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RESEARCH

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Baseline susceptibility of cabbage butterfly, *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae) to *Bacillus thuringiensis* (Berliner) Cry toxins in Meghalaya

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Abstract

Background Cabbage butterfly, *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae), is one of the most important pests of cabbage and other cruciferous crops and accounts for > 40% yield reduction in the crops. An investigation on the baseline susceptibility of *P. brassicae* to *Bacillus thuringiensis* (*Bt*) Cry toxins in Meghalaya was evaluated for future exploitation in *Bt* resistance monitoring. Two different Cry toxins, *Bt* Cry1C and Cry2Ab, were screened against 11 different field populations of *P. brassicae* from the state of Meghalaya. LC_{50} was evaluated based on the response of larval mortality of *P. brassicae* using the leaf-dip bioassay method.

Results The baseline-susceptibility tests conducted on *P. brassicae* in 11 different field populations from Meghalaya revealed that Smit population strains seemed to show less tolerance to both the *Bt* Cry toxins (Cry1C and Cry2Ab). Compared to the Cry1C toxin, Cry2Ab was found more potent against *P. brassicae*. The median lethal concentrations, LC_{50} 72 h, varied from 0.535 to 1.725 µg/ml for Cry2Ab and 0.546–1.803 µg/ml for Cry1C toxin. The screening using leaf-dip bioassay resulted in a tolerance ratio of 3.3-fold and 3.2-fold for Cry1C and Cry2Ab, respectively. The most tolerant strains of *P. brassicae* from Umiam and Pepbah regions were observed to show discriminating concentrations of 19.30 µg/ml for Cry1C and 24.03 µg/ml for Cry2Ab (LC_{99}, 72 h).

Conclusions The Cry2Ab toxin was found to be more virulent than Cry1C toxin for *P. brassicae*. Certain candidate discriminating concentrations for Cry1C and Cry2Ab can be used as benchmarks for future resistance monitoring of *P. brassicae* to *Bt* Cry toxins.

Keywords *Pieris brassicae*, *Bacillus thuringiensis*, Cry1C, Cry2Ab, Baseline-susceptibility

Background

The cabbage butterfly, *Pieris brassicae*, (Linnaeus) (Lepidoptera: Pieridae) is an important oligophagous and destructive pest that attacks cabbage and other cruciferous crops. It is distributed in the Himalayas from Chitral to Bhutan, while in the hills and plains, it has been recorded in Meghalaya, Manipur, West Bengal, Assam, Andhra Pradesh, Bihar, Gujarat, Orissa and Uttar Pradesh in India as well as in many other countries (Das et al. 2018). The most damaging stage of this pest is the larvae which attack during the seedling, vegetative and flowering stages of the crop and alone cause > 40% yield reduction in the crop (Hasan and Ansari 2011), which makes most of the produce unfit for human consumption (Ali and Rizvi 2007).

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Several chemical insecticide residues such as dichlorvos, monocrotophos, phorate, parathion, captafol, permethrin, etc., were found to contaminate the vegetable crops (Nishant and Upadhyay 2016). Continuous and repetitive use of pesticides caused an environmental contamination, and the pests raised insecticidal resistance. Alternatively, other methods such as the use of biocontrol agents are environmentally safe and preferable for sustainable agriculture. Due to the toxicity of *Bt* delta-endotoxins produced in the sporulating cells, biopesticides based on insecticidal crystal protein (ICP) of *Bt* toxin have been widely used in insect control all over the world.

In the insect midgut, inert protoxins are proteolytically broken down to produce active toxins that attach to midgut cells and cause cell lysis through the formation of ion pores in the midgut, leading to the cessation of feeding and death of the insects (Gill et al. 1992). Currently, *Bt* formulations that are promoted for lepidopteran control contain the *Bt* subsp. *aizawai*, and *Bt* subsp. *kurstaki.* These subspecies produce various toxins belonging Cry1 and Cry2 classes, which have been found to be extremely toxic to *P. brassicae* (Mohan et al. 2014). In addition to being used as a bio-pesticide, *Bt* transgenic crops that produce toxins also act as virtually year-round protection against insect damage. Since 20–30% of crop produce is lost due to insect damage, transgenics for insect resistance have been drawing special attention in India also due to the damage caused by the insect pest. (Tuli et al. 2000).

