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Quantative analysis of *Bt* protein on different events of IR-64 plants and insects sample by ELISA

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Abstract
The experiment was undertaken at the transgenic containment facility, Department of Plant molecular biology and Biotechnology, College of Agriculture, Raipur during 2014 and 2015. The primary exposure data indicated that amount of *Bt* protein contained transgenic lines fed by BPH ranged from 0.001 to 0.109µg/g and secondary exposure data indicated that the amount of *Bt* protein contained BPH on transgenic lines fed by Coccinella beetle ranged from 0.005 to 0.044µg/g. On the basis of two years mean, in primary exposure, the maximum (0.106 µg/g) amount of *Bt* protein was expressed in IR64-4 followed by IR64-2 (0.086 µg/g) and minimum in IR64-C (0.001 µg/g) Whereas, in secondary exposure, the maximum (0.044 µg/g) amount of *Bt* protein was expressed in IR64-3 followed by IR64-2 (0.029 µg/g while protein was not in detectable level in IR64-C.The transgenic plants expressing insecticidal properties are becoming environmentally safe alternatives to chemical pesticides.

Keywords: Quantative analysis of *Bt* protein, IR-64 plants, primary exposure and secondary exposure and ELISA test

Introduction
Genetically modified crop containing crystal protein from the bacterium *Bacillus thuringiensis* (Bt) was grown on 26.3 million ha worldwide in the year 2005 (James, 2005) [2]. Bt rice has the potential to eliminate yield losses caused by lepidopteron pests up to 2%-10% of Asia's annual rice yield of 523 million tons (High *et al.*, 2004) [1]. Genetically modified crops had provided economic benefits to growers and also offer a promising alternative to chemical insecticides for control of lepidopteran pests in rice (Zhu, 2001; High *et al.*, 2004) [1]. Service *et al.* (1986) [4] developed a sandwich enzyme-linked immunosorbent assay (ELISA) test to detect blood-meals in insects and identify the host fed on. The test proved both sensitive and specific. Very small quantities of fresh blood (about 0·02 µl) can be detected; in practice, this enables blood in mosquitoes which are about three-quarters gravid to be identified. In trials in both Zambia and Britain, positive reactions were easily identified visually; consequently, this enabled the ELISA technique to be used as a routine field test. In addition to those of mosquitoes, blood-meals of a few Culicoides species were also successfully identified. Torres *et al.* (2006) [6] studied the expression of *B. thuringiensis* Cry1Ac protein in cotton plants, *Spodoptera exigua* (pest) and *Podisusmaculiventris* (predator) (three trophic levels) under the greenhouse cage experiments. They measured in *Bt* cotton plants (first trophic level), Cry1Ac was 0.18 ± 0.03 (mean ± SD of µg/g fresh weight) and decreased to 0.14 ± 0.01, 0.14 ± 0.02 and 0.12 ± 0.03 µg/g fresh weight in 10, 4 and 2-day-old *S. exigua* larvae fed *Bt* cotton plants (second trophic) whereas in *P. maculiventris* (third trophic level) Cry1Ac was 0.02 ± 0.004 µg/g fresh body weight. Predatory heteropterans acquired Cry1Ac from prey fed *Bt*cotton, but acquisition was dependent on the concentration of Cry1Ac conveyed by the prey and the amount of prey consumed.

Materials & methods
Levels of Cry protein expressed in the leaf tissues of the transgenic *Bt* rice lines under study were measured using the ELISA test kit (Envirologix, USA) according to the manufacturer's instructions.

Protein extraction and Enzyme Linked Immune Sorbent Assay (ELISA)
For quantification of Bt protein in plant tissue and insect body samples "sandwich" enzyme linked immune sorbent assay (ELISA) by Envirologix plate kit designed for quantitative detection of Cry1Ac protein was showed that the concentrations of *Cry1Ac*protien in *Bt* rice lines phytophagous insects and predators were different.

Extraction of protein and sample dilution

The protein extraction protocol of Envirologix plate kit was followed to isolated protein; approximately 20 mg of leaf tissues from the young transgenic rice lines and control non-transgenic lines were collected, crushed and mixed thoroughly with 250 µl of extraction buffer using microtiter plates and glass rod at 4°C. Another 250 µl of extraction buffer was added and mixed with tissues thoroughly for 30-40 seconds. The crushed tissues along with the extraction buffer were collected into 1.5 ml micro centrifuge tube. The samples were centrifuged at 10,000 rpm for 5 min at 4°C and supernatant containing soluble proteins was collected and stored at -20°C. Extracted proteins were diluted to 1:11 times with extraction/ dilution buffer before subjected to ELISA test.

