Improvement of sugarcane for borer resistance using Agrobacterium mediated transformation of cry1Ac gene

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ABSTRACT

The sugarcane (Saccharum X officinarum) is one of the most important crops used to produce sugar and raw material for biofuel in the world. One of the main causes for sucrose content and yield losses is the attack by insect. In this investigation, cry1Ac gene was introduced into sugarcane variety GT54-9 (C9) using the Agrobacterium tumefaciens transformation method for transgenic sugarcane production presenting insect-resistance. The A. tumefaciens strain GV1303 including pARTcry1Ac vector was used for the production of transformed sugarcane. The Bacillus thuringiensis cry gene were successfully used to produce transgenic plants used for the improvement of both agronomic efficiency and product quality by acquiring insect resistance. PCR and Southern hybridization techniques were used to confirm the cry1Ac gene incorporation into sugarcane genome. Transformation percentage was 22.2% using PCR analysis with specific primers for cry1Ac and npt-II (Neomycin phosphotransferase) genes. The expression of cry1Ac gene was determined using reverse transcriptase polymerase chain reaction (RT-PCR), QuickStix test, and insect bioassays. Bioassays for transformed sugarcane plants showed high level of toxicity to Sesamia cretica giving 100% mortality of the larvae. Sugarcane insect resistance was improved significantly by using cry1Ac gene transformation.

Introduction

Sugarcane is a paramount sugar plant vastly cultivated in the subtropical and tropical regions. It supplies about eighty percent of the sugar in the world. Moreover, in many countries sugarcane is also considered a main raw material for the production of ethanol. Cultivated sugarcane varieties are hybrids from the cross between Saccharum spontaneum (2 n = 36–128) and Saccharum officinarum (2 n = 20–122) which represent complex aneuploidy. It is an octaploid species have complex genome (x = 10 and 2 n = 80 ~ 270). Insect pests are an essential problem for sugarcane crop all over the world. One of the significant pests of sugarcane is lepidoptera stem borers. The major Lepidopteran insect pests of sugarcane are stem borer (Diatraea saccharalis) in South America, central America, the Caribbean, and the southern United States, root borer (Emmalocera depressalis) in India and Pakistan, sugarcane top borer (Chilo terrenellus) in Bangladesh, Thailand and Australia, pink borer (Sesamia inferens) in ASIA, Cambodia, China, Hong Kong, India, Mexican rice borer (Eoreuma loptini), and pink stem borer Sesamia cretica in Mediterranean basin and extends through the Middle East and Arabia to Pakistan, northern India, and northern Africa extending south to northern Kenya and northern Cameroon. These borers cause yield losses of nearly 25–30%.

To improve economic traits in agriculture many traditional plant breeding techniques can be used but these techniques can be time consuming, especially for genomic complex crop such as sugarcane. Moreover, conventional breeding to develop insect-resistance in sugarcane is limited by the lack of resistance available in the crop germplasm. One of the effective and economic strategies for improvement the resistance of different plants to insects is introduction of insect-resistant genes including Bt genes. Insect resistance could be improve by using genetic engineering approaches and could help in the
development of sugarcane varieties production. *Bacillus thuringiensis* (Bt), is a Gram positive and spore-forming bacteria. During sporulation, it produce a crystalline parasporal body that shows biocidal activity against some invertebrate orders at larval stage including dipteran, *lepidopteran* and *coleopteran* insects. It was first discovered in 1901 by Gill. There are many reports that successfully obtained insect resistant transgenic sugarcane lines through introduced Bt genes. A lot of other crop species has been developed through *cryA(b)* gene introducing; those plants such as rice, cotton, tomato, potato, corn, and sugarcane.

The first research with the aim of introducing Bt gene into sugarcane to produce insect resistance plants used the *cry1Ab* gene. Lately, *cry1Ac* gene was introduced into sugarcane genome by Gao that successfully obtained insect-resistant transgenic events. Wang introduced both the *EPSPS* and *cry1Ab* genes into sugarcane genome and obtained transgenic lines with herbicide tolerance and insect resistance. Recently, many monocotyledonous species used *Agrobacterium* transformation method including rice, maize, wheat, and barley. The transformation by *Agrobacterium* have diverse advantages, including minimal DNA rearrangement in transformants, technical simplicity and the capability to transfer long fragment of DNA. Although the *Agrobacterium* method has been used also in sugarcane, the shortage of a reproducible result has been an obstacle to found effective transformation method for routine genetic manipulation in the crop. The present study aimed to improve the borer resistance in sugarcane plants via introducing the *cry1Ac* gene through *Agrobacterium* transformation.

