Mysore J. Agric. Sci., 57 (4) : 364-371 (2023)

Relative Response of Pink Bollworm, *Pectinophora gossypiella* (Saunders) Population Towards Bollgard-II® *Bt* Cotton Expressing Cry1Ac+Cry2Ab Toxins

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Received : August 2023
Accepted : September 2023

Abstract

The widespread adoption of genetically modified (GM) crops, particularly *Bt* cotton expressing Cry1Ac and Cry2Ab toxins, has revolutionized global agriculture by providing effective solutions to pest-related challenges. However, the Pink Bollworm, *Pectinophora gossypiella*, a highly destructive insect pest threatening cotton cultivation worldwide, has developed resistance to *Bt* cotton, leading to reduced benefits. This study aimed to assess the relative response of Pink Bollworm population collected from different regions in South India to Bollgard-II® *Bt* cotton expressing both Cry1Ac and Cry2Ab toxins. Field populations of Pink Bollworm were collected from major cotton-growing areas in Karnataka (Raichur, Dharwad and Yadgir), Tamil Nadu (Jolarpet) and Gujarat (Vadodara), while a laboratory-susceptible population served as a reference. The results showed that pink bollworm populations from various locations exhibited different levels of resistance to Bollgard-II® *Bt* cotton. Median lethal concentration (LC_{50}) values for field populations ranged from 0.790 to 1.011 ppm, indicating reduced susceptibility compared to the susceptible laboratory colony (LC_{50} = 0.011; χ^2 = 0.829; df = 3; h = 0.414). The highest resistance ratio (RR) of 91.90 was recorded in the population collected from Vadodara (LC_{50}= 1.011; χ^2 = 2.461; df=3; h= 0.820), followed by Raichur (RR = 90.63; LC50 = 0.997; χ^2 = 7.523; df = 3; h = 2.509), Yadgir (RR = 89.63; LC_{50}= 0.986; χ^2 = 3.862; df =3; h = 1.288) and Dharwad (RR = 84.64; LC_{50} = 0.931; χ^2 = 5.334; df =3; h = 1.781). The population collected from Jolarpet (LC_{50} = 0.790; χ^2 = 3.815; df = 3; h = 1.272) showed resistance ratio of 71.81, lowest among the populations tested. These findings highlight the increased resistance of pink bollworm to the combined Cry1Ac and Cry2Ab toxins in Bollgard-II® *Bt* cotton, indicating the need for careful management strategies to delay further resistance development. These results emphasize the importance of sustainable agricultural practices to overcome the challenges posed by insect pests in GM crop cultivation.

Keywords : Bollgard-II, *Bt* resistance, Cry toxins, *Bt* cotton, Pink bollworm

The widespread adoption of genetically modified (GM) crops has significantly impacted global agriculture, offering promising solutions to numerous pest-related challenges. Among these genetically modified crops, *Bt* cotton has emerged as a prominent example due to its effectiveness in controlling lepidopteran pests. One such pest of critical concern is the pink bollworm (PBW), *Pectinophora*

gossypiella (Saunders) (Gelechiidae: Lepidoptera), an insect that poses a substantial threat to cotton cultivation worldwide (Naik *et al.*, 1996 and Tabashnik *et al*., 2023). Conventionally, chemical insecticides have been employed to control pink bollworm infestations, but these methods often lead to several challenges, including environmental pollution, development of insecticide resistance and detrimental effects on non-target organisms. To address these issues, genetically engineered *Bt* cotton expressing Cry1Ac protein (Bollgard-I®) toxic to lepidopteran pests was introduced during 2002 in India. But, soon after its introduction PBW has got resistance to Cry1Ac toxin and continued its menace. To enhance efficacy and durability of GM technology, cotton hybrids pyramided with Cry1Ac and Cry2Ab were commercialized in India during 2006; subsequently it enhanced the quality and yield of the cotton (Choudhary and Gaur, 2015).

