PYRIMETHANIL TOLERANCE OF *BOTRYTIS CINEREA* ISOLATES FROM EGYPT AND HUNGARY

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Botryotinia fuckeliana (de Bary) Whetzel, anamorph *Botrytis cinerea* Pers.:Fr. causes severe damages in vineyards on the yield as well as on quality of grapes at harvest.[1] Pyrimethanil (anilinopyrimidine fungicide class) have been used to control grey mould, the disease caused by this pathogen.[2] The anilinopyrimidine were registered in Switzerland in 1995 to control grey mould in grapes.[3]

A dual mode of action has been described involving the inhibition of methionine biosynthesis[4] and the inhibition of hydrolytic enzyme secretion.[5] The anilinopyrimidines inhibit mycelial growth of fungus. The intensive use of anilinopyrimidines may result reduced sensitivity and loss of efficacy.[6-8] Recently, a reduction in sensitivity to anilinopyrimidines in *B. cinerea* strains was also reported from France.[9]

In the last 35 years *B. cinerea* developed resistance to virtually all the specific fungicides used to control grey mould. Field resistance to benzimidazoles, phenylcarbamates and dicarboximides was detected shortly after their introduction.[10] Therefore, an anti-resistance strategy should be introduced for current fungicides as well. This study was initiated to study the resistance of *B. cinerea* to one of the main important class of modern fungicides, the anilinopyrimidines viz. pyrimethanil. This information will be useful to provide alternative fungicide options to the growers.

Materials and Methods

Isolation of fungus

Twenty three *Botrytis cinerea* isolates were used to study their tolerance against the fungicide pyrimethanil (Mythos®). Twenty one isolates (Bc18, Bc22, Bc29, Bc30, Bc 31, Bc33, Bc34, Bc36, Bc39, Bc41, Bc43, Bc45, Bc46, Bc53, Bc54, Bc55, Bc57, Bc58, Bc59, Bc61 and Bc63) were isolated from diseased grape berries in vineyards located in Eger Grapevine Region (Northern-Hungary) in 2003. Two isolates (Bc3, Bc4) were isolated in Egypt from postharvest fruits of two different host plants, bean and strawberry, respectively. All strains were maintained on PDA.

Assay on mycelium

The mycelial growth of *B. cinerea* isolates were measured on *Botrytis* Minimal Agar (BMA) (glucose 10 g litre^{-1}, K_2HPO_4 1.5 g litre^{-1}, KH_2PO_4 2 g litre^{-1}, $(NH_4)_2SO_4$ 1.0 g litre^{-1}, $MgSO_4$

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(x $7H_2O$) 5.0 g litre^{-1}, agar 20g litre^{-1}). This solid medium amended with a range of the fungicide (pyrimethanil) concentrations (0.75, 0.375, 0.075 and 0.0075 mg litre^{-1}), were poured into 9 cm diameter Petri dishes. Each Petri dish was inoculated with an inverted mycelium plug (10 mm diameter), cut from the margin of a 5-day-old colony. Three replicates were used per treatments and incubation took place at room temperature in the dark. The mycelial growth rate was evaluated by the diameter of fungal colonies, measured daily for 5 days. Tolerance was determined by assessing the concentrations causing a 50% reduction in the growth rate (EC_{50}) by decreasing the relative growth rate (in percentage of control) against the logarithm of the fungicide concentrations.

Assay on conidia

The action of the fungicide pyrimethanil upon the spore germination and germ-tube elongation of *B. cinerea* was tested as follows. The nutrient medium (10g glucose, 2 g K_2HPO_4, 2 g KH_2PO_4, and 10 g agar in 1 litre water) was used in tests involving pyrimethanil. This medium amended with a range of pyrimethanil concentrations (0.75, 0.375, 0.075 and 0.0075 mg litre^{-1}) was poured into 9 cm in diameter Petri dishes. Then a conidial suspension of each *B. cinerea* isolate was dropped onto the surface of the agar plates. After 20-24 hrs incubation at room temperature in the dark, the percentage of spore germination (100-200 conidia for each treatment) and the length of germ-tubes (50-100 germinated conidia for each treatment) were estimated under a microscope supplemented with an ocular micrometer. As in the previous test, the dose response curves allowed determination of the concentrations causing 50% reduction in the germination of spores or in the length of germ tubes (EC_{50}).