Estimation of Cry1Ac protein

1. 100 µL of negative control, 100 µL of each calibrator, and 100 µL of each sample extracts were added on respective wells, as shown in the example plate layouts (Table 3.10).
2. The contents of the wells were mixed thoroughly by moving the strip holder in a rapid circular motion on the bench top for 20-30 seconds.
3. The wells were covered with parafilm and incubate at ambient temperature for 15 minutes. The plates were placed on a shaker at 200 rpm during incubation time.
4. 100 µL of Cry1Ab-Enzyme Conjugate was added to each well. Content of each well were mixed thoroughly by shaking at 200 rpm. The plate was incubated at ambient temperature for 1 hour.
5. After incubation, the content of each well was removed by shaking. Wells of the plate were washed by completely flooding the wells with washing buffer and later to empty. The plate was kept upside down on a paper towel to remove as much wash buffer, as possible.
6. Add 100 µL of substrate was added to each well. Mixed thoroughly by shaking and incubated for 30 minutes at ambient temperature.
7. Add 100 µL of Stop Solution (1N HCL) was added to each well. The plate was subjected to reading within 30 minutes of stop of reaction.

The absorbance of content of each well was recorded by spectrophotometer at 450 nm. For quantitative estimation of Cry1Ac toxic protein following procedure was used:

- The OD of each set of calibration and sample were averaged.
- A standard graph was made by plotting the mean OD of each calibrator against its Cry1Ac concentration.
- The amount of Cry1Ac protein was estimated by following formula and expressed as µg Cry1Ac toxins per gram of tissue.

$$\mu g\ Cry1Ac\ protein = \frac{OD\ Conc.\ X\ (500/\ x\ mg\ of\ leaf\ sample)\ X\ 11}{1000}$$

Table 1: Arrangement of 44 protein samples on an ELISA plate along with positive and negative control

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
B	C1	C1	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
C	C2	C2	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
D	C3	C3	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
E	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
F	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
G	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
H	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44

Result and Discussions

First of all the transgenic nature of plants were analyzed by using Polymerase Chain Reaction (PCR) analysis. The presence of single band of 750 bp for CryIAc confirmed the transgenic nature of plants. The results are presented in table-2and fig. - 1, 2 & 3.

Primary exposure

The primary exposure data indicated that amount of *Bt* protein contained transgenic lines fed by BPH ranged from 0.001 to 0.109µg/g. The maximum amount of *Bt* protein (0.102 µg/g) was expressed in IR64-4 followed by IR64-2 (0.086 µg/g) and minimum in IR64-C (0.001 µg/g) during 2014. Whereas, during 2015, the maximum amount of *Bt* protein (0.109 µg/g) was recorded in IR64-4 followed by IR64-2 (0.085 µg/g) and minimum in IR64-C (0.001 µg/g). On the basis of two years mean, the maximum (0.106 µg/g) amount of *Bt* protein was expressed in IR64-4 followed by IR64-2 (0.086 µg/g) and minimum in IR64-C (0.001 µg/g). whereas protein was not in detectable level in IR64-C. The present finding are similar to the reporting of Tang *et al.*, (2006) [5]. As the amount of Cry1C protein was measured at the heading stage using the enzyme-linked immunosorbent assay (ELISA) kit from Enviro-Logix (Portland, Me.) by for the protein preparation, approximately 20 mg of fresh leaves from the plant was homogenized by grinding in 500 µl of extraction/dilution buffer. After 30 min at room temperature, a 20 µl aliquot of the supernatant was transferred to a tube then 480 µl of buffer was added. The enzyme-linking reaction was conducted following the manufacturer's instructions. The optical density values of the diluted samples were measured using a microplate reader (Multiskan MK3, Lab system, P.R. China) at 450-nm wavelength, and the Cry1C content was calculated based on the reading. These protein contents varied considerably from one line to another. T1c-19 and its hybrid had 1.38 and 1.32 µg per gram fresh leaf at the heading stage, the highest Cry1C protein contents among the five lines and the corresponding hybrids.