On the other hand, benefits of *Bt* cotton have been reduced by subsequent evolution of resistance in PBW (Madhu and Muralimohan, 2022). The global monitoring data revealed the 19 cases of field evolved resistance to *Bt* crops (Tabashnik and Carriere, 2019). Several reports highlighting the *Bt* resistance development in Indian population of PBW was reported from different states (Nair *et al*., 2016; Naik *et al*., 2018; Annepu *et al*., 2023 and Likhita *et al*., 2023). Hence, the present investigation was formulated to assess the relative response of pink bollworm, *Pectinophora gossypiella* population collected from different locations of South India to Bollgard-II® *Bt* cotton which expresses Cry1Ac and Cry2Ab toxins.

Material and Methods

Sample Collection

A comprehensive survey was conducted in major cotton growing areas of Karnataka (Dharwad, Raichur and Yadgir), Tamil Nadu (Jolarpet) and Gujarat (Vadodara) during 2021-22 and 2022-23 to collect the representative field population of pink bollworm (Table 1 & Fig. 1). For this, at least 10 fields were surveyed in each area and around 200 pink bollworm

Table 1
Details of locations surveyed for collection of Pink bollworm population during 2021-22

Location	State	Coordinates	Area (000 ha)	Production (Bales)	Productivity (Kg/ha)	Temperature (ºC)	Relative Humidity (%)	Rainfall (mm)	Altitude (m above MSL)
Dharwad	Karnataka	15.4889° N, 74.9813° E	71.20	42663.00	326	12 - 40	40 - 88	787	750
Jolarpet	Tamil Nadu	12.5687° N, 78.5749° E	2.20	4627.00	96 (fiber lint)	13 - 42	48 - 75	370	186
Raichur	Karnataka	16.2160° N, 77.3566° E	45.41	665.94	367	18 - 45	37 - 74	658	407
Vadodara	Gujarat	22.3072° N, 73.1812° E	76.71	371.80	823.91	20 - 34	56 - 89	922	36
Yadgir	Karnataka	16.7487° N, 77.1309° E	103.87	109.24	316	22 - 45	37 - 74	832	370

*ha- Hectare; ºC- Degree Celsius; Kg/ha- Kilogram per hectare; %- per cent; mm- millimeter; MSL- Mean Sea Level; One bale- 170 kg

Fig. 1 : Geographical map indicating different survey locations

infested bolls were collected. The bolls were brought to the Genomic Resources laboratory of ICAR-NBAIR, Bengaluru for destructive sampling. A susceptible laboratory colony of pink bollworm being maintained at ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru without exposing to insecticides and Bt toxins since 2009 (National Accession Number: NBAII-MP-GEL-02a) was used as a susceptible population.

Population Rearing

The laboratory rearing of field collected pink bollworms was undertaken at the genomic resource's laboratory at ICAR- NBAIR, Bengaluru. The field collected larvae were directly transferred individually to plastic vials (6 mm diameter x 5 mm height) containing semisynthetic diet (Jothi *et al.,* 2016) (Table 2) with necessary modifications to complete the generation.

Later, the male and female pupae were segregated based on the position of genital and anal pores (Between 9th and 10th in Male; 8th and 10th in female). The emerged adult moths were released into the oviposition jars in the ratio of 1(♂): 2(♀). Fresh cotton twigs were provided as oviposition substrate and a piece of cotton dipped in 10 per cent honey solution was offered as adult food. Cotton twigs were replaced once in three days and collected old twigs were kept in separate jar for hatching. Freshly hatched neonates were transferred individually into 2 ml Eppendorf tubes (Spinwin™ Micro Centrifuge Tube) containing 0.5 ml semisynthetic diet prepared as per Table 2. All the population were maintained in the growth chamber with 25±1°C temperature, 60-65 per cent Relative Humidity (RH) and 10:14 hour (Light: Dark) photoperiod.

