectively was required for complete elimination of fungal growth. In contrast, the same fungicide was the least effective against strains dg6 and dg2 (MIC of 2700 and 50 μg mL^{-1} was required). However, Ranvil was the most effective against strain dg2 (MIC of 10 μg mL^{-1} was required). In contrast, results of *in vivo* study revealed that such inhibition was obtained against strains dg5 infecting lemon and dg6 infecting both fruit types when either Benomyl or Ranvil were used (G.J. Kanan, unpublished data). Concerning the mode of action for the DMI (demethylation inhibitors) group of fungicides such fungicides inhibit the enzyme C14-demethylase leading to depletion of ergosterol. Ergosterol serves as a bioregulator of membrane fluidity, symmetry and integrity in fungal cells and it is essential for the development of functional cell wall (Savocchia *et al*., 2004; Sugiura *et al*., 2006; Ma *et al*., 2006). The most common mechanisms by which pathogens can evolve fungicide resistance appears to be alternation of the target site (i.e. when the chemical shows single site of action). This presumably disrupts the binding of the fungus to the active site, rendering the chemical ineffective. In addition, DMI resistant strains may evolve due to mutations in the regulatory sequence of target genes which render the fungicide ineffective (McGrath, 2001; Survilienë and Dambrauskienë, 2006). However, if the evolution of fungicide resistance occurs more gradually then a shift in sensitivity to fungicide can be seen. Such type of resistance is a polygenic (Van Tuyl, 1977; Kalamarakis *et al*., 1987) where the result would appear due to cumulative or additive effects of several mutations in different genes (Georgopoulos and Skylakakis, 1986; De Waard and Van Nisterrooy, 1990; Penrose, 1993). In this kind of resistance (quantitative or continuous resistance) the fungal strain would revert back to be sensitive if the fungicide is no longer applied. Since variation in fungicide treatment during extensive recurrent use of fungicides of the same family (e.g., DMI members) would affect the selection pressure to evolve resistance to the applied fungicide. The doses that provide the most control of the disease also, provide the largest selection pressure to acquire resistance and lower doses will decrease the selection pressure. Additional mechanisms leading to DMI resistance in several important human and plant fungal pathogens have been studied intensively (De Waard, 1996) and these include (i) mutations in the DMI target enzyme, C14 alpha-demethylase (CYP51), leading to a decreased affinity of DMIs to the target protein (Dèlye *et al*., 1997, 1998) (ii) Over-expression or increased copy number of the (CYP51) gene, leading to increased production of the target enzyme (Schnable and Jones, 2001; Ma *et al*., 2006), (iii) Through efficient efflux where over-expression of ATP binding cassette (ABC) transporters with overlapping substrate specificities that function together in order to effectively pump toxic chemicals out of the cell. The strain here may develop resistance to several fungicides using the same mechanism (Hayashi *et al*., 2002; Zwiers *et al*., 2002). Further mechanisms may include acquisition of mutated enzymes that enable metabolism of the fungicide to harmless substance (detoxify the fungicide) or mutated enzyme which became not inhibited by the toxic effect of the fungicide. In addition, failure to activate the

fungicide, or deposition of fungicide in lipid droplets and change in pH leading to protonation of fungicide may cause resistance and this would agree with the suggestions of McGrath (2001). Concerning the effect of benzimidazoles members on tested strains Benomyl was the most effective against the most resistant strain dg6 (MIC of 40 μg mL^{-1} was required). In addition, Benomyl was the most effective for the treatment of all tested strains where an MIC in the range of 40 to 300 μg mL^{-1} was required to completely eliminate fungal growth. Furthermore, Benomyl fungicide has shown selective toxicity to several microorganisms, including fungi (Stringer and Wright, 2006) and had interfered with the intracellular transportation (Spencer *et al*., 1998). The Benomyl's mode of action is mainly related to its transformation into methyl-2-benzimidazole carbamate, which causes morphological distortions in germinating spores. This component is thought to alter mitotic cell division, through its binding to microtubules and inhibiting the β-tubulin assembly (Dalgie, 2005; Spencer *et al*., 1998). Resistance to benzimidazoles fungicides is suggested to be a kind of qualitative resistance, since it is resulted from conformational changes at the target site of the fungus (McGrath, 2001). 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 (i.e., the strain is either resistant or sensitive). This type of resistance leads to diversifying or disruptive selection of biotypes, because resistance in *Penicillium* isolates is evolved as a result of highly stable and persistent single gene mutation (Van Tuyl, 1977; Davidse and Flach, 1978). This kind of mutations will show high selection pressure during fungicidal application, but there is low selection pressure to remove them in the absence of the fungicide and this would agree with the suggestions of McGrath (2001). Concerning the effect of fungicide mixtures, the mixtures of Benomyl/Canvil and Blin exa/Canvil were the most effective against strains since either complete inhibition or the largest zones of inhibition were obtained at the least combined (50:50 μg mL^{-1}) concentrations. In contrast, the *in vivo* study indicated significant effect of fungicides mixtures on inhibition zones and that had mostly resulted in synergistic effects on strains infecting either lemon or orange fruit since complete inhibition of growth was obtained (G.J. Kanan, unpublished data). Recommended doses provided by researcher or manufacturers are normally designed to give the correct balance between controlling the disease and limiting the risk of resistance against the applied chemical. Higher doses increase selection pressure for single site mutations that confer resistance and by this, resistant strains will survive and propagate. However, lower doses greatly increase the risk of polygenic resistance as strains that are slightly less sensitive to the fungicide would survive and propagate. As a result, it is recommended to use a mixture of fungicides or alternate sprays of chemicals having different modes of action, because evolution of resistant strains may decrease due to the fact that the appearance of resistant isolates to one chemical will hopefully be killed by the other. However, if the applied fungicides are of the same family sharing the same mode of action or can be detoxified by the same mechanism then, fungal strain that evolve resistance to one fungicide it automatically generates resistance to others as a result of positive cross resistance. In contrast, negative cross resistance would result when resistance to one chemical class leads to an increase in sensitivity to a different chemical class of fungicides (e.g., DMI and TBZ classes). Because, resistance to two fungicides from different classes or families may result from separate mutational events i.e., two mutations would be required rather than just one to control disease rather than relying on single fungicide. These findings disagreed with the suggestion of Shaw (1993) who stated (depending on theoretical studies of resistance) 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.

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