The evolution of *Bt* or pesticide resistance is a process that restricts the development of new insecticides. Steps should be taken to delay the evolution of resistance after the introduction of an insecticide. *Plodia interpunctella* (McGaughey 1985) was the first insect species to be identified to show resistance or tolerance to *Bt* along with the other pests including *Pectinophora gossypiella* (Saunders), *Plutella xylostella* (Linnaeus.), *Helicoverpa armigera* (Hübner), *Spodoptera frugiperda* (JE Smith), *Trichoplusia ni* (Hübner) and *P. brassicae* (Linnaeus). Several studies have recently been conducted on the development of *Bt* cruciferous crops for controlling various lepidopterous pests like *P. brassicae*, *P. xylostella, C. binotalis* etc. However, the variations in the toxicity response shown by cabbage butterfly to *Bt* Cry toxins have not been thoroughly explored. This communication provides substantial information on the baseline-susceptibility pattern of different insect populations of *P. brassicae* from Meghalaya against *Bt* Cry toxins (Cry1C and Cry2Ab).

Methods

Insect strain

Different stages of *P. brassicae* were collected from the cruciferous fields of 11 different regions of Meghalaya as shown in Table 1.

Table 1 Source description of *Pieris brassicae* populations used to establish baseline susceptibility to *Bt* Cry toxins

Districts	Samples' No	Regions	Collection months	GPS	Stages of pest	Host plants
Ri-Bhoi	1	Umiam	November, 2021	25.68195° N 91.911194° E	Eggs, larvae, pupae, adults	Mustard
	2	Kharpati	January, 2022	25.912974° N 91.934373° E	Eggs, Larvae	Cabbage
	3	Mawpun	January, 2022	25.698759° N 91.93338° E	Larvae	Cauliflower
	4	Nongrah umroi	January, 2022	25.71879° N 91.989126° E	Eggs	Cabbage
	5	Umeit	February, 2022	25.709728° N 91.952581° E	Eggs and larvae	Cauliflower
East Khasi hills	6	Mawklot	October, 2021	25.549284° N 91.823664° E	Larvae	Cabbage
	7	Pepbah	December, 2021	25.512808° N 91.940159° E	Larvae	Cabbage
	8	Smit	June, 2022	25.504187° N 91.907181° E	Eggs	Cabbage
West Jaintia hills	9	Mookyndur	May, 2022	25.504996° N 92.115218° E	Eggs	Broccoli
	10	Sohphoh	May, 2022	25.565753° N 92.163766° E	Eggs and larvae	Cabbage
	11	Wahiajer	May, 2022	25.531982° N 92.158137° E	Larvae	Cabbage

Establishment and maintenance of insect colony

Different field populations of the cabbage butterfly, *P. brassicae* were reared and maintained separately in the Entomology laboratory, School of Crop Protection, CPGS-AS, Umiam, Meghalaya. The insects were maintained under the controlled conditions at 27 ± 1 °C with 60–70% R.H. and 14:10 h (L:D). The eggs collected from the field were kept in sterilized Petri plates (9 cm diameter). The moistened filter papers were kept inside the Petri plates to prevent the desiccation of the eggs. The field-collected larvae were reared on fresh, healthy and tender leaves of cruciferous crops throughout the experiment. After hatching of the eggs, the larvae were kept in Petri plates. After attaining second instar, the larvae were transferred to plastic jars having a size of (15×10 cm^2). Pesticide-free and tender leaves of crucifers were provided regularly when necessary. The jars were covered with muslin cloths using a rubber band. Pupae were kept separately in a separate jar after one day of their formation. After emergence, adult insects were released in the ratio of 1:1 (female: male) for mating in wooden rearing cages having a size of ($45 \times 45 \times 54$ cm^3) containing fresh cabbage seedlings for oviposition. Cotton swab with 20% honey solution + vitamin E was provided as a source of food for the adults. The eggs along with the leaves were collected and placed in clean Petri plates until hatching. Upon hatching, the larvae were provided with fresh leaves for the second generation, and the second instar larvae obtained were used for evaluating baseline data on the susceptibility status to *Bt* Cry toxins (*Bt* Cry1C and Cry2Ab).