Resistance level

Resistance levels were estimated as ratios EC_{50} of each isolate / EC_{50} of the most sensitive isolate.

Results

Effect of the fungicide pyrimethanil on mycelial growth of *Botrytis cinerea*

On the base of *in vitro* sensitivity of mycelial growth of *B. cinerea* isolates against pyrimethanil (anilinopyrimidine fungicide class), three phenotypes were detected. They could be characterized as sensitive (S) (Bc3, Bc29, Bc30, Bc33, Bc34, Bc36, Bc41, Bc43, Bc45), moderately resistant (MR) (Bc4, Bc18, Bc22, Bc31, Bc39, Bc46, Bc54, Bc57, Bc58, Bc61), and highly resistant (HR) (Bc53, Bc55, Bc59, Bc63). Their respective resistance levels (RL) were less than 2.09 (S), more than 2.09 and less than 10.09 (MR), more than 10.09 (HR). Such three phenotypes show EC_{50} values less than < 0.3 mg litre^{-1} (S), $0.3 < EC_{50} < 1.44$ mg litre^{-1} (MR) and $1.44 < EC_{50} < 3.725$ mg litre^{-1} (Table 1 and Fig. 1, 2). Hence, the frequency percentage of sensitive, moderately resistant and highly resistant isolates was 39.3, 38.96 and 21.74%, respectively.

Table 1 Effect of the fungicide pyrimethanil on the mycelial growth of *Botrytis cinerea* isolates

Isolates	$EC_{50\ (mg/l)}$	Resistance Level (RL)	Resistance
Bc3	0.2193	1.049	S
Bc4	0.3294	1.576	MR
Bc18	0.3797	1.817	MR
Bc22	0.3084	1.476	MR
Bc29	0.2126	1.017	S
Bc30	0.2444	1.169	S
Bc43	0.210	1.005	S
Bc45	0.219	1.048	S
Bc31	0.418	2.000	MR
Bc33	0.154	0.737	S
Bc34	0.204	0.976	S
Bc36	0.144	0.689	S
Bc39	0.413	1.977	MR
Bc41	0.273	1.306	S
Bc46	0.321	1.536	MR
Bc53	3.689	17.659	HR
Bc54	0.528	2.527	MR
Bc55	3.679	17.611	HR
Bc57	0.956	4.576	MR
Bc58	1.444	6.912	MR
Bc59	2.137	10.229	HR
Bc61	0.869	4.159	MR
Bc63	2.244	10.741	HR

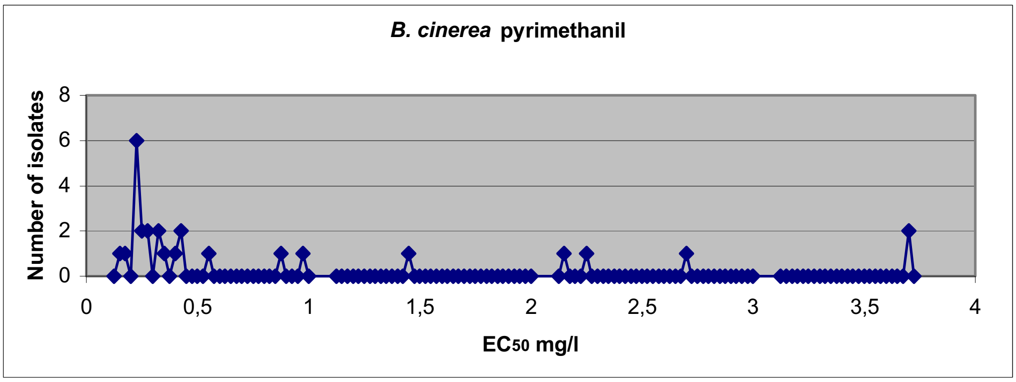

Fig 1 Cumulative frequency baseline distribution for pyrimethanil (EC_{50} 0 – 4 mg/l) and *Botrytis cinerea*

