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V Sunitha
AINP on VPM, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

TVK Singh
Former Dean of Agriculture, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

GB Supriya
Department of Entomology, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

J Satyanarayana
Dean of Agriculture, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
V Sunitha
AINP on VPM, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

Insecticide resistance monitoring of diamondback moth, *Plutella xylostella* (Linn.) in Delhi population

V Sunitha, TVK Singh, GB Supriya and J Satyanarayana

Abstract
Insecticidal resistance studies against third instar larvae of DBM (*Plutella xylostella* L.) were carried out to know the rate of development of resistance from F_1 to F_3 generations in Delhi population. The third instar larvae obtained from field population were tested against acephate, cypermethrin, spinosad, cartap hydrochloride and Cry2Ab by using leaf dip method of bioassay to calculate LC_{50} values. The LC_{50} values of the insecticides were further used to quantify the resistance in *P. xylostella* of parental generation (F_0) from Delhi population. The survivals from F_0 generation were reared to next generation (F_1). Resistance development studies were carried out with third instar larvae of F_1 generation by applying a concentration of 80.00% mortality in every generation for selection from F_1 to F_3 generations against all the test insecticides and Cry2Ab toxin revealed that 1.27 folds resistance was developed against acephate in F_3 generation. In case of cypermethrin, 3.00 folds resistance in F_3 generation. Resistance studies further revealed development of 1.00 folds resistance against spinosad, 1.26 folds resistance against cartap hydrochloride, respectively, in F_3 generation of Delhi populations. In case of Cry2Ab toxin 1.35 folds resistance was recorded in F_3 generation. The rate of development of resistance from F_1 to F_3 generations increased in all the test insecticides and Cry toxin, except against spinosad from F_1 to F_3 generations.

Keywords: Cry2Ab, Delhi population, insecticide resistance, *Plutella xylostella*

Introduction
India is the world's largest cauliflower (*Brassica oleracea* var. *botrytis* L.) grower and second largest cabbage (*B. oleracea* var. *capitata* L.) grower next to China occupying an area of 3,72,000 and 4,02,000 hectares, respectively [1].

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae), is important pest of cruciferous crops and ubiquitous in nature [2]. In India, DBM was reported in 1914 on cruciferous vegetables and is now the most devastating pest of cole crops in the states of Punjab, Haryana, Himachal Pradesh, Delhi, Uttar Pradesh, Bihar, Andhra Pradesh, Tamil Nadu, Maharashtra and Karnataka [3].

The infestation of the pest increases gradually from first fortnight of August and leads to total loss of the crop [4]. In India it causes significant economic losses up to 50% with an estimate of US$ 168 million per year. Absence of effective natural enemies and rapid development of insecticide resistance to many classes of insecticides, which account for 30-50% of the total cost of production are considered to be the major causes of increasing pest status of DBM in most parts of the country.

DBM occupies second position in being resistant to 91 compounds of insecticides [5] and to be the first species to develop field resistance to *Bacillus thuringiensis (Bt)* Cry toxins, and is one amongst three insect species to have developed field resistance to Bt based spray products [6].

It is documented that resistance is inevitable within a span of two to three years for following the introduction of a new insecticide. Recent examples of field resistance developed to relatively selective new compounds, include indoxacarb, avermectins, spinosad, Bt- based products, benzyl ureas and chlorantraniliprole.

Hence, the present study was undertaken for quantifying the resistance levels in DBM from Delhi against four commonly used insecticide groups with diverse modes of action and one toxin.

Material and Methods

Laboratory investigations were carried out during 2011-2012 in the Bt Lab, Department of Entomology, College of Agriculture, Rajendranagar, Hyderabad.

LC_{50} Calculation

Test Insect Population

Cabbage cultivar "Charmant" nursery was raised in the greenhouse and one month old seedlings were transplanted in the main field and raised without any insecticide application. DBM, larvae were collected from farmers' cabbage fields in and around Hyderabad, to establish culture. Leaves were harvested daily washed with tap water and provided as feed to the larvae.