**Results**

In order to use kanamycin as a selectable marker gene in plant transformation, suitable concentration of kanamycin that inhibits explants growth should be determined. Young leaves segments were placed into media containing different concentrations of kanamycin ranging from 25 up to 150 mg/l, whilst the control was placed on kanamycin free medium. The results indicated that the number of survival explants decreased with the increasing of the kanamycin concentration. The lethal kanamycin dose for the leaf segments was found to be 100 mg/L (Figure 1).

![Figure 1](image-url) The effect of different kanamycin on the survival explants.

**Agrobacterium Transformation**

The sugarcane variety GT54-C9 is very popular among Egyptian farmers due to its high yield and other desirable agronomic. Sugarcane variety GT54-9(C9) were used for *Agrobacterium* transformation. Young leaf explants of sugarcane variety were co-cultivated with *Agrobacterium tumefaciens* GV3101 harboring the binary vector pARTcry1Ac. After three days of co-cultivation, the inoculated explants were transferred to MS regeneration medium including 1 mg/l BAP and 2 mg/l NAA. After 48 hours in the dark at 28 ± 2°C, a sterile solution of strength MS medium with 300 mg/L carbenicillin was used to wash the explants then blotted briefly on sterile filter paper in the laminar flow hood. Explants were transferred to regeneration media supplemented with 100 mg/L kanamycin and 300 mg/L carbinicillin. The cultures were incubated under the regeneration conditions. After 30 days of incubation, shoots were subcultured to fresh regeneration medium with the same antibiotics in the selection plates, and were re-incubated under the same conditions. Young leaves from nontransformed sugarcane were used as control. For inducing roots, regenerated shoots (about 7–10 cm) were transferred to MS medium supplemented with 60 g sucrose and 2 mg/l NAA. Regenerated plants with well-developed roots were transferred to pots containing sand, peat-moss and clay (1:1:1) and kept in a greenhouse under shadow for 15–20 days for acclimatization (Figure 2). For further analyzes, sixteen regenerated transgenic sugarcane lines were used. From a single shoot bud, a transgenic line was develop and grew on MS media containing kanamycin. Based on the morphological parameters and molecular evaluation using ISSR marker, no phenotypic
abnormalities appeared in the putative transgenic plants in comparison to the untransformed control plants (data not shown).

Detection of Transgenic Sugarcane

The transformed kanamycin resistant sugarcane shoots were used for DNA isolation. The polymerase chain reaction (PCR) was used to confirm the cry1Ac gene integration into the genetic material of the putative kanamycin resistant shoots (transgenic) of sugarcane cultivar GT54-9(C9) using nptII and cry1Ac specific primers. The selected primers were designed to amplify fragments of 250 and 497 bp of the nptII and cry1Ac genes, respectively. Out of 90 plants examined from kanamycin resistant tissues only 20 gave positive results (The PCR test showed a clear band corresponding to the relevant sequence of both primers) with a percentage of 22.2%.

Southern blot is a commonly used technique to confirm gene integration and copy numbers in transgenic plants. Southern blotting analysis was used to confirm the integration of the cry1Ac gene in the T1 sugarcane plants. A restriction enzyme, KpnI was used to digest sugarcane genome and then digested DNA was hybridized with cry1Ac specific probe that showed the integration of cry1Ac gene in sugarcane genetic material. Liner pARTcry1Ac plasmid DNA was used as positive control. Southern blotting test for transgenic plants showed bands >3256 bp molecular weight as expected (Figure 3).