Bacillus thuringiensis Cry toxins

For the evaluation of baseline susceptibility of *P. brassicae* to *Bt* Cry toxins, the toxins such as Cry1C (Department of Land and Environment, University of Melbourne, Vic3010, Australia) and Cry2Ab (6 mg toxin/gm corn leaf powder, Monsanto Research Centre, Bangalore) were procured from the Division of Entomology, IARI, New Delhi.

Preparation of stock solutions and working solutions of Bt Cry1C and Cry2Ab toxins

Stock solutions (10 ppm) of both the Cry toxins were prepared. The screening was done for all 11 field populations of *P. brassicae* by treating with two different concentrations, i.e., 0.1 ppm and 10 ppm of both Cry1C and Cry2Ab toxins, respectively. The range of working solutions of the test Cry toxins at different concentrations (0.1–6.4 µg/ml) was sorted out and prepared in distilled water based on the reaction of *P. brassicae* to *Bt* Cry1C and Cry2Ab toxins. For the assessment of *P. brassicae* susceptibility, working solutions of *Bt* Cry1C toxin in the range of 0.1–5.0 µg/ml and *Bt* Cry2Ab in the range of 0.1–6.4 µg/ml were used.

Leaf-dip bioassay method

The bioassays were used to assess the toxicity response exhibited by the larvae of *P. brassicae* to *Bt* Cry toxins at different doses (Tabashnik et al. 1991). In this approach, the cabbage or cauliflower leaves were washed thoroughly with distilled water that contains 0.1 per cent Triton X-I00 before being air-dried for approximately 10 min. Using a plastic punch, leaf disks (4.5–5 cm diameter) were cut from the center of the cabbage plants and dipped in different concentrations of respective toxins for 15 s and allowed to dry under the fan for 30 min to one hour at room temperature. Control leaf disks were dipped in distilled water containing Triton X-I00 (50 µg/ml). The leaf disks were spread in individual plastic Petri dishes (7.5 cm diameter) containing moistened filter paper (4.5 cm diameter). Then, on each leaf disk, ten numbers of 5-day-old second instar larvae were subsequently released. Eight concentrations as treatments (including control) were used in each experiment with three replications for the bioassay of each location by taking 30 larvae per treatment. Larvae were allowed to feed on treated leaf disks for 72 h at 26 ± 1 °C and 60–70% relative humidity. A larva was considered dead if it failed to move in a coordinated manner when probed with a blunt needle. The mortality obtained at 24, 48, and 72 h after treatment was recorded till the 14^{th} day and these mortality values were used for data analysis. The mortality was corrected using Abbott's formula wherever needed.

$$\% \text{ corrected mortality} = \frac{X - Y}{X} \times 100 \text{ (Abbott, 1925)}$$

where X Percent of living in the control, Y Percent living in the treated plates, $X - Y$ Percent killed after treatment.

This corrected mortality was used for data analysis. Experiments showing control mortality of more than 20% were discarded and repeated.

Data analysis

Larval mortality data recorded at 72 h after treatment was considered for probit analysis. The data obtained were corrected for control mortality using Abbott's formula (Abbott 1925) wherever necessary. The LC_{50} was estimated for 72-h mortality data using the Maximum likelihood program (Ross 1977). Significant difference between the two LC_{50} values was determined on the basis of the overlap of 95% fiducial limits at 0.05% level (Litchfield and Wilcoxon 1949).

