Study No.18:

Title : Baseline Susceptibility
Organization : Metahelix Life Sciences Private Limited, Bangalore
Status : Year I – Kharif 2007

Objectives

The main objective of this study was to establish the baseline susceptibility of *Spodoptera litura* (F) (Noctuidae) to the Cry1C protein expressed by the *B.t*. cotton developed by Metahelix Life Sciences to the primary target pest, *Spodoptera litura*.

Introduction:

The degree of susceptibility of populations from different geographical area to the Cry1C protein is critical for measuring the trend in temporal and spatial resistance development of the target pest towards the protein of interest. This will help in effectively implementing any insect resistance management program in case needed. A basic requirement for this is to establish the baseline for the populations of the target pest, *S. litura,* from different geographical areas. The main target pest of the Cry1C protein is *Spodoptera litura*.

The toxicity of Cry1C protein to *Spodoptera* species has been reported earlier (Hofte and Whiteley, 1989; Visser et al., 1990) and was shown effective against *S. litura*, upon transgenic expression in tobacco by Liu et al. 2003. However, the information about the baseline susceptibility specifically for Cry1C to *Spodoptera* spp is quite limited. The LD_{50} (Lethal Dose) values have been established using various strains of *Bacillus thuringiensis* containing different Cry proteins for several other species of *Spodoptera* (Martinez et al. 2004). Park *et al* 2000 also showed that the LC_{50} (Lethal Concentration) varies depending on the strain hosting the construct. To our knowledge, the baseline value for the Cry1C protein has been reported for *S. litura* (LC_{50} of 0.19 µg/ml) only by Michiels *et al.* (2001)

Definition for Lethal dose: The LC_{50}/LD_{50}, or lethal Concentration/ Lethal Dose 50, is the concentration of endotoxin which is necessary to kill 50% of the treated population after specified period of time in comparison to the control.

Materials and Methods :

Test Insects:

The test insect *Spodoptera litura* was collected from the cotton areas of Aurangabad, Attur and Bellary. Third to fourth instar larvae were collected from the field and reared on semi synthetic diet. The second generation larvae were used in the bioassay to estimate the baseline susceptibility.

Diet Preparation:

The diet primarily consisted of powdered kabuli channa (10 g), Methyl-p-benzoate (0.2 g), Ascorbic acid (0.32 g), sorbic acid (0.1 g), streptomycin sulphate (0.025 g), Multivitamin and Vitamin E tablets (2 numbers), Yeast (1.3 g) and agar agar (1.28 g) for 100 ml diet. The components were ground in 40 ml sterile water in a blender and mixed with the agar (1.28 g) boiled in 40 ml water. The diet was used to establish a laboratory population from different locations and also used in the bioassay. Adults were released on castor plants in cages and fed with honey solution throughout the egg laying period.

Preparation of *B.t.* insecticidal protein:

The transgenic cotton seed was crushed finely using a blender. The Cry1C protein per gram of seed powder was estimated in ten replicates using the Envirologix quantiplate kit and was estimated to be 2.5 µg /g of tissue. This was the standard source for the Cry1C protein in all the bioassays. The insecticidal protein was prepared by suspending required amount of seed powder in 0.2 % agar to obtain various dilutions and dispensed at the rate of 250 µl into each well. The concentrations bioassayed corroborated to 2.83, 2.12, 1.42, 0.71 and 0.35 ng /cm^2 of diet area.

Bioassay set up.

The assays were all set up in 24 well tissue culture plates. The diet was poured at the rate of 750 µl per well, allowed to dry for 30 min at room temperature. The plates were dried at 30°C for about 2 hours. A simultaneous set of control plates were used where the *B.t*. cotton seed powder was substituted by non *B.t.* seed powder representing the top three concentrations. Control plates with 0.2 % agar alone were also included. It has been established in our laboratory that the growth of larvae when reared on diet overlaid with cotton seed powder (at the rate of 0.08 g of non B.t. cotton seed powder per

10 ml agar) was comparable to that of the neonates grown on control plates with plain agar overlay and without agar overlay.

