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Negatively correlated cross-resistance to phenylcarbamate fungicides in benomyl-resistant Venturia inaequalis and V. pirina*

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Abstract

Isolates of *Venturia inaequalis* and of *V. pirina* sensitive (S) or resistant (R) to benomyl were examined in vitro on media amended with two phenylcarbamate fungicides. There was a negatively correlated cross-resistance (NCCR) to both methyl N-(3,5-dichlorophenyl) carbamate (MDPC) and isopropyl N-(3,4-diethoxyphenyl) carbamate (NPC) in some benomyl-resistant isolates. In *V. inaequalis,* isolates with low benomyl resistance (LR) did not show NCCR to MDPC, whereas isolates with medium (MR), high (HR) and very high (VHR) resistance to benomyl were more sensitive to MDPC than were the benomyl-sensitive isolates. To NPC, MR and VHR isolates showed NCCR whereas LR and HR isolates reacted similarly as sensitive isolates. In *V. pirina* only HR and VHR isolates showed NCCR to MDPC. The VHR isolates were sensitive to NPC, whereas the reactions of S, LR, MR and HR to NPC were similar.

Crosses between benomyl-sensitive and benomyl-resistant *V. pirina* as well as between different resistant isolates showed that NCCR is inheritable and controlled by a single Mendelian gene.

Additional keywords: benzimidazole fungicides, carbendazim, apple scab, pear scab, genetics of fungicide resistance.

Introduction

Pear and apple scab, caused by *Venturia pirina* Aderh. and *V. inaequalis* (Cke) Wint., respectively, are important diseases of pear and apple all over the world. *V. nashicola* is the most serious disease of the Japanese pear (Tanaka and Yamamoto, 1964).

After the introduction of the benzimidazole fungicides, excellent control of these scab diseases was achieved with benomyl and thiophanate-methyl, but after some years the benzimidazole fungicides failed to control scab because *V. inaequalis, V. pirina* and *V. nashicola* had developed resistance against them (Wicks, 1974; Shabi and Ben-Yephet, 1976; Ishii et al., 1984). Kato et al. (1984) reported on fungicides to which benzimidazole-resistant strains of several fungi, including *V. nashicola,* show nega-

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tively correlated cross-resistance. Benomyl-resistant strains were more sensitive to the fungicides methyl N-(3,5-dichlorophenyl) carbamate (MDPC) and isopropyl-N-(3,4-diethoxyphenyl) carbamate (NPC) than were the wild-type strains.

The purpose of this study was to determine the in vitro MDPC and NPC sensitivity of isolates of both *V. pirina* and *V. inaequalis* with different levels of benomyl resistance. The inheritance of the MDPC-sensitivity was also studied in *V. pirina.*

Material and methods

Fungicides. Benomyl (Benlate 50 WP, DuPont de Nemours & Co., Wilmington, DE, USA) was added to the media used in resistance determination, before autoclaving at 120 °C for 20 min. MDPC technical grade and NPC 25 WP were kindly supplied by Dr T. Kato (Sumitomo Chemical Company, Tukatsukasa, Takarazuka, Hyago, Japan). MDPC and NPC were dissolved in methanol and added to the media at 50 °C after autoclaving. The final methanol concentration never exceeded 1%, to avoid effects on fungal growth (Kato et al., 1984).

Media. Cultures were maintained on potato dextrose agar with 250 μg/ml chloramphenicol (PC1). To determine the negatively correlated cross-resistance, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml MDPC (a.i) or 0.5, 1, 5, 10 and 20 μg/ml NPC (a.i) were added to the PC1. To determine the benomyl resistance level, 0.5, 5 and 50 μg/ml were added to the PC1 (Shabi et al., 1983).

Isolates. Monoconidial isolates of *V. pirina* and *V. inaequalis* were obtained by diluting suspensions of conidia from individual scab lesions. The conidial suspensions were plated on PC1 and incubated at 8 °C for 48 h to allow germination.

Uncontaminated germinated spores were picked up with a stainless steel needle under microscopic observation and transferred individually to PC1 plates. The single spore isolates were incubated for 10 days at 8 °C and then colonies were transferred to individual plates.