Results

Variability response in *P. brassicae* populations to Bt Cry1C toxin

The results on the concentration at which 50% of the test population responded, i.e., LC_{50} values of *Bt* Cry1C toxin to 11 different field populations of *P. brassicae* are shown in Table 2. The findings showed that there was a significant variation in LC_{50} values among the different field populations. The median lethal concentrations of *Bt* Cry1C toxin to eleven different field populations of *P. brassicae* ranged from 0.546 to 1.803 µg/ml. The Umiam population, which documented the increased tolerance to Cry1C, was on par with that of Pepbah population and notably different from the Mookyndur, Mawklot, Umroi, Umeit, Mawpun, Kharpati, Sohphoh, Wahiajer and Smit populations in terms of tolerance. The population sampled from Smit region had the lowest LC_{50} value (0.546 µg/ml) with fiducially limits of lower (0.41) and upper (0.71) values. Accordingly, it was determined to be less tolerant based on the LC_{50} value (0.546 µg/ml), while the Umiam population was more tolerant with the LC_{50} value (1.803 µg/ml). Based on the LC_{50}, it was found that the tolerance ratio from the more tolerant population (Umiam) with respect to the less tolerant population (Smit) was 3.30-fold for *Bt* Cry1C toxin. The discriminating dose at which 90–99% of the susceptible population showed a response (LC_{99}) to *Bt* Cry1C toxin was 19.30 µg/ml of the more tolerant strain from Mookyndur, which can also be used for monitoring resistance.

Variability in response of *P. brassicae* populations to Bt Cry2Ab toxin

The variation in the LC_{50} values of *Bt* Cry2Ab toxin to 11 different field populations of *P. brassicae* is indicated in Table 3. The LC_{50} of *Bt* Cry2Ab toxin to 11 different geographic populations of *P. brassicae* ranged from 0.535

Table 2 Susceptibility of larvae of cabbage butterfly, *Pieris brassicae* from different locations to *Bt* Cry1C toxin

Regions	Numbers	LC_{50} (95% FL) µg/ml	Slope ± SE	χ^2 (*df*)	Resistance ratio
Umiam	240	1.803 (1.53–2.04)1.53-	4.26 ± 0.60	5.04(5)	3.30
Kharpati	240	1.564 (1.25–1.83)	3.51 ± 0.55	3.69(5)	2.86
Mawpun	240	1.626 (1.25–1.93)	2.90 ± 0.50	3.09(5)	2.98
Umroi	240	1.650 (1.34–1.90)	3.64 ± 0.55	3.92(5)	3.02
Umeit	240	1.642 (1.35–1.89)	3.91 ± 0.58	2.29(5)	3.01
Mawklot	240	1.674 (1.31–1.98)	3.02 ± 0.51	1.96(5)	3.06
Pepbah	240	1.776 (1.40–2.10)	2.91 ± 0.50	3.82(5)	3.25
Smit	240	0.546 (0.41–0.71)	1.85 ± 0.22	4.61(5)	_
Mookyndur	240	1.720 (1.43–1.97)	3.86 ± 0.57	4.47(5)	3.15
Sohphoh	240	1.532(1.14–1.85)	2.69 ± 0.47	4.92(5)	2.80
Wahiajer	240	1.397 (1.01–1.76)	2.04 ± 0.33	3.97(5)	2.56

Test for heterogeneity; χ^2 value was significant at 5% level of significance for a goodness-of-fit test for each regression

SE, Standard error; N, No. of test insects; 95% FL, 95% fiducial limits

Table 3 Susceptibility of larvae of cabbage butterfly, *Pieris brassicae* from different locations to *Bt* Cry2Ab toxin

