EXPRESSION OF THE INSECTICIDAL PROTEIN GENE *CRY*1C OF *BACILLUS THURINGIENSIS* IN THE PLANT-COLONIZING NITROGEN FIXING BACTERIA

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Abstract

The NMO10 bacterial isolate was isolated from the rhizosphere of cotton plants. This isolate showed high potentiality to fix nitrogen and high ability to colonize the phyllosphere of cotton plants. Based on this criterion, the plasmid pHTNC3 harboring *cry*1C gene (Nahed, 2001) was used to transform the NMO10. The transformed bacterial isolate (tNMO10) proved dual action both as bio-control of the Lepidopteron insect *Spodoptera littoralis* and as bio-fertilizer. The presence of expressed 135 kDa protein of the CryIC in the protein pattern of the tNMO10, the microscopic examination of the bipyramidal crystals that characterized the CryIC protein and the immuonoblot analysis indicated efficient expression of *cry*1C gene in the heterologous host NMO10. Bioassays against the neonate larvae of the cotton leaf worm *S. littoralis* revealed that the protein preparations from the tNMO10 were toxic.

INTRODUCTION

The most abundant and successful microorganism used as biopesticide is *bacillus thuringiensis* (*Bt*), a gram-positive spore-forming bacterium that is characterized for containing a variety of plasmids encoding insecticidal proteins named delta-endotoxins (Hofte and Whiteley, 1989). These proteins are assembled into parasporal crystalline inclusion bodies. Commercial preparations of *Bt* have been used for many years in insect control programmes and shown no toxicity against non-targeted organisms. *Bt* was available only to control Lepidopterous insects, using a highly potent strain (*Bt* var *Kurstaki*). It still constitutes the basis of many *Bt* formulations. The efficient use of these preparations is hindered by several of field conditions (Cohen, 1991), photo inactivation by uv-light, temperature, dew or rain are the major environmental factors affecting stability and efficacy of entomopathogenic toxins (McGuire and Shassha, 1990). Reaching root and stem dwelling insect is another problem with conventional *Bt* biopesticide.

One approach to overcome these problems, is to introduce the *cry* gene into the plant chromosome where the plant cell expresses the Cry proteins. Although the success of this approach, it has certain restrictions. One of it is the target insects are perpetually exposed to toxins and this creates a strong selection pressure for the development of toxin resistance.

A second approach relies on the expression of Cry proteins in different genera of microorganisms, which naturally colonize plants and express Cry proteins at sufficient levels to protect the plant. The introduction of *cry* genes into plant-associated microorganisms has been successfully developed, as this is much quicker and more cost-effective than producing transgenic plants (Bora *et al*, 1994). Moreover, the use of an endophytic bacterium was seen as possible solution to the problem of inaccessibility of conventional *Bt* based products to the interior regions of plant.

In the present study, attempts were made to develop a new delivery system for the insecticidal protein of *Bt* by transferring the *cry*1C gene into the nitrogen fixing bacterial isolate NMO10 that has been isolated from the rhizosphere of cotton (Feibo and Omar, 1998). This soil bacterium, a gram-positive, endospore-forming diazotrophic organism found in association with plant, is capable of atmospheric di-nitrogen reduction. The effect of this bacteria ranging from increased shoot/root ratio, increased seedling emergence to higher yield. NMO10 shown to be a good colonized of the phylloplane of cotton. Thus obtaining of a strong and a potent bioinsecticide, as well as, biofertilizer bacteria was an aim in this work.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

An Egyptian isolate NMO10 was isolated from cotton rhizosphere (Fiebo and Omar, 1998) and selected for molecular study on the basis of its nitrogen fixing activity. The bacterial strain *Bt* subsp *entomocidus* was used as a positive control for Cry1C crystal protein, which is naturally producing only Cry1C toxin protein (Kalman *et al.*,1995). The recombinant strain *Bt*NC3 (Nahed, 2001) was used as a source for the plasmid pHTNC3. All bacterial strains used were grown on T3 media (tryptone 3 gm, tryptose 2 gm, yeast extract 1.5 gm, sodium phosphate buffer 50 mM, Mn Cl_2 0.005 gm, in I L dH_2O) at 30°C for 72 h for spors; and they were grown on LB media (Trypton 10 gm, yeast 5 gm, sodium chloride 5 gm) at 30°C over night (O/N) for vegetative

cells. The plasmid pHTNC3 was isolated from bacterial strain *Bt*NC3 by alkaline lysis method (Sambrook *et al.*, 1989).