Sensitive isolates and isolates with different levels of benomyl resistance were distinguished by the germination of conidia and the growth of the mycelium at 20 °C on media amended with 0, 0.5, 5 and 50 μg/ml benomyl (Shabi et al., 1983). Sensitive isolates (S) are inhibited by 0.5 μg/ml. Isolates that grew on 0.5 μg/ml but were inhibited by 5 μg/ml were designated as the low resistant (LR) phenotype.

Isolates that grew on 5 μg/ml but were inhibited by 50 μg/ml were designated as the medium resistant phenotype (MR). Isolates that had restricted growth on 50 μg/ml benomyl with curled hyphae were designated as the highly resistant (HR) phenotype. Very highly resistant (VHR) isolates grew freely on 50 μg/ml benomyl.

A collection of 38 isolates of *V. pirina* from 11 pear orchards and of 37 isolates of *V. inaequalis* originating from 14 orchards, was tested (Tables 1 and 2). In the spring of 1985 the scab population of five pear orchards and one apple orchard was monitored on 0, 0.5, 5 and 50 μg/ml. Additional single-spore isolates were prepared from those orchards where *V. pirina* or *V. inaequalis* was found to be resistant to benomyl (Table 3).

Determination of negatively correlated cross-resistance (NCCR). Pieces of ca. 1 mm^3

of sporulating mycelia were placed on PC1 with various concentrations of the fungicides and incubated at 20 °C. Germination of the conidia was examined under a microscope after 24 h. The radial growth of the cultures was measured after 12 or 18 days on the PC1, benomyl and MDPC media (Table 1). The growth on NPC was evaluated after 10 days (Table 2).

Inheritance studies. Crosses between pairs of single-spore cultures of *V. pirina* were done on autoclaved pear leaf discs (Shabi et al., 1983). In the genetic studies the parental genotypes were designated Ben^{S}, Ben^{LR}, Ben^{MR}, Ben^{HR} and Ben^{VHR} (Shabi et al., 1986).

The sensitivity to MDPC and to benomyl of the ascospore progeny was determined by placing pieces of single ascosporic cultures on media amended with 0, 1 and 5 μg/ml MDPC and with 0.5, 5 and 50 μg/ml benomyl.

Results

Venturia inaequalis. NCCR was demonstrated for the MR, HR and VHR isolates tested on MDPC (Table 1, Fig. 1). These isolates were all inhibited by 5 μg/ml MDPC. NCCR was not found in the LR isolates. The response of LR isolates was similar to that of the benomyl-sensitive isolates, and all were able to grow on 5 and 10 μg/ml MDPC (Table 1, Fig. 1). On 50 μg/ml MDPC, LR and benomyl-sensitive isolates were also totally inhibited. The HR isolates were less sensitive to MDPC than were the MR

Table 1. Relative growth response of isolates of *Venturia inaequalis* and *V. pirina* on media amended with fungicides[1].

Benomyl resistance level	Fungicide (μg a.i./ml)											
	Benomyl							MDPC				
	0	0.1	0.5	1	5	10	50	0.1	0.5	1	5	10
V. inaequalis												
S (12)[2]	100	0	0	0	0	0	0	94	97	97	86	29
LR (10)	100	100	92	20	0	0	0	92	99	99	95	25
MR (5)	100	100	105	105	58	19	0	87	0	0	0	0
HR (3)	100	100	104	104	104	95	65	94	91	61	0	0
VHR (7)	100	102	99	99	109	99	83	100	0	0	0	0
V. pirina												
S (20)	100	0	0	0	0	0	0	92	93	90	83	52
LR (3)	100	79	54	10	0	0	0	72	72	75	59	59
MR (5)	100	100	93	89	93	30	0	87	89	87	74	59
HR (9)	100	113	105	101	112	98	90	101	105	92	0	0
VHR (1)	100	110	100	99	109	99	90	100	80	20	0	0

[1] Average on fungicide-amended media as percentage of average growth of control after 18 days *(V. inaequalis)* and after 12 days *(V. pirina)* at 20 °C.
[2] In parentheses, number of isolates tested.

