In vitro bioassay to determine the toxicity of cry1F protein against sugarcane early shoot borer (*Chilo infuscatellus* Snell).

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Abstract - The insecticidal cry gene (cry1F) was cloned into pET28a(+) vector and expressed in E. coli strain DH5α with IPTG induction. SDS-PAGE confirmed the expression of ~60 kDa protein size. The insect bioassay with 2nd instar larvae of *Chilo infuscatellus* was carried out in glass petri dishes containing varying concentrations of cry1F protein (3.14, 6.28, 9.43, 12.57 and 15.72 µg of protein/cm2) and insect mortality rate was subsequently determined for 15 days with a 3 day time interval respectively. The cry1F protein had a considerable impact on the survival rate of shoot borer and highest mortality (%) was achieved at 15.72 µg protein/cm2 on 9th day. Moreover, the dosage mortality probit analysis for cry1F toxic protein indicated the LD50 to be 6.11 µg protein/cm2 on 6th day against *Chilo infuscatellus* larvae by diet surface treatment method. The results suggested that feeding of diet containing partially purified cry1F protein shows significant insecticidal activity against Chilo infuscatellus..

Keywords - *Chilo infuscatellus*, Cry protein, cloning, insect bioassay, protein expression, SDS-PAGE, Sugarcane.

1 INTRODUCTION

Sugarcane (Saccharum officinarum L.) is a monocot plant widely spread and economically important in many regions around the world. It is an important cash crop which contributes to around 70–80% of sugar production globally [1]. It is world largest crop by production quantity (MT). The annual production of sugarcane in the world is ~1.84 billion tons [2]. In all over the world India is second in terms of sugar production and sugarcane yield. In 2013 average sugarcane yield was 70.77 tons per hectare worldwide.

There are many factors like drought; climatic conditions, diseases and pest are responsible for low sugarcane yield. Among all of them more significant loss due to the severe attach of insect pest at early stages of sugarcane crop. Sugarcane borer belongs to order Lepidoptera include early shoot borer (Chilo infuscatellus), Internode borer (Chilo saccharifagus indicus) and Top Borer (Scirpophaga excerptalis) are the major and most injurious among all other factors [3]. The damage caused by early shoot borers not only reduces cane yield but also affects the percentage of sugar recovery. Early shoot borer (*Chilo infuscatellus* Snell.) is a major pest of sugarcane in India, causing extraordinary agricultural and industrial losses annually, especially in Maharashtra, Tamilnadu, and Uttar Pradesh region. Larva causes damage by feeding on tender leaves and further tender shoots and causing "dead hearts". The pest causes loss up to 10-20% of young shoot. It may be as high as 70% in certain cases. The attack does not produce dead hearts after the formation of canes and the damage is confirmed to a few internodes only. Control of sugarcane borere is very difficult and expensive due to the typical feeding behavior of the larvae into the sugarcane stem [4] and Trichogramma sp. and Beauveria basiana have been unsuccessfully tried to control the sugarcane borer in field conditions [5].

Now a day's use of pesticide is expensive and extreamly toxic to non target organism and often harmful to human and animal health. One alternative to chemical insecticide is the use of GM crops that express insecticidal proteins in economically proven cultivars. Bt represents a useful alternative to conventional insecticides, formulated in bio-insecticides or delivered in transgenic plants. Bacillus thuringiensis (Bt) is a gram positive, soil borne, aerobic bacterium that has been used as a biopestiside due to the its ability to produce insecticidal crystal proteins (δ-endotoxins) and cytolitic (Cyt) toxins [6]. The cry proteins are classified separately according to its effect on particular class of insect [7]. Cry proteins are active against lepdopteran insect in many crops [8].

Cry protiens are solubilized and proteolytically activated in the larval midgut. The biocidal activity spectrum of these toxins is very narrow because of their mode of action, which is based on specific receptor-recognition and membrane insertion of the active Cry proteins bringing about the paralysis of the transepithelial transport [9]. The advantages of biodegradability and total safety both for humans and the environment have led to an increase in their use to control agricultural pests. The different cry genes deployed in transgenic plants,

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cry1F is an important one with transgenic plants developed in several crops like cotton [10,11] and maize [12]. Bt cry1F gene is known to be effective against different insects including Lepidoptera.

