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Geographical variation in larval susceptibility of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* spore–crystal mixtures and purified crystal proteins and associated resistance development in India

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Abstract

The susceptibility of larvae of the diamondback moth, *Plutella xylostella* Linnaeus to purified crystal proteins and spore–crystal preparations of *Bacillus thuringiensis* was investigated for 13 populations from seven states in India. The LC_{50} (µg ml^{-1}, 48 h) values of Cry proteins for different populations of *P. xylostella* ranged from 0.14–3.74 (Cry1Aa), 0.007–1.25 (Cry1Ab), 0.18–2.47 (Cry1Ac) and 0.12 – 3.0 (Cry1C). The LC_{50} (mg (ai) l^{-1}, 48 h) of spore-crystal preparations ranged from 0.02–0.98 (HD-1) and 0.06–2.14 (HD-73). Significantly higher LC_{50} values for all tested toxins and strains were obtained with populations collected from Iruttupallam and Ottanchathiram in the southern state of Tamil Nadu, whereas some of the populations collected from the northern part of India were more susceptible than the susceptible IARI 17–65 population. The high levels of resistance in the Iruttupallam and Ottanchathiram populations to Cry1Ab suggested selection pressure by Cry1Ab, which is the predominant toxin in *B. thuringiensis* formulations used in India. Cry1Ab was found to be more toxic than the other toxins. The population from Iruttupallam showed increased resistance following selection with Cry1Ab in the laboratory (LC_{50} from 1.25 to 4.31 µg ml^{-1} over two generations) and also showed cross resistance to Cry1Aa and Cry1Ac. The resistance to Biobit® in the field population from Iruttupallam declined slowly; requiring *c.* 33 generations for an overall 10-fold decline in LC_{50} when the insects were reared in the laboratory without exposure to *B. thuringensis*.

Introduction

Bacillus thuringiensis Berliner (Eubacteriales: Bacillaceae) is a gram-positive, spore forming soil bacterium pathogenic to insects. It produces parasporal crystal (Cry) proteins during sporulation which are toxic to insects. There are five major classes of Cry toxins with specific insecticidal activity, namely, Cry1 (Lepidoptera), Cry2 (Lepidoptera and Diptera), Cry3 (Coleoptera), Cry4 (Diptera) and Cry5 (Lepidoptera and Coleoptera) (Cannon, 1996; Crickmore *et al.*, 1998).

Several developments, such as the ill-effects of conventional pesticides, have indirectly led to an expanding usage of *B. thuringiensis* products which are effective against

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many insect pests and safe to higher animals, including humans. Moreover, recent advances in genetic engineering have led to the development of transgenic microbes and crop plants expressing *cry* toxin genes, and improved *B. thuringiensis* strains for controlling insect pests (Entwistle *et al*., 1993; Kumar & Kapur, 1998; de Maage *et al*., 1999). It is projected that US$ 2.7 billion of 8.1 billion spent annually on insecticides worldwide could be replaced by *B. thuringiensis* transgenic crops alone (Krattiger, 1997).

Despite use of *B. thuringiensis* since the 1960s, no field level resistance in insects has been found until the last decade. Although laboratory studies have shown development of resistance in several insect species to *B. thuringiensis* (Tabashnik, 1994), the diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) is the only insect species that has evolved resistance under field conditions in many countries (Zhao *et al.*, 1993; Tabashnik, 1994; Perez & Shelton, 1997).

Diamondback moth is a key pest of cruciferous vegetables throughout the world with an annual cost of control estimated at US$ 1 billion (Talekar, 1992). In India, it is a serious pest of cabbage, cauliflower and other cruciferous crops throughout the country (Chelliah & Srinivasan, 1986; Gujar, 1999). The first record of insecticide resistance in *P. xylostella* was observed in 1966 in Punjab (Verma & Sandhu, 1968). Since then, resistance has been reported against many insecticides namely, organochlorines, organophosphates, synthetic pyrethroids and cartap hydrochloride across the subcontinent (Raju, 1996). *Bacillus thuringiensis*-based products, such as Biobit®, Bioasp®, Biolep®, Dipel® and Halt® are now available in India for use against *P. xylostella*. The effectiveness of *B. thuringiensis* formulations alone or with other insecticides for the control of *P. xylostella* under laboratory, glasshouse and field conditions has been reported (Narayanan *et al*., 1970; Rajamohan & Jayaraj, 1978; Rabindra *et al.*, 1995; Sannaveerappanavar & Viraktamath, 1997). The increasing use of *B. thuringiensis*-based insecticides in recent years provides the potential for resistant alleles to emerge, in view of the development of resistance reported elsewhere (Chandrasekaran & Regupathy, 1996; Gujar & Kalia, 1999). Mohan & Gujar (2000) reported baseline susceptibility of diamondback moth populations collected from all over the country to Biobit®, a commercial formulation of *B. thuringiensis* subsp. *kurstaki* Kurstak, and showed development of resistance in some populations. The present communication reports studies on the pattern of susceptibility of different geographical populations of *P. xylostella* to strains and purified toxins of *B. thuringiensis*, for use in developing effective integrated pest management strategies against this species.