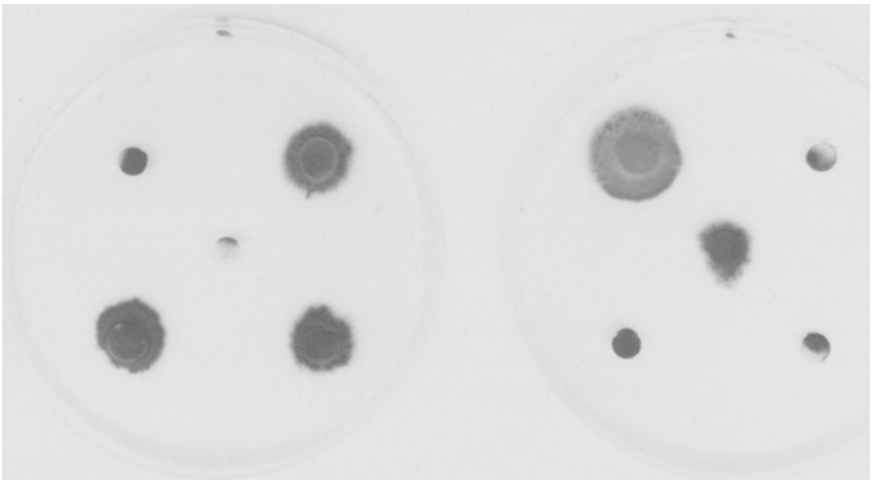

Fig. 1. Growth response, after 27 days at 20 °C, of five *Venturia inaequalis* isolates on 5 μg/ml of benomyl (left) or MDPC (right). Each plate was inoculated with 4-mm mycelial discs of benomyl-sensitive (centre) and benomyl-resistant isolates: LR-upper left, MR-upper right, HR-lower left and VHR-lower right.

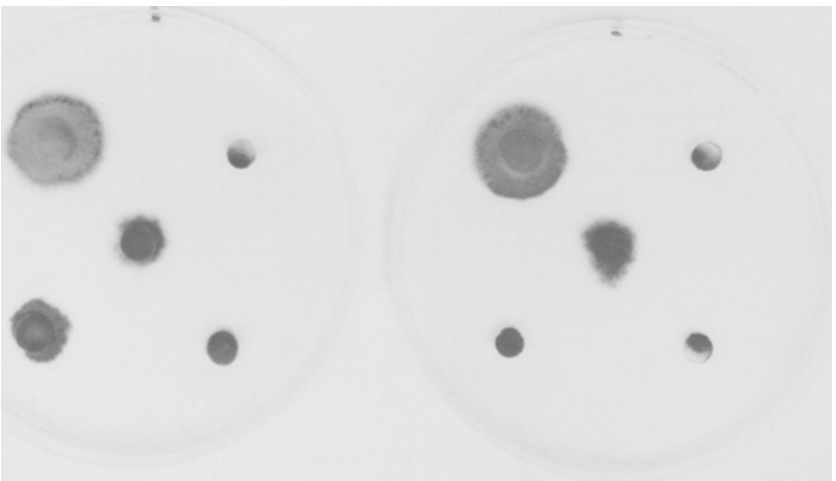

Fig. 2. Growth response of *Venturia inaequalis* isolates on 1 (left) and 5 (right) μg/ml of MDPC. Note the growth (reduced sensitivity) of the HR isolate (lower left) on 1 μg/ml as compared with the MR (upper right) and the VHR (lower right) isolates.

and VHR isolates. MR and VHR isolates were not able to grow on 0.5 μg/ml MDPC, while HR isolates were not sensitive to 1 μg/ml MDPC (Table 1, Fig. 2). The difference between HR and MR or VHR isolates was even more obvious on NPC (Table 2). On 20 μg/ml NPC the HR isolates were still able to grow like the benomyl-sensitive and

Table 2. Effect of isopropyl N-(3,4-diethoxyphenyl) carbamate (NPC) on growth of *Venturia inaequalis* and *V. pirina*[1].

Benomyl resistance level	NPC (μg a.i./ml)					
	0	0.5	1	5	10	20
V. inaequalis						
S (12)[2]	+	+	+	+	+	+
LR (10)	+	+	+	+	+	+
MR (5)	+	—	—	—	—	—
HR (3)	+	+	+	+	+	+
VHR (7)	+	—	—	—	—	—
V. pirina						
S (20)	+	+	+	+	+	+
LR (3)	+	+	+	+	+	+
MR (5)	+	+	+	+	+	+
HR (9)	+	+	+	+	+	+
VHR (1)	+	—	—	—	—	—

[1] +, growth as in control; —, growth totally inhibited, after 10 days at 20 °C.
[2] In parentheses, number of isolates tested.

