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Natural Variation among Rice Yellow Stem Borer, *Scirpophaga incertulas* (Walker) and Rice Leaffolder, *Cnaphalocrocis medinalis* (Guenee) Populations to *Bacillus thuringiensis* δ-endotoxins

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Abstract: Natural variability in susceptibility of field collected rice yellow stem borer (*Scirpophaga incertulas)* and rice leaffolder (*Cnaphalocrocis medinalis*) to *Bacillus thuringiensis* (Bt) δ-endotoxins 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 rice and commercial formulations. Populations of *S. incertulas* and *C. medinalis* were evaluated for their susceptibility to purified Bt Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry2A toxins. The range of LC_{50}'s among selected populations in response to variety of different Bt toxins were between 19-88 fold for *S. incertulas* and 2-20 fold for *C. medinalis*. The data provide a strong basis for monitoring changes in susceptibility of *S. incertulas* and *C. medinalis* populations to future use of Bt toxins in Bt rice program.

Key words: Rice, scirpophaga incertulas, rice leaf-folder

Introduction

The development of insecticides based on *Bacillus thuringiensis* 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*., 1995a, b). 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, 1994). 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 available, these new microbial and plant products will play an important role in insect management (Karim and Riazuddin, 1997).

Selection for rice yellow stem borer (*Scirpophaga incertulas*) (Lepidoptera: Pyralidae) and rice leaffolder (*Cnaphalocrocis medinalis*) (Lepidoptera: Pyralidae) to Bt toxins is expected to be intense and is likely to result in the evolution of resistance to Bt endotoxins (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).

S. incertulas and *C. medinalis* are important economic targets 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 *S. incertulas* and *C. medinalis* field populations collected from Basmati rice growing area (Kalar Tract) in Punjab province of Pakistan.

Materials and Methods

S. incertulas and *C. medinalis* were collected from 4 administrative districts representing traditional Basmati rice growing areas (Kalar Tract) of Punjab, Pakistan (Fig. 1, Table 1). Field collections were made from booting stage of local Basmati rice cultivars. Bt has never been used for insect control in these areas (Karim and Riazuddin, 1999a). Therefore, these populations had no previous Bt exposure. Adult female moths of *S. incertulas* settled on top of rice plants were captured in the early morning hours. Moths were immediately transferred to TN1 rice plants in plastic cages. Moths were oviposit for one night and egg masses were cut from rice leaves. Egg masses were kept in scintillation vials and incubated in the Insectary at 28 ± 2°C (Karim *et al*., 1994) for hatching of larvae. Adult moths of *C. medinalis* were collected by netting and infested on 40 days old TN1 rice plants in their vegetative stage covered with plastic cages (Karim *et al*., 1999a). Collections of *C. medinalis* moths were carried out in bright sunlight. Larvae infested on rice plants were kept in the Insectary for egg laying. Eggs were allowed to hatch on the rice plants. Second instar larvae of *C. medinalis* maintained on TN 1 plants and neonatal stage of *S. incertulas* were used for bioassay studies. The Cry1Ac, Cry1Ab, Cry1Ac, Cry1C and Cry2A proteins were 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 (50 mM Sodium carbonate, 10 mM Dithiothreitol, pH 9.5) for 2 hrs at 37°C. The solubilized protoxin of Cry2A was dialyzed against 50 mM 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).

SDS-PAGE was carried out by the method reported by Laemmli (1970). Toxins were run on 10% Polyacrylamide gel and stained with Coomassie brilliant blue stain (0.25% Coomassie brilliant blue, 45.5% methanol and 9% Glacial acetic acid) at 65°C and destained at the same temperature (Fig. 2).

'TN1' rice seedlings, 18-25 days old were used for the toxicity assays of *Cnaphalocrocis medinalis* larvae. Roots of five tillers were wrapped in wet cotton to prevent drying. The leaves of rice seedlings were initially dipped in 0.02% Triton x-100 and then rinsed in water for several times. After air drying, seedlings were dipped in different dilutions of trypsin activated Bt Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry2A toxins. Seedlings were dried under ceiling fan and were placed in plastic petriplates lined with moist filter paper to keep the plant materials fresh. These petriplates were then infested with 2nd instar *Cnaphalocrocis medinalis* larvae. In case of *Scirpophaga incertulas* fresh 'TN1' rice stems of equal length were inoculated with different dilutions of Bt toxins and were placed in petri plates. The neonatal larvae were allowed to bore in the inoculated stems and incubated at 28 ± 2°C and 65% humidity. Untreated stems and seedlings were used as control. Mortality was recorded after 3 days.

