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Original Research Article

Controlling Pigeon pea Pod borer (Heliocoverpa armigera) by Natural Toxin(s) isolated from Microbes

Sonam Singh, N.A. Khan, Shambhoo Prasad*, Poonam, Raja Hussain and K.N. Singh

Department of Plant Molecular Biology and Genetic Engineering, Narendra Deva University of Agriculture and Technology, Narendra Nagar (Kumarganj), Faizabad-224229 (UP), India
**Corresponding author*

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A B S T R A C T

Pigeon pea (*Cajanus cajan* L. Millspough) is an important legume crop in semi arid-tropical areas of the world. It is second most important pulse crop of India. Among various constraints pigeon pea production, the insect pest has one of the prime importance. *Helicoverpa armigera* is one of the most divesting pests of this crop affecting most variety of this crop. Excessive use of chemical pesticides has resulted in environmental degradation, adverse effects on human health and other organism. Eradication of beneficial insect researchers have shifted the pest control programme from chemical to biology use of microbial is regarded today as one of the suggest bio control agent for a variety of pests. The naturally killed larvae of *Helicoverpa* were crushed and streaked on LB agar. Growth on LB agar indicates that the *Helicoverpa* larvae dead due to bacterial micro-organism. The bacterial protein isolated and purified through Sephadex G-75 column chromatography. The effect of fractionated protein on weight and mortality of pod borer larvae was also studied in artificial bioassay. SDS-PAGE analysis indicated that very low molecular weight of bacterial protein was responsible for death of larvae of *Helicoverpa armigera*.

Introduction

Pigeon pea is an important pulse/grain legume and commonly known as toordal, red gram or arhar. It is the rich source of protein, iron, iodine and essential amino acids likewise methionine, lysine, tyrosine (Alykroid and Doughty, 1964) and also supply vitamins-B, minerals and fats. d agriculture. Globally, it is cultivated on 4.79 M ha in 22 countries (FAO, 2008) but with only a few major producers. In Asia, India (3.58 M ha), Myanmar (560,000 ha), and Nepal (20,703 ha) are important pigeonpea producing countries. In African continent, Kenya (196,261 ha), Malawi (123,000 ha), Uganda (86,000 ha), Mozambique (85,000 ha), and Tanzania (68,000 ha) produce considerable amounts of pigeonpea. The Caribbean islands and some South American countries also have reasonable areas under pigeonpea cultivation. India accounts for 78% of global output with current production of 2.45 million tonnes from 3.80 million hectare recording average yield of 686 kg ha^{-1}.

Among various constrains of pigeonpea production, the insect pest are of prime important. Pigeon pea is crop of intermediate plant type with long duration hence serves as an ideal host for many insect

pests. More than 200 insect species have been reported infesting this crop. Major pest of pigeon pea are pod fly and pod borer. In India 3 species of *Helicoverpa* are found viz. *H. armigera*, *Helicoverpa assulta* and *H. peltigera* .The larvae of *H. armigera* destroys the buds, flowers and pods. It bores inside the pod of pigeon pea during vegetative state and damages inside the pod. In the absence of pod larvae feeds on foliage. The worldwide annual losses caused by *H. armigera* in pigeon pea are $ 317 million (Shanower et al., 1999). In India yield losses of pigeon pea due to insect and disease ranged from 470 to 988 kg $ha^{-1,}$ which accounts for 29-85% of grain yield in different cultivars.

Like other legumes, pigeonpea seeds also contain some anti-nutritional factors. These include oligosaccharides (raffinose and verbascose), polyphenols (phenols and tannins), phytolectins, and enzyme inhibitors (trypsin, chymotrypsin, and amylase). Pigeonpea seeds also have some amounts of unavailable carbohydrates which adversely affect the bio-availability of certain vital nutrients. Some of the anti-nutritional factors such as phytolectins are heat sensitive and are destroyed during cooking (Kamath and Belavady, 1980).

Godbole et al. (1994) reported protease inhibitors in seven day old seeds while Ambekar et al. (1996) found that such inhibitors are either not synthesized or inactive up to 28 days of the seed development. No other plant part except seed exhibited trypsin or chymotrypsin inhibitors.