Materials and methods

Collection and maintenance of P. xylostella *populations*

Field populations of *P. xylostella* were collected from different localities in various states across India, from north to south, covering a distance of about 2500 km (fig. 1). Insects were collected from cauliflower or cabbage in the form of larvae or pupae between 1997 and 1999. The susceptible IARI 17–65 population was founded from insects collected from cauliflower fields at Iruttupallam (15 km east of Coimbatore) during February 1997. Since then, this population has been reared in the laboratory at the Indian Agricultural Research Institute, New Delhi. Adult moths were fed 10% honey solution fortified with multivitamins and provided with mustard seedlings for egg laying. The larvae were transferred to cabbage/cauliflower leaves and maintained at 28°C with more than 60% rh and a 14L:10D photoperiod. Under such conditions, the life cycle of *P. xylostella* lasts 22–25 days with a total larval period of 9–10 days. With the exception of the laboratory colony, larvae used in bioassays belonged mostly to F_1 and sometimes F_2 or F_3 generations.

B. thuringiensis *strains and toxins tested*

The bioassays were performed with the following seven *B. thuringiensis* toxins and formulations: acetone powder formulations of spore–crystal mixtures of *B. thuringiensis* subsp. *kurstaki* HD-1 and HD-73; *Escherichia coli* expressed *B. thuringiensis* toxins, Cry1Aa, Cry1Ab, Cry1Ac and Cry1C and a commercial formulation of *B. thuringiensis* subsp. *kurstaki*, Biobit® 6.4% ai (a gift from Rallis India Ltd, Mumbai). The HD-1 and HD-73 strains of *B. thuringiensis* subsp. *kurstaki* were obtained from the Pasteur Institute, Paris, France and the Bacillus Genetic Stock Center, Ohio State University, Columbus, USA, respectively. *Escherichia coli* strains producing Cry1Aa, Cry1Ab, Cry1Ac and Cry1C were gifts from Professor Donald Dean, Ohio State University, Columbus, USA and Dr P. Anand Kumar, National Research Centre for Plant Biotechnology of the Indian Agricultural Research Institute.

Preparation of B. thuringiensis *subsp.* kurstaki *HD-1 and HD-73 spore-crystal mixture*

Cultures of *B. thuringiensis* subsp. *kurstaki* HD-1 and HD-73 were grown separately in nutrient broth medium for 96 h at 30°C in flasks with constant shaking at 150 rpm. The autolysed culture was centrifuged (Model 3K18, Sigma Laborzentrifugen GmbH, Osterode, Germany) at 4492 × *g* for 10 min and the resulting pellet was resuspended in 0.5 M sodium chloride for 15 min to avoid exoprotease activity. After centrifugation, the pellet was washed with sterile distilled water twice and finally it was suspended in 6% lactose for 30 min under constant stirring (1/10–1/20 volume based on original broth). At the end, four volumes of ice-cold acetone were added slowly while stirring, as per Dulmage *et al*. (1970). After 10 min, it was filtered under suction. The residue containing spore–crystal mixture was dried overnight under partial vacuum and stored in aliquots at −4°C until further use.

The number of viable spores present in the preparations was determined by plating appropriately diluted samples on agar plates after heat shocking the stock solution for 10 min at 80–85°C to kill vegetative stages. The number of colonies formed were counted after 24 h and expressed in terms of spores per 100 mg product. The number of spores observed per 100 mg in HD-1 and HD-73 were 89.3×10^{10} and 147×10^{10}, respectively.

Purification of recombinant B. thuringiensis *δ-endotoxins*

The crystal proteins, Cry1Aa, Cry1Ab, Cry1Ac and Cry1C produced by *E. coli* strains were purified according to the method previously described by Lee *et al*. (1992). Cells were cultured in nutrient broth containing 50 μg ml^{-1}

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Fig. 1. Sampling locations of *Plutella xylostella* in India 1997–1999 (Najafgarh, a suburb of Delhi not shown).

ampicillin for 48 h at 37°C. The cells were harvested by spinning at 4492 × *g* for 10 min, the pellet was suspended in 50 ml of lysis buffer (50 mM Tris-hydrochloride, 50 mM ethylene diamine tetra-acetic acid, 15% sucrose, pH 8.0, 2 mg ml^{-1} lysozyme) and incubated for 12 h at 37°C. The cell suspension was sonicated (Labsonic L, B. Braun Biotech International, Melsungen, Germany) on ice (50% duty cycle with output control 8 for 2 min) and centrifuged at 4492 × *g* for 10 min. The pellet was washed three times with ice cold 2% Triton X-100, 0.5 M sodium chloride, then five times with 0.5 M sodium chloride, and three times with sterile distilled water. In all steps protease inhibitor phenyl methyl sulfonyl fluoride was added at 1 mM concentration. The pellet containing inclusion bodies was solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 10 mM dithiothreitol at 37°C for 3–4 h. Then it was centrifuged at 9168 × g for 10 min, and the supernatant containing the solubilized protoxin was stored at −20°C until further use.