Expression of cry1Ac Gene in Transgenic Sugarcane Plants

The stable expression of the cry1Ac gene in the transgenic sugarcane lines was confirmed by using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extract from PCR-positive putative transgenic lines and also from the nontransgenic plants (as negative control). Extracted RNA was used as a template in RT-PCR for synthesizing the cDNA followed by amplification of the cry1Ac gene with cry1Ac specific primer (Table 1). The results showed that a RT-PCR fragment with a molecular size of about 497 bp was amplified from total RNA

Table 1. The primer sequences of transgenes used for confirmation of T-DNA integration in putative transformed plantlets and RT-PCR.

| Genes | Sequences 5' - 3' | Expected Size |
|-------|------------------|---------------|
| nptII | F: CGCAGGTTGTTCGCCGGCTTGG | 250 bp |
|      | R: GACCTCGCCGTCCTGAACGCCGACGA |
| cry1Ac| F: GCATCTCCGGCCGCTCCCAGTC | 497 bp |
|      | R: ACGCGGCTCCAGGCCGGGTGTTGA |

Figure 2. The regeneration stages of transformed sugarcane plants via direct organogenesis. (1) Leaf young explant taken from 6- to 8-month-old sugarcane (2, 3, and 4) stages of young leaf explant. (5) Shoots formation. (6) Root formation. (7 and 8) Acclimatization of transformed sugarcane plants.

Figure 3. Southern blot hybridization of genomic DNA isolated from transgenic sugarcane plants, transformed by the cry1Ac gene. P: the liner pARTcry1Ac plasmid (positive control) and lanes 1–8 are the cry1Ac transgenic sugarcane plants.
isolated from transformed plants (Figure 4). The RT-PCR analysis for the sugarcane plants showed the occurrence of the mRNA for the cry1Ac gene in 6 out of 8 (75%) PCR-positive plants for sugarcane.

Survey with Trip Tests for the Cry1Ac Protein

The QuickStix test was done with Cry1Ac to detect the expression of the Cry1Ac protein in eight transgenic sugarcane lines. In the strip containing lines 6 and 8, only the assay band was observed. This indicated the absence of Cry proteins and presence of analyze. It also indicates that two spurious bands are not formed due to analyze. In samples containing the cry1Ac (lines 1, 2, 3, 4, 5, and 7) both assay band and expression band were detected as shown in Figure (5). This indicated that those lines containing the Cry1Ac protein.

Bioassay

It is important to evaluate potential insect-resistant transgenic plants for insect resistance against target pest(s) at field conditions and conducting insect laboratory bioassays. Figure (6) shows the mortality percentage of Sesamia cincta caused by transgenic plants expressing cry1Ac toxin. The results indicated that the lethal concentration 50 (LC50) value for cry1Ac toxin protein from transformed sugarcane plants were 500 ppm (line 12, 15) and 300 ppm (line 5, 8, 14, 16) against the Sesamia cincta in 6 transformed plants. The mortality percentage of cry1Ac toxin expressed in all transgenic plants against Sesamia cincta were 100% with 1000 ppm compared to the negative control (Table 2). This data indicated the high expression of cry1Ac gene in the transformed sugarcane plant. The transgenic plants showed higher resistant to the target pests. However, the line 8 showed the highest toxicity to the larvae at lower concentrations followed by line 16. Therefore, these two lines are recommended for

![Figure 4](image1.png) **Figure 4.** RT-PCR isolation of the cry1Ac gene of transformed sugarcane. M: 100 bp DNA ladder marker. Lanes (1, 2, 3, 4, 5, and 7) represent putative transformed Sugarcane plants, Lane (-) (nontransformed sugarcane) negative control, Lane (+) (pARTcry1Ac vector) positive control.

![Figure 5](image2.png) **Figure 5.** Cry1Ac protein expression in transgenic sugarcane on QuickStix Combo strips. Lines 6 and 8 showed negative results (one band). Lines 1, 2, 3, 4, 5, and 7 showed positive results (two bands).

![Figure 6](image3.png) **Figure 6.** Determine the mortality percentage of Cry1Ac toxin protein against Sesamia cincta. (a) Mortality reached 100% at 1000 ppm of dried transformed sugarcane leaves compared to the (b) control.

| Selected sugarcane lines | 1000 | 700  | 500  | 300  | 200  | 50 (LC50) |
|--------------------------|------|------|------|------|------|-----------|
| Nontransformed           | 0%   | 0%   | 0%   | 0%   | 0%   | 0%        |
| 5                        | 100% | 80%  | 60%  | 50%  | 40%  | 300 ppm   |
| 8                        | 100% | 100% | 80%  | 60%  | 40%  | 300 ppm   |
| 12                       | 100% | 70%  | 50%  | 40%  | 20%  | 500 ppm   |
| 14                       | 100% | 70%  | 60%  | 50%  | 30%  | 300 ppm   |
| 15                       | 100% | 80%  | 60%  | 40%  | 30%  | 500 ppm   |
| 16                       | 100% | 90%  | 70%  | 50%  | 20%  | 300 ppm   |

Table 2. Mortality percentages of Sesamia cincta fed on nontransgenic (control) and six transformed sugarcane of expressing the cry1AC gene.
using to further experiments to control the steam borer _Sesamia reticulata_.