Transformation

NMO10 bacterial cells were transformed by electroporation as described by (Chang *et al.*, 1992). 500 ng of the plasmid DNA, pHTNC3 that derived from the recombinant strain *Bt*NC3 were added to 0.8 ml of NMO10 competent cells and placed in sterile pre chilled electroporation cuvettes (0.4 cm interelectrode gap) and hold on ice for 5 min. Electroporation was carried out with a Bio-rad Gene pulser at a field strength 2.5 KV, resistance 150 Ω (Ohms) and capacitance 25 µf. The electroporated cells were added to 1.5 ml of LB medium, incubated for 1 h at 37°C, plated on LB medium containing 100 µg/ml kanamycin and then incubated at 30°C for 24 to 48 h. One of the Kanamycin resistant (Kan^r) transformants was analyzed for the presence of the pHTNC3 plasmid.

Expression of cry 1C Toxin gene in transformed NMO10 cells

Total cellular proteins of sporulated bacterial cells from the transformed NMO10 (tNMO10) and the parent bacterial isolate were prepared. Those bacterial cells were grown on T3 medium for 72 h in incubator shaker at 30°C. SDS-PAGE was carried out as described by (Laemmli, 1970). The transformed cells were collected by centrifugation and treated with sample buffer composed of (50 mM Tris-HCl (pH 6.8), 2% (W/V) SDS, 2% (V/V) 2-mercaptoethanol, 10% (v/v) glycerol and 0.0025% (w/v) bromophenol blue) and boiled at 100 °C for 5 min. Samples were applied to a 10% polyacrylamid gels and run at 200 v for 45 min at room temperature in mini protein Biorad cell. Protein bands on gels were visualized with coomassie brilliant blue R-250.

Western Blot

The presence of Cry1C delta-endotoxin was detected in crude extracts of transformed cells by a western blot (immunoblot) analysis (Lampel *et al.*, 1994). Total cellular proteins were prepared and solubilized by boiling in sample buffer and separated by electrophoresis on 10% polyacrylamid gels. The gels were electrophoretically blotted onto pre wet PVDF membrane. The membranes were blocked in blocking buffer containing 1% bovine serum albumin (BSA), then membranes were incubated in blocking buffer contained the toxin (Cry1C) for 2 h. Anti-truncated 65 k Da from *Bt kur-* HD-1 serum (1:1000 dilution) was used as primary antibody and was incubated with membranes in the blocking buffer O/N at 4°C. The membranes were incubated with alkaline phosphates conjugated secondary antibody (1:1000 dilution). CDP-chemiluminescent substrate was used and the emitted light was captured on X-ray film.

Microscopic Examination

Microscopic examination of different transformed (tNMO10) bacterial cells compared to the parent isolate (NMO10) was carried out for the presence of bipyramidal crystals that characterized the presence of Cry1C toxin protein. A smear of sporulated culture, that were grown in T3 media on shaking incubator for 72 h at 30°C, was taken, spread on clean slide and stained with spore-stain solutions. Slide was stained first with malachite green for 20 min over a boiling water bath, and then the slide was rinsed with water, dried and stained with safranine for 5 min.

Polymerase Chain Reaction(PCR) and Oligonucleotide primers

Two pairs of specific primers to *cry*1C gene were used to testing the presence of *cry*1C in the transformed bacterial cells. The two primers IAF and IAR (Ragev *et al.* 1996). IAF, 5' ACGGAGGATCCATATGGAGGAAAATAATCAAAATC3' and IAR, 5' CTCTTGGATCCTAACGGGTATAAGCTTTTAATTTC3', that give 2.2 kb PCR product. The reaction conditions were performed according (Regev *et al.*, 1996), where the PCR mixture was in a total volume of 25 µl contained 1 µg of total DNA, 50 pmol of each primer, 0.2 mM deoxy nucleoside triphosphates, 2.5 µl of the Taq polymerase enzyme, 2.5 µl of 10 X enzyme buffer and 2.5 µl $MgCl_2$. The amplification reaction was carried out using 35 cycles of 94 °C (45 sec), 48 °C (45 sec) and 72 °C (120 sec) and then a 7-min termination at 72 °C. The same reaction conditions were used with the second pair of primers IAF and ICR, 5'TTATTCCTCCATAAGGAGTAATTCC3' (Nahed, 2001), that give 3.7 k bp PCR product. The *gln* B specific pair of primers that define the nitrogen regulatory gene were used, to detect that gene in both the NMO10 isolate and the transformed tNMO10. *Gln* B up 5'GCCATCATTAAGCCGTTCAA3' and *gln* B do 5'AAGATCTTGCCGTCGCCGAT. The reaction conditions were as described by (Potrich *et al.*, 2001), 250 bp PCR products are amplified by this pair of primers.