Quantification of toxins

A weighed amount of acetone powder of HD-1 and HD-73 was dissolved in ice cold 2% Triton X-100, 0.5 M sodium chloride to avoid any exoprotease activity. It was then sonicated, washed and centrifuged for 10 min. The pellet was suspended in sodium carbonate solubilizing buffer, and the protoxin obtained as described previously for *E. coli* expressed toxins. Protoxins from either acetone powders or recombinant *E. coli* strains were converted to toxins by treatment with trypsin according to Lee *et al*. (1992).

The protein profiles of toxin preparations were analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the discontinuous system of Laemmli (1970) using Bio-Rad Mini-Protean II Cell assembly with 8% resolving gel. The gel was fixed in 15% trichloroacetic acid and stained with 0.25% Coomassie brilliant blue (CBB) R 250 in methanol–acetic acid–water (40:10:50) for 2 h and destained using methanol–acetic acid–water (40:10:50) till the bands were clearly visible.

Toxin bands were identified by comparing with standard molecular weight markers. Quantification was done by elution of CBB R 250 dye from stained bands as described by Ball (1986). Gel pieces containing the toxin band (5 × 5 mm) were excised and placed in test tubes containing 1 ml of 3% SDS in 50% isopropanol. The tubes were capped with parafilm and incubated at 37°C for 24 h without shaking. The absorbance of supernatant was determined at 595 nm using the extraction solution with similarly sized gel piece as

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a blank. Concentration of toxin present in a preparation was established after plotting on the standard curve of bovine serum albumin (electrophoresis-marker grade from Sigma) obtained by the similar protocol. The toxin content (ai) estimated for HD-1 and HD-73 preparations was 0.538% (0.538 mg per 100 mg powder) and 0.282% (0.282 mg per 100 mg powder), respectively. Toxin protein estimated from Cry1Aa, Cry1Ab, Cry1Ac and Cry1C was 4.50, 0.87, 0.22 and 0.35 mg ml^{-1}, respectively.

Bioassays

A leaf-dip bioassay method was followed as described by Tabashnik *et al*. (1991) using fully opened cabbage leaves. The leaves were first washed thoroughly with distilled water containing 0.1% Triton X-100 and allowed to dry. A leaf disc of 4.5 cm diameter was cut and dipped in solutions of different concentrations prepared with spore–crystal mixture or individual Cry toxins. At least six appropriate concentrations (mortality spanning 20–80% at 48 h) between 5.38–0.0053 mg (ai) l^{-1} for HD-1; 28.2–0.0056 mg (ai) l^{-1} for HD-73; 2255–0.022 μg ml^{-1} for Cry1Aa; 8.8–0.00004 μg ml^{-1} for Cry1Ab; 8.8–0.04 μg ml^{-1} for Cry1Ac and 56.0–0.02 μg ml^{-1} for Cry1C were used. Each disc was dipped for 5–10 s and allowed to air dry for a period of 1 h. Then the discs were placed individually into small Petri dishes (5 cm diameter). Eight to ten, 6-day-old larvae were released in each disc with four to five replications. The entire bioassay with all replicates including control was done on the same day and same time to minimize any variation in results. A minimum of 40 larvae was used for each concentration (Moermans & van Hecke, 1995). Larvae were allowed to feed for 72 h at 28°C and > 60% rh. Larval mortality was recorded every 24 h. Forty-eight hour mortality data were found to be the most reliable and reproducible for estimation of median lethal concentrations (LC_{50}) as high control mortality was occasionally observed at 72 h. Experiments showing control mortality of more than 20% were discarded and repeated.

Laboratory selection and cross resistance

The F_1 generation of the field population obtained from Iruttupallam was selected for investigating the development of resistance to Cry1Ab toxin over four generations under laboratory conditions using the bioassay described previously. A concentration of 0.8 μg ml^{-1} of Cry1Ab toxin was used for the first two generations, which was then increased to 1.6 μg ml^{-1} for the third and fourth generations of selection. More than 200 larvae from each filial generation were allowed to feed on treated leaf discs for 24 h. The survivors were then transferred to untreated leaf discs to complete the rest of their life cycle. Larval mortality was recorded at various time intervals after exposure. Full bioassays were carried out with Cry1Ab against the F_2 and F_3 generations only. The larvae belonging to the F_3 generation were also used for testing their susceptibility to Cry1Aa and Cry1Ac to investigate the development of cross resistance.

Stability of field-evolved resistance under laboratory conditions

The *B. thuringiensis*-resistant field population from Iruttupallam was used to investigate the stability of resistance to *B. thuringiensis* by rearing it over successive generations in the laboratory in the absence of selection pressure as per Tabashnik *et al*. (1994). Biobit® was used for bioassays. The parameter R was used to quantify the rate of change in median lethal concentration (LC_{50}) when rearing under selection pressure was stopped. $R = [\log(\text{final } LC_{50}) - \log(\text{initial } LC_{50})]/n$; where n is the number of generations without exposure to insecticide; final LC_{50} is the LC_{50} after n generations without selection, and initial LC_{50} is the LC_{50} before n generations without selection. Negative values of R reflect decreases in LC_{50}; the inverse of R is the number of generations required for a 10-fold change in LC_{50} (Tabashnik, 1994).