**Discussion**

Transgenic plants expressing _cry_ genes from _Bacillus thuringiensis_ could drastically minimize the use of broad-spectrum insecticides against insect pests. Endotoxins produced by _Bt_ strains have insecticide effect for some of the main pests of important crop plants.\(^28\) Genetically modified plants expressing _Bt_ genes are more effective in controlling to pests than _Bt_ formulations. In 1995, the Environmental Protection Agency (EPA) in the United States approved the commercial production and distribution of the _Bt_ crops: corn, cotton, potato, and tobacco.\(^29\) Recently, _Bt_ soybean varieties expressing the _cry1Ac_ gene has been approved for commercial use in Latin America\(^30\) and _Bt_ sugarcane CTC175 has been approved for commercial use in Brazil.\(^31\) The use of synthetic insecticides was significantly reduced by using commercialization _Bt_ crops.\(^32\) A lot numbers of _cry_ genes have been tested and described against main insect pests.\(^33\) Many research suggested that the toxicity of _cry_ toxins was due to either their pore formation ability\(^34\) or the signal transduction pathway by receptor binding.\(^35\) The mode of action model of _Bt_ genes suggest that _cry_ toxins pass through a successive binding mechanism with several insect gut proteins leading to membrane insertion, pore formation, and toxin oligomerization.\(^36\) The _cry_ toxin produced as protoxin in _Bt_ bacterial cells. A high yield of an active three-domain toxin of _Bt_ _cry_ toxins can be produced by insect gut proteases enzymes. Both _cry1A_ protoxin and activated toxin binds to cadherin receptor forming distinct oligomers that insert into the membrane forming lytic pores. The outcome of this double mode of action is the decreasing of possibility evolution of resistance and possibly to expand the spectrum of insect targets. For the continues use of _Bt_ crops, it is probable that obtaining a stable expression of _cry_ full length proteins will have the same outcome, delaying resistance and protection from a broad number of insect pests.\(^37\) The first reported of the _cry1Ac_ gene expression using _Agrobacterium_ transformation method was in cotton for insect resistant to bollworm (_Helicoverpa armigera_).\(^37\) In sugarcane, _Agrobacterium_ mediated genetic transformation is considered to be reliable method and more efficient than direct biolistic gene transfer method.\(^38\) _Agrobacterium_ mediated genetic transformation has traditionally been the preferred method to generate events with low transgene copy number. Standard biolistic gene transfer method, in which large quantities of whole plasmid constructs are introduced, typically result in the integration of multiple transgene copies as well as vector backbone sequences into the plant genome.\(^39\) In this investigation, sugarcane variety GT54-9(C9) was transformed with _A. tumefaciens_ GV3101 that have pARTcry1Ac binary vector. The target gene integration and expression in the plant genomic DNA are important reasons for success of transformation as well as its inheritance in progeny plants. The stable integration of the _cry1Ac_ gene into sugarcane DNA was assured by using the PCR and Southern blot analysis. The results showed that only twenty plants from ninety gave specific bands (250, 497 bp) corresponding to both _nptII_ and _cry1Ac_ specific primers, repressively about 22.2% were transformed sugarcane plants. The copy number determination of transgenes in transgenic plants is important due to the effect of the copy number on the gene expression level and genetic stability. Southern blot analysis is considered the traditional method to assess copy number of exogenous genes in transgenic plants. In this study Southern results showed the integration of _cry1Ac_ gene in the sugarcane genetic material. However, the gene copy number was integrated in one to three position in sugarcane plants that were transformed using _Agrobacterium_ method.\(^40,41\) The _cry1Ac_ toxin demonstrated to be expressed by transgenic sugarcane plant and it remains biologically active when absorb by the target insects. Quickstix was used to quantification determination of _cry_ proteins. RT-PCR analysis was used to confirm the expression of the _cry1Ac_ gene. RT-PCR technique can be used to determine the presence or absence of specific transcript and the steady-state RNA levels. Falco and Silva-Filho\(^42\) used RT-PCR in transformed sugarcane plants to reveal the expression of _cry1Ac_ gene and detected that all plants expressed mRNA of the transgene.

