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FINAL REPORT

PROJECT NO. I-750-84

Forecasting, Detection and Evaluation of Resistance to Ergosterol Biosynthesis-Inhibiting Fungicides in Pathogens of Apple Scab, Pear Scab

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Standard BARD Cover Page for Scientific Reports

Date: 14 March 1990

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Bet Dagan, ISRAEL

BARD project No. 1 750-84

Title

Forecasting, Detection and Evaluation of Resistance to Ergosterol

Biosynthesis-inhibiting Fungicides in Pathogens of Apple scab and pear scab.

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Project's starting date: 13 November 1985

Type of Report: 1st Annual 2nd Annual Final X

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630.72 ; 632.952 ; 634.11 ; 632.44 ; 634.13

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Table of Contents

	Page
Abstract	2
Objectives	4
Report	6
Cooperation & Achievements	15
Publications	

Forecasting, Detection and Evaluation of Resistance to Ergosterol Biosynthesis-inhibiting Fungicides in Pathogens of Apple Scab and Pear Scab.

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Abstract

Strains of Venturia inaequalis with reduced sensitivity to EBI could be detected by their ability to grow on PDA amended with 1.5 µg/ml of fenarimol.

Monitoring for strains of V.inaequalis less sensitive to EBI fungicides, revealed a few isolates from orchards in Michigan, that grew on medium containing 1.5 µg/ml fenarimol.

No strains with reduced sensitivity to EBI fungicides were detected in apple orchards in Israel, although commercial apple orchards have been treated with EBI fungicides for almost ten years and failures in the control of apple scab were noted.

Isolates of V.pirina were obtained from pear orchards in Michigan and Israel. All isolates grew well on media containing 1.5 and 3.0 µg/ml fenaromil and slowly on media containing 6.0 µg/ml fenarimol. Experiments with isolates of V.pirina obtained from pear trees which had never been treated with fungicides, showed that races 1, 3, 4 and 5 of V.pirina were more sensitive to EBI than race 2 which prevails in the commercial pear orchards in Israel.

From the analysis of crosses between EBI sensitive and less sensitive isolates of V.inaequalis and among the less sensitive strains, it was concluded that reduced sensitivity to the EBI fungicides was determined by a single Mendelian gene.

A study on the genetics of negatively correlated cross-resistance to diethofencarb in benomyl-resistant V. inaequalis indicated linkage between the gene for sensitivity to diethofencarb and the allele for very high resistance to benomyl. Examination of progeny from crosses between sensitive and resistant phenotypes indicated that sensitivity to diethofencarb was determined by a single Mendelian gene.

Objectives

- a. Determination of threshold of sensitivity to EBI fungicides in Venturia inaequalis, V. pirina, Podosphaera leucotricha, Uncinula necator, and Sphaerotheca fuliginea.
- b. Detection of EBI-resistant strains in pathogen populations.
- c. Mutagenesis and selection of EBI-resistant mutants of Venturia spp.
- d. Characterization of the EBI-resistant strains.
- e. Competitiveness of EBI resistant Venturia strains.
- f. Genetics of EBI resistance in Venturia spp.
- g. Practical tactics for avoiding or delaying resistance to EBI.

The original proposal included a proposed budget according to our estimations of the needs. The reduced budget approved by BARD, made it necessary to change our initial research program accordingly. Therefore, our research activities were focused on the two scab pathogens Venturia inaequalis and V. pirina.

Due to the fact that we did not detect strains less-sensitive to EBI fungicides in the populations of V. inaequalis and V. pirina in Israel, we were not able to study competitiveness. Inoculation of untreated apple and pear plants in the greenhouse by mixtures of EBI-resistant and sensitive isolates had to be used for fitness evaluation. Since we were concerned to avoid the import and spread of foreign less-sensitive strains, such tests could not be conducted.

Practical tactics for avoiding or delaying the development of less-sensitive strains could not be worked out within the time limits of this project. Although spray tactics such as fungicide mixtures and alternations were used in our fungicide tests in apple and pear orchards, we have not been able to draw conclusions on tactics, as long as less-sensitive strains did not appear.