Statistical analysis

Bioassay results were analysed using the maximum likelihood programme (Ross, 1987). The LC_{50} was estimated for each population on the basis of 48 h mortality data. The resistance ratio was calculated by dividing the LC_{50} of a *P. xylostella* field population with the LC_{50} of a susceptible IARI 17–65 population. Two populations were considered significantly different if their 95% fiducial limits did not overlap (Litchfield & Wilcoxon, 1949).

Results

Susceptibility pattern of P. xylostella *to* B. thuringiensis *subsp.* kurstaki *HD-1 and HD-73*

Bioassays with seven populations of *P. xylostella* revealed field-evolved resistance to the *B. thuringiensis* subsp. *kurstaki* HD-1 formulation in populations from Iruttupallam and Ottanchathiram (table 1). The LC_{50} values for these two populations were significantly higher, i.e. 57.6- and 46.8-fold respectively, when compared with the susceptible IARI 17–65 population.

The populations of *P. xylostella* from Iruttupallam and Ottanchathiram showed significant differences in their susceptibility to *B. thuringiensis* subsp. *kurstaki* HD-73 (resistance ratios of 17.2- and 24.3-fold, respectively) compared with the susceptible IARI 17–65 population. The populations from Iruttupallam and Ottanchathiram showed *c*. 25.3- and 35.6-fold level of resistance relative to the most susceptible field population from Amritsar. Larvae obtained from Najafgarh (a suburb of Delhi) and Varanasi were significantly more resistant to *B. thuringiensis* subsp. *kurstaki* HD-73 than the susceptible laboratory colony, but not as resistant as the populations from Iruttupallam and Ottanchathiram. The populations obtained from localities such as Delhi, Jalandhar, Phagwara and Amritsar, were similar in susceptibility to HD-1 and HD-73 as the laboratory population.

Individual Cry toxins: Cry1Aa, Cry1Ab, Cry1Ac and Cry1C

Bioassays revealed that the population obtained from Delhi was the most susceptible to purified Cry1Aa toxin (LC_{50} 0.14 μg ml^{-1}) (table 2) followed by the population from Pune and the susceptible IARI 17–65 population (LC_{50} 0.85 and 1.11 μg ml^{-1}, respectively). The population from Iruttupallam was the least susceptible to Cry1Aa (LC_{50} 3.74 μg ml^{-1}) followed by the populations from Guntur (2.32 μg ml^{-1}), Phagwara (1.89 μg ml^{-1}), Nagpur (1.43 μg ml^{-1}) and Ottanchathiram (1.32 μg ml^{-1}). The levels of resistance of

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Table 1. Response of various populations of *Plutella xylostella* to *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 spore crystal formulations.

Collection site	Date of collection	Date of bioassay	LC_{50} (mg ai l^{-1})	Slope ± S.E.	Fiducial limits (95%)		RR[a]
					Lower	Upper	
			B. thuringiensis subsp. *kurstaki* HD-1				
Iruttupallam	29 Sept. 98	11 Dec. 98	0.98	1.33 ± 0.16	0.63	1.50	57.6
Ottanchathiram	28 Oct. 98	16 Nov. 98	0.79	1.52 ± 0.41	0.20	1.44	46.7
Phagwara	28 Nov. 98	10 Jan. 99	0.04	1.76 ± 0.23	0.03	0.05	2.3
Jalandhar	28 Nov. 98	10 Jan. 99	0.04	1.78 ± 0.24	0.03	0.05	2.2
Delhi	15 Nov. 98	3 Dec. 98	0.36	1.61 ± 0.21	0.03	0.05	2.1
Amritsar	28 Nov. 98	6 Jan. 99	0.03	2.12 ± 0.35	0.02	0.04	1.7
IARI 17-65	28 Feb. 97	29 Nov. 98	0.02	1.03 ± 0.19	0.006	0.03	1.0
			B. thuringiensis subsp. *kurstaki* HD-73				
Iruttupallam	29 Sept. 98	16 Nov. 98	1.52	1.49 ± 0.22	0.99	2.20	17.3 (25.3)
Ottanchathiram	28 Oct. 98	25 Nov. 98	2.14	1.35 ± 0.21	1.40	3.13	24.3 (35.7)
Varanasi	15 Nov. 99	25 Nov. 99	0.53	2.60 ± 0.52	0.37	0.72	6.0 (8.8)
Najafgarh	9 Nov. 99	21 Nov. 99	0.21	1.21 ± 0.12	0.14	0.33	2.3 (3.5)
IARI 17-65	28 Feb. 97	29 Nov. 98	0.09	1.84 ± 0.32	0.05	0.14	1.0 (1.5)
Phagwara	28 Nov. 98	10 Jan. 99	0.08	1.08 ± 0.12	0.05	0.13	– (1.3)
Delhi	15 Nov. 98	27 Nov. 98	0.07	1.57 ± 0.22	0.04	0.11	– (1.1)
Jalandhar	28 Nov. 98	10 Jan. 99	0.07	1.33 ± 0.15	0.04	0.10	– (1.1)
Amritsar	28 Nov. 98	7 Jan. 99	0.06	1.34 ± 0.55	0.04	0.09	– (1.0)

[a]Resistance ratio: LC_{50} of field population/LC_{50} of susceptible IARI 17–65 population.
Figures in brackets refer to resistance ratios in relation to the Amritsar population.