LR phenotypes. However, the MR and VHR phenotypes of *V. inaequalis* were very sensitive to NPC and growth was arrested at even 0.5 μg/ml NPC. Single-spore isolates obtained from an apple orchard that in 1985 was found to be infected with benomyl-resistant *V. inaequalis* showed the same response to MDPC and NPC as the isolates from the collection examined in the previous test. The HR isolates were less sensitive than the VHR isolates to MDPC. The VHR isolates were not able to grow on 0.5 μg/ml NPC, whereas the HR isolates were insensitive to even 20 μg/ml NPC (Table 3).

Venturia pirina. LR and MR isolates behaved similarly to the benomyl-sensitive isolates on MDPC and were able to grow on 10 μg/ml. The growth of the HR and VHR phenotypes was inhibited on 5 μg/ml MDPC, demonstrating NCCR in *V. pirina* (Table 1). HR and VHR isolates reacted differently on 1 μg/ml MDPC: the former grew freely, and the latter was sensitive. This distinction was even more obvious on NPC: the VHR phenotype was sensitive to 0.5 μg/ml, whereas the HR isolates were insensitive to even 20 μg/ml NPC (Table 2).

Single spore isolates from five pear orchards infected with benomyl-resistant *V. pirina* since 1975 had a similar response to MDPC and NPC as the isolates in the previous test. There was again NCCR for HR and VHR isolates on MDPC. NCCR to NPC was obtained only for VHR isolates, while HR isolates were able to grow on 20 μg/ml NPC like the benomyl-sensitive, LR and MR isolates (Table 3).

Inheritance of the MDPC-sensitivity in V. pirina. Ascospores obtained from 22 crosses consisting of ten combinations of Ben^{S}, Ben^{LR}, Ben^{MR}, Ben^{HR} and Ben^{VHR} genotypes were analyzed. Ascosporic cultures obtained from crosses 1-8 (Table 4) be-

Table 3. Effect of fungicides on single-spore isolates of *Venturia pirina* and *V. inaequalis* from several orchards throughout Israel.

Orchard	Benomyl resistance level[1]	Fungicide concentration (μg a.i./ml)							
		MDPC					NPC		
		0	0.5	1	2	5	0.5	1	20
V. pirina									
Tel Zofit	VHR (10)	+[2]	NT	—	—	—	—	—	—
Idmit	HR (4)	+	NT	+	+	—	+	+	+
Matitiyahu	HR (5)	+	NT	+	+	—	+	+	+
Metulla	HR (1)	+	+	+	+	—	+	+	+
Ramot Naftali	S (10)	+	+	+	+	+	+	+	+
Ramot Naftali	MR (5)	+	+	+	+	+	+	+	+
Ramot Naftali	HR (9)	+	+	+	+	—	+	+	+
V. inaequalis									
Majdal Shams	HR (10)	+	+	+	—	—	+	+	+
Majdal Shams	VHR (9)	+	—	—	—	—	—	—	—

[1] Benomyl resistance determined by response to 0.5, 5 and 50 μg/ml benomyl. In parentheses, number of tested single-spore isolates.
[2] + free growth, — no growth after 10 days at 20 °C; NT not tested.

tween Ben^S, Ben^{LR} and Ben^{MR} genotypes were not sensitive to MDPC and were growing like the parental isolates on MDPC. In 14 combinations it was proved that NCCR is inheritable (Table 4, crosses 9-22). In two crosses (Table 4, crosses 9 and 10) where the parents were from the same genotype, Ben^{HR}, no segregation was found, and the parental level of sensitivity to MDPC was retained by the progeny. The progeny segregation of crosses 11-22 fitted a 1:1 ratio proving that the MDPC sensitivity is controlled by a single Mendelian gene (Table 4). In the 788 ascospores obtained from these 12 crosses the degrees of sensitivity to MDPC and benomyl of each progeny were similar to those of one of its parents; namely the two traits were inseparable by crossing.