In Michigan, Dr. Jones was not able to detect less-sensitive strains again in the research orchard at MSU, where a few isolates with reduced sensitivity to EBI fungicides had been detected in 1986. This may be due to low scab pressure in the following years.

Description of the research results

Monitoring for less-sensitive strains of *Venturia inaequalis*. to monitor for strains of *V. inaequalis* with reduced sensitivity to the EBIs, germinated spores from lesions on leaves from three experimental apple orchards in Michigan were tested on media amended with 0.5 ug/ml bitertanol and 1.5 ug/ml fenarimol. These concentrations prevented colony formation by germinated spores of sensitive strains but not of less-sensitive strains (Stanis and Jones, 1985, *Phytopathology* 75:1098-1101).

Some 15 of 58 conidia from one orchard produced colonies on fungicide amended media whereas all samples from other orchards yielded only sensitive isolates. This orchard has received experimental treatments with EBIs for 8 years including 1986. Less-sensitive strains were detected in trees sprayed with curative schedules but not on trees sprayed on a regular 7-day schedule. The frequency of less-sensitive strains in the population was low. We detected them because a few leaf lesions were never inhibited by repeated sprays with EBIs.

Cultures with reduced sensitivity to EBIs were tested by transferring mycelial bits to media containing a concentration series of fenarimol. All 15 isolates grew at 1 ug/ml fenarimol while sensitive isolates failed to grow. Among the 15 isolates, 11 grew at 3 ug/ml fenarimol and 4 grew at 6 ug/ml fenarimol. These isolates grew at higher concentrations than isolates with reduced sensitivity from Germany. Four isolates with reduced sensitivity, when tested for cross-resistance to pyrifenoX (Ro 15-1297), bitertanol (Baycor), flusilazol (Nustar), myclobutanil (RH-3866), and SAN-617, showed reduced sensitivity to each fungicide. Also, three of these isolates exhibited low resistance, nine exhibited high resistance, and five were sensitive to benomyl.

Determination of threshold of sensitivity to EBI fungicides. In the first year of the project, it was determined that strains of Venturia inaequalis with reduced sensitivity to EBI's could be detected based on their growth on Difco potato dextrose agar (PDA) containing 1.5 ug of fenarimol per milliliter. In 1987, the response of 856 single ascospore and conidia isolates from 11 commercial apple orchards in Michigan, USA, and 1217 single conidia isolates from 10 commercial apple orchards in Israel were evaluated on fungicide amended media. No strains with reduced sensitivity were detected. In four of the orchards in Michigan, fenarimol was used for the first time in 1987 with poor results. These control failures could not be attributed to the presence of less-sensitive strains. The data will serve as a base line of future years.

Nine isolates of V. pirina obtained from one Bartlett pear orchard in Michigan and 412 conidial isolates from two Spadona pear orchards in Israel were tested for sensitivity to 1.5, 3.0, and 6.0 ug/ml of fenarimol. All isolates grew well on media containing 1.5 and 3.0 ug/ml fenarimol and slowly on media containing 6.0 ug/ml fenarimol. Field strains of V. inaequalis failed to grow on medium containing 1.5 ug/ml fenarimol. These results indicate that wild-type strains of V. inaequalis are normally more sensitive to EBI's than wild-type strains of V. pirina.

Mycelial suspensions (four replicates of 0.02-ml drops) of monoconidial cultures were pipetted onto PDA amended or not with 0.0625-8 ug/ml a.i. of bitertanol. After 3-week incubation at 18°C, colony diameter was measured, and mycelial growth was rated as dense or sparse.

Two parameters were used to express bitertanol sensitivity of each isolate: (1) ED₅₀ was estimated by calculating the fungicide concentration which reduced the increase in colony diameter by 50% compared to control plates. By this method, mycelial density is not considered.

(2) The transition from dense to sparse mycelial growth is presented as the highest concentration which allowed dense growth, regardless of colony diameter.

The results are presented in Fig. 1.