Secondary exposure

The secondary exposure data indicated that the amount of *Bt* protein contained BPH on transgenic lines fed by Coccinella beetle ranged from 0.005 to 0.044µg/g. The results are presented in table 4.4and fig.4.4.The maximum amount of *Bt* protein (0.044 µg/g) was expressed in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005 µg/g) during 2014. Whereas, during 2015, the maximum amount of *Bt* protein (0.044 µg/g) was recorded in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005 µg/g). On the basis of two years mean, the maximum (0.044 µg/g) amount of *Bt* protein was expressed in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005µg/g)

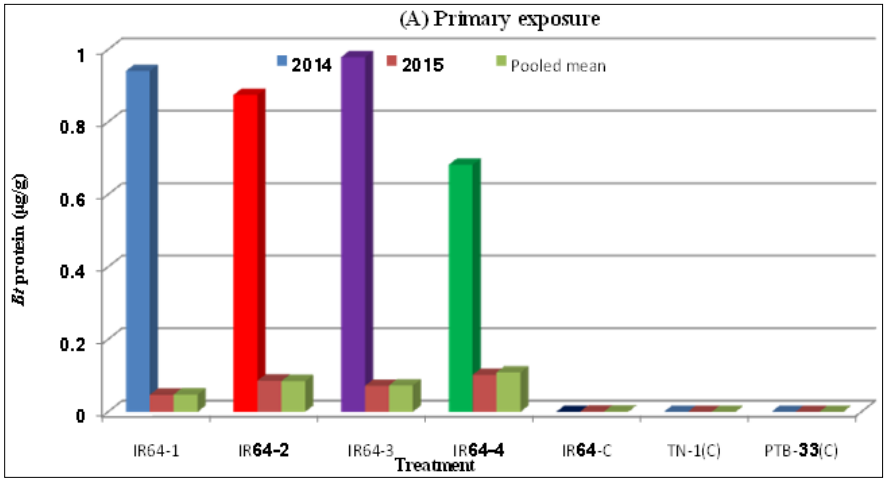
Whereas, protein was not in detectable level in IR64-C.Levels of Cry protein expressed in the leaf tissues of the transgenic *Bt* rice lines under study were measured using the ELISA test kit (Envirologix, USA) according to the manufacturer's instructions. Amount of Bt protein (µg/g of leaf tissue) of rice transgenic lines containing *mcry*IAc gene was studies under the green house conditions against rice insect-pests. During 2014 and 2015, the amount of Bt protein (µg/g of leaf tissue) ranged from 0.682 to 0.979. Levels of Cry protein expressed in the leaf tissues of the transgenic *Bt* rice lines under study were measured using the ELISA test kit (Envirologix, USA) according to the manufacturer's instructions. The quantification of plant and insect immunoassay by Enzyme-linked Immuno-sorbent Assay (ELISA) showed that the concentrations of *Cry1Ac*protien in *Bt* rice events, phytophagous insects and predators were different. For quantification of Bt protein in plant tissue and insect body samples "sandwich" enzyme linked immune sorbent assay (ELISA) by Envirologix plate kit designed for quantitative detection of Cry1Ac protein was showed that the concentrations of *Cry1Ac*protien in *Bt* rice lines phytophagous insects and predators were different.

The primary exposure data indicates that the amount of *Bt* protein contained transgenic lines fed by BPH ranged from 0.001 to 0.109µg/g. The maximum amount of *Bt* protein (0.102 µg/g) was expressed in IR64-4 followed by IR64-2 (0.086 µg/g) and minimum in IR64-C (0.001 µg/g) during 2014. Whereas, during 2015, the maximum amount of *Bt* protein (0.109 µg/g) was recorded in IR64-4 followed by IR64-2 (0.085 µg/g) and minimum in IR64-C (0.001 µg/g). On the basis of two years mean, the maximum (0.106 µg/g) amount of *Bt* protein was expressed in IR64-4 followed by IR64-2 (0.086 µg/g) and minimum in IR64-C (0.001 µg/g). whereas protein was below detectable level in IR64-C. The secondary exposure data indicates that the amount of *Bt* protein contained BPH on transgenic lines fed by Coccinella ranged from 0.005 to 0.044µg/g. The maximum amount of *Bt* protein (0.044 µg/g) was expressed in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005 µg/g) during 2014. Whereas, during 2015, the maximum amount of *Bt* protein (0.044 µg/g) was recorded in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005 µg/g). On the basis of two years mean, the maximum (0.044 µg/g) amount of *Bt* protein was expressed in IR64-3 followed by IR64-2 (0.029 µg/g) and minimum in IR64-C (0.005 µg/g). Whereas, protein was below detectable level in IR64-C.