Therefore, the goal of this work was to check insectisidal activity of partially purified cry1F protein against larvae of sugarcane early shoot borer (*Chilo infuscatellus* Snell.). Isolated expressed cry1F protein from E. coli was used to check relative efficiency.

2. Material and Methods

2.1 Bacterial strains and plasmid

The cry1F gene fragment was isolated from the vector pBinAR and cloned in pET28a(+) expression vector for transformation in to Escherichia coli (DH5α). The transformed E. coli were grown in LB broth medium containing kanamycin (50 mg/l) and incubated at 37 ºC for overnight on continuous shaking at 180 rpm.

2.2 Plasmid DNA isolation, manipulation and confirmation of cry1F

Plasmid DNA from E. coli (pBinARCry1F and pET28a(+)) was isolated according to alkaline lysis method reported by Sambrook and Russell [13] and purified by using Qiagen-tip 100 column. The recombinant plasmid pBinAR-cry1F and pET28a(+) vectors were digested with the Xba I and Sal I. Approximately 1.8 kb DNA fragment of cry1F gene and 5.2 kb DNA fragment of pET28a(+) plasmid were purified after agarose gel electrophoresis and ligated using T4 DNA ligase at 16 ºC for 5 h incubation to achieve recombinant pET28a(+)-cry1F (7 kb) expression construct. The ligated product was transformed in E .coli and colonies obtained under the selection of Kanamycin (50 mg/l) were subjected to colony PCR using cry1F gene specific primers (FP: 5'-ATC CAG AAT CAA TGC GTC CC-3' and RP: 5'-GAA AGA GCT CAG AAG GCG TAG-3') to amplify 1.8 kb fragment. The PCR mixture (20 µl) consisted pricked colony, 1X Taq buffer, 250 µM dNTP's, 1.5 mM $MgCl_2$, 0.25 µM each forward and reverse primers and 1U Taq DNA polymerase. The thermal cycler conditions were initial denaturation at 95 °C for 4 min, followed by 35 cycle at 94 °C for 45 s of denaturation, 59 °C for 30 s of annealing, 72 °C for 90 s of extension and final extension at 72 °C for 10 min. Amplified PCR products were analyzed by using electrophoresis on 0.8% (w/v) agarose gel. The recombinant pET28a(+)-cry1F was also confirmed with restriction analysis.

2.3 Expression of recombinant protein (cry1F) in E. coli (DH5α)

Escherichia coli strain DH5α clone harboring pET28a(+)-cry1F was grown in 5 ml LB medium containing kanamycin (50 mg/l) at 37 ºC until OD600 reaches to 0.6. Further the same culture was transferred into 50 ml fresh LB medium and induced by adding isopropyl-β-D-1-thiogalactoside (IPTG) (1 mM) at 37 °C for 12 h. The E. coli clone harboring pET28a(+) vector was used as a control. The cells were then harvested by centrifugation at 7500 rpm for 5 min at 4 ºC and pellets obtained were washed with distilled water and resuspended in 4 ml of extraction buffer (pH 8.0) containing Tris-HCl (30 mM), EDTA (1 mM) and phenyl methane sulfonyl fluoride (PMSF) (1 mM) lyse on ice by ultra sonication (VibraCell™ sonicator) at 20 KHz with pulse of 10 seconds for 6 times with an interval of 5 s. The pellet was washed twice with NaCl (0.5 M) plus Triton-X (2%) followed by NaCl (0.5M) and sterile distilled water. Un-solubilized protein i.e. supernatant was isolated by centrifugation at 15,600 g at 4 °C for 10 min.

The expressed protein extract from E. coli lysate was separated by SDS/Polyacrylamide gel (10%) electrophoresis (Amersham Biosciences miniVE vertical electrophoresis system apparatus, UK). The separated polypeptides were visualized by staining with comassie brilliant blue and gel image was recorded using gel documentation unit. The total protein isolated from cell lysate estimated by Bradford [14] method, using the BSA as a standard protein.

