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NATURAL VARIATION AMONG *HELICOVERPA ARMIGERA* (HÜBNER) POPULATIONS TO *BACILLUS THURINGIENSIS* Cry1Ac TOXIN

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Abstract: Natural variation in susceptibility of field collected cotton American bollworm (*Helicoverpa armigera*) to purified *Bacillus thuringiensis* (Bt) Cry1Ac toxin was investigated to establish a geographic baseline for comparison of future population responses to the increased use of Bt based insect control products like Bt transgenic crops and commercial formulations. Populations of *H. armigera* were evaluated for their susceptibility to purified Bt Cry1Ac toxin. The range of LC_{50}'s among selected populations from Lahore, Multan, Rahim Yar Khan and Hyderabad in response to Cry1Ac toxin was more than 16 fold. The data provide a strong basis for monitoring changes in susceptibility of *H. armigera* populations to future use of Bt toxins in Bt transgenic crops program in Pakistan.

Key words: Insect control, BT toxins, American bollworm, susceptibility, future responses.

INTRODUCTION

The development of insecticides based on *Bacillus thuringiensis* (Bt) δ-endotoxin proteins has increased in response to the need of efficacious, environmentally safe and selective pesticides with unique modes of action. Advances in Bt formulation technology and genetic engineering, and the discovery of Bt strains with a broader spectrum of activity, have resulted in new microbial products with increased potency and greater stability (Khan *et al.*, 1995, 1995a). In addition, genetically modified crops such as cotton, corn and potato have already been commercialized (James and Krattiger, 1996). The advantages of genetically engineered plants include more efficient delivery of active ingredient to the target pest, less input in terms of the costs of pesticide application, and reduced environmental impact from pesticide use. Although, genetically altered plants producing their own protective insecticides provide an exciting new approach to insect control, a large scale introduction of these crops could rapidly lead to the development of resistance to Bt toxins within pest populations (Tabashnik, 1993). The combined impact of transgenic rice and increased use of Bt formulation for rice lepidopteran pests management increase the likelihood for resistance development which would negate the economic and environmental benefits of this important management option. Once commercially

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available, these new microbial and plant products will play an important role in insect management (Karim and Riazuddin, 1997).

Selection for *Helicoverpa armigera* to Bt toxins is expected to be intense and is likely to result in the evolution of resistance to Bt endotoxins (for details, see Karim *et al.*, 1999a). An effective resistance management program will also be needed to foresee the long-term utility of Bt technology (Karim and Riazuddin, 1999b). The US Environmental Protection Agency (EPA) has approved conditional registrations for several Bt crops and is requiring the development of scientifically sound resistance management strategy by the year 2001 (Mellon and Rissler, 1998). The currently favored resistance management strategy for Bt crops is the "high dose/refuge strategy" (Gould, 1994, 1998).

Helicoverpa armigera is an important economic target for many Bt based products, but variation in susceptibility among populations has not been investigated. One of the most important research needs currently in Bt resistance management programs is the development of baseline susceptibility studies of key insect pests in all ecosystems where Bt will be used (Whalon, 1992). The present study reports the results from experiments to determine if differences exist in susceptibility to Cry toxins among *H. armigera* field populations collected from Lahore, Multan, Rahim Yar Khan and Hyderabad.

MATERIALS AND METHODS

Insects

Helicoverpa armigera larvae were collected from cotton growing areas like Multan, Rahim Yar Khan and Hyderabad. Larvae were reared on an artificial diet (Makhdoom *et al.*, 1998) at 28±2°C and a photoperiod of 16:8 (light : dark). Field collected larvae were the generous gift from IRAC, Pakistan. The bioassays with *H. armigera* were carried out with neonatal first instar larvae on artificial diet. A total of 100 µl of toxin dilutions were layered on diet in glass vials and air-dried. One larvae each was placed per vial because of their cannibalistic behavior. A total of 30 larvae per toxin dilution were used in each set of experiment. The mortality rates were recorded after 5 days and lethal concentration of toxin (LC_{50}) 95% fiducial limits was calculated using Probit analysis (Leora Software, 1987). At least five concentrations per toxin were used to estimate LC_{50} value. Each set of experiment was repeated at least 4 times.