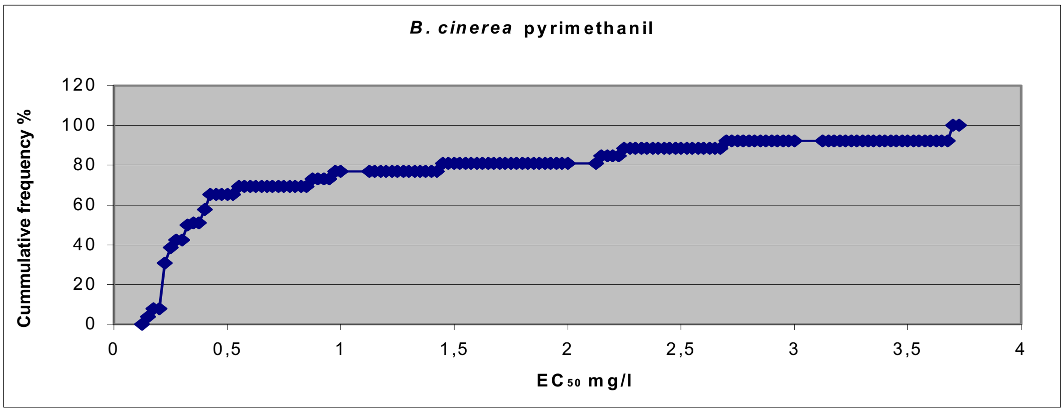

Fig 2 Isolate number baseline distribution for pyrimethanil (EC_{50} 0 – 4 mg/l) and *Botrytis cinerea*

Effect of the fungicide pyrimethanil on spore germination of *Botrytis cinerea*

The effect of pyrimethanil on spore germination shows that all isolates of *B. cinerea* were sensitive (S) except four ones, two of which (Bc18, Bc22) were moderately resistant (MR) and the other two isolates (Bc33, Bc59) were highly resistant (HR). Resistance levels (RL) for sensitive (S), moderately resistant (MR) and highly resistant (HR) isolates were less than 1.36, more than 1.36 and less than 1.82, more than 1.82, respectively. On the other hand, their respective EC_{50} values were less than 0.3 mg litre^{-1} (S), $0.3 < EC_{50} < 0.4$ mg litre^{-1} (MR), and $0.4 < EC_{50} < 0.5$ mg litre^{-1} (HR) (Table 2 and Fig. 3, 4). Hence, the frequency percentage of sensitive, moderately resistant and highly resistant isolates was 82.6, 8.7 and 8.7%, respectively.

Table 2 Effect of the fungicide pyrimethanil on spore germination of *Botrytis cinerea* isolates

Isolates	$EC_{50\ (mg/l)}$	Resistance Level (RL)	Resistance
Bc3	0.2553	1.165	S
Bc4	0.2219	1.013	S
Bc18	0.326	1.488	MR
Bc22	0.3799	1.734	MR
Bc29	0.1998	0.912	S
Bc30	0.2264	1.033	S
Bc31	0.1998	0.912	S
Bc33	0.4151	1.895	HR
Bc34	0.2115	0.965	S
Bc36	0.2038	0.930	S
Bc39	0.2602	1.188	S
Bc41	0.2102	0.959	S
Bc43	0.2038	0.930	S
Bc45	0.2143	0.978	S
Bc46	0.202	0.922	S
Bc53	0.2885	1.317	S
Bc54	0.2219	1.013	S
Bc55	0.2219	1.013	S
Bc57	0.2059	0.940	S
Bc58	0.2038	0.930	S
Bc59	0.5336	2.436	HR
Bc61	0.2097	0.957	S
Bc63	0.2097	0.957	S