Bioassay

Bacterial isolates were grown until sporulation in liquid T3 media for 72 h. Cultures were centrifuged and the pellets were washed once with Tris-HCl pH 8.00 containing 1 M NaCl, and lyophilized. The dried cells were used directly for bioassay. A stock concentration of 1000 ppm was made by dissolving 1 gm of lyophilized cells in 1000 ml H_2O (Dulmage, 1971). 500 ppm, 400 ppm, 250 ppm, 100 ppm, 75 ppm, 50 ppm, 25 ppm, and 10 ppm different concentrations were added to the surface of solidified artificial medium (dry powdered Lima beans 150 gm, dry yeast 15 gm, Ascorbic acid 3 gm, Nipagin 3gm, agar-agar 6 gm and 600 ml dd H_2O) (Loutfy 1973)

and kept for 2 h at room temperature. 10 neonate larvae of *Spodoptera littoralis* were added to each cup, the mortality was recorded every 24 h until 72 h.

RESULTS

The aim of this study was to introduce of the *Bt* toxin gene (*cry*1C) into a plant colonizing bacteria to protect the toxin protein from the environmental factors that affect its activities and also to prolong its efficiency.

Electroporation

The shuttle vector pHT7593 harboring a copy of *cry*1C gene, the entire *cry*1C gene represented in a 4.1 kb *Hind* III DNA fragment was cloned into the *Hind* III sites of the plasmid pHT7593 (Nahed, 2001), was used to transform the bacterial isolate NMO10. The transformed bacterial cells resulting from electroporation were selected primarily according to their growth on 100 µg/ ml kanamycin plates.

Expression of the cry 1C gene in NMO10 bacterial isolate

SDS-PAGE analysis of protein from sporulated cultures from transformed NMO10 (tNMO10), the parent isolate NMO10, and from recombinant bacterial cells containing the *cry*1C as a positive control, revealed the presence of 135 kDa protein in both the tNMO10 and the positive control but it was absent in the parent isolate NMO10. The 135 kDa protein produced by tNMO10 and the positive control, recombinant bacteria was confirmed as the Cry1C protein by the immunoblot analysis as shown in (Fig 1 A&B) Antibody-antigen immuno reaction was detected between anti-60 k Da toxin antiserum and antigen expressed by the tNMO10. Reactions also were done with the parent isolate. The data showed that the recombinant proteins from tNMO10 and from the recombinant bacteria containing Cry1C toxin protein, gave sharp bands where the homologous antiserum reacted strongly to its respective homologous toxin protein.

PCR The presence of the target gene (*cry*1C gene) in the transformed tNMO10 bacterial strain was confirmed via PCR. Two pair of primers IAF & IAR and IAF & ICR specific for *cry*1C gene, were used to amplify 2.2 kb and 3.7 kb fragments respectively (Fig 2 A). On the other hand a specific pair of primers for detection of *gln* B gene (the nitrogen regulatory gene) that amplify 250 bp fragments from *gln* gene was used with the parent isolate NMO10. The expected PCR product that appeared in the (Fig 2 B) indicated the presence of *gln* gene in that isolate. This test was used to show that the parent isolate is nitrogen-fixing bacteria.

Light microscopy

Microscopic examination of spore-crystal stained smears from transformed bacteria tNMO10 and from the parent isolate NMO10 were illustrated. Fig (3 A) showed the spores from sporulating cells of the isolate NMO10, and (Fig 3 B) showed the bipyramidal crystal in the transformed bacteria tNMO10. The data revealed and confirmed the expression of *cry1C* in the transformed bacterial isolate.

Insecticidal activity

Bioassays against neonatal larvae were performed. In the toxicity assays, the insecticidal activity of Cry1C proteins synthesized by the transformed bacteria tNMO10 was compared with that of the crystal protein Cry1C isolated from the native *entomocidus* strain. Table (1) showed that the recombinant toxin, displayed a low LC_{50}, it was nearly half of the LC_{50} of *Bt entomocidus*. The parent isolate NMO10 showed no mortality at all and it was like negative control, where no toxins were added. Using the propet analysis the data were analyzed in (table 1). Fig (4 B) showed how the parent isolate had no effect on larvae while the transformed tNMO10 had 100% mortality at 100 ppm (Fig 4 C).