Data generated through biotoxicity assays of *S. incertulas* and *C. medinalis* were statistically analysed through Probit analysis using computer programme Quantal software (LeOra Software, 1987). IRRISTAT software was used to estimate ANOVA and correlation for individual toxin and insect strain effect (IRRI, 1997).

Results

Insect population sensitivity was investigated to *Bacillus thuringiensis* Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry2A toxins. 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 populations of *S. incertulas* and *C. medinalis* to Cry toxins. However, no obvious geographic trends in susceptibility for *S. incertulas* and *C. medinalis* were observed (Table 2-6), nor was the level of population susceptibility to one Bt toxin consistently predictive of the toxins relative susceptibility to the other.

The sensitivity of *S. incertulas* populations to Bt Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry2A toxin proteins is mentioned in Table 2-6. LC_{50s} ranged from 31.13 ng/ml (Sheikupura) to 1633 ng/ml (Narowal); this difference represented an 52-fold in susceptibility to Cry1Aa toxin (Table 2). The range (19-23 fold) in sensitivity to Cry1Ab, Cry1Ac and Cry2A toxins was narrower than Cry1Aa and Cry1C toxins. Sialkot population was found most susceptible to Cry1Ac and Cry2A toxins with LC_{50} values 28.09 and 33.83 ng/ml respectively. Narowal population of *S. incertulas* was least susceptible to all toxins. Cry1C toxin was found the most potent and also high variation in its toxicity. ANOVA on the basis of LC_{50} revealed significant variation among *S. incertulas* populations for their susceptibility (F 1.980 > Prob 0.162) and among Bt toxins for their efficacy (33.079 > 4.403) (Table 7-8). Results were evaluated at 0.05, 1-tail with critical value +/- 0.8221. Variation in susceptibility to purified toxins was comparatively less than that of *S. incertulas* populations from Kalar tract in Punjab. LC_{50} value of Cry1Aa for *C. medinalis* ranged 3 fold from 31.37 ng/ml (Narowal) to 112.58 ng/ml (Sheikupura) (Table 2). Cry1C toxin found most toxic to *C. medinalis* with LC_{50} value of 8.74 ng/ml and as high range of 21 fold with 175.13 ng/ml for Sialkot population. Cry1C toxin also found the most potent and also highly variable among *C. medinalis* populations. ANOVA on the basis of LC_{50} also revealed significant variation among *C. medinalis* populations For their susceptibility (F 1.708 > Prob 0.218) and among Bt toxins for their efficacy (F 0.821 > Prob 0.536)(Table 9-10). Correlation among different populations for their behavior towards Bt d-endotoxins was also evaluated at 0.05, 1-tail with critical value +/- 0.8221.

Discussion

Significant differences in susceptibility to *Bacillus thuringiensis* δ-endotoxins exist among the *S. incertulas* and *C. medinalis* 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 *S. incertulas* and *C. medinalis* and may be responsible for differences in susceptibility unrelated to prior exposure of Bt toxin.

The difference in susceptibility among *S. incertulas* and *C. medinalis* populations was almost 3-fold minimum and 88-fold maximum (Table 2-6). Correlation coefficient provided a qualitative evaluation for the behavior of insect population towards Bt toxins (Table 7-10) 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 (1994). 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) (Kinsinger 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) and Spruce budworm, *Christoneura fumiferana* (Clemens) (Tortricidae) (Van Frankenhuyzen *et al*., 1995). 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.

The results obtained from a limited number of *S. incertulas* and *C. medinalis* populations sampled suggest that variability in susceptibility to Bt may exist among geographically distinct populations (Table 2-6). However, large unexplained variations in susceptibility of field populations have been observed (Sawicki, 1987; Ffrench-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 recommend that stably transformed Bt rice be used as standards for comparison with purified proteins.