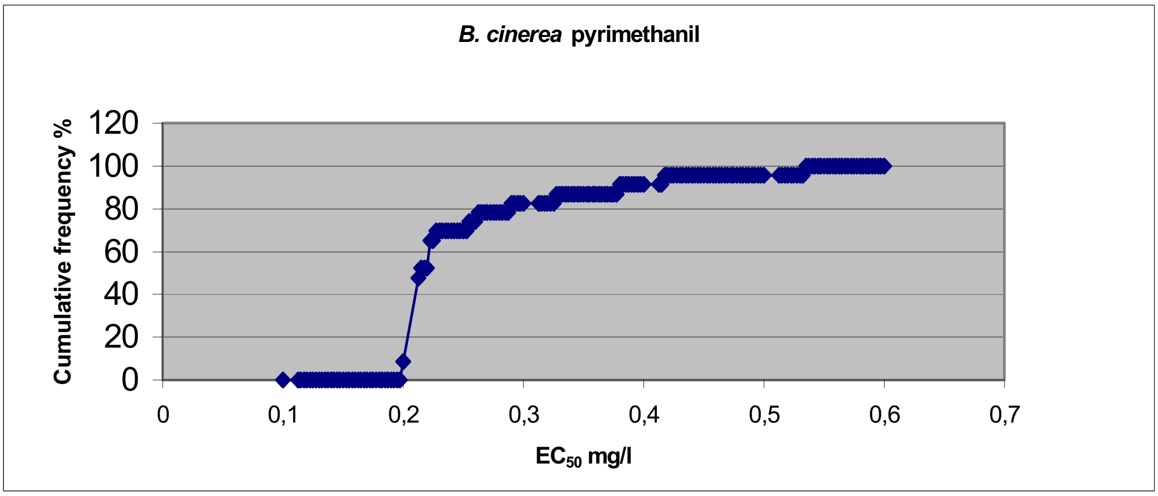

Fig 3 Cumulative frequency baseline distribution for pyrimethanil (EC_{50} 0 – 0.7 mg/l) and *Botrytis cinerea*

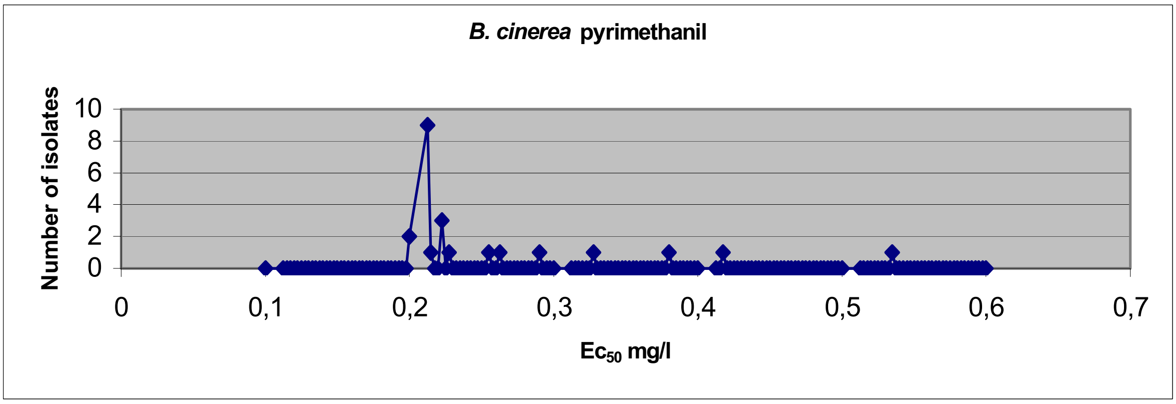

Fig. 4 Isolate number baseline distribution for pyrimethanil (EC_{50} 0 – 0.7 mg/l) and *Botrytis cinerea*

Effect of the fungicide pyrimethanil on germ-tube elongation of *Botrytis cinerea*