The white seeded pigeonpea cultivars contain relatively less amounts of polyphenols. Such cultivars are preferred in many countries where dehulling facilities are not available and whole Seeds are consumed. In comparison to the white seeded cultivars the red seeded types contain three times greater quantity of polyphenols (Singh et al., 1984). Similarly, the enzyme inhibition activity was also greater in the colored seeds of pigeonpea.

Pigeonpea leaves also contain other compounds such as hordenine, juliflorine, betulinic acid, stigmasterol, beta-sitosterol, etc. In recent years, extensive research is being carried on various antibacterial, anti-fungal, anti-viral, anti-cancer and anti-inflammatory properties of these flavonoids (Matsuda et al., 2003; Srinivas et al., 2003 and Kim et al., 1999).

The non-judicious use of chemical insecticides has led to the development of resistance to one or more multiple insecticides in over 500 species of insects (Akhurst and Heckel, 2002). Protein isolated from dead larvae can be pathogenic and killed *Helociverpa armigera*. Exploring new insecticides with lesser residues and lower environmental threat has become imperative. In recent years, newer compounds with novel modes of action are being evolved to check infestation by this insect pest. The present study is aimed at evaluating the efficacy of new bio-insecticides against the pod borer in pigeonpea ecosystem. Determination of the composition and toxicity of the parasporal crystals, by means of SDS-PAGE analysis and bioassay is useful complement for gene identification.

Materials and Methods

Collection of naturally killed larvae (Helicoverpa armigera) for identification of micro- organism: The naturally killed larvae of pigeon pea pod borer (*Helicoverpa armigera*) were collected from the experimental site of Genetics and Plant

Breeding Department and Student Instructional Farm. Naturally killed pod borer (*Helicoverpa armigera*) larvae were collected from pigeon pea field and homogenized in 0.1 M sodium phosphate buffer (pH 7.0) with the help of mortar and pestle. The homogenized sample of dead larvae was streaked over the LB agar plate with the help of inoculation loop and incubated at 37^0C for overnight. Small colonies occured on L.B agar plate indicates the bacterial growth.

Isolation of protein from bacteria and naturally killed larvae: A single colony from the LB agar plate was inoculated into 250 ml LB media and incubated at 37^0C for overnight. The overnight grown culture was centrifuged at 10000 xg for 10 minutes at 4^0C and the pellets was resuspended in 0.1 M Tris buffer (pH-6.8) containing 15% glycerol and SDS to final concentration of 1% w/v. The sample was heated in boiling water bath for 10 minute and centrifuged at 6000 xg for 5 minute. The supernatant containing bacterial protein was collected in a microcentrifuge tube and stored at 4^0C till the use.

Protein estimation by Folin Lowry's method: The concentration of bacterial protein was estimated as the method described by Folin and Lowry's (1951). The O.D. of sample was taken at 660 nm by UV visible spectrophotometer and calculation was done with the help of standard curve prepared by using Bovine Serum Albumin (BSA), as standard protein.

3. Purification of appropriate fraction on sephedex G-75 column: Sephadex G-7 was suspended in large volume of water and left it over night. Untill, the gel was fully swollen after this the bottom of column was plugged by the glass wool and stands up right the column (60 cm. long and diameter 3 cm). After making a good slurry of the matrix (stationary phases) in the phosphate buffer, the matrix was poured in the column with the help of same buffer. After proper setting the gel in the column properly the buffer was passed through the column to over night for better settlement of the gel in the column. After this, bacterial protein sample was loaded in the column and it was subsequently eluted by sodium phosphate buffer. The flow rate was controlled at 1 ml/minute. Serial of test tube in the 1 ml per test tube was collected. After collecting 25 fractions, each fraction was observed at 280 nm to monitor the protein content in the test tube. After this, the maximum peaks fractionation samples were used as the source of protein.