Purification and activation of Bt Cry1Ac δ-endotoxin

The Cry1Ac protein was obtained as recombinant proteins expressed in *Escherichia coli* (Karim *et al.*, 1999b). Bt δ-endotoxins were purified from *E. coli* as documented (Karim *et al.*, 1999b). Inclusion bodies from Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry2A were solubilized in Alkalic buffer (50mM Sodium carbonate, 10mM Dithiothreitol, pH 9.5) for 2 hrs at 37°C. The solubilized protoxin of Cry2A was dialyzed against 50mM sodium carbonate buffer (pH 10.5). The purity of protoxins and toxins was examined by Sodium dodecyl sulfate 10% Polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentrations of the protoxins and toxins were determined by the Bradford method (Bradford, 1976).

Statistical analysis

Data generated through biotoxicity assays of *H. armigera* were statistically analysed through Probit analysis using computer program Quantal software (LeOra software, 1987). IRRISTAT software (1997) was used to estimate ANOVA and correlation for individual toxin and insect strain effect.

RESULTS AND DISCUSSION

Significant differences in susceptibility to *Bacillus thuringiensis* Cry1Ac toxin exist among the *H. armigera* populations tested in the present study. Briese (1981), Georghiou (1988) and Rossiter *et al.* (1990) suggested that if the natural variation in susceptibility within a population is heritable, resistance can develop. Because of the extensive resistance of insects to chemical insecticides monitoring for the potential development of resistance to Bt is crucial. The results of present investigation indicate that similar natural variation exists among *H. armigera* and may be responsible for differences in susceptibility unrelated to prior exposure of Bt toxin.

Insect population sensitivity was investigated to *Bacillus thuringiensis* Cry1Ac toxin. Figure 2 shows a 10% SDS-PAGE containing the trypsin activated Cry toxins used in these studies. The significant difference was found in sensitivity of population of *H. armigera* to Cry1Ac toxin. However, no obvious geographic trends in susceptibility for *H. armigera* were observed (Table I). The sensitivity of *H. armigera* populations to Bt Cry1Ac toxin protein is mentioned in Table I. LC_{50} ranged from 411 ng/cm^2 (Lahore) to > 6400 ng/cm^2 (Multan); this difference represented an 16-fold in susceptibility to Cry1Ac toxin (Table I). Hyderabad population was found most susceptible to Cry1Ac with LC_{50} value 256 ng/cm^2. Multan population of *H. armigera* was least susceptible to Cry1Ac. Hyderabad population was also susceptible to Bt toxins with LC_{50} value 518 ng/cm^2.

Table I: Susceptibility of field collected *H. armigera* to Bt Cry1Ac toxin.

Location	LC_{50} ng/cm^2	Fiducial limits	Slope ± SE
Lahore	411	369-713	4.84±2.46
Multan	>6400	6400-51952	38.13±5.23
Rahim Yar Khan	518	399-599	6.11±2.11
Hyderabad	256	352-497	7.85±3.11

The difference in susceptibility among *H. armigera* populations was almost 1-fold minimum and 16-fold maximum (Table I). Correlation coefficient provided a qualitative evaluation for the behavior of insect population towards Bt toxins (Table II) in relation to each other. These populations were collected from an area not known to have received prior exposure to Bt, and probably do not represent a resistant population. Surveys of susceptibility to Bt in other pest species have shown that considerable inter-population variation exists (reviewed by Tabashnik, 1993). Although, we have not done an extensive sampling of target pests, variation has been reported in other Lepidoptera including the diamond back moth, *Plutella xylostella* (Tabashnik *et al.*, 1990), Indian meal moth, *Plodia interpunctella* (Hubner) (Pyralidae) (Kingsinger and McGaughey,