The data provide an important baseline information on the susceptibility of *S. incertulas* and *C. medinalis* 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

Table 1: *Scirpophaga incertulas* and *Cnaphalocrocis medinalis* colonies tested for susceptibility to purified Bt δ-endotoxins from rice Basmati growing area in Pakistan

District	Location	Specie	Crop/Cultivar	Plant growth stage	Date	Moths #
	RRI farm, Kala shah Kaku	*S. incertulas*	Rice/Basmati 385	Booting	Sept. 10, 99	300
Sheikupura		*C. medinalis*	Rice/ Basmati 385	Booting	Sept. 10, 99	750
Narowal	Budda Dolla	*S. incertulas*	Rice/ Basmati 385	Booting	Sept. 18, 99	250
		C. medinalis	Rice/ Basmati 385	Booting	Sept. 18, 99	719
Sialkot	GT Road	*S. incertulas*	Rice/ Basmati 386	Booting	Sept. 20, 99	250
		C. medinalis	Rice/ Basmati 386	Booting	Sept. 20, 99	750
Gujranwala	Awan Chowk	*S. incertulas*	Rice/Super Basmati	Booting	Sept. 21, 99	150
		C. medinalis	Rice/Super Basmati	Booting	Sept. 21, 99	534

Table 2: Susceptibility of field collected *S. Incertulas* and *C. medinalis* to Bt Cry1Aa δ-endotoxin

Location	*Scirpophaga Incertulas*		*Cnaphalocrocis medinalis*	
	LC_{50} (ng/ml)	Slope ± SE	LC_{50} (ng/ml)	Slope ± SE
Sheikupura	31.13	3.94 ± 3.35	112.58	2.07 ± 2.26
Narowal	1633.12	4.65 ± 2.34	31.37	10.40 ± 4.12
Sialkot	128.66	11.10 ± 3.79	71.27	2.28 ± 1.82
Gujranwala	420.69	4.00 ± 2.49	55.07	0.73 ± 1.82

Table 3: Susceptibility of field collected *S. Incertulas* and *C. medinalis* to Bt Cry1Ab δ-endotoxin

Location	*Scirpophaga Incertulas*		*Cnaphalocrocis medinalis*	
	LC_{50} (ng/ml)	Slope ± SE	LC_{50} (ng/ml)	Slope ± SE
Sheikupura	58.30	10.89 ± 3.97	84.53	3.47 ± 2.83
Narowal	1085.91	4.51 ± 2.39	91.67	19.18 ± 4.26
Sialkot	72.27	2.61 ± 2.62	19.05	10.05 ± 4.25
Gujranwala	219.92	6.19 ± 3.10	16.39	12.27 ± 4.40

Table 4: Susceptibility of field collected *S. Incertulas* and *C. medinalis* to Bt Cry1Ac δ-endotoxin

Location	*Scirpophaga Incertulas*		*Cnaphalocrocis medinalis*	
	LC_{50} (ng/ml)	Slope ± SE	LC_{50} (ng/ml)	Slope ± SE
Sheikupura	75.86	4.31 ± 3.07	50.21	3.46 ± 3.02
Narowal	671.06	0.29 ± 0.95	43.49	6.32 ± 3.63
Sialkot	28.09	12.99 ± 4.28	26.04	1.77 ± 2.71
Gujranwala	141.64	4.02 ± 2.52	46.38	2.67 ± 4.40

Table 5: Susceptibility of field collected *S. Incertulas* and *C. medinalis* to Bt Cry1C δ-endotoxin

Location	*Scirpophaga Incertulas*		*Cnaphalocrocis medinalis*	
	LC_{50} (ng/ml)	Slope ± SE	LC_{50} (ng/ml)	Slope ± SE
Sheikupura	13.12	58.25 ± 4.87	103.22	5.01 ± 3.11
Narowal	1155.58	1.18 ± 1.32	8.74	9.42 ± 4.49
Sialkot	51.37	5.91 ± 3.51	175.13	0.83 ± 1.53
Gujranwala	469.79	7.42 ± 3.10	117.13	7.01 ± 3.40

Table 6: Susceptibility of field collected *S. incertulas* and *C. medinalis* to Bt Cry2A δ-endotoxin

Location	*Scirpophaga Incertulas*		*Cnaphalocrocis medinalis*	
	LC_{50} (ng/ml)	Slope ± SE	LC_{50} (ng/ml)	Slope ± SE
Sheikupura	81.27	4.80 ± 3.15	178.79	7.83 ± 3.39
Narowal	754.15	3.05 ± 2.09	20.19	3.97 ± 3.56
Sialkot	33.82	8.18 ± 3.92	38.42	2.04 ± 2.67
Gujranwala	183.26	7.81 ± 3.38	52.67	1.67 ± 2.37