Table 2. Response of various populations of *Plutella xylostella* to purified Cry toxins.

Collection site	Date of collection	Date of bioassay	LC_{50} (µg ml^{-1})	Slope ± S.E.	Fiducial limits (95%)		RR[a]
					Lower	Upper	
			Cry1Aa				
Iruttupallam	29 Oct. 98	1 Feb. 99	3.74	0.89 ± 0.12	1.76	6.91	3.4 (26.7)
Guntur	28 Jan. 99	10 Feb. 99	2.32	0.69 ± 0.10	0.81	5.05	2.1 (16.6)
Phagwara	28 Nov. 98	31 Jan. 99	1.89	0.81 ± 0.10	0.89	3.46	1.7 (13.5)
Nagpur	10 Feb.99	1 Mar. 99	1.43	0.85 ± 0.11	0.66	2.59	1.1 (10.2)
Ottanchathiram	28 Oct. 98	30 Jan. 99	1.31	1.71 ± 0.26	0.80	1.95	1.2 (9.4)
IARI 17-65	28 Feb. 97	2 Feb. 99	1.11	1.07 ± 0.13	0.57	1.99	1.0 (7.9)
Pune	20 Jan. 99	4 Feb. 99	0.85	0.95 ± 0.13	0.39	1.61	– (6.1)
Delhi	15 Nov. 98	24 Jan. 99	0.14	0.80 ± 0.14	0.027	0.41	– (1.0)
			Cry1Ab				
Iruttupallam	29 Oct. 98	18 Nov. 98	1.25	0.56 ± 0.10	0.42	8.07	169.0
Ottanchathiram	28 Oct. 98	13 Dec. 98	0.86	1.28 ± 0.18	0.59	1.33	116.2
Phagwara	28 Nov. 98	31 Jan. 99	0.15	0.40 ± 0.07	0.06	0.51	20.3
Bangalore	11 July 98	11 Oct. 98	0.43	1.52 ± 0.25	0.25	0.69	58.1
Guntur	28 Jan. 99	1 Mar. 99	0.03	0.85 ± 0.12	0.02	0.04	3.51
Pune	20 Jan. 99	2 Feb. 99	0.02	1.14 ± 0.13	0.02	0.03	2.97
Delhi	15 Nov. 98	3 Dec. 98	0.02	1.05 ± 0.15	0.02	0.03	4.46
IARI 17-65	28 Feb. 97	2 Jan. 99	0.007	0.08 ± 0.09	0.003	0.02	1.00
			Cry1Ac				
Ottanchathiram	28 Oct. 98	30 Nov. 98	2.47	0.70 ± 0.09	1.26	4.34	13.7
Phagwara	28 Nov. 98	2 Feb. 99	1.66	1.25 ± 0.17	1.14	2.44	5.03
Iruttupallam	16 June 99	2 July 99	0.82	1.17 ± 0.15	0.55	1.20	4.56
Pune	20 Jan. 99	1 Mar. 99	0.64	1.62 ± 0.23	0.42	0.89	3.56
Delhi	15 Nov. 98	23 Jan. 99	0.33	1.37 ± 0.17	0.22	0.47	1.83
IARI 17-65	28 Feb. 98	31 Nov. 98	0.18	1.56 ± 0.18	0.13	0.25	1.00
			Cry1C				
Iruttupallam	16 June 99	3 July 99	3.00	0.85 ± 0.11	1.76	5.30	13.0 (25.0)
Varanasi	15 Nov. 99	25 Nov. 99	0.63	0.68 ± 0.08	0.33	1.16	2.70 (5.2)
IARI 17-65	28 Feb. 97	14 July 99	0.23	1.43 ± 0.24	0.13	0.37	1.00 (1.9)
Najafgarh	9 Nov. 99	27 Nov. 99	0.12	1.15 ± 0.14	0.08	0.19	– (1.0)

[a]Resistance ratio: LC_{50} of field population/LC_{50} of the susceptible IARI 17–65 population. Figures in brackets refer to ratios of LC_{50} of a field population/LC_{50} of the Delhi population for Cry1Aa and LC_{50} of a field population/LC_{50} of the Najafgarh population for Cry1C, respectively.

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the Iruttupallam population were estimated to be 3.4-fold and 26.7-fold relative to the IARI 17–65 and Delhi populations, respectively.

Wide variation in the susceptibility of different populations of *P. xylostella* to Cry1Ab toxin was observed (table 2). The IARI 17–65 population was the most susceptible (LC_{50} 0.007 μg ml^{-1}). The field-collected population from Iruttupallam was the most tolerant of Cry1Ab (1.25 μg ml^{-1}) followed by populations from Ottanchathiram, Bangalore and Phagwara (LC_{50}s of 0.86, 0.43 and 0.15 μg ml^{-1}) respectively. The populations of *P. xylostella* obtained from Delhi, Pune and Guntur were similar to IARI 17–65 in susceptibility. The highest levels of resistance to Cry1Ab, 169× and 116×, were shown by the populations from Iruttupallam and Ottanchathiram, respectively.