Regions	Numbers	LC_{50} (95% FL) µg/ml	Slope ± SE	χ^2 (*df*)	Resistance ratio
CPGS-AS, Umiam	240	1.473 (1.06–1.86)	1.92 ± 0.31	3.50(5)	2.75
Kharpati	240	1.564 (1.25–1.83)	3.51 ± 0.55	3.69(5)	2.92
Mawpun	240	1.555 (1.18–1.86)	2.92 ± 0.50	4.60(5)	2.91
Umroi	240	1.374 (0.99–1.68)	2.71 ± 0.48	5.22(5)	2.57
Umeit	240	1.418 (1.14–1.65)	4.06 ± 0.61	1.76(5)	2.65
Mawklot	240	1.534 (1.53–1.80)	3.44 ± 0.55	3.24(5)	2.87
Pepbah	240	1.725 (1.42–1.99)	3.70 ± 0.56	4.56(5)	3.22
Smit	240	0.535 (0.39–0.71)	1.59 ± 0.20	1.25(5)	_
Mookyndur	240	1.497 (1.12–1.80)	2.85 ± 0.49	2.11(5)	2.80
Sohphoh	240	1.619 (1.29–1.89)	3.39 ± 0.53	0.87(5)	3.03
Wahiajer	240	1.627 (1.30–1.90)	3.33 ± 0.53	4.76(5)	3.04

Test for heterogeneity; χ^2 value was significant at 5% level of significance for a goodness-of-fit test for each regression

SE, Standard error; N, No. of test insects; 95% FL, 95% fiducial limits

to 1.725 µg/ml. Pepbah population recorded a maximum LC_{50} value (1.725 µg/ml) with corresponding fiducially limits of lower and upper (1.42–1.99 µg/ml) values, followed by the population strain collected from Sohphoh (1.619 µg/ml) and Mawpun (1.555 µg/ml). The population sampled from the Smit region had the lowest LC_{50} value (0.535 µg/ml) with respective fiducially limits of lower and upper (0.39–0.71 µg/ml) values. Therefore, the Smit population strain of *P. brassicae* was found to be more vulnerable based on the lowest LC_{50} value of 0.535 µg/ml and Pepbah population was found to be more tolerant based on the highest LC_{50} value of 1.725 µg/ml.

Based on the LC_{50}, the tolerance ratio evaluated from the more tolerant population with respect to the least tolerant population was 3.22-fold for *Bt* Cry2Ab toxin. The discriminating dose at which 90–99% of the susceptible population showed a response (LC_{99}) to *Bt* Cry2Ab toxin was 24.03 µg/ml (95% fiducially limits, 12.90–77.00 µg/ml) of the most resistant strain from Umiam, which can also be used for monitoring resistance. The above study summarizes that Cry2Ab toxin was more virulent to *P. brassicae* having a lower LC_{50} value of 0.535 (fiducially limits, 1.42–1.99 µg/ml) when compared to Cry1C having LC_{50} value of 0.546 (fiducially limits, 0.41–0.71 µg/ml). The least tolerant strains of *P. brassicae* for both the Cry toxins (Cry1C and Cry2Ab) were found to be from Smit population with that of more tolerant strains from Umiam and Pepbah for Cry1C and Cry2Ab, respectively.

Discussion

Evaluation of baseline susceptibility of 11 different field populations of *P. brassicae* (2^{nd} instar larvae) to two different *Bt* Cry toxins (Cry1C and Cry2Ab) revealed significant natural variability in response to the Cry toxins as shown by the difference in LC_{50} values of both the Cry toxins. In the present study, the median lethal concentrations, LC_{50} (72 h), were reported to be in the range of 0.546–1.803 µg/ml for Cry1C toxin and a similar toxicity response was also proved by Ningthoujam et al. 2020 on *H. armigera* larvae susceptibility to *Bt* Cry1C toxin. Insecticidal Crystal Protein's (ICPs) from other strains of *Bt* (*Bt* subspecies *aizawai*) are equally effective against many of the lepidopterous pests like *P. brassicae*, *T. ni* etc.