Discussion

Four levels of benomyl resistance were found in isolates of *V. inaequalis* and *V. pirina.* In *V. inaequalis,* HR and VHR isolates were identified by the restricted growth on 50 μg/ml benomyl of the HR phenotypes as compared with the free growth of the VHR phenotypes (Shabi et al., 1983). In *V. pirina,* unlike the situation with *V. inaequalis,* the response of HR and VHR phenotypes on 50 μg/ml benomyl was not obvious (Shabi et al., 1986). The use of media amended with the phenylcarbamate fungicides enabled us to distinguish between HR and VHR isolates of *V. pirina.* The NCCR to 1 μg/ml MDPC or 0.5 μg/ml NCP of the VHR phenotypes, confirmed that in *V. pirina* Ben^{HR} and Ben^{VHR} are different alleles which can be distinguished more clearly on

Table 4. Analysis of crosses between benomyl-sensitive and benomyl-resistant genotypes of *Venturia pirina* and the segregation of single-ascosporic cultures on methyl N-(3,5-dichlorophenyl) carbamate (MDPC).

Cross No.	Parental genotypes	Total	Growth on MDPC				
			numbers		$\chi^2_{(1:1)}$[a]	%	
			1 μg/ml	5 μg/ml		1 μg/ml	5 μg/ml
1	$Ben^S \times Ben^S$	15	15	15	NS	100	100
2	$Ben^{MR} \times Ben^{MR}$	25	25	25	NS	100	100
3	$Ben^S \times Ben^{MR}$	40	40	40	NS	100	100
4	$Ben^{LR} \times Ben^{MR}$	50	50	50	NS	100	100
5	$Ben^{LR} \times Ben^{MR}$	40	40	40	NS	100	100
6	$Ben^{LR} \times Ben^{MR}$	40	40	40	NS	100	100
7	$Ben^{LR} \times Ben^{MR}$	32	32	32	NS	100	100
8	$Ben^{LR} \times Ben^{MR}$	20	20	20	NS	100	100
9	$Ben^{HR} \times Ben^{HR}$	65	65	0	NS	100	0
10	$Ben^{HR} \times Ben^{HR}$	39	39	0	NS	100	0
11	$Ben^S \times Ben^{HR}$	35	35	20	0.71	100	57
12	$Ben^{LR} \times Ben^{HR}$	40	40	26	3.60	100	65
13	$Ben^{LR} \times Ben^{HR}$	29	29	16	0.31	100	55
14	$Ben^{LR} \times Ben^{HR}$	40	40	19	0.10	100	48
15	$Ben^{MR} \times Ben^{HR}$	35	35	17	0.03	100	49
16	$Ben^{MR} \times Ben^{HR}$	60	60	33	0.60	100	55
17	$Ben^{MR} \times Ben^{HR}$	40	40	25	2.50	100	62
18	$Ben^{MR} \times Ben^{VHR}$	98	47	47	0.16	48	48
19	$Ben^{MR} \times Ben^{VHR}$	89	46	46	0.10	52	52
20	$Ben^{MR} \times Ben^{VHR}$	79	35	35	1.03	44	44
21	$Ben^{HR} \times Ben^{VHR}$	126	55	0	2.03	44	0
22	$Ben^{HR} \times Ben^{VHR}$	117	55	0	0.42	47	0

[a] Expected value for a 1:1 ratio at $P = 0.05$ is 3.84. NS = no segregation. In all the crosses (excluding nos 1, 2, 9 and 10, which were the same parental genotypes) the chi-square values for segregation on benomyl were less than 3.84.

NPC than on MDPC. NCCR to MDPC or NPC occurred predominantly in the isolates with higher levels of benomyl resistance, and not in isolates with a low level of resistance. In *V. inaequalis*, isolates with medium resistance (MR) to benomyl were more sensitive than the HR phenotypes to the phenylcarbamate fungicides. The sensitivity of these MR phenotypes was similar to that of the VHR isolates of *V. inaequalis* to MDPC and NPC. Therefore, the NCCR of MR and VHR phenotypes of *V. inaequalis* to 0.5 μg/ml of either MDPC or NPC cannot be used to discriminate between the two alleles Ben^{MR} and Ben^{VHR} of *V. inaequalis*. This bimodal response in *V. inaequalis* is rather different from the response of our *V. pirina* isolates and of *V. nashicola* isolates (Ishii et al., 1984).