Field populations showed moderate levels of resistance to Cry1Ac toxin. The population from Ottanchathiram was the least susceptible to Cry1Ac with an LC_{50} of 2.47 μg ml^{-1} followed by the populations from Phagwara and Iruttupallam (LC_{50}s of 1.66 and 0.82 μg ml^{-1}) respectively. The populations from Delhi and the IARI 17–65 were similar in susceptibility to Cry1Ac (table 2). The level of resistance of the Ottanchathiram population relative to the IARI 17–65 was 13.7-fold.

The populations obtained from Najafgarh and Varanasi and the susceptible IARI 17–65 population, were significantly more susceptible to the Cry1C toxin (LC_{50}s of 0.12, 0.63 and 0.23 μg ml^{-1}) than the field population obtained from Iruttupallam (LC_{50} 3.00 μg ml^{-1}) (table 2). Levels of resistance to Cry1C in this population were estimated to be 13× and 25× relative to the IARI 17–65 and Najafgarh populations, respectively.

Response to selection with Cry1Ab

Selection of the Iruttupallam population of *P. xylostella* with Cry1Ab toxin under laboratory conditions led to an increase in larval survival in each subsequent generation (table 3) and a reduction in activity of Cry1Ab from F_1 to F_3 generation (table 4). The highest mortality in the F_5 generation following selection was only 5.9% after 96 h at a concentration of 1.6 μg ml^{-1} Cry1Ab. Overall susceptibility declined with the LC_{50} value increasing from 1.25 μg ml^{-1} (F_1) to 4.31 μg ml^{-1} in the F_3 generation, with a corresponding increase in slope from 0.56 ± 0.1 (F_1) to 2.33 ± 1.1 (F_3 generation) (table 4).

Cross resistance pattern

The F_3 larval population from Iruttupallam showed a degree of cross resistance to other Cry1A toxins following selection with Cry1Ab. Levels of resistance in the selected Iruttupallam population to Cry1Ab, Cry1Aa and Cry1Ac increased by 3.4, 16.8 and 4.9×, respectively over two generations (table 4). Selection with Cry1Ab thus led to a substantially larger increase in cross resistance to Cry1Aa than to Cry1Ac.

Stability of field-evolved resistance

The susceptibility of the Iruttupallam population to Biobit® increased from an LC_{50} of 11 mg (ai) l^{-1} (fiducial limits, 7.7–16.4) to 2.76 mg (ai) l^{-1} (fiducial limits, 1.9–4.0) over 14 generations in the absence of any selection with *B. thuringiensis* under laboratory conditions. The LC_{50}

Table 3. Changes in susceptibility of *Plutella xylostella* (Iruttupallam population) over four generations of selection with Cry1Ab toxin.

Generation	Dose (μg ml^{-1})	Treatment duration	Corrected percentage mortality			
			24 h	48 h	72 h	96 h
F_2	0.8	24 h	25.0	40.8	42.2	44.2
F_3	0.8	24 h	12.0	22.2	22.9	25.0
F_4	1.6	24 h	27.7	27.7	27.8	39.5
F_5	1.6	24 h	0.69	3.8	5.9	5.9

The susceptibility of F_1 generation larvae is presented in table 2 and also in table 4.

Table 4. Response of the selected Iruttupallam population of *Plutella xylostella* to Cry1Ab and cross resistance pattern to Cry1Aa and Cry1Ac toxins.

Generation	Cry1Ab		Cry1Aa		Cry1Ac	
	LC_{50} (μg ml^{-1})	Slope ± S.E.	LC_{50} (μg ml^{-1})	Slope ± S.E.	LC_{50} (μg ml^{-1})	Slope ± S.E.
Unselected (F_1)	1.25 (0.42–8.07)	0.56 ± 0.10	3.74 (1.76–6.99)	0.89 ± 0.12	0.82 (0.55–1.20)	1.17 ± 0.15
Selected (F_2)	2.98 (1.52–9.50)	0.75 ± 0.16	–	–	–	–
Selected (F_3)	4.31 (2.97–120)	2.33 ± 1.09	62.9 (30.8–116)	0.84 ± 0.12	4.03 (2.51–13.3)	1.55 ± 0.46

Values in brackets are 95% fiducial limits.

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subsequently declined to 0.93 mg (ai) l^{-1} (fiducial limits, 0.5–1.4 after *c*. 34 generations under laboratory conditions. The R value indicated a relatively fast decline in LC_{50} over the first 14 generations (−0.04) compared to subsequent (F_{14}–F_{35}) generations (−0.02). This indicated that, on average, *c*. 24 and 45 generations were required for each 10-fold decrease in LC_{50} of Biobit® respectively. Overall, an average of *c*. 33 generations of *P. xylostella* (R value of −0.03 for F_1–F_{35} generations) was required for a 10-fold decline in LC_{50} of Biobit® when insects were reared without exposure to *B. thuringiensis*.