The amount of Cry1C protein was measured at the heading stage using the enzyme-linked immunosorbent assay (ELISA) kit from Enviro-Logix (Portland, Me.) by Tang *et al.,* (2006) [5]. For the protein preparation, approximately 20 mg of fresh leaves from the plant was homogenized by grinding in 500 µl of extraction/dilution buffer. After 30 min at room temperature, a 20 µl aliquot of the supernatant was transferred to a tube to which 480 µl of the buffer was added. The enzyme-linking reaction was conducted by following the manufacturer's instructions. The optical density values of the diluted samples were measured using a microplate reader (Multiskan MK3, Labsystem, P.R. China) at 450-nm wavelength, and the Cry1C content was calculated based on the reading. These protein contents varied considerably from one line to another. T1c-19 and its hybrid had 1.38 and 1.32 µg per gram fresh leaf at the heading stage, the highest Cry1C protein contents among the five lines and the corresponding hybrids. It is also clear that Cry1C protein contents did not seems to be dependent on the dosage of the gene; the hybrids produced approximately the same amounts of the Cry1C protein as the restorer lines. Not accurate but similar type finding given by Manley, G.V. (1977) on laboratory studies and field trial for staphylinid beetle, *Paederus fuscipes* Curtis to determine natural field density level, feeding patterns, behaviour and biology. He observed that *P. fuscipes* was found to be an aggressive leaf hopper predator in rice fields and feeding on leaf hopper nymphs appears to be density dependent within certain population levels.

Table 2: Quantative analysis of *Bt*protein (µg/g) on different events of IR-64 plants and insects sample by ELISA during 2014 and 201

Transgenic line	leaf	Primary exposure			Secondary exposure		
		BPH			Coccinella		
		2014	2015	Mean	2014	2015	Mean
IR64-1	0.941	0.047	0.048	0.048	0.015	0.014	0.015
IR64-2	0.875	0.086	0.085	0.086	0.029	0.029	0.029
IR64-3	0.979	0.072	0.073	0.073	0.043	0.044	0.044
IR64-4	0.682	0.102	0.109	0.106	0.019	0.02	0.020
IR64-C	NDL	0.001	0.001	0.001	0.005	0.005	0.005
TN1-C	-	-	-	-	-	-	-
PTB 33-C	-	-	-	-	-	-	-



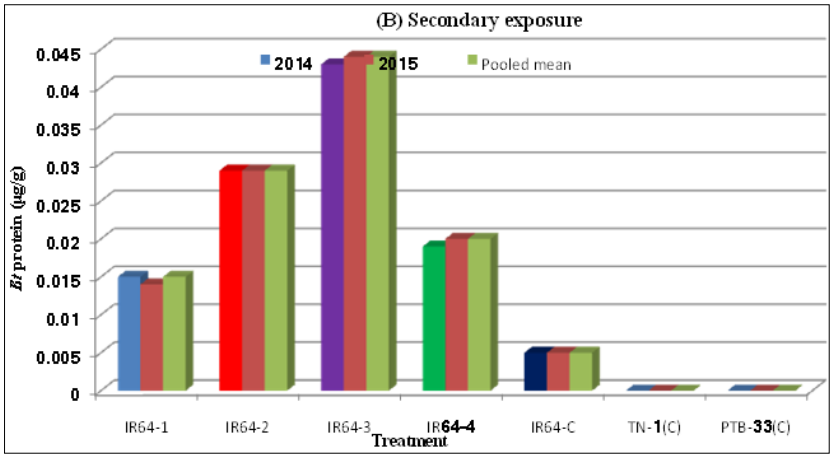


Fig 1: Quantative analysis of *Bt*protein (µg/g) on different events of IR-64 plants and insects sample by ELISA during 2014 and 2015