Larvae were allowed to pupate in the jars and the pupae were kept in petri plates and placed in a cage for moth emergence. The emerged adult moths were allowed to lay eggs on mustard seedlings. Adults were provided with 10% honey solution fortified with multivitamins and proteinex on a cotton swab for better egg laying. Mustard seedlings with eggs of DBM were collected from the cage and kept in glass jars for hatching. The neonates were reared on insecticide free cabbage leaves. At every successive instar, the larvae were shifted to clean jars and fresh cabbage leaves were provided. Larvae in the third instar stage were used in bioassay studies.

Test Insecticides and Cry toxin

To determine the LC_{50} values of insecticides and Cry toxin against DBM larvae, four insecticides *viz.*, acephate (Organophosphate), cypermethrin (Synthetic pyrethroid), spinosad (Spinosyn), cartap hydrochloride (Neries toxin) and Cry2Ab were used.

One hundred ml of one per cent stock solution of all the above test insecticides were used for the preparation of serial dilutions. Initially broad range concentrations were tested for each test insecticide and toxin depending on the 20 to 80% mortality observed, narrow range concentrations were tested. A control was also maintained at each time of experimentation and the mortality data was corrected by using modified Abbotts formula [7]. Bioassay was repeated for treatments wherein control mortality exceeded 20%.

Stock Solution Preparation for Cry toxin

The technical formulation of Cry2Ab (3.93 mg/g) was supplied by CICR, Nagpur. 100 mg of the toxin was dissolved in 5 ml distilled water to obtain a stock solution of 60 µg/ml concentration. The stock solution was subjected to serial dilutions to obtain different concentrations and a drop of Tween-80 was added. Similarly a drop of Tween-80 was added to control also.

Bioassay

Bioassays were conducted with third instar larvae of *P. xylostella* by using a standard leaf dip method [8]. A bioassay was conducted to deduce the LC_{50} of all the four test insecticides and Cry2Ab toxin, this LC_{50} concentration was used in assessment of resistance among different populations of DBM from parental generation F_0 to F_3 generation.

Leaf discs (5 cm) were used for bioassay studies. The leaf discs were dipped in 10ml of aqueous solution of various concentrations of test insecticides and Cry toxin, whereas, control leaf discs were immersed in distilled water having a drop of Tween-80 for about fifteen seconds and shade dried before transferring onto a moistened filter paper in a petri plate. Ten third instar larvae were released on each treated leaf disc in each concentration. Each treatment was replicated thrice. Larval mortality was recorded at 24, 48 and 72 hours after treatment (HAT) by counting the larvae as dead or moribund when they did not resume activity after repeated proddings. The mortality at 72 HAT was considered as end point for the assessment of toxicity of test insecticides and Cry toxin [9]. LC_{50} values of all test insecticides and Cry2Ab toxin were determined by probit analysis [10]. The calculated LC_{50} was used in quantifying the resistance in different populations by inducing selection pressure.

Quantification of Insecticidal Resistance in Delhi Population

To assess the resistant levels in different DBM populations, larvae were supplied from Division of Entomology, IARI, Delhi and reared on insecticide free cabbage leaves in the laboratory. All the three populations were reared separately and larvae in the third instar were used for bioassay studies.

Bioassay and Lab Selection

The larvae obtained from the field collected population were designated as F_0 population and the subsequent generations (obtained from previous generations) were designated as F_1 (First generation), F_2 (Second generation), F_3 (Third generation). The process of selection pressure for insecticides and Cry toxin was initiated in the parental generation (F_0) and continued up to F_3 generation.

The calculated LC_{50} values of each insecticide and Cry2Ab toxin was subjected to preliminary bioassay for all the three populations separately. Individual DBM population was subjected to five concentrations (LC_{50}, two concentrations higher than LC_{50} and two concentrations lower the value of LC_{50}) of each individual insecticide and Cry2Ab toxin and a control with ten third instar larvae per treatment and replicated thrice. Larval mortality was recorded at 24, 48 and 72 HAT. The concentration (LC_{80}) that gave 80% mortality was selected from the preliminary bioassay and the survivals at other concentrations were rejected. Using this LC_{80} concentration of all the test insecticides and Cry2Ab subsequent bioassays were conducted with all the three different DBM populations using 100 third instar larvae per treatment (individual insecticide and Cry2Ab) and replicating the same thrice for inducing selection pressure from the parental generation (F_0) onwards along with a control. The survivals in the bioassay were raised to first generation (F_1) again during third instar F_1 larvae were subjected to bioassay in the above mentioned manner till F_3 generation.