According to Davidse and Flach (1977), sensitivity of fungi to benomyl is governed by the affinity of spindle tubulin to carbendazim, the active principle of benomyl. It

seems, therefore, that changes in tubulin, which strongly reduce the affinity of carbendazim, may increase the affinity to MDPC or NPC, but changes in tubulin which decrease affinity to carbendazim only slightly, have little or no effect on affinity to MDPC or NPC. According to our inheritance study in *V. pirina,* we can conclude that the affinity of spindle tubulins to carbendazim and MDPC is inheritable and is controlled by the same gene.

The question has been put forward whether NCCR might offer possibilities to prevent development of fungicide resistant in practice. However, this will be effective only if all strains resistant to the first fungicide are more sensitive to the second fungicide than the wild type pathogen, and vice versa (Dekker, 1984). From our observations with *V. inaequalis* and *V. pirina* it appears that this is not so, as not all benomyl-resistant strains are more sensitive to MDPC or NPC. Thus, when pathogen strains with various levels of resistance are present in the orchard, application of the two compounds together will cause a shift toward those benomyl-resistant strains which do not show NCCR to MDPC or NPC. An increase in the frequency of these isolates in the pathogen population may then still cause failure of disease control. If only strains with very high benomyl resistance occur, then application of MDPC or NPC might provide scab control, but even then it cannot be ruled out that benomyl-resistant mutants – with little sensitivity to MDPC or NPC – will appear, so that the possibilities for use of NCCR in practice will be limited and will be dependent on the ability to monitor the population of the fungi involved. By monitoring the benomyl-resistant isolates, strains with cross-resistance (such as Ben^{LR}) to benzimidazole and phenylcarbamate fungicides could be detected and before crop losses occur, other effective fungicides for scab control could be used.

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Samenvatting

Negatief gecorreleerde kruisresistentie tegen phenylcarbamaat fungiciden in Venturia inaequalis en V. pirina

Benomyl-gevoelige en -resistente isolaten van *Venturia inaequalis* en *V. pirina* werden in vitro onderzocht op media met de fungiciden methyl N-(3,5-dichlorophenyl) carbamate (MDPC) en isopropyl N-(3,4-dichlorophenyl) carbamate (NPC). Een aantal benomyl-resistente isolaten van deze pathogeen bleken een negatief gecorreleerde kruisresistentie (NCCR) te vertonen ten opzichte van MDPC en NPC.

Isolaten van *V. inaequalis* met matige (MR), hoge (HR) en zeer hoge (VHR) benomyl-resistentie vertoonden NCCR. Ten opzichte van NPC vertoonden alleen MR en VHR isolaten NCCR, en niet de LR en HR isolaten. In *V. pirina* vertoonden HR en VHR isolaten NCCR ten opzichte van MDPC, maar alleen de VHR isolaten ten opzichte van NPC.

Kruisingen tussen benomyl-gevoelige en -resistente *V. pririna,* en tussen verschillende benomyl-resistente isolaten onderling, toonden aan dat NCCR erfelijk is en berust op een enkel gen.

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Book review

A. Krieg, 1986. *Bacillus thuringiensis*, ein mikrobielles Insektizid: Grundlagen und Anwendung. Acta Phytomedica 10. Paul Parey Scientific Publishers, Berlin and Hamburg. In German, with 16 figures and 10 tables, 191 pp. Price DM 58.

Insect-pathogenic bacteria with a potential for plant protection and control of human disease vectors are limited to three species of spore-forming bacteria., viz. *Bacillus thuringiensis*, *B. sphaericus* and *B. popilliae*. The first two species are characterized by the presence of an insecticidal proteinaceous inclusion body in the spores. This body contains a substance highly toxic to the gut epithelium of insects; it usually kills the insect rapidly. The toxin gene is plasmid-borne and this convenience allows, in principle, the manipulation of bacterial strains by conjugation or genetic engineering in order to produce more strains with extended host range and/or increased virulence. The main emphasis so far has been on *B. thuringiensis* and this bacterium has been advocated by the Food and Agricultural Organization and the World Health Organization as a biological insecticide for a long time and has gained world-wide acclaim in insect control.

B. thuringiensis (*Bt*) was first isolated and described by Ernst Berliner in 1911 from diseased larvae of the Mediterranian flour moth, *Ephestia kühniella*. Since then, over 800 strains of *Bt* have been described and they are effective against many noxious Lepidoptera, Diptera and Coleoptera. A wealth of information on *Bt* is available now in the literature and warrants a com-