Genetics of EBI resistance in *Venturia inaequalis*. Monoconidial isolates MSU-20, MSU-33, MSU-37, and MSU-40 of *V. inaequalis* with reduced sensitivity to fenarimol were crossed with sensitive isolates MSU-3, WB, W10, and GAVIN 43. Isolates with an MSU designation were collected from an experimental orchard near East Lansing, MI, where EBI fungicides were tested for 8 years. Except for MSU-3, each sensitive isolate was from an orchard where EBI fungicides had never been used. Less-sensitive isolates 86R2 and BF1R were crossed with sensitive isolates WB, MSU-3, and WRR. The less-sensitive isolates were from a commercial orchard in France and were supplied by T.S. Thind and J.M. Olivier. Additional crosses were also made, including crosses between strains with reduced sensitivity, but these crosses failed to produce pseudothecia with ascospores. For each fertile pairing, 8 to 10 pseudothecia were removed from the agar substrate and crushed in 1 ml of sterile distilled water in a 7-ml tissue grinder. Portions of the resultant ascospore suspensions were plated on PDA. After 24 hr, single germinated ascospores were transferred to individual culture tubes. After 2-3 weeks of growth, bits of mycelium were taken from the margin of the colony and inoculated on PDA amended with 1.5 ug/ml of fenarimol.

Single-ascospore isolates derived from fertile pairings were readily separated into two groups based on growth on PDA containing 1.5 ug/ml of fenarimol. When crosses were made between sensitive isolates, isolates MSU-3, WB, and W10 were compatible with MSU-12 and isolate MSU-3 was compatible with MSU-4. From the four isolates, there were 752 sensitive progeny out of 761

ascospores tested. Although nine ascospores were recorded as less-sensitive, their growth was weak.

To determine whether reduced sensitivity was controlled by more than one gene, 2037 ascospore were tested from 12 crosses between sensitive and less-sensitive isolates. In all but one cross, sensitive progeny were found in a 1:1 ratio with less-sensitive progeny. Similar results were obtained with the isolates from the USA and from France. The only case of deviation from the 1:1 ratio was that of cross MSU-20 x GAVIN 43. Whether some ascospores from this cross had aborted or were not viable was not determined. GAVIN 43 differed from the other sensitive isolates in being resistant to both dodine and benomyl.

None of the pairings between less-sensitive isolates produced progeny. In retrospect, it was determined that all less-sensitive strains from the USA that were used in the study were of the same mating type. Therefore, less-sensitive progeny from crosses between less-sensitive and sensitive strains were crossed with less-sensitive isolates. Isolates BF1R and 86R2 were compatible with four of the less-sensitive progeny (Table 1). From the four crosses, there were 790 ascospores tested. Because no sensitive progeny were detected, we conclude that the mutations for reduced sensitivity to EBI fungicides occurred at the same locus. We also noted that 25 (3%) of the 790 less-sensitive progeny had much higher growth rates than either parent. Whether these strains were more fit or were more resistant was not determined. From the analysis of crossed between sensitive and less-sensitive isolates, and among the less-sensitive isolates, it was concluded that, in the isolates studies, reduced sensitivity to the EBI fungicides was determined by a single gene.

Biochemistry of ergosterol biosynthesis in *Venturia inaequalis*. The mode of action of the C-14 demethylation-inhibiting fungicides in *V. inaequalis* was.

investigated by labeling neutral lipids in mycelium with (U-¹⁴C) acetate and then separating the lipid components using thin-layer chromatography. In both a sensitive and a less-sensitive isolate treated with fenarimol, the amount of radioactivity in sterols migrating to the region of C-4 desmethyl sterols (ergosterol) was markedly reduced compared to untreated mycelium. In addition, radioactivity in the region of C-4 methyl and dimethyl sterols increased. The effects of fenarimol on incorporation of ¹⁴C-acetate into lipids were evident as early as 2 hr and at fungicide concentrations of 0.2-2.0 ug/ml. Sterol patterns in the two isolates did not differ qualitatively. These preliminary results suggest that the fungitoxic action of fenarimol in V. inaequalis is due to inhibition of sterol demethylation. Also, reduced sensitivity is probably not related to the nature of sterols present, but possibly due to some other adaptive mechanism.

Table 1. Segregation of single-ascospore isolates of Venturia inaequalis into less-sensitive and sensitive phenotypes according to growth on potato-dextrose agar amended with fenarimol at 1.5 ug/ml.