2.4 Insect Bioassay with cry1F

The first instars larvae of early shoot borer (*Chilo infuscatellus* Snell.) were collected from Sugarcane farm, VSI, Pune. Insect bioassay was performed at Molecular Biology and Genetic Engineering section, VSI, Pune. The artificial diet for larvae raring was prepared as described by Taneja and Nwanze [15] which consisted of kabuli gram flour (60 g), casein (22.5 g), ascorbic acid (9 g), yeast tablets (9 g), methyl parahydroxy benzoate (MPH) (2 g), sorbic acid (1 g), sucrose (19 g), hostacycline (1.5 g), multivitaplex capsule (5 nos.), formaldehyde -

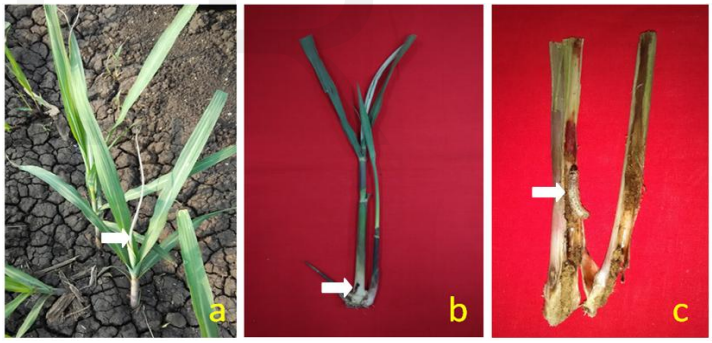

Figure 1. Crop damage due to early shoot borer (Chilo infuscatellus). (a) dried leaf spindle, (b) borer larvae entre hole, (c) *Chilo infuscatellus* larvae feed on the soft tissue and make cavities extending to the sets.

10% (v/v) (2 ml), sugarcane shoot powder (75 g) and agar (9 g). The molted nutrient agar (400 ml water) was supplemented with the above mixture. The artificial diet was dispensed in glass petriplates (45×10 mm) with varying concentration (3.14, 6.28, 9.43, 12.57 and 15.72 µg toxin/cm^2) of partially purified cry1F proteins smeared on the surface of the diet. Protein extracted from E. coli harboring pET28a(+) vector served as negative control and artificial diet without protein served as a control. Fifteen larvae were used for each concentration and each set was replicated trice and mortality was recorded after every three days till 15 days after initiation. Cumulative mortality was determined by measuring number of dead larva per treatment as

Cumulative mortality (%) = (Total number of larve survived) /(Total number of larve released) × 100

2.5 Data recording and statistical analysis

In the in-vitro bioassay, the concentration of cry1F protein as microgram per unit area of diet surface, cumulative mortality percentage and LC50 value was analyzed as per Finney [16]. The data was analyzed using Microsoft Excel 2007 and SPSS (software package version 16). Mortality rate was expressed as the proportion of dead larvae to the released larvae subjected to analysis. One way ANOVA was applied to test mean differences of all treatments.

3 Results and Discussion

3.1 Construction of the pET28a(+)-cry1F vector

In order to develop pET28a (+) -cry1F, plasmid DNA of pBinARcry1F and pET28a(+) was digested with SalI and XbaI. This digestion resulted into two separate bands each in pBinAR-cry1F i.e 1.8 kb, >10 kb (Fig. 2a) and pET28a(+) i.e 166 bp and 5.4 kb (Fig. 2b). These separated bands were eluted from the gel using Quigen QIAEX®II Gel extraction kit. These fragments (1.8 kb band of cry1F and 5.2bp band of pET28a(+)) were further ligated using T4 DNA ligase and then further transfected into E. coli (DH5α) resulting 7 kb DNA fragment. The transformation in bacteria was confirmed by colony PCR using gene specific primer for cry1F. PCR amplified product confirmed the ligation of 1.8 kb fragment in the pET28a(+)-cry1F vector (Fig. 2c). The reconfirmation of the pET28a(+)-cry1F vector was assured by restriction digestion with XbaI and SalI as these yielded two separate and expected bands (1.8 kb and 5.2 kb) (Fig. 2d).