In the present findings, the least tolerant strain to *Bt* Cry1C among the eleven different insect populations of *P. brassicae* was from the Smit region. The toxicity response of the remaining 10 field populations to *Bt* Cry1C followed a gradual increase in tolerance with the change in the variation of LC_{50} values which was mostly due to the natural variability shown by the insect populations. It was also noticed that there was not much variability shown pertaining to Cry1C toxin. These findings were in accordance with the toxicity tests conducted using the *Bt* Cry1C toxin against *P. xylostella* (Zhao et al. 2001), *C. suppressalis* (Tang et al. 2018) and *S. littoralis* (Khalil et al. 2021) resulted in a relatively high ppm showing its evolving resistance toward the Cry toxin. *P. brassicae* was more sensitive to Cry1C toxin at a very low concentration and resulted in various changes in the biological parameters of the pest which are in agreement with the earlier work done by Ningthoujam et al. (2017a) on *H. armigera* which resulted in some changes in both adult and pupal emergence. The toxicity of *Bt* Cry1C reached up to 0.546 µg/ml for *P. brassicae* strains smit region and was the least tolerant population strain among all the 11 different insect populations.

The tolerance ratio of Cry1C toxin was 3.30-folds which was relatively low in contrast to the highest fold showed by the F_2 of *H. armigera* from the baseline studies given by Ningthoujam et al. (2017b). The discriminating concentration for resistance monitoring of *P. brassicae* to *Bt* Cry1C toxin was evaluated as 19.30 µg/ml (LC_{99}, most tolerant strain) which is in agreement with the findings reported by Kranthi et al. (2009). Similar to this, the present studies also found toxicity of Cry2Ab toxin to *P. brassicae* with LC_{50} values ranging from 0.535 to 1.725 µg/ml which are at par with findings reported by Chaitra and Kalia (2020). The least tolerant strain to *Bt* Cry2Ab among the 11 different insect populations of *P. brassicae* was from the Smit region. The toxicity response of the remaining ten field samples to *Bt* Cry2Ab followed a gradual increase in tolerance with the increase in the variation of LC_{50} values which is also due to the natural variability shown by the insect populations. *P. brassicae* strains from Wahiajer and Sohphoh showed significant variation in susceptibility to *Bt* Cry2Ab due to the different agro-ecological conditions. The tolerance ratio of Cry2Ab toxin of the most tolerant strain with that of the least tolerant strain were 3.22-folds. The discriminating concentration (Roush and Milller 1986) for insect resistance to *Bt* Cry2Ab toxin was evaluated as 24.03 µg/ml (LC_{99}, the most tolerant strain).

Comparatively, the toxicity potential of Cry2Ab to *P. brassicae* was more than the Cry1C toxin. The fold tolerance shown by both toxins was observed to be very low which is equally reported in the studies of Shelton et al. (2009). Obtained findings provide the baseline information of two different Cry toxins (*Bt* Cry1C & Cry2Ab) against *P. brassicae,* and the present studies also suggested the need for continuous monitoring of resistance for the future exploitation of *Bt* transgenic crops to become a major component in an overall integrated pest management program.

Conclusion

The present study concluded that among the pest complex of cruciferous crops, the cabbage butterfly, *P. brassicae* is considered as a key insect pest in Meghalaya. The worldwide adoption of *Bt* cry toxins in the management of lepidopteran insect pests has been well-documented. However, the threat of resistance development is a major problem imposed by the continuous adoption of *Bt*. Therefore, constant monitoring for any changes in susceptibility of the pests against *Bt* cry toxins is necessary for which the present research was undertaken on the baseline susceptibility of cabbage butterfly to *Bt* Cry toxins viz., Cry1C and Cry2Ab. The Cry2Ab toxin was found to be more potent than Cry1C against *P. brassicae*. The variation observed in the baseline susceptibility among the *Pieris* populations from different geographical regions of Meghalaya will provide information about the probability of the development of resistance and devise suitable management options.

Abbreviations

Bt	*Bacillus thuringiensis*
ICP	Insecticidal crystal protein
RH	Relative Humidity
LC_{50}	50% Lethal concentration
LC_{99}	99% Lethal concentration
L:D	Light:dark
ppm	Parts per million

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Author contributions

The concept and design of the experiment were prepared by all authors. SSM conducted the experiments, analyzed the data and prepared the original manuscript. KN contributed to supervision, editing, conceptualization, analyzing and interpretation of the data. All the authors also contributed to reviewing of the manuscript. All authors read and approved the manuscript.

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Declarations

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Competing interests

The authors declare that they have no competing interests.

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