Cross	Progeny tested (no.)			Chi-square values (1:1) ^a
	Total	Phenotype		
		Less- sensitive	Sensitive	
Sensitive x sensitive				
MSU-3 x MSU-12	209	1	208	
WB x MSU-12	187	0	187	
MSU-3 x MSU-4	163	2	161	
W10 x MSU-12	202	6	196	
Totals	761	9	752	
Less-sensitive x sensitive				
MSU-20 x MSU-3	141	70	71	0.01
MSU-33 x MSU-3	40	21	19	0.10
MSU-40 x WB	183	91	92	0.01
MSU-37 x W10	232	113	119	0.16
MSU-20 x GAVIN 43	156	43	113	34.40
MSU-37 x MSU-3	200	92	108	1.28
MSU-33 x W10	157	89	68	2.80
86R2 x WB	194	105	89	1.38
86R2 x MSU-3	214	115	99	1.99
BF1R x WB	132	67	65	0.03
BF1R x WRR	206	112	94	1.57
BF1R x MSU-3	182	96	86	0.55
Totals	2,037	1,014	1,023	0.34
Less-sensitive x less-sensitive				
BF1R x (86R2 x WB39)	169	169	0	
BF1R x (BF1R x MSU #51)	187	187	0	
BF1R x (BF1R x MSU #52)	165	165	0	
86R2 x (86R2 x MSU #33)	269	269	0	
Totals	790	790	0	

^aThe expected chi-square value, $P = 0.01$, is 6.63

Fig 1. Effect of bitertanol on growth of *Venturia pirina* & *V. inaequalis* (method 1)

Fig 2. Effect of bitertanol on growth of *Venturia pirina* & *V. inaequalis* (method 2)

Fig.1 (cont.)

Method 1 : ED 50

Method .2 : dense --> sparse transition

A: V.pirina, race 2, from pear cv. 'Spadona'.

B: V.pirina, races 4 and 5.

C: V.inaequalis from apple cv. 'Anna'.

D: v.inaequalis from apple cv. 'Red Delicious'.

A,C and D: Isolates from commercial orchards treated with EBI for several years.

B: Isolates from untreated pear trees.

Populations A,B,C,D were significantly different in a multiple range test ($\underline{p}=0.01$) by both method 1 and 2. Using method 1, isolates MSU 33-1 and B-2-1 were less sensitive to bitertanol than the V.inaequalis isolates from populations C and D, whereas isolates MSU 18-1 and B-9-2 fell within the range of sensitivity of these populations. Using method 2, the sensitivity of the isolates mentioned above did not differ significantly from that of populations C and D.

Description of cooperation and evaluation of the research achievements.

The project collaborators were able to meet several times to discuss the research activities. Dr. Shabi spent two weeks of a visit with Dr. Jones at the end of the first year of the project (September 1986). During the visit joint research discussions and coordination of the research plans were fruitful and beneficial. Dr. Jones had planned to spend several weeks in Israel and to be involved in the research activities, mainly in competitiveness tests. Since such tests were not conducted due to the lack of less-sensitive strains in Israel, Dr. Jones' visit was cancelled.

The cooperation between the researchers was beneficial to both countries. The fact that even after several years of intensive use of EBI fungicides in commercial orchards in Israel, crop losses due to resistance had not been evident, indicated that there is no immediate resistance risk in the use of EBI fungicides in apple and pear orchards.

All the three investigators were being involved in the past with research on problems of fungicide resistance. It seems that the case of reduced sensitivity to EBI fungicides in the populations of V. inaequalis and V. pirina is different from cases of fungicide resistance which the three investigators of this project had studied (benzimidazoles, phenylamides and dicarboximides). We may conclude that the attribution of crop-losses to reduced sensitivity to EBI fungicides in V. inaequalis and V. pirina have not been verified. As long as the use of these efficient and beneficial fungicides will be based on rational decisions of the growers, accompanied by monitoring for strains less-sensitive to EBI fungicides, we may assume that sudden failures in the control of apple scab and pear scab due to EBI resistance are not likely to occur.

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