The concentrations were adjusted in subsequent generations depending on the per cent larval survivals obtained in the previous generation. In each generation at least 4-5 DBM larvae from Delhi that survived in the bioassays were stored in 100% alcohol for genetic variation studies.

Assessment of Insecticidal Resistance in *P. xylostella*

The degree of development of resistance through different generations was determined by working out LC_{50} values in each generation and thus computing the resistance ratio (RR) by dividing the LC_{50} value for F_n generation with LC_{50} value of the F_1 generation [11].

$$\text{Resistance ratio (RR)} = \frac{LC_{50} \text{ value of } F_n \text{ generation}}{LC_{50} \text{ value of } F_1 \text{ generation}}$$

Results and Discussion

The initial LC_{50} calculated for the DBM population collected around Hyderabad cabbage agro-ecosystem for all the four test insecticides and Cry2Ab was acephate (0.1%), cypermethrin (0.008%), spinosad (0.003%), cartap hydrochloride (0.01%) and Cry2Ab (0.3µg/ml). These LC_{50} values were used for assessing the resistance development for Delhi population.

Resistance development in DBM against acephate in Delhi population

The concentrations of acephate which was used in bioassay varied from 0.05% to 0.25%, 0.10% to 0.30% and 0.10 to 0.30%, in F_1, F_2 and F_3 generations, respectively. The documented LC_{80} against third instar larvae of DBM in F_1, F_2 and F_3 generations were 0.20%, 0.25% and 0.20% respectively.

The calculated LC_{50} values obtained in F_1, F_2 and F_3 generations were 0.088%, 0.130% and 0.112% for Delhi population. Resistance ratios obtained in F_2 and F_3generations in comparison to F_1 generation were 1.47 and 1.27 folds. The results obtained in the present study indicate that Delhi population developed resistance against acephate because the resistance ratio was more than one (Table 1).

The results showed that the LC_{50} values increased from F_1 to F_2 generations and again decreased from F_2 to F_3 generations. Among the three generations the highest LC_{50} value was recorded in F_2 generation (0.130%). The results obtained in the present study indicate that Delhi population developed resistance against acephate because the resistance ratio was more than one.

Resistance development in DBM against cypermethrin in Delhi population

LC_{50} concentration of 0.008% was obtained in bioassay using DBM population sampled from cabbage agro-ecosystem in and around Hyderabad the same was used to obtain the survivals of DBM in Delhi with cypermethrin.

Concentrations in the range of 0.001% to 0.032% were used bioassays in all three different generations (F_1, F_2 and F_3) to get LC_{80} and LC_{50} against DBM third instar larvae of Delhi population. The calculated LC_{80} for applying selection pressure were 0.008%, 0.008%, 0.016% in F_1, F_2 and F_3 generations, respectively. The LC_{50} recorded from the bioassays were 0.001%, 0.002%, 0.003%, for Delhi population in F_1, F_2 and F_3 generations, respectively. Resistance ratios in F_2 and F_3 generations over the F_1 generation were 2.00 and 3.00 fold for Delhi population in F_2 and F_3 generations, respectively (Table 2).

Resistance development in DBM against spinosad (Spinosyns) in Delhi population

The median lethal concentration of 0.003% for spinosad was recorded in the bioassay conducted with DBM larval population from Hyderabad. Using this LC_{50} bioassays were conducted with third instar larvae of DBM collected from Delhi population to get 80% larval mortality and for inducing selection pressure for resistance development. Concentrations ranging from 0.0015% to 0.004% were used in bioassays for all F_1, F_2 and F_3 generations, respectively. LC_{80} obtained were 0.0030%, 0.0035%, 0.0035% in F_1, F_2 and F_3 generations, respectively. The LC_{50} calculated for Delhi population displayed similar LC_{50} values of 0.002% in F_1, F_2 and F_3 generations (Table 3).