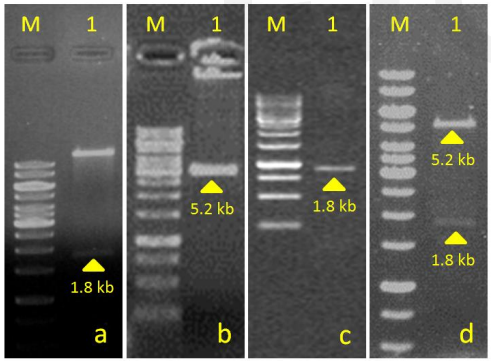

Figure 2. Cloning of cry1F fragment in pET28a(+). (a) pBinAR-cry1F digested with XbaI and SalI released 1.8 kb fragment of cry1F, (b) pET28a(+) digested with XbaI and SalI showing 5.2 kb fragment, (c) PCR amplification in modified pET28a(+):cry1F showing presence of cry1F gene and (d) Restriction digestion of pET28a(+):cry1F with XbaI and SalI released the 1.8 kb fragment of cry1F and 5.2 kb backbone of pET281(+).

3.2 Expression of recombinant cry1F protein expressed in *E. coli*

The cry1F gene was cloned from pBinAR-cry1F into pET28a(+) vector and the recombinant clones were confirmed by PCR using cry1F gene specific primers and restriction digestion with XbaI and SalI (Fig. 2c,d). After induction (IPTG) protein was extracted from E. coli harboring pET28a(+)-cry1F showed expected band size of ~60 kDa which was absent in negative control on SDS-PAGE (Fig. 3) and corresponded to the estimated size for cry1F based on predicted amino acid sequence of the protein. Similar findings were noticed during isolation and expression of cry protein from E. coli [17,18,19,20,21].

Saraswathy et al., [22] carried out the expression study of two fused Bt genes viz. cry1Ac (toxic to Helicoverpa armigera and *Plutella xylostella*) and Vip3Aa14 (active against *Spodoptera litura*) yielded a recombinant protein active against all the respective insects.

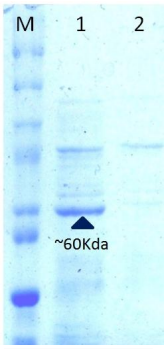

Figure 3. SDS-PAGE analysis (Coomassie brilliant blue stained 10% gel) of protein expression of cloned pET28a(+)-cry1F (1), Crude extract of E. coli cells containing pET28a(+) vector (2). Molecular marker (M).

3.3 Response of early shoot borer (*Chilo infuscatellus* Snell.) against recombinant cry1F protein

Insect pest constitute a major biotic stress in all sugarcane growing countries including India. Among all insect and pest, early shoot borer *Chilo infuscatellus* Snell damaged crops in early stage leading to reduction in yield and ultimately sugar recovery [23]. These pests are solely responsible for losses of 19.63% in yield of sugarcane and 1.72 - 8.02% in sugar recovery [24]. Now a day's use of pesticide is not only expensive and extremely toxic to non target organism and often harmful to human and animal health. One alternative to chemical insecticide is the use of GM crops that express insecticidal proteins in economically proven cultivars. Cry proteins are active against lepdopteran insect in many crops [9]. Therefore, to ascertain the efficacy of cry1F protein against Chilo spp. before going for transformation experiment is mandatory. Insect bioassay was carried out with the partially purified cry1F protein using sugarcane early shoot borer (*Chilo infuscatellus* Snell) larvae. The present investigation was carried out using different concentrations of partially purified recombinant protein (pET28a(+)-cry1F) isolated from E. coli DH5α and did insect bioassay with 2nd instar larvae of C. infuscatellus. Protein from pET28a(+) used as negative control and artificial medium without any protein served as control. The lethal concentration of the cry1F protein at the concentration of 15.72 µg/cm2 was most effective showing LD100 at 9th day after 12th day after release (Fig. 4 and 5d). LD50 of this protein at the concentration of 6.28 µg/cm2 was observed on 6th day after release of larvae on diet and 9th day at 3.14 µg/cm2. release (Fig. 5e) followed by 12.57 µg/cm2 showing LD100 at