Table 7: ANOVA for the Variation in the susceptibility among *S. incertulas* populations to different Bt δ-endotoxins and among the toxicity of individual Bt toxins

SOV	SS	D.F.	MSS	F-ratio	Prob.
YSB Strain	3391799.07	3	1130599.69	33.08	4.403E-06
Bt Toxins	270647.01	4	67661.752	1.980	0.1619
Error	410145.68	12	34178.807		
Total	072591.77	19			

Table 8: Correlation among the susceptibility of *S. incertulas* populations to different Bt δ-endotoxins

	Sheikupura	Narowal	Sialkot	Gujranwala
Sheikupura	1.0000			
Narowal	-0.75647	1.0000		
Sialkot	-0.53645	0.95510	1.0000	
Gujranwala	-0.97262	0.79883	0.58732	1.0000

for potential resistance genes in *S. incertulas* and *C. medinalis* 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 *S. incertulas* and *C. medinalis* 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

Table 9: ANOVA for the Variation in susceptibility among *C. medinalis* populations to different Bt δ-endotoxins and among the toxicity of individual Bt toxins

SOV	SS	D.F.	MSS	F-ratio	Prob.
Leaffolder strain	11660.802	3	3886.934	1.708	0.2184
Bt Toxin	7472.722	4	1868.181	0.821	0.5364
Error	27314.338	12	2276.195		
Total	46447.863	19			

Table 10: Correlation among the susceptibility of *C. medinalis* populations to different Bt δ-endotoxins

	Sheikupura	Narowa	Sialkot	Gujranwala
Sheikupura	1.00000			
Narowal	-0.44786	1.000		
Sialkot	0.07914	-0.66404	1.000	
Gujranwala	0.02781	-0.82787	0.90778	1.00000

Fig. 1: Map of collection sites in Basmati growing area of Pakistan

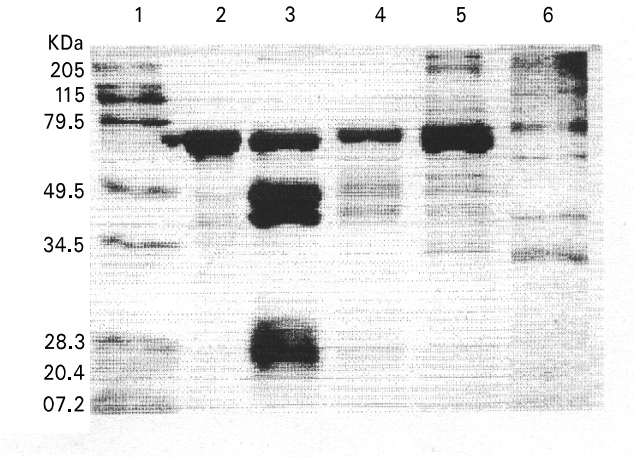

Fig. 2: Commassie Blue-stained 10% SDS-PAGE, Lane 1: high molecular weight protein markers, land 2-5: proteolytically activated Cry1Aa, Cry1Ac and Cry1C, lane 6: solubilized Cry2A toxin

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 (Anwar *et al.*, 1999; Altosaar *et al.*, 1999) rice genetically modified to contain a Bt δ-endotoxin protein has consistently demonstrated efficacy against both *S. incertulas* and *C. medinalis*. These initial trials indicate that genetically modified Bt rice will be effective throughout rice growing countries. The development of strategies for implementing genetically modified Bt rice within integrated pest management offers an exciting challenge to pest management specialists and a considerable potential benefit to rice growers. We analyzed natural variation to variety of different Bt toxins in field collected insects of *S. incertulas* and *C. medinalis*. These results suggest that natural tolerance to Bt toxins is present in four different populations of both insects towards Bt toxins. 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? These results could have important implications for resistance management of rice pests in Bt rice. The currently proposed resistance management strategy for Bt rice, the high dose/refuge strategy (Cohen *et al.*, 1999) 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 rice. In presence of natural variations, the high dose/refuge strategy is subject to a number of stringent prerequisites that may be difficult to meet in practice. More robust resistance management options are imperative.

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