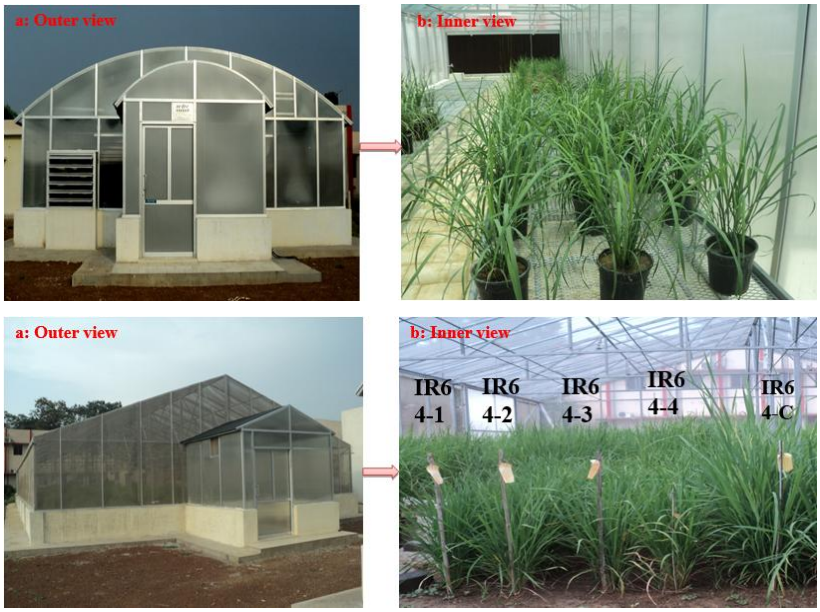


Fig 2: View of Bt rice lines grown in the transgenic contain the facility, IGKV, Raipur

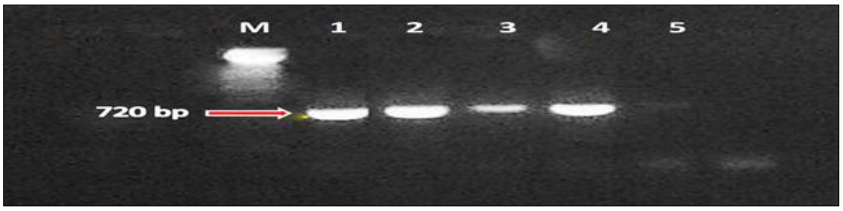


Fig 3: PCR analysis of IR64 transgenic rice lines for mcryIAc

Conclusion

Out of these five genotypes, 4 genotypes were proved as positive lines with single band and one was negative line with no bands. The overall mean of two years, the highest amount of Bt protein (µg/g of leaf tissue) 0.979 was observed in IR64-3 and lowest (0.682) in IR64-4.Quantification of CryIAc protein in plant, primary and secondary expressed insect was analyzed through ELISA (Enzyme-linked Immunosorbent Assay) technique. The ELISA data indicates that the amount of *Bt* protein in IR64-3, (0.97 µg/g in leaf tissue) and IR64-1, (*i.e.* 0.94 µg/g in leaf tissue)is greater than the other *Bt* rice events whereas protein is not in detectable level in IR64-C.

References

1. High SM, Cohen MB, Shu QY, Altosaar L. Achieving successful deployment of *Bt* rice. Trends in plant science. 2004; 9:286-292.
2. James C. Global status of commercialized biotech/GM crops: 2005. ISAAA briefs, no. 37, ISAAA: Ithaca, New York, 2005.
3. Manley GV. *Paederus fuscipes* (Col.: Staphylinidae) - A predator of rice in West Malaysia. Entomophaga. 1977; 22(1):47-59.
4. Service MW, Vollera A, Bidwella DE. The Enzyme-Linked Immunosorbent Assay (ELISA) test for the identification of blood-meals of haematophagous insects. Bulletin of Entomological Research. 1986; 76(2):321-330.
5. Tang W, Chen H, Xu C, Li XH, Lin Y, Zhang Q. Development of insect-resistant transgenic indica rice with a synthetic *cry1C** gene. Mol. Breed. 2006; 18(1):1-10.
6. Torres JB, Ruberson JR, Adang MJ. Expression of *Bacillus thuringiensis* Cry1Ac protein in cotton plants, acquisition by pests and predators: a tritrophic analysis. Agricultural and Forest Entomology. 2006; 8:191-202.