Discussion

Variation in susceptibility to different strains and purified toxins of *B. thuringiensis* was investigated by bioassays on *P. xylostella* larvae collected from locations ranging from Tamil Nadu in the South to Punjab in the North and from Maharashtra in the West to Uttar Pradesh in the East covering a substantial portion of the diamondback moth distribution across India. In general, the susceptibility of *P. xylostella* to *B. thuringiensis* subsp. *kurstaki* strains and their toxins as well as Cry1C was found to be significantly lower in populations collected from the South, compared to ones from western and northern regions. This suggested geographical differences in susceptibility of *P. xylostella* to *B. thuringiensis*, possibly due to differences in genetic make-up and resistance to the field level usage of *B. thuringiensis* formulations. Although the current usage of *B. thuringiensis*-based insecticides is small, *c*. 30 tons per annum; most of it is being used for the control of insect pests of vegetable crops, especially cole crops (Sawant, 1998), suggesting the possibility of resistance development in *P. xylostella* in regions where *B. thuringiensis* is regularly used.

The populations of *P. xylostella* sampled from India showed a higher mean susceptibility to *B. thuringiensis* subsp. *kurstaki* HD-1 (LC_{50} 0.03 ± 0.15 mg (ai) l^{-1}) than to HD-73 (LC_{50} 0.53 ± 0.24 mg (ai) l^{-1}), supporting the findings of Dilawari *et al*. (1996). The susceptibility of *P. xylostella* varied with respect to individual Cry toxins; Cry1Aa (LC_{50} 1.60 ± 0.38 μg ml^{-1}), Cry1Ab (LC_{50} 0.34 ± 0.17 μg ml^{-1}), Cry1Ac (LC_{50} 1.02 ± 0.36 μg ml^{-1}) and Cry1C (0.99 ± 0.68 μg ml^{-1}) on the basis of all populations tested. Ballester *et al*. (1994) reported that the susceptibilities of different field populations of *P. xylostella* in Philippines to Cry1Ab, Cry1Ac and Cry1B were similar, but lower than that of Cry1Aa by one order of magnitude compared with the laboratory strain (lab V).

The Iruttupallam and Ottanchathiram populations showed relatively high resistance to various individual toxins compared to the susceptible IARI 17–65 population. The resistance levels to Cry1Ab found in some of these populations were relatively high compared to other individual toxins. The propensity of the Iruttupallam population to develop resistance to Cry1Ab was further demonstrated in the laboratory over four generations. Populations of *P. xylostella* did not show as high resistance to *B. thuringiensis* subsp. *kurstaki* HD-1 as to Cry1Ab, although the former has been extensively used in India for pest management. This is probably due to the presence of a mixture of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab toxins expressed in formulations of *B. thuringiensis* subsp. *kurstaki* HD-1 (Koziel *et al*., 1993). For example, Dipel®, a formulation of *B. thuringiensis* subsp. *kurstaki* HD-1 widely used in India, is reported to contain 28% Cry1Aa, 53% Cry1Ab and 19% Cry1Ac (Liu *et al*., 1996). Since the Cry1Ab content is about 50% of the total Cry1A toxins, it might have acted as a major selection agent for development of resistance. Ferré *et al*. (1991) also found a 200-fold resistance in *P. xylostella* to Cry1Ab, due to use of Dipel® under field conditions in the Philippines.

In the present investigation, the Iruttupallam population that showed relatively high resistance to Cry1A toxins also exhibited a 13-fold resistance to the Cry1C toxin. Cry1C toxin is present in *Bacillus thuringiensis* subsp. *aizawai* Bonnefoi & de Barjac, but not in *B. thuringiensis* subsp. *kurstaki* (Iqbal *et al*., 1996). Insecticide use surveys indicate that the *B. thuringiensis* subsp. *aizawai*-based formulations are rarely used in India. Thus, the observed variation in susceptibility to Cry1C may be attributed largely to natural tolerance to the toxin, though this may be conferred by a measure of cross resistance due to use of other *B. thuringiensis*-based products.

Variation in development of resistance in some populations of *P. xylostella* may be due to resistance to *B. thuringiensis* formulations and other agrochemicals used in crop protection acquired over a period of time. Cole crops are grown extensively in Iruttupallam (a suburb of Coimbatore) and Ottanchathiram (a suburb of Dindugal) in Tamil Nadu throughout the year. Products containing *B. thuringiensis* subsp. *kurstaki*, such as Dipel®, Delfin® and Biobit®, are regularly used by farmers in these areas, together with other insecticides, for the control of insect pests of cole crops. Resistance to conventional insecticides in *P. xylostella* is extensively reported from many cole crop growing areas in India (Raju, 1996; Renuka & Regupathy, 1996). Interestingly, *P. xylostella* populations obtained from some States, such as Delhi, Punjab and Maharashtra, showed a high level of susceptibility to *B. thuringiensis* toxins despite their extreme resistance to synthetic insecticides. Increased susceptibility to *B. thuringiensis* in sheep lice *Bovicola ovis* (Schrank) (Phthiraptera: Trichodectidae) is reported to be associated with resistance to synthetic insecticides, possibly through raised levels of mono-oxygenases present in resistant individuals (Drummond *et al*., 1995).