The effect of pyrimethanil on germ-tube elongation of *B. cinerea* showed four phenotypes which were characterized as sensitive (S) (Bc43, Bc45), low resistant (LR) (Bc3, Bc4, Bc18, Bc30, Bc31, Bc33, Bc34, Bc41, Bc55, Bc61), moderately resistant (MR) (Bc29, Bc53), and highly resistant (HR) (Bc22, Bc36, Bc39, Bc46, Bc54, Bc57, Bc58, Bc59, Bc63). Their respective resistance levels (RL) were less than 1.58 (S), more than 1.58 and less than 3.96 (LR), more than 3.96 and less than 5.28 (MR), and more than 5.28. On the other hand, their EC_{50} values were less than 0.06 mg $litre^{-1}$ (S), 0.06 < EC_{50} < 0.15 mg $litre^{-1}$ (LR), 0.15 < EC_{50} < 0.2 mg $litre^{-1}$ (MR) and 0.2 < EC_{50} < 7.85 (Table 3 and Fig. 5, 6). Hence, the frequency percentage of sensitive, low resistant, moderately resistant and highly resistant isolates was 8.69, 43.47, 8.69 and 39.13%, respectively.

Table 3 Effect of the fungicide pyrimethanil on germ-tube elongation of *Botrytis cinerea* isolates

Isolates	$EC_{50\ (mg/l)}$	Resistance Level (RL)	Resistance
Bc3	0.0688	1.8177	LR
Bc4	0.1212	3.2021	LR
Bc18	0.099	2.6155	LR
Bc22	0.0917	2.4227	LR
Bc29	0.1998	5.2787	MR
Bc30	0.0917	2.4227	LR
Bc31	0.0663	1.7516	LR
Bc33	0.113	2.9854	LR
Bc34	0.1023	2.7027	LR
Bc36	0.2079	5.4927	HR
Bc39	0.2079	5.4927	HR
Bc41	0.0851	2.2483	LR
Bc43	0.0474	1.2523	S
Bc45	0.0283	0.7476	S
Bc46	0.2277	6.0158	HR
Bc53	0.1904	5.0303	MR
Bc54	0.2153	5.6882	HR
Bc55	0.0685	1.8097	LR
Bc57	0.2218	5.8599	HR
Bc58	0.2282	6.0290	HR
Bc59	0.2144	5.6644	HR
Bc61	0.0792	2.0924	LR
Bc63	0.2709	7.1571	HR

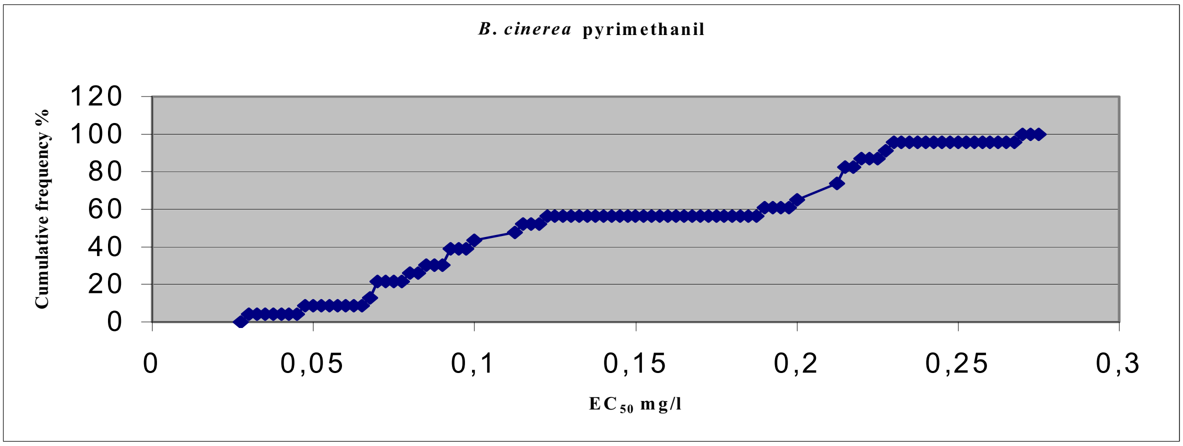

Fig. 5 Cumulative frequency baseline distribution for pyrimethanil (EC_{50} 0 – 0.3 mg/l) and *Botrytis cinerea*