TABLE 2

Semisynthetic diet composition used for rearing of pink bollworm larvae

Ingredients	Quantity
Chickpea flour	25 g
Yeast	2.5 g
Agar	3 g
Wheat germ	6.25 g
Casein	5 g
Sucrose	5 g
Cholesterol	0.187 g
Sorbic Acid	0.21 g
Methyl Paraben (MPB)	0.412 g
Ascorbic Acid	0.66 g
Wesson's salt	1.25 g
Streptomycin sulphate	0.093 g
Multivitamin mix	0.125 g
Formaldehyde	1.68 g
Distilled water	200 ml

Toxin Source

Since our aim was to assess relative response of pink bollworm to Bollgard-II® Bt cotton expressing combined Cry1Ac and Cry2Ab toxins, Bt seed powder containing both the proteins was used as a toxin source (Variety: MRC 7918 BG-II; Developer: Mahyco Pvt. Ltd.). Prior to the assay, the quantification of Cry1Ac and Cry2Ab protein was carried by Enzyme-Linked Immunosorbent Assay (ELISA) using DesiGen™ Quan-T ELISA 96-well plate kit (DesiGen, Jalna, India). Every gram of Bt seed powder contained 3.25 µg Cry1Ac and 182.32 µg Cry2Ab protein toxins.

Bioassay Protocol

The early 2nd instar larvae were used in dose response bioassays by diet incorporation method. The diet was prepared with five appropriate concentrations (4.27, 1.43, 0.47, 0.15 and 0.05 ppm for field populations; 0.31, 0.12, 0.04, 0.01 and 0.004 ppm for Lab-Susceptible population) of combined Cry toxin (Cry1Ac + Cry2Ab) along with a control (Non Bt seed powder). The concentrations were decided based on the pre-bioassay bracketing results. One ml of diet containing respective concentrations was poured into each well in a 128 well bioassay tray (C-D International, Pitman, NJ). The early 2nd instar individual larvae were placed in each well using small camel hair brush after diet get air dried. The wells were covered with air-vented lids and the bioassay trays were kept in growth chamber for seven days (Temperature- 25±1 °C: RH- 60-65%: Photoperiod- 10 (L) : 14 (D) h). In each concentration 30 larvae were tested and repeated on alternate days.

Statistical Analysis

Probit analysis was used to evaluate the dose response mortality for each population (Finney, 1971) using Polo-PC® LeOra software Petulama, California, USA. Before the analysis, the larval mortality was corrected based on the survivorship in control using Abbott's formula (Abbott, 1925). Values estimated included the median lethal concentrations (LC_{50}), the lethal concentration that kills 90 per cent of tested larvae (LC_{90}) and model parameters (slope and intercepts). Goodness of fit statistics was calculated for each population. Resistance Ratio (RR) was calculated by using the formula, RR= (LC_{50} of field population)/ (LC_{50} of susceptible population). Rest of the analysis and graphical representation was done using R-program (Version 4.2.1).

Results and Discussion

Pink bollworm, *Pectinophora gossypiella* is one of the destructive insect pests of cotton and has known to cause 2.80 to 61.90 per cent loss in seed cotton yield, 2.10 to 47.10 per cent loss in oil content and 10.70 to 59.20 per cent loss in normal boll opening (Patil, 2003). In the later period it also become one of the major pests in Bt cotton through evolution of resistance against both the toxins (CRY1Ac + Cry2Ab) at varied rate. With this background, the present investigation was carried out to understand the present resistance level in pink bollworm population from different regions of southern India.

The representative population of pink bollworm from different locations indicated the varied level of susceptibility to Bollgard-II® *Bt* cotton expressing Cry1Ac and Cry2Ab. Median lethal concentration (LC_{50}) values from dose response assay ranged from 0.790 to 1.011 ppm for the field population whereas, lab reared susceptible population of pink bollworm registered LC_{50} value of 0.011 (χ^2 = 0.829; df =3; h = 0.414) ppm against combined Cry1Ac and Cry2Ab toxin (Table 3). The highest LC_{50} value of 1.011 (χ^2 = 2.461; df = 3; h = 0.820) ppm indicating least susceptibility to combined toxins was recorded in the population collected from Vadodara, followed by Raichur (LC_{50}= 0.997; χ^2 = 7.523; df = 3; h = 2.509), Yadgir (LC_{50}= 0.986; χ^2 = 3.862; df =3; h = 1.288) and Dharwad (LC_{50}= 0.931; χ^2 = 5.334; df =3; h= 1.781). Whereas, the least value indicating more (compared to other population) susceptibility was recorded in Jolarpet population (LC_{50}= 0.790; χ^2 = 3.815; df = 3; h = 1.272). The accuracy, precision and sensitivity of different pink bollworm population towards Cry1Ac + Cry2Ab was depicted through the residue graph and probit response curve (Fig. 2).