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 1.00 and 1.00 folds.

The resistance ratios of F_2 and F_3 generations in relation to F_1 generation was equal to one, which indicated that resistance has not developed.

Resistance development in DBM against cartap hydrochloride (Nereistoxin) in Andhra Pradesh, Karnataka and Delhi population

The median lethal concentration of 0.01% for cartap was recorded in the bioassay conducted with DBM larval population from Hyderabad. Using this LC_{50} bioassays were conducted with third instar larvae of DBM collected from Andhra Pradesh, Karnataka and Delhi populations to get 80% larval mortality and for inducing selection pressure for resistance development. Concentrations ranging from 0.005% to 0.045% were used in bioassays for all the three populations studied in F_1, F_2 and F_3 generations, respectively. LC_{80} obtained was 0.02% in all three generations of Andhra Pradesh population, 0.025%, 0.030% and 0.03%, in F_1, F_2 and F_3 generations for Karnataka population while in case of Delhi population it was 0.035%, 0.04% and 0.04% in F_1, F_2 and F_3 generations, respectively. The LC_{50} calculated for Andhra Pradesh population was 0.009%, 0.011% and 0.012% in F_1, F_2 and F_3 generations. LC_{50} values for Karnataka population were 0.007%, 0.015%, 0.016% in F_1, F_2 and F_3 generations. While that of Delhi population, LC_{50} values were 0.023%, 0.027%, 0.029% in F_1, F_2 and F_3 generations, respectively (Table 4).

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 1.22 and 1.33 for Andhra Pradesh population, 2.14 to 2.28 fold for Karnataka population and 1.17 and 1.26 folds for Delhi population respectively. The results clearly showed that in all the three populations the resistance ratios were more than one in F_3 generation, which indicates that resistance developed against cartap hydrochloride in all three populations.

Resistance development in DBM against Cry2Ab in Delhi population

The median lethal concentration of 0.3µg/ml for Cry2Ab was recorded in the bioassay conducted with DBM larval population from Hyderabad. Using this LC_{50} bioassays were conducted with third instar larvae of DBM collected from Delhi to get 80% larval mortality and for inducing selection pressure for resistance development. Concentrations ranging from 0.1µg/ml to 1.0µg/ml were used in bioassays F_1, F_2 and F_3 generations, respectively. LC_{80} obtained was 1.00µg/ml, 0.8µg/ml, 1.00µg/ml in F_1, F_2 and F_3 generations, respectively. The LC_{50} calculated were 0.337µg/ml, 0.289µg/ml and 0.456µg/ml in F_1, F_2 and F_3 generations, respectively (Table 5).

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 1.17, 1.26 folds for Delhi population respectively. The results clearly showed that the resistance ratios were more than one in F_3 generation, which indicates that resistance developed against Cry2Ab in Delhi population.

Discussion

Variation in susceptibility pattern of *P. xylostella* larvae collected from Delhi was investigated by bioassays against insecticides conferring diversified mode of action. The study was carried out with four conventional insecticides acephate

(organophospahte), cypermethrin (synthetic pyrethroid), spinosad (spinosyns), cartap hydrochloride (Nereistoxin) and a Bt toxin, Cry2Ab. Susceptibility pattern was quantified in terms of median lethal concentration for all the insecticides and Cry2Ab toxin by inducing selection pressure of magnitude LC_{80} from F_0 till F_3 generation and resistance ratios were calibrated over the F_1 generation in terms of LC_{50} obtained in every generation. In general, the susceptibility of *P. xylostella* to conventional insecticides and to toxin were lower.

The median lethal concentration for the insecticides and Cry2Ab toxin against third instar larvae sampled from Delhi showed less variation in their susceptibility patterns. The highest median lethal concentration for acephate was documented LC_{50}-0.13% in F_2 generation.