DISCUSSION

In recent years, the need for environmentally safe pesticides has encouraged the replacement of these chemicals with biological approaches, which are friendlier to the environment. Strategies are being developed to control a variety of phytopathogenic agents including the development of transgenic plants expressing delta-endotoxin of *Bt*. This has led to the production of insect resistant *Bt*-transformed lines of tobacco, cotton, corn, potatoes, maize, tomatoes and others (Frutos, *et al.*, 1999). *Cry* genes have been transferred into other plant associated microorganisms to improve stability and efficacy obtaining maximum insect control on aerial and subterranean surfaces of plants (Downing, *et al.*, 2000). Thanabalu, *et al.* (1992) tried *Caulobacter crescentus* as an ideal carriers for biological toxins instead of *Bt*. Watrud *et al.* (1983), also tried to clone *Bt* delta-endotoxin gene into *Pseudomonas fluoprescens*, that colonize roots to protect toxin from environmental factors that affecting its efficiency. Manasherob *et al.*, (2002), were expressed *Bt* toxin in the nitrogen fixing bacteria *Anabaena*, to protect toxin from damage by UV-B, sunlight component. Also (Bainton *et al.*, 2004) had modified a strain of *Pseudomonas* with dual biocontrol mechanisms. Theoduloz *et al.*, (2003) had expressed *Bt* toxin in *B. subtilis* and

B.licheniformis that naturally colonize phylloplane of tomato. These all attempts and the progress in this trend lead us to say, microorganisms that naturally colonize the plant phylloplane could be a source of new microbial expression systems of a delta-endotoxin gene of *Bt*. Furthermore, we have presented that delivery of toxin genes of *Bt* to other microorganisms seems to be a practical and quicker alternative instead of the production of transgenic plants. In our study insertion of plasmid *Bt*NC3 that harboring the *cry*1C toxin gene into the plant colonizing bacteria NMO10 was accomplished by electroporation. Polymerase chain reaction (PCR) was used to detect the presence of *cry*1C gene in the transformed strain tNMO10. (Fig 2 A). PCR also was used to study the presence of *gln* B gene, the nitrogen regulatory gene, in the parent isolates NMO10 and data in (Fig 2 B) showed that NMO10 contained *gln* B gene. This was a confirmation, on the molecular level, that our isolate NMO10 is a nitrogen-fixing bacterium. The expression of the cloned *cry*1C gene was examined and analyzed on SDS-PAGE, and the results showed that the cloned *cry*1C gene was highly expressed in the isolate NMO10. Immunoblot analysis revealed the success of the reaction of antiserum with the 135 kDa from the tNMO10, saying clearly that the Cry1C is present and well expressed in the transformed strain (Fig 2 B). The microscopic examination of sporulating cells of tNMO10 showed the bipyramidal crystals (Fig 3 B) emphasized the high expression of Cry1C toxin protein in the heterologous bacterial isolates tNMO10. Expression of the *cry*1C gene and its toxicity effect were examined by determining the activity of the tNMO10 strain against the cotton leaf worm *S. littoralis* in an artificial diet bioassay. The activity was measured by mortality of the *S. littoralis* larvae compared with activity of the *Bt entomocidus* strain that is a native donor of *cry*1C, the data in (table 1) and (Fig 4) showed that the activity of tNMO10 against *S. littoralis* larvae was two times higher than that of the native strain and the LC_{50} of tNMO10 was half of that of *entomocidus*. Moreover, the toxin proteins of the tNMO10 showed quick mortality while we were reading the experiment every 24 h. On the other hand the toxin proteins from *entomocidus* were slow in its effect on the larvae however, after three days (72 h), its real effect appeared. This activity was very promising that our strain tNMO10 is strong bioinsecticidal agent. In conclusion, the cloned *cry*1C gene in NMO10 bacteria synthesized a polypeptide of 135 kDa that cross-reacted with anti-truncated 65 kDa from *Bt* HD-1 serum. The transformed tNMO10 showed higher toxicity to larvae of *S. littoralis* than that of *Bt entomocidus* strain that contain native Cry1C. Thus our results revealed that we have a strong biopesticide strain as well as a biofertilizer one