Clear effects of _cry1Ac_ expression were tested by the death-rate of _Sesamia reticulata_ when it was fed on transgenic _Bt_ sugarcane. These results showed that a large amount of _Bt_ protein was found in all sugarcane transgenic lines and that for target lepidopteran insect pests management the plants...
expressing cry1Ac gene could be used. The mortality percentage of cry1Ac toxin expressed in all transgenic plants against Sesamia cretica were 100% with 1000 ppm compared to the negative control. This data indicated the high expression of cry1Ac gene in the transformed sugarcane plant. The results indicated that sugarcane line 8 showed the highest toxicity to the larvae at lower concentrations followed by line 16 this may be due to the integration of only one cope number of the gene due to the result of Southern blot analysis. Many earlier researchers found that the multiple T-DNA insertions had exhibited less expression levels than single copy transgenes. They also are similar to Lin et al, they found that bioassays with cry1Ac transformed transgenic tobacco plants showed high level of toxicity toward (Spodoptera littura) giving rate of 76.9 to 100% mortality of the larvae after 72 hr. Earlier, sugarcane cultivars, CoJ 64 and Co 86032 was modified by the cry1Ab gene. The percentage of cry1Ab protein in several transgenic lines ranged from 0.007% to 1.73% for total soluble protein in leaves. At the seedling stage, transgenic plants had significantly less dead rate and there was a negative relation between the dead rate and protein expression. Weng et al. found that only resistance was shown in transgenic sugarcane lines expressing the cry1Ac protein more than 9 ng/mg when they analyzed pest resistance of cry1Ac. A positive correlation between pest resistance and the cry1Ac content was also detected.

**Conclusion**

The transgenic sugarcane with cry1Ac gene that has been inserted into sugarcane genetic material by Agrobacterium transformation method, showed resistant to insects and high productivity. From different molecular analyzes confirmed that crystal protein gene is stable integrated into transgenic sugarcane genome.

**Materials and Methods**

**Plant Material**

Sugarcane variety GT54-9(C9) was obtained from Sugar Crops Research Institute, Agricultural Research Center, Giza, Egypt. Young leaf explants (apical part of the shoot) 3 cm in length of several layers of leaves taken from 6- to 8-month-old sugarcane (Saccharum officinarum cv. GT54-C9). The outer leaves were removed to expose the six inner leaves. innermost six leaves were removed in 70% ethanol for one min, disinfected in 40% clorox solution for 20 minutes 40% (v/v), then washed four times in sterile distilled water followed by removing the outer 6th and 5th leaves. Eight cm segments from the bases of the innermost three or four leaves were cut into small transverse sections (2–3 mm) and used as explants.

**Bacterial Strains and Vector**

Agrobacterium tumefaciens GV1303 strain containing pARTcry1Ac plasmid was used to transform sugarcane explants for produce insect resistance plants. The plasmid was constructed by Prof. Dr. Naglaa Abdallah from cloning the synthetic cry1Ac gene accession number AF023672.1 that was kindly provided by Dr. Pamela Green (it was modified for plants to achieve higher expression) into the pART27 binary vector and under the control of 35S promoter and was used for transforming sugarcane explants using Agrobacterium method (Figure 7).

**Transformation and Regeneration Conditions**

Young leaf explants were soaked in Agrobacterium solution for fifteen min, left to dry on a sterilized filter paper, and then co-cultivated on the shooting formation media (MS including 2 mg/l NAA and 1 mg/l BAP) for three days. After co-cultivation, the

![Figure 7. The map of pARTcry1Ac vector.](image)
explants were transferred to the same medium supplemented with 300 mg/l carbinicillin to inhibit Agrobacterium growth in addition to 100 mg/l kanamycin for transgenic shoots selection. Developed shoots were re-cultured on the optimized elongation medium (MS supplemented 0.1 mg/l BAP and 2 mg/l Kin) to reach suitable length. The cultures were kept in the growth chamber at 28 ± 2°C under 16 hours photoperiod of 3000 Lux provided with white cool fluorescent lamps. Then the shoots were transferred to appropriate rooting media (MS including 2 mg/l NAA) and plantlets were acclimatized in the greenhouse.47

Survival Curve of Kanamycin

To set the minimal lethal dose of kanamycin, different concentrations 0, 25, 50, 75, 100, 125, 150 mg/l kanamycin was added to MS medium and 90 explants were used for each concentration. Kanamycin was sterilized using disposable filters (0.22 µm) and mixed with precooled (45–50°C) autoclaved MS medium. The percentages of explants survival (kanamycin resistant) were recorded after 21 days from culturing.