Artificial Bioassay of pod borer (Helicoverpa armigera) larvae with fractionated purified protein: The effect of fractionated purified (maximum O.D.) protein on weight and mortality of pod borer larvae was studied in artificial bioassay. Two treatments were setup, one for control and another twenty five for fractionated protein. The pre weighted individual pod borer larvae were transferred into the petridishes (90 mm diameter) with pigeon pea pod in control (without protein), and dipped in fractionated protein and incubated at 37^0C in the dark. Moisture was maintained by putting pre soaked whatmann grade no. 1 filter paper in water at the bottom in each petri dish. The pigeon pea pod used as feeding material for pod borer larvae. The weight gain/reduction and mortality was recorded before and after treatment of feeding till 48 hours.

SDS-PAGE: The fractionated protein sample showing the mortality of pod borer (*Helicoverpa armigera*) larvae in minimum time duration was characterized on 12% SDS-PAGE as the method described by Laemmli, (1970).

Results and Discussion

Bacterial crude protein was extracted from naturally killed larvae using 0.1 M phosphate buffer (pH 7) and concentration of bacterial crude protein was determined by Lowry method. This protein sample was fractionated on Sephadex G-75 with the help of 0.1M sodium phosphate buffer (pH-7). Twenty five fractions were collected by passing the protein sample (17.52 mg/g) from column with the help of phosphate buffer, each fraction was observed at 280 nm to monitor the protein content in the test tube. The column's flow rate is 0.5 ml/minute. In bacterial protein sample maximum peak were observed in fraction no.3,4,6,10,12 and 18. Highest O.D., 0.380 was observed in fraction no.3 and lowest O.D was observed in fraction no.13 whereas highest protein concentration was found in fraction no. 6 (Fig. 1).

The effect of same dosages (200µl) of protein sample was observed against the larvae of pod borer by determining the weight before treatment and after treatment in order to find out the increase or decrease in weight of larvae of pod borer (Fig. 2). In control, the weight of larvae (gm) increased from 0.249 to 0.255, 0.262, 0.293 and 0.315 when observed at different periods *i.e.* at 4, 8, 12, and 24 hours after treatments. In fraction no. 1 the weight of larvae increased from 0.269 to 0.271 and 0.286 after 4 and 8 hours treatment and the weight of larvae decreased 0.202 to 0.195 after 12 to 24 hours treatment respectively. In fraction no. 2 the weight of larvae increased from 0.163 to 0.184, 0.256, 0.262 and 0.271 after 4,8,12 and 24 hours treatment respectively. In fraction no. 3 the weight of larvae increased from 0.268 to 0.309 after 4 hours treatment. In fraction no. 4 the weight of larvae increased from 0.257 to 0.305 and 0.341 after 4 and 8 hours treatment respectively. In fraction no. 5 the weight of larvae increased from 0.254 to 0.301, 0.342, 0.387 and 0.402 after 4, 8, 12 and 24 hours treatment respectively. In fraction no. 6 the weight of larvae increased from 0.282 to 0.372, 0.389, 0.401 and 0.402 after 4, 8, 12 and 24 hours treatment respectively. In fraction no. 7, the weight of larvae increased from 0.260 to 0.317, 0.327 and 0.298 after 4, 8 and 12 hours treatment respectively and the weight of larvae decreased to 0.256 after 24 hours treatment. In fraction no. 8 the weight of larvae increased from 0.246 to 0.307 after 4 hours treatment and the weight of larvae decreased from 0.261 to 0.182 and 0.151 after 8, 12 and 24 hours treatment respectively. In fraction no. 9 the weight of larvae increased from 0.282 to 0.307 and 0.321after 4 and 8 hours treatment and the weight of larvae decreased 0.250 to 0.242 after 12 and 24 hours treatment respectively. In fraction no.10 the weight of larvae increased from 0.105 to 0.107, 0.163, 0.172 and 0.191 after 4, 8, 12, and 24 hours treatment respectively. In fraction no.11 the weight of larvae increase from 0.292 to 0.303 and 0.320 after 4 and 8 hours treatment and the weight of larvae decreased 0.228 to 0.225 after 12 and 24 hours treatment. In fraction no.12 the weight of larvae decreased from 0.425 to 0.414, 0.407 and 0.353 after 4, 8 and 12 hours respectively and the weight of larvae increased to 0.368 after 24 hours treatment. In fraction no.13 the weight of larvae increased from 0.241 to 0.267 and 0.332after 4 and 8 hours treatment and decreased from 0.306 to 0.292 after 12 and 24 hours treatment. In fraction no. 14 the weight of larvae is increased from 0.125 to 0.154 and 0.147 after 4 and 8 hours treatment and the weight of larvae is decreased from 0.286 and 0.252 after 12 and 24 hours treatment. In fraction no.15 the weight of larvae is increased from o.168 to 0.181 and 0.332 after 4 and 8 hours