Active neonates less than 12 h old were released at the rate of one larvae per well. 12 larvae were released per replication and the assay was replicated four times. The plates were then incubated at dark with relative humidity of 70% and temperature of 28ºC ± 0.5ºC. The mortality and the numbers of larvae which molted into third instars and above on fifth day were recorded.

Results and Conclusion:

Spodoptera litura from all locations were assayed for the dose mortality response using cotton seed powder containing Cry1C insecticidal protein. The results indicate the LD_{50} values to *S. litura* populations from different regions varies from 2.41 to 5.26 ng/cm^2

Table 1: Dose Mortality Response of *Spodoptera litura* to Cry1C (ng/cm^2)*

Location	LD_{50} (ng/cm^2)	LD_{50}-Upper limit	LD_{50}- Lower limit	Slope +SE
Aurangabad	5.26	6.37	4.14	0.42 ±0.30
Attur	3.10	3.65	2.55	0.35±0.22
Bellary	2.41	2.76	2.05	0.67±0.24

*Probit analysis was done using the software StatPlus 2007

The LC50 values were estimated using the neonates whereas Lereclus *et al.* (2005) reported an LC_{50} of 70 ng/cm^2 at 5 days for *S. littoralis* larvae of second stage with Cry1C endotoxin. An LC_{50} value of 0.19 µg/ml was reported for second stage *S. litura* by Michiels et al. in 2001. The LD_{50} reported earlier for *Spodoptera frugiperda* for Cry1C was of 31 ng/cm^2 (Aranda *et al.* 1996) Visser *et al* in 1998 reported an LC_{50} value of 68 ng/cm^2 against *Spodoptera exigua.* Cry1C and Cry1D were reported to be more toxic to *Spodoptera* spp by Aranda et al 1996, Van Rie et al. 1990 and Bohorovo *et al.*1997.

The larval development was visibly affected by the presence of Cry1C protein in the seed material as seen by the growth retardation. The larval stunting was also associated with the reduction in larval weight. The mean weight of the surviving larvae was measured and is presented in Table 2.

Table 2: Weight of surviving larvae

No	Cry1C ng/sq.cm area of diet	Average weight of surviving larvae (mg)		
		Aurangabad	Attur	Bellary
1	2.83	4.38	8.11	6.42
2	2.12	6.33	9.05	8.92
3	1.42	6.86	16.68	8.62
4	0.71	9.66	26.55	8.78
5	0.35	18.96	27.69	16.26
6	control	22.35	38.25	22.35

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PCR and ELISA CONFIRMATION OF *B.T.* & NON *B.T.* COTTON SEED

Objective: Quality control of the seed material from Cry1C-9124 intra hirsutum cotton hybrids to be used for the biosafety studies at NDRI, Karnal and baseline susceptibility studies conducted at Metahelix Life Sciences Pvt. Ltd., Bangalore.

1. Confirmation of the transgenic nature by PCR based testing
2. Confirmation of presence of protein by ELISA and Quantification of Cry1C protein in the seed material.

1. PCR confirmation:

PCR confirmation was done using the following primers and conditions:

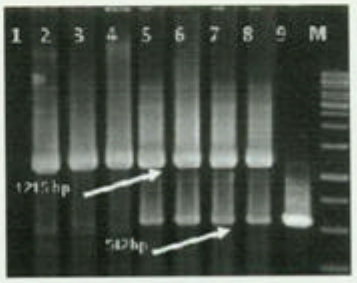
Primers Used
Internal Control: (1) Primer 229 Gh 2S alb U and (2) Primer 230 Gh 2S alb L
Cry1C Specific: (1) Primer 117 MH1CGh2-U and (2) Primer 118 MH1CGh2-L

PCR Conditions: (Eppendorf master Cycler)

Step	Temp°C	Duration	No. of cycles
1	94	2 min	1
2	94	15 sec	
3	60	20 sec	Minus 0.5 deg for 10 cycles
4	72	1 min	
5	94	15 sec	
6	55	20 sec	30 cycles
7	72	1 min	
8	72	5 min	1
9	END		

