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# Nitrogen fertility and planting date effects on *Bt* ä-endotoxin and mortality of *Helicoverpa armigera* (Hüb) in Bollgard II cotton genotype

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**ABSTRACT :** Toxin expression of Cry1Ac was studied in plant parts of Bollgard II cotton genotype MRC 7031 sown on different planting dates and receiving different treatments of nitrogen application. The expression was quantified by using Cry1Ac/Ab ELISA kit. Mean per cent mortality of one day old larvae of *Helicoverpa armigera* was observed on different plant parts of Bollgard II and its respective non *Bt* cotton genotype. The studies indicated that at higher nitrogen doses *i.e* 225 and 300 kg/ha, maximum expression of Cry1Ac was observed, while the expression was minimum at low nitrogen doses *i.e* 150 kg/ha. Among different planting dates, the expression of Cry1Ac was found to be maximum during late sown (15th May) as compared to early sown crop (Ist May and 15th April). Quantitative expression of Cry1Ac was found to be variable among different plant parts, it was more in leaves followed by squares and bolls. Mortality of *H. armigera* was significantly more at higher nitrogen doses *i.e* 225 and 300 kg/ha than 150 kg/ha. Statistically non significant difference in mortality was observed between different planting dates.

**Keywords:** Bollgard II cotton, Cry1Ac, *Helicoverpa armigera,* nitrogen doses, planting dates

Nitrogen is widely considered as one of the major essential nutrients for plant growth. Being a constituent of proteins, purines, pyrimidines and many coenzymes, a deficiency of N interferes with protein synthesis and thus in growth of plants. *Bt* cotton is considerably effective for lepidopteran pests and is beneficial by reducing insecticide sprays and preserving population of beneficial arthropods. The insecticidal properties of *Bt* are owing to crystalline proteins (ä-endotoxin) that are solubilized and activated in the highly alkaline midgut environment of lepidopteran insects. Efficacy of *Bt* cotton against lepidopteran pests is not consistent over the growing season (Adamczyk and Sumerford, 2001), with plant age (Wan *et al.*, 2005), plant parts, type of genes and gene insertion sites (Gore and Adamczyk, 2004; Jackson *et al.*, 2004), environmental conditions (Mahon *et al.*, 2002). Variation in the efficacy of *Bt* cotton and the involved mechanisms need to be understood fully, so as to plan rational resistance management strategies to retard the rate of the development of resistance and to control target pests effectively by enhancing endotoxin expression through genetic or agronomic practices. For this reason, some well valiadated bioassay techniques and enzyme linked immunosorbent assays (ELISA) (Holt *et al.*, 2002) have been established to quantify changes in the concentration of *Bt* toxin proteins. Keeping in view, the present study was conducted to find out the effect of nitrogen doses and planting dates on expression of Cry toxin as well as on mortality of *H. armigera*.

## MATERIALS AND METHODS

MRC 7031 Bollgard II (BG II) genotype alongwith its non *Bt* genotype were sown following factorial complete randomized design (FCRD) with three different planting dates *viz.,*15th April ($D_1$), 1st May ($D_2$) and 15th May ($D_3$) at Entomological Research Farm, PAU, Ludhiana during 2006 and 2007. Doses of nitrogen *viz.,* recommended (150 kg/ha) $N_1$, 1.5 times recommended (225 kg/ha) $N_2$, 2.0 times recommended (300 kg/ha) $N_3$ and Control (without any dose) $N_0$ were applied in different plots. The expression of Cry1Ac was recorded in leaves, squares and bolls of Bollgard II cotton genotype sown under different treatments. Samples were collected at different time intervals *viz.,* leaves: 60, 90, 120, 140 and 180 days after sowing (DAS); squares: 60, 90, 120 and 140 DAS and bolls: 90, 120, 140 and 180 DAS. For sampling, different plant parts were collected

and brought to the laboratory under cool and dark conditions. Estimation of Cry1Ac was done with ELISA kit of ENVIROLOGIX 500 (Riverside Industrial Parkway Port Land Marine, U.S.A) as per manufactures protocol.

**Estimation of Cry1Ac toxin :** Toxin was estimated by using Cry1Ac/Cry1Ab ELISA Kit that contained its own negative control solutions, different calibrators, enzyme conjugate, substrate and stop solution. For extraction of Cry1Ac, 20 mg tissue of leaf, square and boll was collected in the sample extractor and extracted with 500 ìl of 1x extraction/dilution buffer. Samples were grinded until tissues were crushed completely. From this, 50 ìl of Cry1Ac supernatant was taken and again diluted with 500 ìl of 1x extraction/dilution buffer. Later to 100 ìl negative control, 100 ìl of each calibrator and 100 ìl of each sample extract were poured to their respective wells of ELISA plate. The wells were covered with para film to prevent evaporation and the contents were mixed thoroughly by moving the ELISA plate in a rapid circular motion for 15 min. Then, 100 ìl of Cry 1Ab- enzyme conjugate was added to each well and again the wells were covered with para film and kept on orbital shaker for shaking at 200 rpm for 1 h. Para film was removed thereafter and the contents were discarded quickly. By using the washing buffer, the wells were given three washings. Later on, the ELISA plate was slapped on a paper towel to remove remaining wash buffer, followed by addition of 100 ìl of alkaline phosphatase substrate. The wells were again covered with para film and placed on an orbital shaker for 30 min. Blue colour developed in all wells except for the well having negative solution. At the end, 100 ìl of stop solution was added to each well and mixed thoroughly. This turned the well contents yellow. The absorbance of each of the well solution was read at 450 çm using micro titer plate reader. The standard curve was prepared while using different concentrations of Cry1Ac. The concentration of each sample was determined by using the regression equation of the standard curve and the result was multiplied with dilution factor used during extraction and the data was presented as micrograms toxin/g of tissue.