LC_{50} values depicting a slow resistance development over the three generations supporting the findings of [12] who found moderate resistance to organophosphates (acephate and methomyl) and cartap hydrochloride in four strains of *P. xylostella* (Okinawa selected, Okinawa unselected, Osaka selected and Osaka unselected), contradictory to this were the results obtained in the present study wherein the DBM populations of Delhi, revealed a gradual decrease in the median lethal concentrations from F_1 generation to F_2 and from F_2 to F_3, proving an increase in susceptibility.

A high level of resistance to organophosphate insecticides in DBM has been reported from various parts of the world -2096 folds resistance to malathion in Malaysia [13], -305 to -735 fold resistance to malathion in Thailand [14], - 20 to -75 fold resistance to chlorpyriphos, methyl parathion, malathion, methamidophos and diazinon [15]. Resistance is inevitable by inducing selection pressure for several generations with insecticides in insects even for the susceptible strains and laboratory strains [16-19] but low level of resistance documented in the present study can be attributed to the fact that selection pressure was induced at LC_{80} concentration and for only three generations. Lack of insecticidal exposure for generations together can deplete resistance development in [20-21] but certain reasons like feeding behavior of the larvae in the bioassay, methodology of bioassay undertaken, precision in performing bioassay and other physiological conditions may have caused the increase in susceptibility pattern for Delhi populations and ultimate reduction in the resistance ratios.

Though usage of acephate for the management of DBM has been replaced by new insecticides that are commercially available, recent studies of [22] is in confirmation with the present study where only 2.3-fold resistance was documented by the resistant *P. xylostella* strain over the susceptible strain against prothiofos.

DBM population sampled from Delhi showed gradual increase in LC_{50} values depicting a slow resistance development over the period of three generations against cypermethrin. DBM population developed resistance folds in range of 3.00 fold resistance, in F_3 generation. Enzymatic role of mixed function oxygenases coupled with target site nerve insensitivity (Kdr) [23-24] are regarded as the most common mechanisms of resistance by DBM to synthetic pyrethroids, reduced insecticide penetration through the cuticle is also cited to occur. The resistance folds developed by DBM against cypermethrin are in accordance with other reports - 144 fold against cypermethrin in DBM at Panipat (Haryana) and -115 fold resistance to pyrethroids at Delhi and Karnataka [25], -25 fold resistance against pyrethroids [26], -26507 folds resistance against cypermethrin by DBM population sampled from Bangalore [27]. In the present study susceptibility pattern was decreased till the F_3 generation (as depicted by increase in LC_{50} values) showing a moderate level of resistance development. Further studies are required with regards to calibration of variation in enzyme titres *viz.*, mixed function oxidases, glutathione S transferases using specific substrates that play vital role in resistance development against synthetic pyrethroids as reported in other insects as such. The present results are in corroboration with studies conducted elsewhere who reported resistance ratio of 21 fold for Peng Hu strain and 899 fold for Ban-Chu strain in China [28], Nicaragua [29], Taiwan [30], USA [15] and Pakistan [31].

Resistance development studies for DBM against spinosad showed no resistance development in the *P. xylostella* from Delhi since the median lethal concentration remained unchanged even after three generations. The reason for the *P. xylostella* populations developing no resistance may be due to the fact that spinosad being a novel insecticide and the usage pattern and selection pressure by this insecticide is relatively new in cabbage agro ecosystem, alternatively the pest was never pre-disposed to spinosad sprays in these areas as such. The findings of the present studies are in corroboration with the findings of [32], [33] and [34] who earlier reported the higher field efficacy of spinosad against *P. xylostella*.

The present data confirms the findings of [35] who determined the toxicity ratio of 1.3 to 1.2 from seven zones and 0.8 to 316 from six zones in Geneva. [36] reported LC_{50} of 24.06 ppm and 26.77 ppm from Lu Chu and His-hu strain, respectively, in China during 2001. [37] showed tolerance ratio of more than 100 to spinosad in DBM population of California (USA), which indicated high levels of resistance than the present study. [38] showed no significant resistance in field population of diamondback moth (New Zealand) which is in conformity with the findings of the present study.