treatment and the weight of larvae decreased from 0.301 to 0.300 after 12 and 24 hours treatment respectively. In fraction no. 16 the weight of larvae is increased from 0.325 to 0.368 after 4 hour treatment, the weight of larvae is decreased from 0.176 after 8 hour treatment and frequently the weight of larvae is increased from 0.307 to 317 after 12 to 24hour respectively. In fraction no. 17 the weight of larvae is increased from 0.305 to 0.325, 0.385 after 4 and 8 hours treatment and the weight of larvae decreased 0.315 to 0.305 after 12 and 24 hours treatment respectively. In fraction no. 18 the weight of larvae is increased from 0.358 to 0.368 after 4 hour treatment and the weight of larvae is decreased 0.229 after 8 hour treatment and after 12 hour weight is increased 0.309 and decreased 0.302after 24 hour treatment respectively. In fraction no. 19 the weight of larvae is increased from 0.254 to 0.265, 0.320 after 4 and 8 hour treatment and the weight of larvae decreased 0.225 and 0.203 after 12 and 24 hours treatment respectively. In fraction no. 20 the weight of larvae increased 0.263 to 0.273, 0.357 after 4 and 8 hour treatment and weight of larvae decreased 0.192 and 0.175 after 12 and 24 hour respectively. In fraction no.21 the weight of larvae is increased from 0.262 to 0.264, 0.265 and 0.265 after 4, 8, and 12 hours treatment and the weight of larvae decreased 0.264 after 24 hour treatment respectively. In fraction no. 22 the weight of larvae is increased from 0.273 to 0.274, 0.276, 0.337 and 0.348 after 4,8,12 and 24 hours treatment respectively. In fraction no. 23 the weight of larvae is increased from 0.156 to 0.176, 0.299, 300 and 302 after 4, 8, 12 and 24 hours treatment respectively. In fraction no 24 the weight of larvae is increased from 0.244 to 0.255 after 4 hour treatment, weight of larvae decreased 0.295 after 8 hour treatment and weight of larvae increased 0.295 to 0.313 after 12 and 24 hours treatment respectively. In fraction no. 25 the weight of larvae is increased from 0.243 to 0.258, 0.286 after 4 and 8 hours treatment and weight of larvae 0.285 is same after 12 and 24 hours treatment respectively.