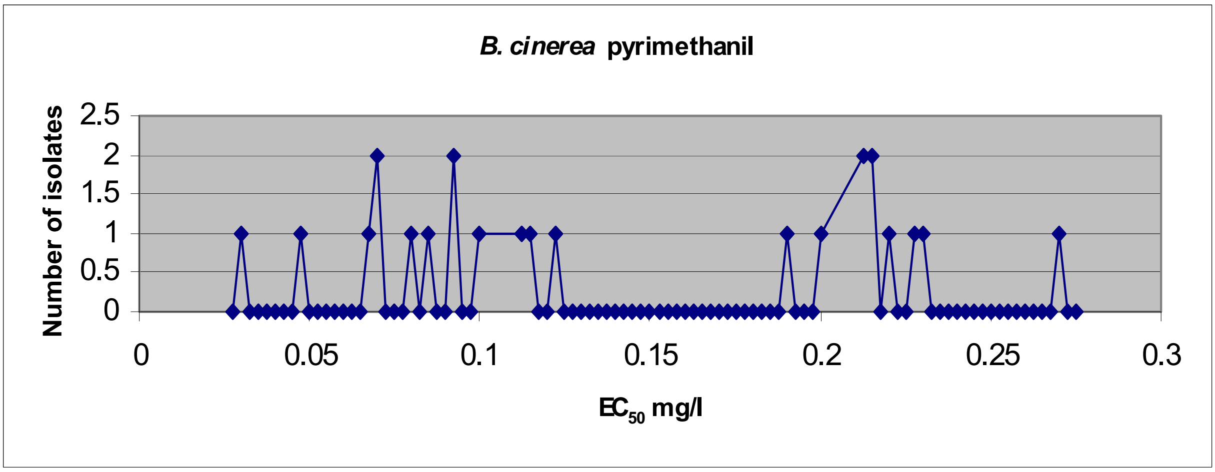

Fig. 6 Isolate number baseline distribution for pyrimethanil (EC_{50} 0 – 0.3 mg/l) and *Botrytis cinerea*

Discussion and Conclusions

The development of tolerance to fungicides is mainly due to selection pressure exerted on resident populations of the pathogen by heavy applications of a single fungicide.[11] When fungal populations are subjected to fungicides, sensitive individuals are selectively killed. Those individuals that harbour a mutation for tolerance may multiply without computation from the normal population.[12] This new tolerant population then becomes dominant, and crop losses can occur despite of continued fungicide application. The pyrimethanil tolerance by *B. cinerea* usually develops over time. Use of single fungicide is discouraged because the pathogen rapidly develops tolerance.[13]

Our results revealed an increase in frequency percentage of isolates resist to pyrimethanil against mycelial growth and germ-tube elongation. The frequency percentages of moderately resistant isolates (MR) and highly resistant ones (HR) against mycelial growth were 38.96 and 21.74%, respectively. In parallel, the frequency percentage of low, moderately and highly resistant isolates against germ-tube elongation were 43.47, 8.69 and 39.13%, respectively **(**Fig. 1 and 5). Such results support those which were experienced that anilinopyrimidines inhibited the germ-tube elongation and mycelial growth of wild type strains (AniS) at low concentrations (EC_{50} values below 0.1 mg litre^{-1}).[14] On the other hand, a decrease in frequency percentage of isolates resisting to anilinopyrimidines, pyrimethanil, against spore germination was recorded. The frequency percentages of moderately and highly resistant isolates against spore germination were 8.7 and 8.7% (Fig. 3). This observation coincides with those variable results of anilinopyrimidine towards the spore germination.[15] Consequently, this stage of fungal development should not be taken into consideration in further studies involving anilinopyrimidine-resistant strains.

This effect on pyrimethanil efficacy might be due to the frequent treatments of pyrimethanil which result increase of the resistant population, which may eventually lead to failure of disease control. Altering usage of different classes of fungicides that have different modes of action and high activity against *B. cinerea* together with limiting the applied number of fungicide treatments might be suggested as an effective way to control *B. cinerea*.

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References

1 Rüegg J, Lauber HP, Siegfried W, Viret O and Hilber UW, Experiences with anilinopyrimidines in Switzerland. *Pestic Outlook* **8**:28-33 (1997).