The present study indicated that *P. xylostella* in India remain susceptible to spinosad. However, resistance to spinosad occurred in Hawaii (2000), Georgia (2001) and California (2002) as a consequence of multiple years of extensive application. A major reason for the rapid resistance development to spinosad in Hawaii was the lack of suitable alternatives and the unsynchronized use of insecticide classes that led to continuous population exposure to spinosad as it happened in South East Asia [39-40] and North America [35].

The propensity for the selection of spinosad resistance may have arisen from pre - existence of resistance alleles from the past use of organochlorine insecticides as the mode of action of organochlorine and spinosad as GABA/nicotinic acetyl choline receptor as a target [41-42]. However, the possibility of *P. xylostella* carrying spinosad resistance allele may have been introduced from other areas via transportation of cabbage also cannot be ruled out [31]. Our results indicated that Delhi population of *P. xylostella* was susceptible to spinosad. From this it can be construed that spinosad can be used commercially as an alternative to particularly those insecticides against which *P. xylostella* has developed resistance.

Studies pertaining to resistance development for the *P. xylostella* populations from Delhi showed considerable decrease in susceptibility pattern over the three generations against cartap hyrochloride. The development of resistance to cartap hydrochloride by *P. xylostella* is reported in India [43] and elsewhere globally *viz.*, Japan [44-46], Taiwan [47], China [48] and Korea [49]. The present study is in accordance with that of

[50] who found resistance levels (expressed as % survival) varied from 17.9 to 52.4 to cartap hydrochloride and [51] who recorded the moderate survival percentage (1.11) of DBM treated with cartap hydrochloride [52]. reported the development of resistance to cartap hydrochloride at recommended field concentrations.

The resistance ratio developed by the DBM populations in present study are in accordance with [53] who obtained resistance ratio in range of 2.8 to 7.1 for the most resistant strain that received multiple sprays of cartap hydrochloride. Likewise [54] documented low to moderate level of resistance to cartap hydrochloride against DBM populations from 3-12 locations of Karnataka. The results of the present studies, by and large, fall in line with those obtained by [55] who obtained LC_{50} values 0.015% to 0.020%. with cartap against multi-resistant *P. xylostella* population from Punjab. In the present study in LC_{50} of cartap in comparison with doses of other test insecticides and Cry2Ab was in the range of 0.007-0.029 which proves the effectiveness of cartap in the mangement of DBM.

The resistance development in *P. xylostella* populations sampled from Delhi gradually decreased from F_1 to F_2 generations and again increased from F_2 generation to F_3 generation, although geographical differences in susceptibility of *P. xylostella* to *Btk* products in India has also been reported by [56-57].

In general the susceptibility of *P. xylostella* to *Bt* strains and their toxins was found to be significantly lower in populations that originated from Southern India followed by those from Western and Northern India [57]. This suggests the possibility of diamondback moth adaptation in the populations where *Btk* formulations are regularly used [58]. However, in our studies the susceptibility patterns indicate some changes in susceptibility. This may be due to the fact that baseline susceptibility of local insect populations depends not only on the extent of selection pressure (amount of insecticide used) but the other factors like relative dominance of resistant alleles, level of immigration of susceptible individuals (gene flow), population structure, and exposure to the pesticide time of year are also responsible. In addition, insect behaviour also plays a major role [59]. Therefore slight differences reported in susceptibility of insect populations to Cry toxins are likely due to differences in gene level among the populations and agro ecological conditions at different locations. Similar difference in susceptibility to *Bt var. kurstaki* have been reported by [56], [60], [61], [62] and [63]. Further studies are required in understanding the mechanism underlying the resistance though reduced binding of Cry toxin to BBMV's and reduced activation of proteases coupled with faster degradation of proteases are already documented to be resistance mechanism [64]. Studies with regards to type of mid-gut proteases involved in activation of Cry toxin for these populations are required to understand cross resistance mechanisms and for successful management of DBM using IRM strategies.

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