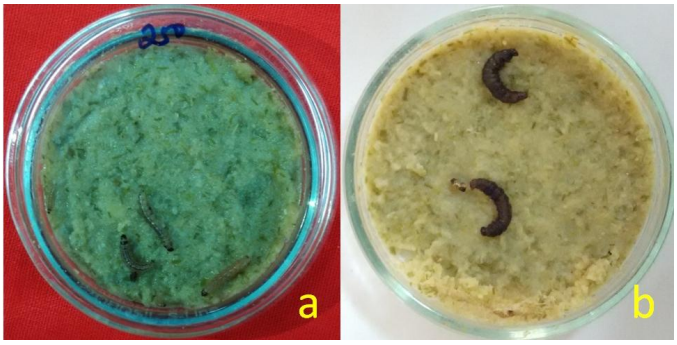

Figure 4. Insect bioassay for Chilo infuscatellus. (a) Larvae on artificial medium containing protein extract, (b) Dead heart larvae.

larvae on control diet or the diet smeared with the protein isolated from the E. coli cells carrying only basal pET28a(+) vector showed highest 23.63% mortality in concentration of 15.72 µg/cm2 on 15th day (Fig. 5e) and the lowest mortality of 7.87% in the concentration of 9.43 µg/cm2 (Fig. 5a) on 9th day. Dosage mortality probit analysis for cry1F toxic protein against *C. infuscatellus* larvae by diet surface treatment method indicated that LD50 at 6.11 µg/cm2 on 6th day (Fig. 6). Reddy et al., [21] also reported that cry protein (cry1Ab) at 1.85 µg toxins/cm2 of diet was as effective at 2.32 µg toxin/cm2 of diet and cumulative mortality was found 97% after 5 days of feeding. However, larvae on control diet containing pET28a(+) vector does not showed any dead larvae. Therefore the result was concluded that cry1F gene could be potential for developing transgenic sugarcane effective against lepidopteron pest.

Cheng et al., [26] studied four cry genes (cry1Aa, cry1Ab, cry1C and cry1Da) were cloned separately from a commercial product Xentari (based on Bacillus thuringiensis var. aizawai) and expressed in a crystalliferous Bt (Cry-B) respectively. Total proteins caused more than 94% mortality against 3rd-instar larvae of Plutella xylostella (Lepidoptera) and 96% mortality against 2nd-instar larvae of Trichoplusia (Lepidoptera). It has been found that cry1Aa3 from Bacillus thuringensis are active against controlling cotton pests and is the active ingredient of many formulated bio-insecticides for the control of cotton pests including Earias insulana [26].

By the end of the toxicity test, all the treated larvae remained in the first instar, whereas most of the control larvae molted to the third instar. The toxicity of B. thuringiensis variety kurstaki Cry1-A insecticidal property is also reported against other pests like tomato pinworm where different concentrations were used and significant mortality was seen [28]. In another study of fusion proteins having toxicity to multiple insect pest species the entomocidal activity of the fusion proteins were determined [23]. In the same study bioassay was performed on larvae of Helicoverpa armigera, Plutella. xylostella and S. litura and it was showed that the fusion protein was toxic to H.armigera, *P. xylostella* which was similar to Cry1Ac toxicity. The toxicity of the fusion protein showed only growth reduction and was not much toxic to Spodoptera litura. The loss in the toxicity of Vip proteins was may be due to limitations posed by in-vitro folding of the toxin, as insufficient folding to the native state leads to the loss of toxicity [23].