**Laboratory bioassay :** Laboratory bioassay with one day old larvae of *Helicoverpa armigera* was conducted on different plant parts *viz.,* leaves, squares and bolls. The plant samples were washed with running water and cleaned with muslin cloth. Then samples of Bollgard II and its respective non *Bt* genotype sown under different treatments were put separately in the vials containing agar solution. Later on, 10 larvae were released separately on different plant parts and it has been replicated thrice. The plant parts were changed every day and mortality was recorded after every 24 h interval till 7 days of release. Corrected mortality was calculated as/ Abbott's formula.

**Statistical analysis :** Observations recorded on different parameters were analyzed after suitably transforming the original values.

## RESULTS AND DISCUSSION

Effect of nitrogen doses and planting dates on Cry1Ac expression Among different nitrogen doses *viz.,* 150kg, 225 kg, 300 kg/ha and control (no nitrogen applied), the expression of Cry1Ac in different plant parts indicated that maximum toxin was expressed at 225 kg or high dose of nitrogen/ ha (Table 1). The mean expression of Cry1Ac at 225 kg/ha was 4.29 ìg/g in leaves, 4.14 ìg/g in squares and 4.03 ìg/g in bolls. The similar trend was reported by Pettigrew and Adamczyk (2006), plants that received the higher nitrogen application (150 lbs/ac) exhibited 14 per cent greater leaf *Bt* conc than those plants which only received 100 lbs of nitrogen/ac. Pandher (2006) also examined three levels of nitrogen *viz.,* recommended (150 kg/ha) $N_2$, $N_1$ (112.5 kg/ha) and $N_3$ (187.5 kg/ha) on expression of Cry1Ac and found that mean content of Cry protein was 5.83, 3.94 and 6.71µg/g, respectively which also supported the present study as with the increasing dose of nitrogen, the expression of toxin was also raised. Among different sowing dates *viz.,* 15$^{th}$ April, 1$^{st}$ May and 15$^{th}$ May, the expression of Cry1Ac in different plant parts *i.e.* leaves, squares and bolls indicated that it was significantly more in 15$^{th}$ May sown crop than in 1$^{st}$ May and 15$^{th}$ April sown crop. Pettigrew and Adamczyk (2006) also observed that *Bt* endotoxin level in cotton planted during early April was 12

**Table 1:** Effect of nitrogen doses and planting dates on expression of Cry1Ac in different plant parts

| Nitrogen dose (kg/ha) | Variation in Cry1Ac (ig/g) expression | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaves | | | | Squares | | | | Bolls | | | |
| | 15th April | 1st May | 15th May | **Mean** | 15th April | 1st May | 15th May | **Mean** | 15th April | 1st May | 15th May | **Mean** |
| **$N_1$**(150) | 3.63 | 3.78 | 3.91 | **3.77** | 3.56 | 3.68 | 3.81 | **3.68** | 3.38 | 3.58 | 3.74 | **3.57** |
| **$N_2$**(225) | 4.15 | 4.31 | 4.41 | **4.29** | 4.02 | 4.13 | 4.29 | **4.14** | 3.91 | 4.03 | 4.16 | **4.03** |
| **$N_3$**(300) | 4.15 | 4.29 | 4.44 | **4.29** | 4.02 | 4.19 | 4.30 | **4.17** | 3.89 | 4.03 | 4.17 | **4.03** |
| **$N_0$**(Control) | 3.08 | 3.22 | 3.34 | **3.21** | 2.91 | 3.06 | 3.17 | **3.05** | 2.86 | 2.99 | 3.06 | **2.97** |
| **Mean** | **3.75** | **3.90** | **4.03** | **3.89** | **3.63** | **3.76** | **3.89** | **3.76** | **3.52** | **3.66** | **3.78** | **3.95** |

P=0.05

| **Plant parts** | **Date of sowing** | **Nitrogen doses** | **Date of sowing x Nitrogen doses** |
|---|---|---|---|
| Leaves | 0.02 | 0.02 | 0.04 |
| Squares | 0.02 | 0.02 | 0.05 |
| Bolls | 0.02 | 0.02 | 0.04 |

per cent lower than planted in early May, presumably due to remobilization of the leaf N to support the larger developing boll load in the early April planted cotton. The interaction between planting dates and doses of nitrogen indicated that maximum toxin expressed in 15th May sown crop at 225 and 300 kg of nitrogen/ha.