Figure.1 O.D. of bacterial protein fractions purified through Sephadex-G column

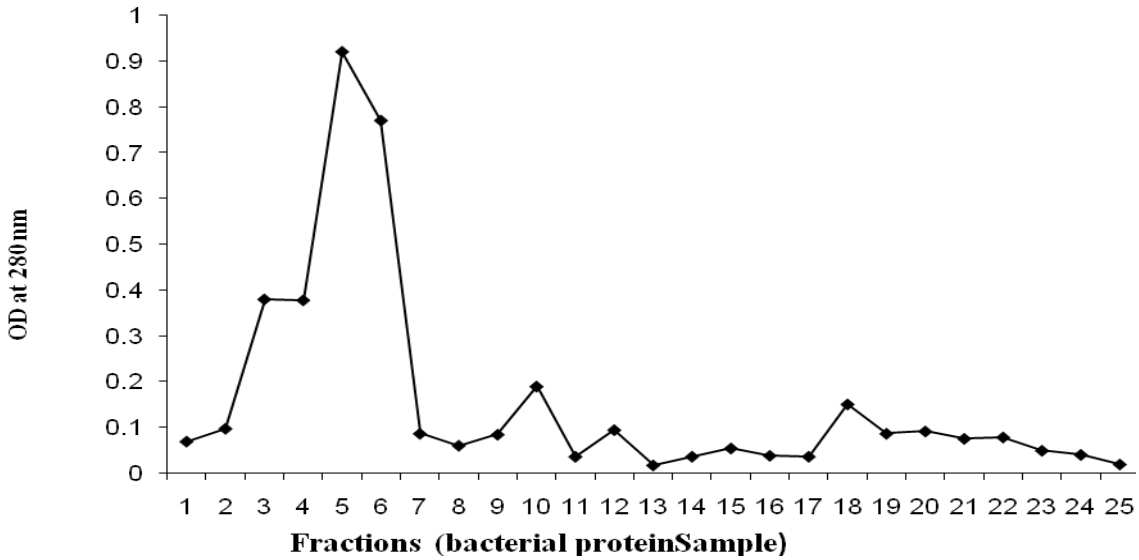
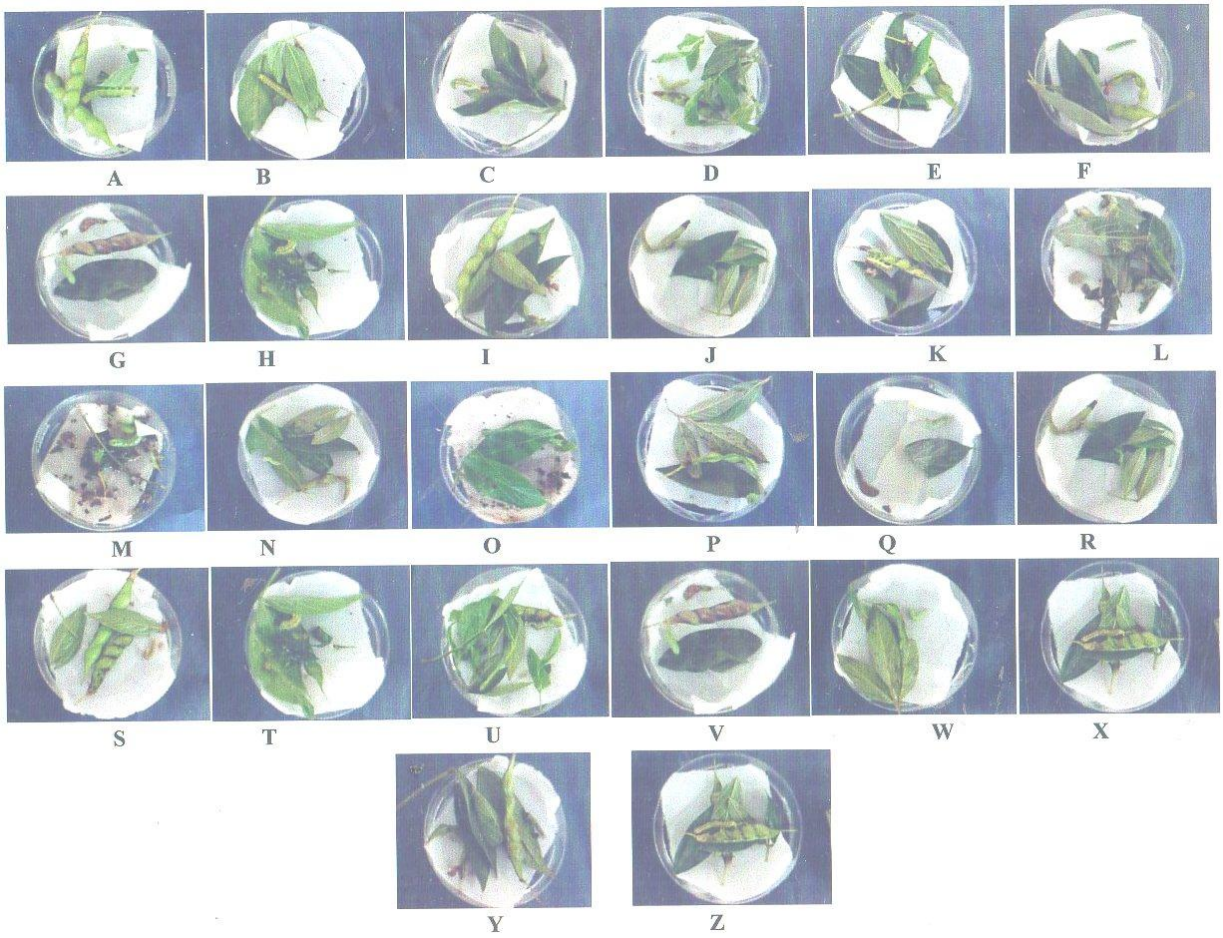