The Resistance Ratio (RR) is a crucial parameter used to assess and monitor the development of insecticide resistance in insect population. It is been considered as one of the important parameter to express relative susceptibility of a population to the assayed insecticide comparing with its reference susceptible colony. In the present study, the highest RR value of 91.90 indicating pronounced resistance against combined toxin of Cry1Ac + Cry2Ab was recorded in the representative population collected from Vadodara, followed by Raichur (90.63), Yadgir (89.63) and Dharwad (84.64); whereas, the least (compared to other population) RR value of 71.81 was noted in the

population collected from Jolarpet (Fig. 3). From the calculated heterogeneity (*h* value), it was found that all the field population were less sensitive to *Bt* toxins present in the Bollgard-II *Bt* cotton indicating pronounced resistance against Cry1Ac + Cry2Ab (Table 3).

Increased *Bt* resistance in Indian PBW population was being studied since from *Bt* cotton introduction to India. Since, this is a first kind of study to investigate the response of pink bollworm population towards combined toxins of Cry1Ac and Cry2Ab in South India, the previous findings available to discuss with the present results are scanty. Despite the individual toxin effect of Cry1Ac and Cry2Ab on pink bollworm survival was studied extensively, Dhurua and Gujar (2011) made an attempt to study the relative response

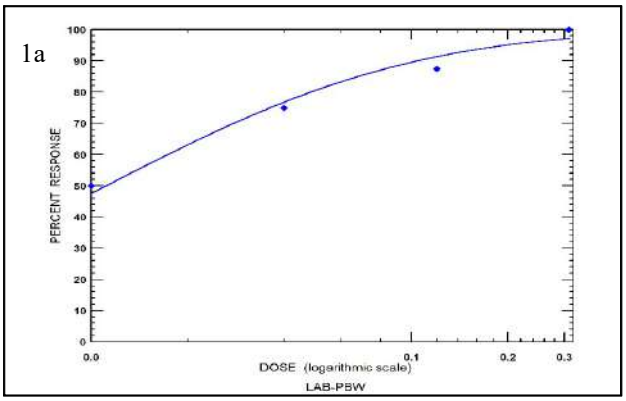
TABLE 3

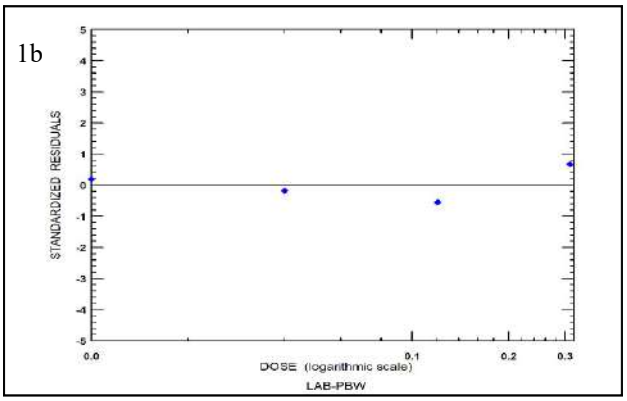
Probit response of pink bollworm, *Pectinophora gossypiella* populations to Bollguard® II *Bt* cotton expressing Cry1Ac + Cry2Ab