Expected Band Sizes: 1215 (internal band) & 542 bp (Cry1C specific band)

Legend
1. Water control
2. Non transgenic leaf DNA (-ve)
3. Non *B.t.* seed DNA 1
4. Non *B.t.* seed DNA 2
5. *B.t.* seed DNA 1
6. *B.t.* seed DNA 2
7. Transgenic cotton leaf DNA 1
8. Transgenic cotton leaf DNA 2
9. Plasmid cry1c



Conclusion: The expected 542 bp amplicon has been observed in the transgenic seed powder DNA only, 1215 bp cotton internal control amplicon was observed in all the cotton DNA samples, as expected water and negative controls were clear.

Metahelix Life Sciences Private Limited
Plot No. 3, KIADB 4th Phase, Bommasandra, Bangalore 560 099, India.
Tel: +91-80-787 0236, 783 6086 Fax: +91-80-783 6084 www.meta-helix.com

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2. ELISA confirmation and Quantification

Confirmation and quantification of Cry1C protein was done using the Quantiplate kit for Cry1C (Envirologix, USA, Catalog number AP 007)

No.	Sample	Concentration
1	Blank	NA
2	Std 1 ppb	1.20 ng /ml
3	Std 5 ppb	5.17 ng/ml
4	Std 10 ppb	9.71 ng /ml
5	B.t. -5 X diluted	2.32 µg/g
6	B.t. 10X diluted	2.55 µg / g
7	Non B.t. 5X diluted	NA

*All blank reduction values

Result: The absorbance value observed for the Non B.t. cotton seed sample was similar to the blank values and the colour development was not seen. Blue colour development was seen in transgenic samples, which was clearly absent in negative controls and non transgenic cotton seed sample. The average amount of Cry1C protein in the seed samples was 2.44 µg / g of seed powder.

Declaration:
I hereby declare that the certificate of quality presented in the above results are true to my knowledge and is made on the basis of experiments conducted at our facility

17th Oct 2007
(Vai. Ramanathan)
Head- Genomics

METAHELIX LIFE SCIENCES PRIVATE LTD. BANGALORE

Metahelix Life Sciences Private Limited
Plot No. 3, KIADB 4th Phase, Bommasandra, Bangalore 560 099, India.
Tel: +91-80-787 0236, 783 6086 Fax: +91-80-783 6084 www.meta-helix.com

Baseline study - *Spopdoptera litura*- against Cry1C in seed powder.
Reference: SOP-0044-ENT-PRO-01-Bioassay for Baseline estimation of Cry proteins using transgenic plant tissue as diet overlay
Plan of set up

Area of one well in 24 well assay plates = 1.766 sq. cm

Use only 0.2 % agar for all suspensions

10-Jan-07		sq cm well
Bt- g		1.766
concn per 10 ml	ng /250 ul	ng /sq cm
0.15	9.375	5.31
0.1	6.25	3.54
0.08	5	2.83
0.06	3.75	2.12
0.04	2.5	1.42
0.02	1.25	0.71
0.01	0.625	0.35
agar		
nbt		
0.15	na	na
0.1	na	na
0.08	na	na

Date of Assay : 11 Jan 2008
Date of final Observation : 16 Jan 2008 (fifth day)

Observations:

Location: BELLARY
Test Insect : *Spodoptera litura*
Date of assay: 11 Jan 08
Date of final Observation: 16 Jan 2008
*CSP- Cotton Seed Powder

CSP* per 10 ml of 0.2 % agar	Cry1C ng /250 ul of 0.2 % agar	Cry1C ng/sq. cm area of diet	total	dead	alive	2nd	3rd and above	% 3rd instar	avg. larval wt (live)	% mortality
0.08	5	2.83	38	20	18	16	2	5.26	8.11	52.63
0.06	3.75	2.12	35	17	18	15	3	8.57	9.05	48.57
0.04	2.5	1.42	34	12	22	14	8	23.53	16.68	35.29
0.02	1.25	0.71	32	3	29	15	14	43.75	26.55	9.38
0.01	0.625	0.35	23	1	22	8	14	60.87	27.69	4.35
agar		0	20	1	19	5	15	75.00	38.25	5.00