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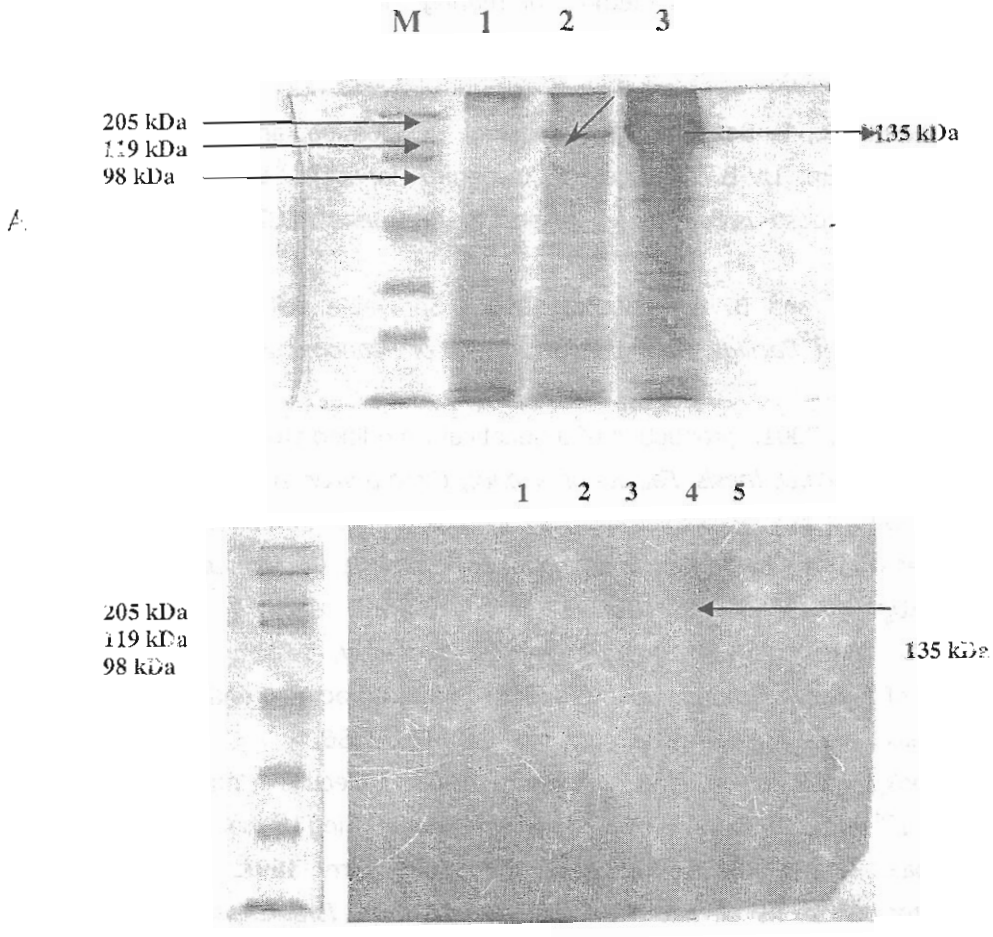


Fig 1.. SDS-PAGE and Immunodetection of crystal proteins in transformants obtained from the tNMO10 compared to the parent isolate NMO10 and the positive control Cry1C.

A: Samples of 25 µl of total cellular proteins were run on 10 % polyacrylamide gels. The gels were stained with coomassie blue. Lane M: Pre stained broad range SDS-PAGE standards. Lane 1: the parent isolate NMO10, Lane 2: the tNMO10, (the arrow refer to the 135 kDa of Cry1C that expressed in tNMO10) Lane 3: the positive control, recombinant bacteria containing Cry1C.

B: Western blot analysis of the proteins synthesized by the transformed bacteria tNMO10, recombinant bacteria containing *cry*1C as positive control and the parent isolate NMO10 with polyclonal antibodies for the *B. thuringiensis* crystal protein after SDS-PAGE. Lanes 1&3: tNMO10. Lanes 2&4: parent isolate NMO10 where no reaction. Lane 5: recombinant bacteria containing Cry1C as positive control. The

protein marker that found on the left had been cut from the gel before transfer on the membrane and was stained alone like the gels of the SDS-PAGE