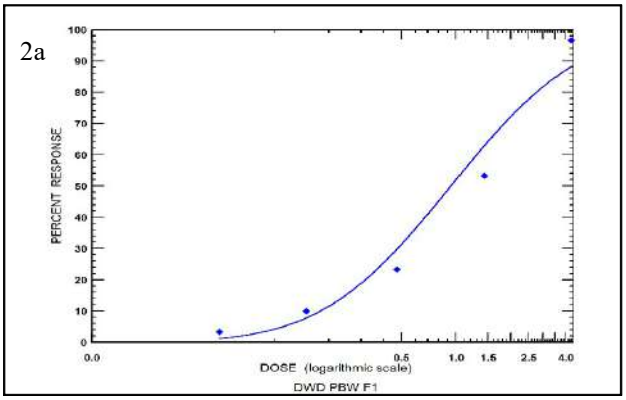
Population	LC_{50} (95% FL)	LC_{90} (95% FL)	Slope ± SE	χ^2	h
Lab-susceptible	0.011 (0.002-0.023)	0.105 (0.051-0.672)	1.317 ± 0.393	0.829	0.414
Dharwad	0.931 (0.423-2.623)	4.843 (1.937-86.386)	1.790 ± 0.247	5.334	1.781
Jolarpettai	0.790 (0.385-1.947)	5.746 (2.228-69.438)	1.487 ± 0.209	3.815	1.272
Raichur	0.997 (0.372-4.300)	4.993 (1.788-449.175)	1.832 ± 0.252	7.523	2.509
Vadodara	1.011 (0.646-1.762)	12.481 (5.508-54.217)	1.174 ± 0.187	2.461	0.820
Yadgir	0.986 (0.511-2.236)	5.362 (2.331-44.144)	1.742 ± 0.240	3.862	1.288

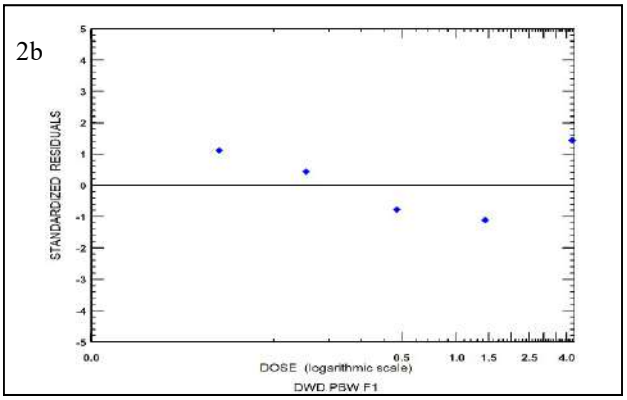
*LC_{50} (95% FL)- Median Lethal Concentration in ppm (95% Fiducial Limits); SE- Standard Error; RR- Resistance Ratio

* LC_{90} (95% FL)- Concentration of toxin in ppm that kills 90 % of tested larvae (95% Fiducial Limits)