PCR Conformation

The genetic material of the putative transgenic tissues were extracted via CTAB method according to Lassner.48 Two specific oligonucleotides primers for cry1Ac and npt-II genes were used to confirm the stable integration of the T-DNA into sugarcane genome in PCR reaction (Table 1). The DNA Synthesizer 392, Applied Biosystems at AGERI, ARC, Giza, Egypt was used for primers manufacturing. The PCR reaction was prepared in a 50 µl consisting of a final concentration of each of the following: 200 µM of each of dNTPs (dGTP, dCTP, dATP, and dTTP), 1 pmole from each of the used primers, 1X PCR buffer, 0.04 U Taq DNA polymerase, 2 ng of plant DNA (as template), 2.5 mM MgCl2 and dH2O. Amplification cycle program of the synthetic Bt gene was performed as following: 95°C for 5 min, followed by 35 cycles at 94°C for 60 sec, 55°C for 60 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min. 1.5% agarose gel electrophoresis was used for PCR analysis by loading 15 µl of PCR product with 3 µl loading buffer

Southern Blotting Analysis

Total DNA was isolated from transformed sugarcane as described previously by Lassner48 method. 10 µg of DNA was digested using restriction enzyme, separated by electrophoresis (using 1% agarose) and transferred to Hybond NC nylon membrane (Amersham, RPN119B, Netherlands) as described by Sambrook.49 Prehybridization and hybridization conditions were in strict accordance with the manufacturer’s recommendations. PCR produced from cry1Ac gene (497 bp) was used as a probes. Biotin Chromogenic Detection kits (#K0661 & #K0662, Ferments Life Sciences, USA) was used for hybridization and detection according to instructions provided by the manufacturer.

Reverse Transcription PCR (RT-PCR) Reaction

Total RNA was extracted from PCR positive plants using SV Total RNA Isolation System (Promega, cat. #Z3100, USA). RT-PCR analysis was performed using Robust I RT-PCR kit, Finnzymes, Finland. The analysis were carried out on both putative transformed (PCR positive) and nontransformed plants using the cry1Ac gene specific primers (Table 1) and the PCR products were separated in 1.5% agarose gels.

Survey with Trip Tests for the Cry1Ac Protein

QuickStix Combo Kit for Cry1A and Cry2A (cat. #AS 012 LS, EnviroLogix, Portland, Oregon, USA) was used to detect the presence of cry1Ac protein in transgenic sugarcane leaves. The samples and protein extract were performed according the manufacturer’s instructions.

Two leaf punches were taken from each plant by snapping cap of eppendorf tube and were grounded by rotating pestle until fine grinding. Extraction buffer (0.5 ml) was added to the tube and mixed with the leaf tissue. QuickStix strips were dipped in the leaf and examined after 10 min for the appearance of the final bands on strip and results were recorded.

Bioassay

Sesamia cretica larvae’s obtained from a laboratory culture were reared on an artificial medium diet as method described by Dulmage.50 Transformed
sugarcane plants with positive PCR results for **nptII** and **cry1Ac** were subjected for bioassay test. One gram of dried grounded sugarcane leaves was suspended in 100 ml water to give 1000 ppm and further diluted to prepare different concentrations of 1000, 700, 500, 300, and 200 ppm. A volume of 500 µl of each dilution was added onto the surface of each cup containing artificial media and three replicates from each dilution were prepared. After the toxin was completely dried and the surface become dry, 10 neonate larvae were placed on the media surface and were monitored for 96 hrs. The cups were covered with aluminum foil and kept at 26°C (± 2°C). The leaves of nontransformed sugarcane plant with 500 µl water were used as negative control. The mortality was recorded every 24 hours for four days.

**Statistical Analysis**

For statistical analysis, ANOVA program was used for variance analysis of data. For all treatments, significance at 5% level was used to test differences among means by using Duncan’s new multiple range test as described by Snedecor and Cochran. Means followed by the same letter are not significantly different at *p* ≤ 0.05.

**Disclosure Statement**

The authors declare that they have no conflict of interest.

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