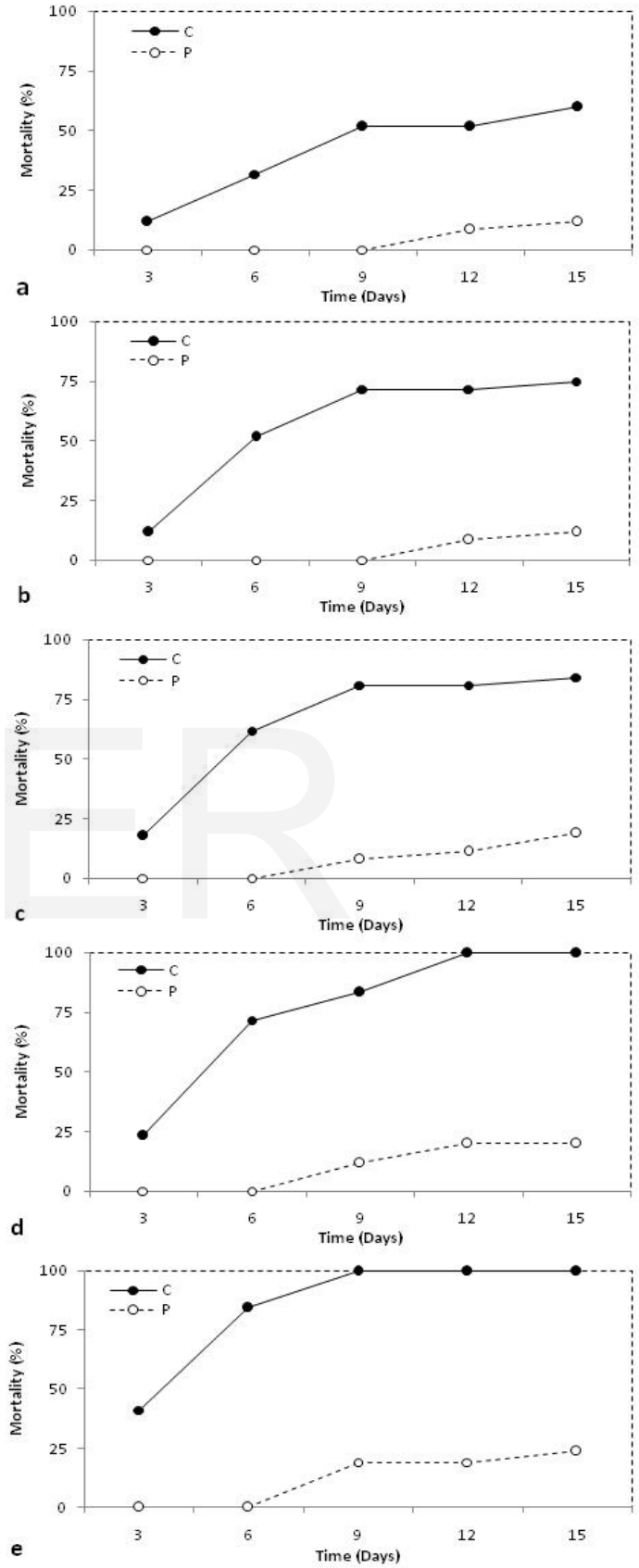

Figure 5. Percentage of cumulative mortality of *Chilo infuscatellus* with respect to the time intervals after their release on artificial diet supplemented with isolated protein in respective

treatment. (a) 3.14 µg/cm2, (b) 6.28 µg/cm2, (c) 9.43 µg/cm2, (d) 12.57 µg/cm2 and (e) 15.72 µg/cm2. (C- pET28a(+):cry1F, P- pET28a(+)).

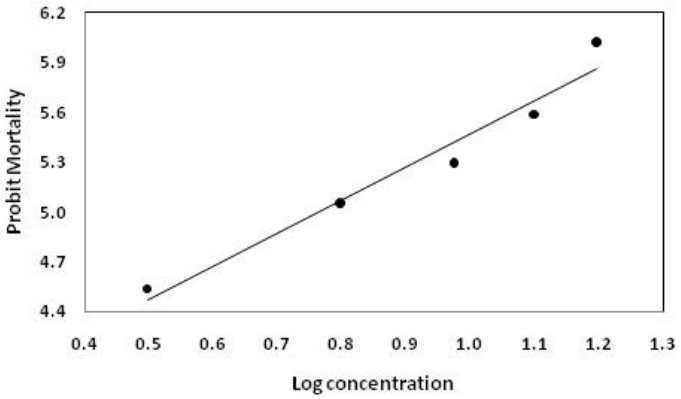

Figure 6. Dosage mortality probit regression line for cry1F against sugarcane early shoot borer Chilo infuscatellus.

The toxicity and larval growth inhibition proteins of Bt were evaluated against neonate larvae of Helicoverpa armigera, a major pest of important crops in Spain, by a whole diet contamination method. The toxin added was in the concentration of 1, 2, 4, 8 and 16µg of toxin/ml of the diet and Cry1Ac4 and Cry2Aa1 were the most active toxins at the concentrations tested. Cry1Ac4, Cry2Aa1, Cry9Ca, Cry1Fa1, Cry1Ab3, Cry2Ab2, Cry1Da and Cry1Ja1 produced a significant growth inhibition, whereas Cry1Aa3, Cry1Ca2, and Cry1Ea had no effect [27].

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