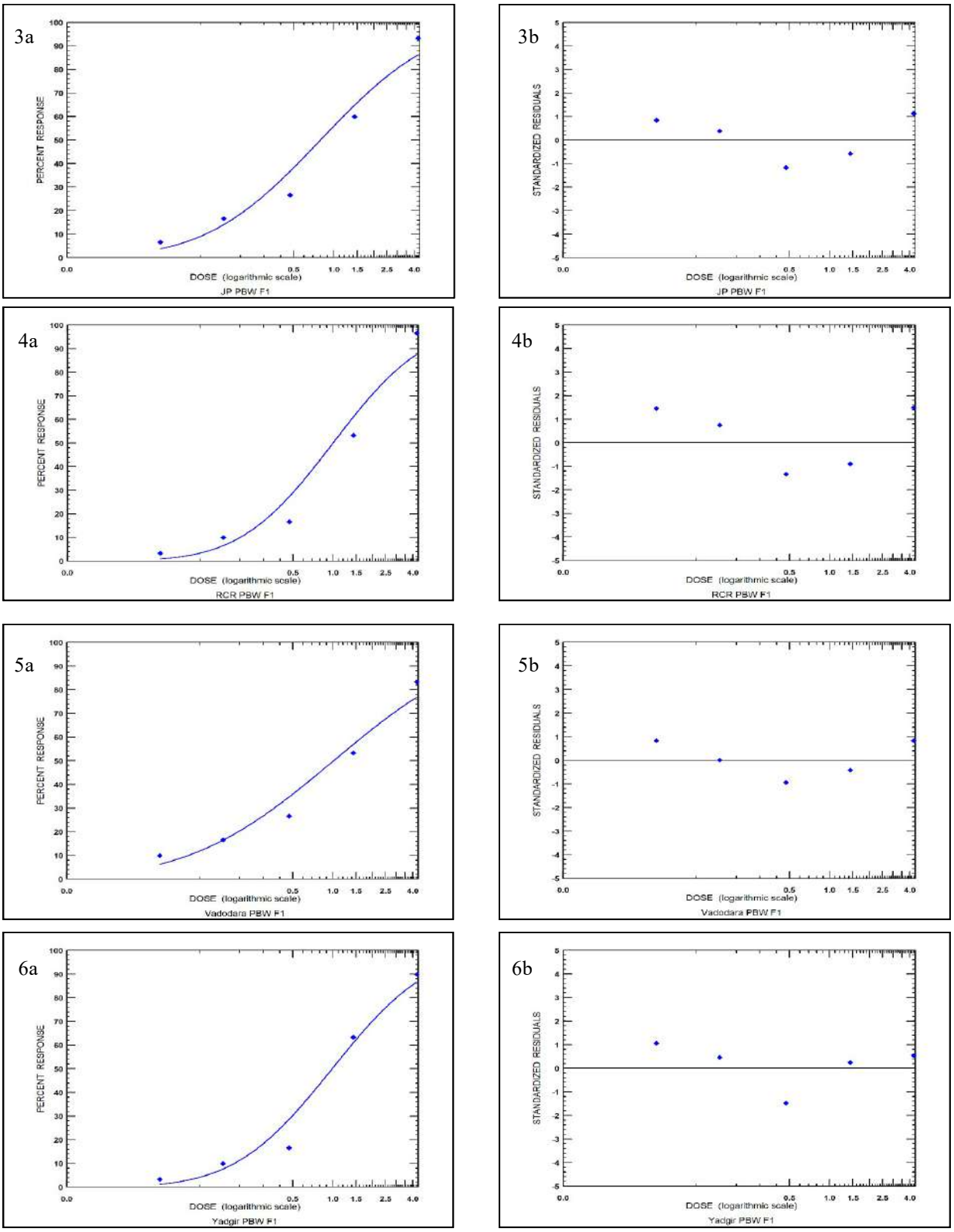


Fig. 2 : Dose response curve and residue graph for different pink bollworm populations (1a & b- Susceptible lab population; 2a & b-Dharwad population; 3a & b- Jolarpet population; 4a & b- Raichur population; 5a & b- Vadodara population; 6a & b-Yadgir population)