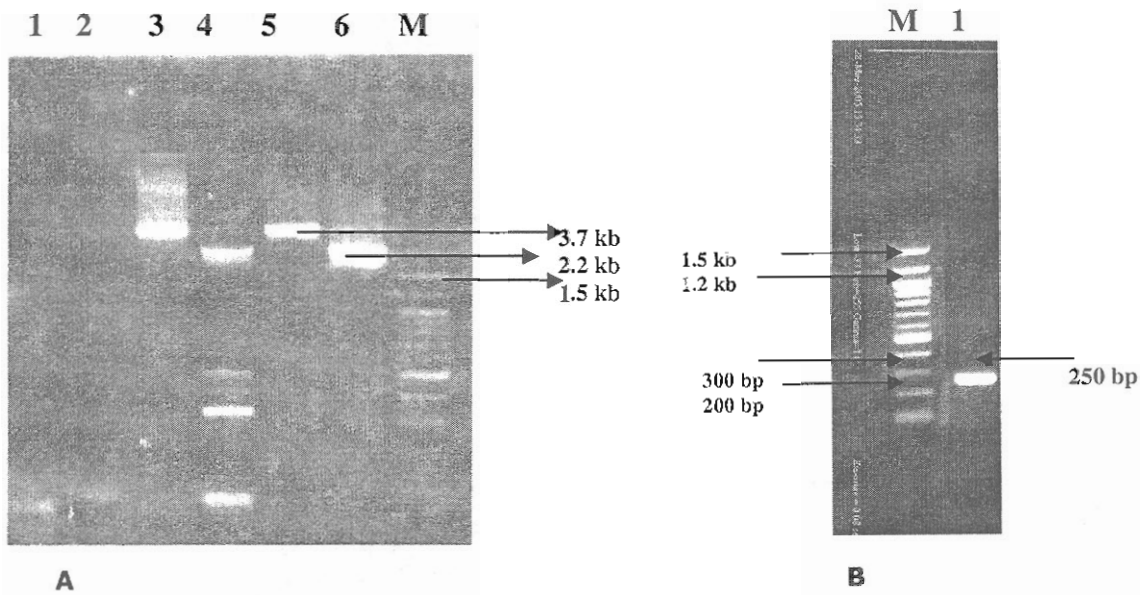
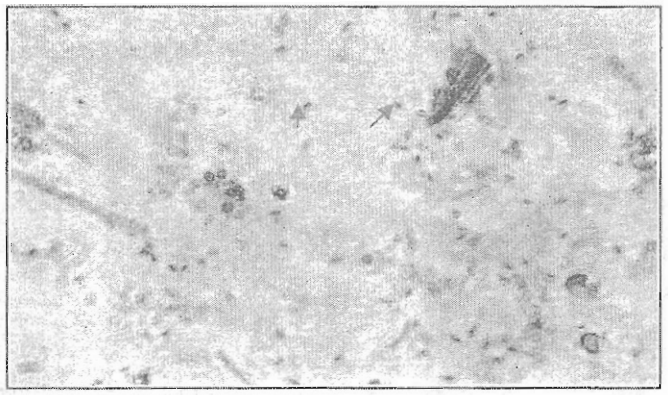


Fig 2. Agarose gel electrophoresis for amplified PCR products from DNA of transformed tNMO10 and parent isolate NMO10.

A: The primer sets IAF&IAR that give 2.2 kb and IAF&ICR that give 3.7 kb PCR products with *cry*1C gene were used. Lanes 1&2: parent isolate NMO10 with IAF&ICR and IAF&IAR respectively (no PCR products). Lanes 3&4: tNMO10 with IAF&ICR and IAF&IAR respectively where the 2.2 kb and 3.7 kb expected PCR products are found. Lanes 5&6: *cry*1C (positive control) with IAF&ICR and IAF&IAR respectively. M: 100 bp DNA ladder marker

B: The primer set *gln* B up&*gln* B do that give 250 bp PCR product with the *gln* B gene. Lane 1: *gln* B up&do specific primers with the parent isolate NMO10. M: 100 bp DNA ladder marker.