Figure.2 Artificial bioassay of of pigeonpea pod borer (*Helicoverpa armigera*)



A to Y : twenty five fractions of bacterial protein
Z : control

Figure.3 Effect of bacterial protein fractions on weight and mortality of pigeonpea pod borer (*Helicoverpa armigera*) larvae

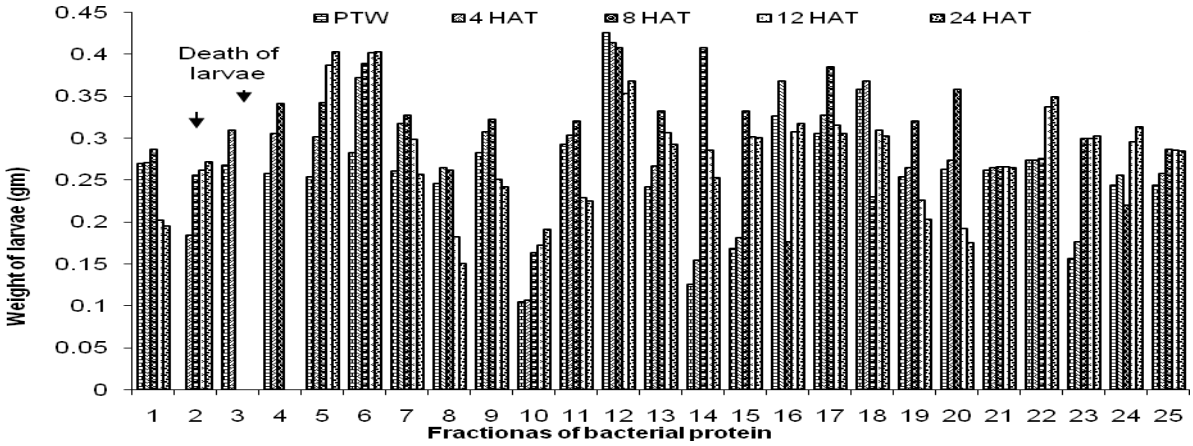
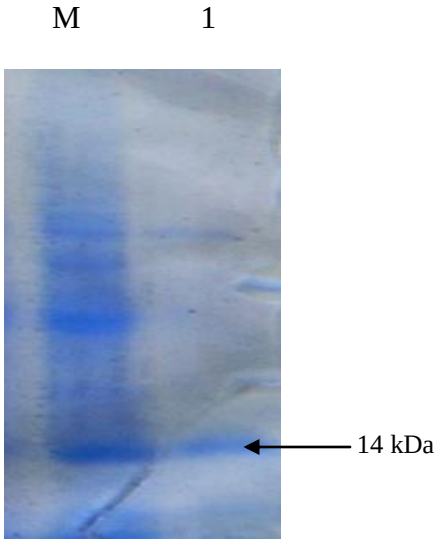


Figure.4 Protein fraction on 12% SDS-PAGE



Lane M: Protein Marker and Lane 1: bacterial protein

Out of 25 fractions, effect of same dosage of protein sample (200 µl) showed that the mortality of larvae was found in fraction no.3 after 10 minute whereas in fraction no. 4 mortality was observed after 1 ½ hour after treatment. Mortality of larvae after 24 hour of treatment was found in fraction no. 7 (Fig. 3). From artificial bioassay it seems that the fraction protein samples have highest O.D. was lethal to pod borer larvae and it is an alternative option to *Bacillus thuringenesis* endotoxin for controlling the insect pest. Now it was aimed to obtain molecular weight of specific protein which caused death of pod borer (*Helicoverpa armigera*) larvae. The isolated bacterial protein showing the mortality of pod borer larvae within 10 minutes was electrophoresed on 12% SDS-PAGE (Fig.4). The SDS-PAGE analysis shows the subunit molecular weight of protein sample from fraction no. 3 was 14 kDa, responsible for insect death in artificial bioassay.