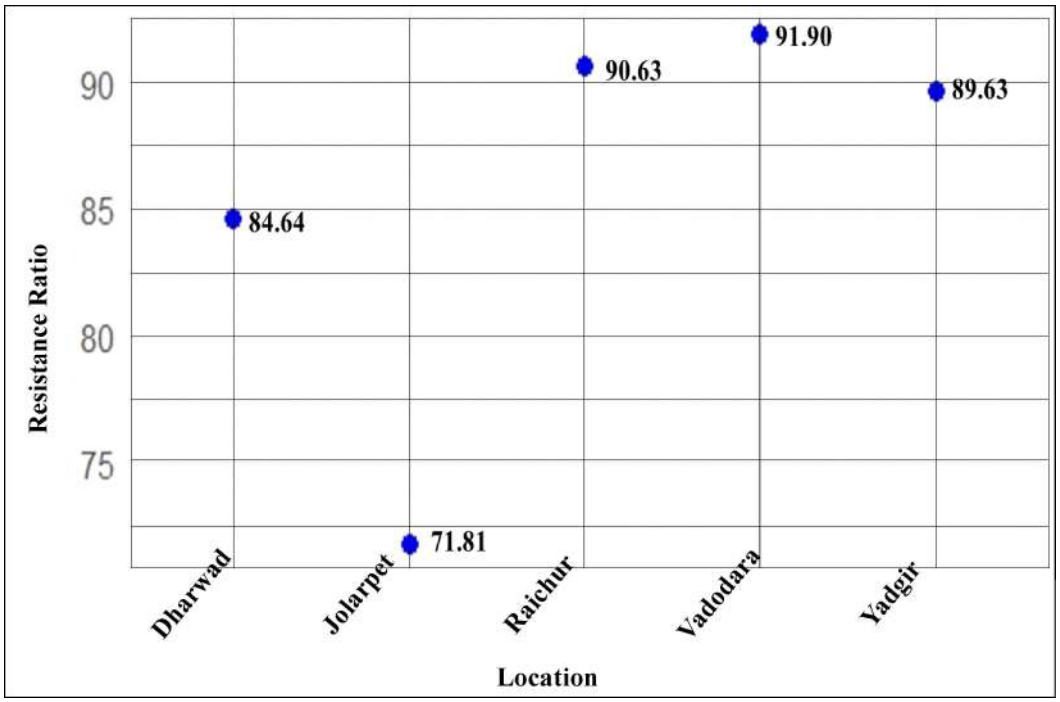


Fig. 3 : Scatter plot indicating resistance ratio of pink bollworm, *Pectinophora gossypiella* population against Bollgard-II® *Bt* cotton expressing Cry1Ac + Cry2Ab

of pink bollworm population collected from Gujarat to Bollgard-II® producing Cry1Ac and Cry2Ab in comparison with *Bt* cotton producing only Cry1Ac and found that the average resistance ratio for Cry1Ac was 44, but it was only two for the seed powder producing both Cry1Ac and Cry2Ab indicating the susceptibility of pink bollworm towards Bollgard-II® *Bt* cotton. After 12 years of this study, the present investigation enumerating the increased resistance level confirmed by higher resistance ratios in all the pink bollworm population collected from Karnataka, Tamil Nadu and Gujarat.

In the same study (Dhurua and Gujar, 2011), the first field evolved resistance in pink bollworm against Cry1Ac was reported from India during 2008 in the population collected from Gujarat. In the previous study, Muralimohan *et al.* (2009) conducted a 21 days bioassay for pink bollworm against Cry1Ac and Cry2Ab; they confirmed the extreme sensitivity of Indian pink bollworm population to *Bt* proteins contained in transgenic cotton varieties, Bollgard and Bollgard-II® with LC_{50} values of 0.040 ppm Cry1Ac and 0.051 ppm Cry2Ab.

In the later studies, Nair *et al.* (2016) reported the higher LC_{50} value of 2.50 µg/ml of diet with 243.52-fold resistance development against Cry1Ac in field population collected from Maharashtra and Madhya Pradesh in comparison with the LC_{50} value of 0.010 µg/ml diet recorded for the susceptible population. The pink bollworm population collected from central and southern India showed the increased LC_{50} of Cry1Ac from a mean of 0.330 µg/ml diet in 2013 to a mean of 6.938 µg/ml diet in 2017, whereas the LC_{50} value for Cry2Ab increased from a mean of 0.014 µg/ml diet in 2013 to a mean of 12.51 µg/ml diet in 2017 (Naik *et al.,* 2018). In the same study, Cry1Ac resistance was increased from 47.12 folds in 2013 to 1387 folds in 2017; whereas Cry2Ab resistance was aggravated from 5.4 folds in 2013 to 4196 folds in 2017. Likewise, the present investigation affirms the field evolved resistance in pink bollworm against Bollgard-II® Bt cotton technology expressing Cry1Ac + Cry2Ab at varied level in major cotton growing areas of South India.

This study enables the present status of resistance level in pink bollworm, *Pectinophora gossypiella* population collected from different locations

of Karnataka, Tamil Nadu and Gujarat against Bollgard-II® Bt cotton expressing both Cry1Ac and Cry2Ab toxins. It was found that the pink bollworm population from different locations exhibited different level of resistance against Bollgard-II®. Hence it is recommended to follow the prescribed integrated pest management and integrated resistance management practices to delay the rapid development of resistance in pink bollworm against Bt cotton. Meanwhile, it is also necessary to monitor the resistance level from time to time for validating and updating the management tools.

Acknowledgement : We extend our heartfelt gratitude to Director, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru for providing all the necessary facilities to conduct this experiment. The first author gratefully acknowledges the award of DST-INSPIRE fellowship (IF190948) by Department of Science and Technology, Govt. of India.

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