**Effect of nitrogen doses and planting dates on mortality of *H. armigera* :** Plant samples *viz.,* leaves, squares and bolls collected during different intervals were fed to one day old larvae of *H. armigera* as to study the effect of nitrogen doses and planting dates. The perusal of the data in Table 2 revealed that when the leaves, squares and bolls were fed to one day old larvae of *H. armigera*, the per cent mean mortality differed non significantly at higher nitrogen doses *viz.,* 225 kg/ha (leaves: 86.29, squares: 87.15 and bolls: 79.90%) and 300 kg/ha (leaves: 86.89, squares: 87.87 and bolls: 79.18%), while the mortality was significantly low at $N_1$ (leaves: 82.12, squares: 82.04 and bolls: 77.30 %) and $N_0$ (leaves: 78.35, squares: 78.10 and bolls: 71.19 %). The present study also corroborate with the study of Pandher (2006) who reported that increased dose of nitrogen from 112.5 to 187.5 kg/ha further enhanced the per cent mortality of *H. armigera* neonate larvae (52.26 to 72.17 % at 60 DAS and 31.93 to 43.98% at 120 DAS), respectively.

While during different sowing dates, the mortality of *H. armigera* larvae on different plant parts showed statistically non significant differences. The interaction between planting dates and nitrogen doses statistically differed non significantly.

It was concluded that increase in nitrogen level enhanced the expression of toxin and the expression was more in late sown than

**Table 2.** Effect of nitrogen doses and planting dates on mortality of one day old larvae of *H. armigera* in different plant parts

| Nitrogen dose (kg/ha) | Per cent mortality* of one day old larvae of *H. armigera* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaves | | | | Squares | | | | Bolls | | | |
| | 15th April | 1st May | 15th May | **Mean** | 15th April | 1st May | 15th May | **Mean** | 15th April | 1st May | 15th May | **Mean** |
| **$N_1$**-150 | 80.57 | 82.63 | 83.17 | **82.12** | 80.62 | 82.48 | 83.04 | **82.04** | 76.71 | 77.23 | 77.95 | **77.3** |
| | -64.32 | -66.59 | -67.05 | **-64.99** | -64.35 | -67.49 | -67.91 | **-66.58** | -61.54 | -61.97 | -62.4 | **-61.97** |
| **$N_2$**-225 | 87.57 | 85.51 | 85.79 | **86.29** | 87.78 | 86.45 | 87.22 | **87.15** | 79.18 | 79.7 | 80.23 | **79.7** |
| | -73.98 | -70.23 | -70.47 | **-71.56** | -72.33 | -71.28 | -71.82 | **-71.81** | -63.27 | -63.63 | -64.08 | **-63.66** |
| **$N_3$**-300 | 85.03 | 87.79 | 87.87 | **86.89** | 86.34 | 88.39 | 88.88 | **87.87** | 78.98 | 78.98 | 79.57 | **79.18** |
| | -69.9 | -74.15 | -74.22 | **-72.76** | -71.18 | -72.76 | -73.17 | **-72.37** | -63.11 | -62.95 | -63.47 | **-63.18** |
| **$N_0$** | 78.88 | 79.32 | 79.55 | **78.35** | 77.78 | 78.04 | 78.48 | **78.1** | 70.78 | 71.43 | 71.93 | **71.19** |
| (Control) | -63.18 | -63.46 | -63.64 | **-62.27** | -62.16 | -62.37 | -62.71 | **-62.41** | -57.37 | -57.79 | -58.11 | **-57.75** |
| **Mean** | **82.64** | **83.57** | **84.04** | **83.42** | **83.13** | **83.84** | **84.41** | **85.79** | **76.41** | **76.84** | **77.42** | **73.56** |
| | **-65.52** | **-66.2** | **-66.25** | **-65.99** | **-67.51** | **-68.48** | **-68.9** | **-64.96** | **-61.32** | **-61.59** | **-62.02** | **-61.64** |

P=0.05

| **Plant parts** | **Date of sowing** | **Nitrogen doses** | **Date of sowing x Nitrogen doses** |
|---|---|---|---|
| Leaves | NS | 2.02 | NS |
| Squares | NS | 1.94 | NS |
| Bolls | NS | 3.36 | NS |

* Corrected mortality; Figures in parenthesis are arc sine transformation

in early sown crop. Varying the levels of nitrogen influenced the Cry1Ac protein and mortality of *H. armigera*. The mean expression of Cry1Ac in leaves varied from 3.21 to 4.29 (µg/g) with increasing nitrogen levels to 225 kg/ha. The trend was similar in squares and bolls. However, expression of gene was more in leaves as compared to other parts. The mean mortality of *H. armigera* in leaves ranged from 78.35 to 86.29 per cent, in squares 78.10 to 87.15 per cent and in bolls 71.19 to 79.70 per cent and showed the direct correlation with the N level upto 225 kg/ha. While, non significant difference in mortality was observed during different sowing dates.

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