Several other workers have recorded regional differences in susceptibility to *B. thuringiensis*. Perez & Shelton (1997) reported 13–20, 9–77 and 4–18× resistance in field populations of *P. xylostella* to *B. thuringiensis* subsp. *kurstaki* (Javelin®) from Costa Rica, Honduras and Guatemala, respectively, relative to a susceptible Geneva 88 population. Similarly, four-fold variation in susceptibility of *P. xylostella* to *B. thuringiensis* was reported in Chile (Garrido & Araya, 1997). Several cases of field resistance in *P. xylostella* to *B. thuringiensis* have been reported from USA (Tabashnik *et al*., 1990; Shelton *et al*., 1993; Tang *et al*., 1996), Japan (Hama *et al*., 1992), the Philippines (Ferré *et al*., 1991), Malaysia (Syed, 1992) and China (Zhao *et al*., 1993) which could be attributed to the high level usage of *B. thuringiensis* products in these countries. The differences in susceptibility of *P. xylostella* to *B. thuringiensis* subsp. *kurstaki* and some of the toxins were reported earlier from India (Chandrasekaran & Regupathy, 1996; Gujar & Kalia, 1999; Mohan & Gujar, 2000). The present findings are in agreement with those reported earlier by ourselves, and confirm the fears that some populations of *P. xylostella* possess appreciably high levels of resistance to individual toxins, especially Cry1Ab.

The resistance to *B. thuringiensis* in *P. xylostella* was found

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to extend broadly to other sub-classes of Cry1A toxins. For example, the F_3 generation of the Cry1Ab-selected Iruttupallam population developed a high level of cross resistance to Cry1Aa and moderate resistance to Cry1Ac. Cross resistance between different Cry toxins of *B. thuringiensis* has been documented in various insect species (Tabashnik, 1994). Recently, Ballester *et al*. (1999) reported cross resistance amongst Cry1Aa, Cry1Ab, Cry1Ac and Cry1F owing to the presence of a common binding site in the midgut epithelium of *P. xylostella*. However, resistance did not extend to Cry1B and Cry1C due to the presence of separate binding sites in the midgut epithelium of *P. xylostella*.

As *P. xylostella* responds quickly to artificial selection under laboratory conditions, and in view of the possibility of resistance development under field conditions, the question looms large as to whether the stability of *B. thuringiensis* resistance in *P. xylostella* is similar to that of conventional insecticide resistance (Tabashnik *et al*., 1991). The resistance to Biobit® declined when a field population was reared in the laboratory in the absence of *B. thuringiensis* over several generations. A similar decline in resistance in *P. xylostella* to Dipel® was reported by Tabashnik *et al*. (1991, 1994) and to Javelin® by Tang *et al*. (1997). The decline in resistance in the absence of *B. thuringiensis* is measured in terms of the genetic parameter, R, which is the rate of decline in resistance per generation. The reports of wide variation in R values (-0.06 to -0.50) for *P. xylostella* (Tabashnik, 1994; Tang *et al*., 1997) suggest that decline in resistance depends on the initial level of resistance and the number of generations of rearing in the absence of any selection pressure of *B. thuringiensis*. Laboratory-selected highly resistant populations of *P. xylostella* appeared to lose their resistance faster than the field-selected moderately resistant populations (Tabashnik *et al*., 1994). Tang *et al*. (1997) reported a rapid decline in resistance to Javelin® in the resistant *P. xylostella* population (Loxa A) over the first three generations followed by a plateau for at least seven generations. The relatively low initial, final and overall R values of -0.04, -0.02 and -0.03 based on LC_{50}s in F_1 and $F_{14;}$ F_{14} and $F_{35;}$ and F_1 and F_{35} generations, respectively in the present study may be attributed to generally lower levels of field resistance in India than in the USA. This implies that the initial phase required about 24 and the final phase about 45 generations for each 10-fold decrease in LC_{50}. On average, *c*. 33 generations were required for a 10-fold decrease in LC_{50}. The most likely cause of instability of resistance to *B. thuringiensis* is a fitness cost associated with resistance, such as low survival, low fecundity or reduced egg hatching. These fitness costs decline rapidly as susceptibility reverts back in the absence of selection pressure (Groeters *et al*., 1994; Tabashnik, 1994). By contrast, Sayyed & Wright (2001) did not find any association of fitness costs with Cry toxin resistant alleles in *P. xylostella*, suggesting the possibility of a trade-off between different fitness factors.

The results of this study confirm the development of resistance to *B. thuringiensis* subsp. *kurstaki* in *P. xylostella* and emphasize the need for monitoring temporal and spatial changes in the susceptibility of this species in relation to *B. thuringiensis* use in India in order to maximize crop production. The results may also be useful in selecting suitable regions for introducing transgenic cole crops now under review. Further studies on the genetic basis and mechanism of resistance at a molecular level will also be important for the development of an effective resistance management strategy against this species (Ferré & van Rie, 2002).

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