In conclusion, the larvae (*Helicoverpa armigera*) were responsible for maximum damage of pod in pigeonpea. The micro-organism (bacteria and fungi) when attacked on *Helicoverpa armigera* secrete the toxin which cause the death of *Helicoverpa armigera*. It is an alternative option to *Bacillus thuringienois* endotoxin for controlling the insect pest (Cannan, 1996). Now it was aimed to obtain to molecular weight of specific protein which caused of death of (*Helicoverpa*) larvae. For this isolated crude protein of bacteria and used this crude protein sample was electrophorsed in gel electrophoresis. After an electrophoresis very high molecular weight protein band was observed which was easily identification, so results now further to repeat for any conclusion. Determination of the composition and toxicity of parasporal crystals, by means of SDS-PAGE analysis and bioassay, is a useful complement for gene identification.

References

Akhurst, R. and Heckel, D.G. (2002). Toxicity of Bt insecticidal pralines for *H. armigera* and *H. punctigera* (*Lepidoptera Noctoidae*), major pests

of cotton. *J. Invertebr Pathol.*, 80, 55-63.

Alykroid W.R. and Doughty .J. (1964). Legumes in human nutrition, FAO *Nutritional Studies*, 19, 45-47.

Ambekar, S.S., Patil, S.C., Giri, A.P. and Kachole, M.S. (1996). Trypsin and amylase inhibitors in pigeonpea seeds. *International Chickpea and Pigeonpea Newsletter*, 3, 106-107.

Cannon, R.J.C. (1996). *Bacillus Thuringiensis* use in agriculture: A molecular prospective. *Biological Review*, 71: 561-636.

FAO (2008). Food and Agricultural Organization website http ://faostate.fao-org.update of 06 July, 2008.

Godbole, S.A., Krishna, T.G. and Bhatia, C.R. (1994). Changes in protease inhibitory activity from pigeonpea (*Cajanus cajan* (L.) Millspaugh) during seed development and germination. *Journal of Science Food and Agriculture*, 66, 497-501.

Kamath, M.V. and Belavady, B. (1980). Unavailable carbohydrates of commonly consumed Indian foods. *Journal of Science of Food and Agriculture*, 31, 194-202.

Kim, H.K., Cheon, B.S., Kim, Y.H., Kim, S.Y. and Kim, H.P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochemical Pharmacology*, 58, 759-765.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the had of bacteriophage T_4. Nature, 227, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, and Randall, R.J. (1951). Protein measurement with the phenol reagent *J. Biol. Chem.*, 193:265

Matsuda, H., Morikawa, T., Ando, S., Toguchida, I. and Yoshikawa, M. (2003). Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. *Bio-Organic and Medicinal Chemistry*, 11, 1995-2000.

Shanowar (1997). Survival growth fecundity and behaviors of *H. armigera* (*Lepidoptera*: *Noctuidae*) on pigeonpea and two wild *Carjanus* spp. *J. Economic Entom*., 90, 837-841.

Singh, U., Jain, K.C., Jambunathan, R. and Faris, D.G. (1984). Nutritional quality of vegetable pigeonpeas [*Cajanus cajan* (L.) Millspaugh]: Dry matter accumulation, carbohydrates and proteins. *Journal of Food Science*, 49, 799-802.

Srinivas, K.V.N., Rao, S.K., Mahender, Y.I., Das, B., Krishna, K.V.S.R., Kishore, K.H. and Murty, U.S.N. (2003). Flavonoids from *Caesalpinia pulcherrima*. *Phytochemistry*, 63, 789-793.