1979), cotton bollworm, *Heliothis virescens* (F.) (Noctuidae), corn earworm, *Helicoverpa zea* (Boddie) (Noctuidae) (Stone and Sims, 1993) and gypsy moth, *Lymantria dispar* L. (Lymantridae) (Rossiter *et al.*, 1990), European Corn borer, *Ostrinia nubilalis* (Pyralidae) (Siegfried *et al.*, 1995), Spruce budworm, *Christoneura fumiferana* (Clemens) (Tortricidae) (Van Frankenhuyzen *et al.*, 1995) and rice pests (*Scirpophaga incertulas* and *Cnaphalocrocis medinalis*) (Alam *et al.*, 2000). Such differences in tolerance are probably the result of natural variation among the populations (Robertson *et al.*, 1995) and unrelated to prior exposure to the Bt based insecticide.

Table II: ANOVA for the variation in the susceptibility among *H. armigera* populations to Bt Cry1Ac toxin.

Source	Sum of squares	D.F.	Mean square	F-ratio	Prob.
Toxin	7183945	1	7183945.125	1.592	0.2963
Population	13539862.375	3	4513287	1.000	0.5000
ERROR	13539862.375	3	4513287.458		
TOTAL	34263669.875	7			

Significant difference was found in *Helicoverpa* populations for their susceptibility towards Cry1Ac toxin (Fcal. 1.0 < Ftab. 0.5).

The results obtained from a limited number of *H. armigera* populations sampled suggest that variability in susceptibility to Bt may exist among geographically distinct populations (Table I). However, large unexplained variations in susceptibility of field populations have been observed (Sawicki, 1987; Ffranch-Constant and Roush, 1990). Further investigation is required to establish that differences in susceptibility are genetically controlled and not the result of other unidentified factors. We recommended that stably transformed Bt cotton be used as standards for comparison with Bt purified proteins.

The data provide an important baseline information on the susceptibility of *H. armigera* to variety of different purified Cry toxins. However, the relationship between population susceptibility to Bt and the potential for field resistance remains to be established. Further monitoring for potential resistance genes in *H. armigera* populations would facilitate the establishment of a discriminating dose based on the frequency of resistance alleles in the populations. This information is critical to the development of an effective resistance monitoring program and implementation of resistance management strategies. However, it is important to document the extent of this variability throughout the known range of *H. armigera* to establish a diagnostic Bt concentration or dose that could be used in more extensive resistance monitoring program. Further, a baseline susceptibility against a variety of Bt toxins, as reported here, provides a basis for determining if resistance is developing as a result of increased exposure to Bt toxins as might be expected with increased use of Bt based products. Such information is essential to develop resistance management strategies designed to maintain the efficacy of these environmentally sound and economically important management options.

In lab, greenhouse and field assays (Halcom *et al.*, 1996; Sachs *et al.*, 1998) cotton genetically modified to contain a Bt δ-endotoxin protein has consistently demonstrated

efficacy against *Heliothis* species. These field trials indicate that genetically modified Bt cotton has been effective throughout cotton growing countries. The development of strategies for implementing genetically modified Bt crops within integrated pest management offers an exciting challenge to pest management specialists and a considerable potential benefit to cotton growers. We analyzed natural variation to variety of different Bt Cry1Ac toxin in field collected insects of *H. armigera*. These results suggest that natural tolerance to Bt toxins is present in four different populations of both insects towards Bt Cry1Ac toxin. Therefore, it is suggested that high dose/refuge strategy should be evaluated on the basis of natural variations among distinct populations of insect pests. Now, a question arises, if natural variation among pest populations are present, then what would be the range of high dose? Does it vary from locality to locality for same pest? The currently proposed resistance management strategy for Bt cotton, the high dose/refuge strategy (Gould, 1998) requires a) plant tissue express enough Bt toxin to kill all heterozygotes, b) the resistance alleles be very rare, and c) susceptible insects are within an effective mating distance of resistant insects. The high dose/refuge strategy would not be useful for resistance management if high natural tolerance exit among different populations of insect pests. However, at higher dosages all individuals are expected to be susceptible. The practical importance of this natural variation will depend on whether these insects can survive on Bt cotton. In presence of natural variations, the high dose/refuge strategy is subject to a number of stringent pre-requisites that may be difficult to meet in practice. More robust resistance management options are imperative.