2 Leroux P, Recent developments in the mode of action of fungicides. *Pestic Sci* **47**:191-197 (1996).

3 Baroffio CA, Siegfried W and Hilber UW, Long-term monitoring for resistance of *Botryotinia fuckeliana* to anilinopyrimidine, phenylpyrrole and hydroxyanilide fungicides in Switzerland. *Plant Dis* **87**:662-666 (2003).

4 Pillonel C and Meyer T, Effect of phenylpyrroles on glycerol accumulation and protein kinase activity of *Neurospora crassa*. *Pestic Sci* **49**:229-236 (1997).

5 Miura I, Kamakura T, Maeno SS, Hayashi S and Yamaguchi I, Inhibition of enzyme secretion in plant pathogen by mepanipyrium a novel fungicide. *Pestic Biochem Physiol* **48**:222-228 (1994).

6 Hilber UW and Schüepp H, A reliable method for testing the sensitivity of *Botryotinia fuckeliana* to anilinopyrimidines *in vitro*. *Pestic Sci* **47**:241-247 (1996).

7 Hilber UW and Hilber-Bodmer M, Genetic basis and monitoring of resistance of *Botryotinia fuckeliana* to anilinopyrimidines. *Plant Dis* **82**:497-500 (1998).

8 Forster B and Staub T, Basis for use strategies of anilinopyrimidines and phenylpyrrole fungicides against *Botrytis cinerea*. *Crop Prot* **15**:529-537 (1996).

9 Leroux P, Chapeland F, Desbrosses D and Gredt M, Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyard. *Crop Prot* **18**:687–697 (1999).

10 Leroux P, Progress and problems in the control of *Botrytis cinerea* in grapevine. *Pestic Outlook* **6**:13-19 (1995).

11 Ogawa JM, Manji BT and Chastagner GA, Field problems due to chemical tolerance of plant pathogens. *Proc Am Phytopath Soc* **3**:47-53 (1976).

12 Dekker J, Acquired resistance to fungicides. *Ann Rev Phytopathol* **14**:405-428 (1976).
13 Miller MW, Fletcher JT, Benomyl tolerance in *Botrytis cinerea* from greenhouse crops. *Trans Brit Mycol Soc* **62**:99-103 (1974).
14 Leroux P, Chapeland F, Giraud T, Brygoo Y and Gredt M, Resistance to sterol biosynthesis inhibitors and various other fungicides in *Botrytis cinerea*. in: *Modern fungicides and antifungal compounds II.* ed by Lyr H, Russel PE, Dehne HW and Sisler HD, Intercept, Andover, UK, pp 297-303 (1999).
15 Leroux P and Lagouarde P, Etude in vitro de la sensibilite de *Botrytis cinerea* vis a vis du pyrimethanil. in: 4th International Conference on Plant Disease. ANPP, Paris, France, pp 1409-1416 (1994).

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Pyrimethanil (anilinopyrimidine group) fungicides have been used widely to control grey mould, *Botrytis cinerea* fungus causes severe damages in vineyards. Resistance development in *B. cinerea* populations for pyrimethanil resulted decreasing the effectiveness on these areas.
Twenty three isolates of *B. cinerea* were isolated from Hungary and Egypt, and presence of fungicide resistance development was studied *in vitro*. Different decreasing effects of pyrimethanil on mycelial growth, spore germination, and germ-tube elongation were observed. Effect on mycelial growth showed that 39.13% (9 isolates), 43.48% (10 isolates), and 17.39% (4 isolates) of the tested isolates were sensitive, moderately and highly resistant, respectively. While the effect on spore germination showed that 82% (19 isolates), 8.69% (2 isolates), 8.69% (2 isolates) were sensitive, moderately and highly resistant, respectively. On the other hand, the effect on germ-tube elongation showed 8.69 (2 isolates), 43.47 (10 isolates), 8.69 (2 isolates) and 39.13% (9 isolates) were sensitive, low resistant, moderately resistant and highly resistant, respectively.
The increased presence of pyrimethanil fungicide resistance of *B. cinerea* strains in both countries initiates to take into consideration the decreased fungicide effectiveness in the anti-resistance strategies as well as providing alternative fungicide options.