Values taken from 0.08 in LD_{50} calculation

Location: ATTUR
Test Insect : *Spodoptera litura*
Date of assay: 11 Jan 08
Date of final Observation: 16 Jan 2008
*CSP- Cotton Seed Powder

CSP per 10 ml of 0.2 % agar	Cry1C ng /250 ul of 0.2 % agar	Cry1C ng/sq. cm area of diet	total	dead	alive	2nd	3rd and above	% 3rd instar	avg. larval wt (live)	% mortality
0.08	5	2.83	41	17	24	21	3	7.32	6.42	41.46
0.06	3.75	2.12	39	14	25	20	5	12.82	8.92	35.90
0.04	2.5	1.42	36	12	24	12	5	13.89	8.62	33.33
0.02	1.25	0.71	42	11	31	22	9	21.43	8.78	26.19
0.01	0.625	0.35	41	6	35	21	14	34.15	16.26	14.63
agar		0	35	2	33	11	22	62.86	22.35	5.71

Values taken from 0.08 in LD50 calculation

Location: AURANGABAD
Test Insect : *Spodoptera litura*
Date of assay: 11 Jan 08
Date of final Observation: 16 Jan 2008
*CSP- Cotton Seed Powder

CSP per 10 ml of 0.2 % agar	Cry1C ng /250 ul of 0.2 % agar	Cry1C ng/sq.cm area of diet	total	dead	alive	2nd	3rd and above	% 3rd instar	avg. larval wt (live)	% mortality
0.08	5	2.83	20	3	17	17	0	0.00	4.38	15.00
0.06	3.75	2.12	18	2	16	16	0	0.00	6.33	11.11
0.04	2.5	1.42	19	1	18	17	1	5.26	6.86	5.26
0.02	1.25	0.71	15		15	14	1	6.67	9.66	0.00
0.01	0.625	0.35	23		23	14	9	39.13	18.96	0.00
agar		0	35	1	34	11	24	68.57	22.35	2.86

Values taken from 0.08 in LD_{50} calculation

Location: BATHINDA
Test Insect : *Spodoptera litura*
Date of assay: 11 Jan 08
Date of final Observation: 16 Jan 2008
*CSP- Cotton Seed Powder

CSP per 10 ml of 0.2 % agar	Cry1C ng /250 ul of 0.2 % agar	Cry1C ng/sq.cm area of diet	total	dead	alive	2nd	3rd and above	% 3rd instar	avg.larval wt (live)	% mortality
0.1	6.25	0.12	17	9	8	8	0	0.00	6.37	52.94
0.08	5	2.83	18	5	13	8	5	27.78	20.77	27.78
0.06	3.75	2.12	21	5	16	12	4	19.05	19.68	23.81
0.04	2.5	1.42	19	4	15	8	7	36.84	23.44	21.05
0.02	1.25	0.71	15	1	14	5	10	66.67	25.32	6.67
0.01	0.625	0.35	17	0	17	6	11	64.71	30.14	0.00
agar		0	20	1	19	5	15	75.00	38.25	5.00

Values taken from 0.08 in LD_{50} calculation

avg.larval wt (live) - mg

Cry1C ng/sq.cm area of diet	Average weight of surviving larvae (mg)			
	Aurangabad	Attur	Bellary	
2.83	4.38	8.11	6.42	
2.12	6.33	9.05	8.92	
1.42	6.86	16.68	8.62	
0.71	9.66	26.55	8.78	
0.35	18.96	27.69	16.26	
control	22.35	38.25	22.35	
	LD50	LD50-Upper limit	LD50- Lower limit	Slope +SE
Aur	5.26	6.37	4.14	0.42 -0.30
Attur	3.1	3.65	2.55	0.35-0.22
Bellary	2.41	2.76	2.05	0.67-0.24