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REFERENCES

ALAM, S.I., KARIM, S. AND RIAZUDDIN, S., 2000. Natural variation among rice yellow stem borer, *Scirpophaga incertulas* (Walker) and rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) population to *Bacillus thuringiensis* δ-endotoxins. *Pakistan J. Biol. Sic.*, **3**: 000-000.

BRADFORD, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein using the principle of protein dye binding. *Anal. Biochem.*, **72**: 248-254.

BRIESE, D.T., 1981. Resistance of insect species to microbial pathogens. In: *Pathogenesis of invertebrate microbial diseases* (ed. E.W. Davidson), pp.511-545. Allenheid, Osmon, Totowa, NJ.

FFRENCH-CONSTANT, R.H. AND ROUSH, R.T., 1990. Resistance detection and documentation: the relative roles of pesticidal and biochemical assays. In: *Pesticide resistance in arthropods* (eds. R.T. Roush and B.E. Tabashnik), pp.4-38. Chapman and Hall, NJ. 303 pp.

GEORGHIOU, G.P., 1988. Implications of potential resistance to biopesticides, in biotechnology. In: *Biological pesticides, and novel plant-pest resistance for insect pest management* (eds. D.W. Roberts and R.R. Grandus), pp. 137-145. Boyce Thompson, Ithacca, NY.

GOULD, F., 1994. Potential problems with high dose strategies for pesticidal engineered crops. *Biocontrol Sci. Technol.*, **4**: 451-461.

GOULD, F., 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Ann. Rev. Entomol.*, **43**: 701-726.

HALCOMB, L.J., BENEDICT, H.J., COOK, B. AND RING, R.D., 1996. Survival and growth of bollworm and tobacco budworm on non-transgenic and transgenic cotton expressing a Cry1A insecticidal protein (Lepidoptera : Noctuidae). *Environ. Ent.*, **25**: 253-255.

IRRISTAT, 4.0, 1997. Developed by the Biometrics Unit, International Rice Research Institute, P.O. Box. 933, 1099 Manila, Philippines.

JAMES, C. AND KRATTIGER, A.F., 1996. Global review of the field-testing and commercialization of transgenic plants, 1986-1995: the first decade of crop biotechnology. ISAAA Briefs No. 1 ISAAA: Ithacca, NY, pp.31.

KARIM, S. AND RIAZUDDIN, S., 1997. *Bacillus thuringiensis* δ-endotoxins: Molecular mechanism of action and pest management. *Proc. Pakistan Acad. Sci.*, **34**: 135-155.

KARIM, S. AND RIAZUDDIN, S., 1999a. Bt cotton: gene pyramiding and resistance management strategies. In: *Proc. of ICAC-CCRI Regional Consultation on resistance management strategies*, June 28 - July 1, Multan, Pakistan (in press).

KARIM, S., RIAZUDDIN, S. AND DEAN, D.H., 1999b. Interaction of *Bacillus thuringiensis* δ-endotoxins with midgut brush border membrane vesicles of *Helicoverpa armigera* (Lepidoptera : Noctuidae). *J. Asia-Pacific Ent.*, **2**: 153-162.

KHAN, E., KARIM, S., MAKHDOOM, R. AND RIAZUDDIN, S., 1995. Abundance, distribution and diversity of *Bacillus thuringiensis* in Pakistan environment. *Pakistan J. Sci. Ind. Res.*, **38**: 192-195.

KHAN, E., MAKHDOOM, R., KARIM, S. AND RIAZUDDIN, S., 1995a. Entomocidal activity of indigenous Bt isolates against two important pests, *Tryporhyza incertulas* and *Cnaphalocrocis medinalis*. In: *Proceedings of International Symposium on Biotechnology for Sustainable Development* (eds. K.A. Malik, A. Nasim and M. Khalid), pp.145-153. Faisalabad, Pakistan.

KINSINGER, R.A. AND McGAUGHEY, W.H., 1979. Susceptibility of populations of Indian meal moth and almond moth to *Bacillus thuringiensis*. *J. Econ. Ent.*, **72**: 346-349.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

LeORA SOFTWARE, 1987. *Polo-PC: a user's guide to probit or logit analysis*. LeOra Software, Berkeley, California.

MAKHDOOM, R., KARIM, S. AND RIAZUDDIN, S., 1998. Pathogenicity of locally isolated *Bacillus thuringiensis* δ-endotoxin against *Earias vitella* and *Helicoverpa armigera*. *Pakistan Ent.*, **19**(1-2): 1-5.

MELLON, M. AND RISSLER, J. (eds.), 1998. Now or Never, Serious New Plans to Save a Natural Pest Control, Union of Concerned Scientists. Cambridge, MA, pp.149.

ROBERTS, J., PRIESLER, H.K., NG, S.S., HICKLE, L.A. AND GELERNTER, W.D., 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. *J. Econ. Ent.*, **88**: 1-10.

ROSSITER, M., YENDEL, W.G. AND DUBOIS, N.D., 1990. Resistance to *Bacillus thuringiensis* in gypsy moth (Lepidoptera : Lymantridae): genetic and environmental causes. *J. Econ. Ent.*, **83**: 2211-2218.

SACHS, S.E., BENEDICT, H.J., TAYLOR, F.J., STELLY, M.D., ALTMAN, W.D., BERBERICH, A.S. AND DAVIS, K.S., 1998. Expression and segregation of genes encoding Cry1A insecticidal proteins in cotton. *Crop Science*, **38**: 1-11.

SAWICKI, R.M., 1987. Definition, detection and documentation of insecticide resistance. In: *Combating resistance to xenobiotics, biological and chemical approaches* (eds. M.G. Ford, C.W. Holloman, B.P.S. Khanbay and R.M. Sawicki), pp.105-177. Ellis Horwood, Chichester, England, pp.320.

SIEGFRIED, B.D., MARCON, P.C.R.G., WITKOWSKI, J.F., WRIGHT, R.J. AND WARREN, G.W., 1995. Susceptibility of field populations of the European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera : Pyralidae) to *Bacillus thuringiensis* (Berliner). *J. Agric. Ent.*, **12**: 267-273.

STONE, T.B. AND SIMS, S.R., 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera : Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Ent.*, **86**: 989-994.

TABASHNIK, B.E., 1993. Evolution of resistance to *Bacillus thuringiensis*. *Ann. Rev. Entomol.*, **39**: 47-49.

TABASHNIK, B.E., CUSHING, N.L., FINSON, N. AND JOHNSON, M.W., 1990. Field development of resistance to *Bacillus thuringiensis* in diamond back moth (Lepidoptera : Plutellidae). *J. Econ. Ent.*, **83**: 1671-1676.

VAN FRANKENHUYZEN, K., NYSTROM, C.W. AND TABASHNIK, B.E., 1995. Variation in tolerance to *Bacillus thuringiensis* among and within populations of the spruce budworm (Lepidoptera : Tortricidae) in Ontario. *J. Econ. Ent.*, **88**: 97-105.

WHALON, M.E., 1992. Research needs to combat Bt resistance. In: *Global management of insecticide resistance in the 90's Seminar Proc.*, pp.119-121. Abbott Laboratories, pp.313.

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