A



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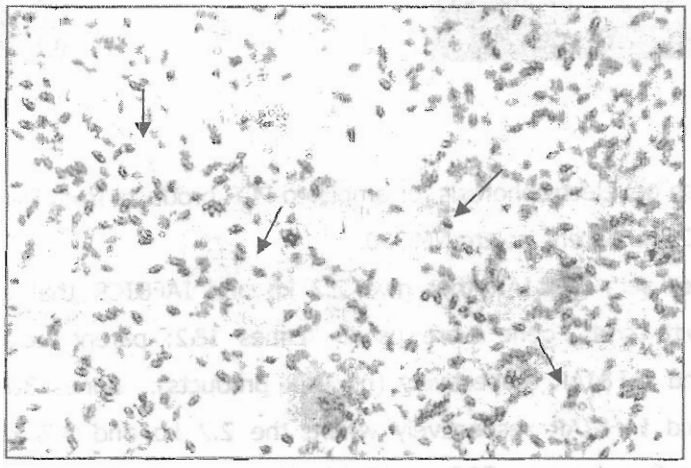
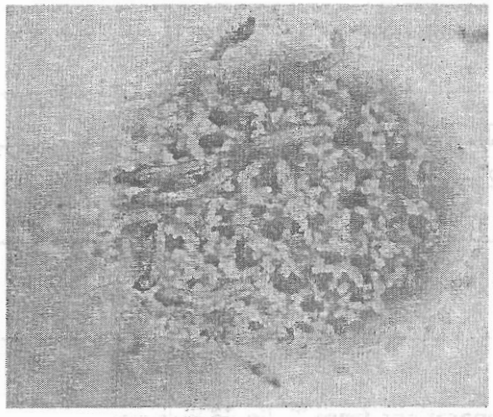


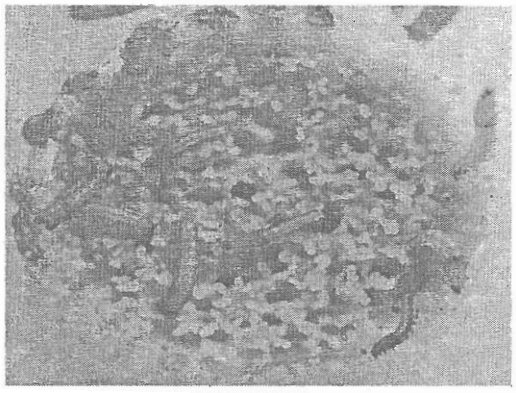
Fig 3. Microscopic examination of sporulating cells from both tNMO10 and parent isolate NMO10.

A: Sporulating cells of parent isolate NMO10. It was grown on T_3 media at 30°C for 72 h. (The red arrows refer to the spores).

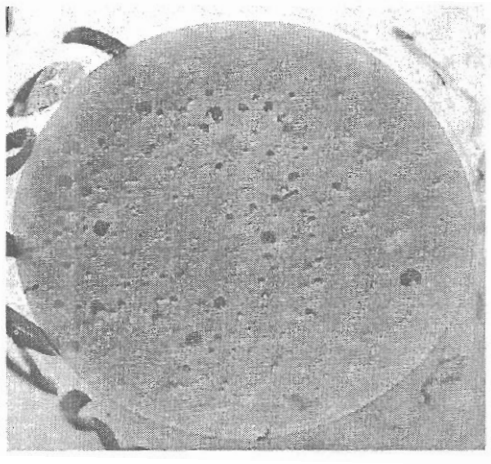
B: Sporulating cells from tNMO10, it was grown on T_3 media at 30 °C for 72 h. The photo shows the spores and the bipyramidal crystals as a result of expression of *cry*1C gene (the black arrows refer to the crystals).



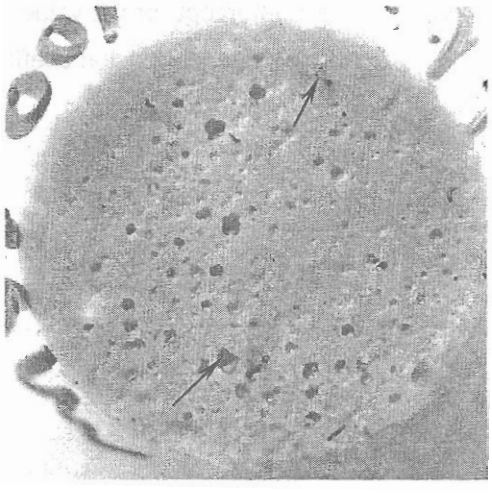
(A) Negative control (no toxin added)



(B) Parent isolate NMO10
(2000 ppm)



(C)
transformed bacterial toxin of tNMO10
(100 ppm)



(D)
Toxin of *Bt entomocidus*
(100 ppm)

Fig 4. The effect of toxicity of tNMO10 compared with that of *Bt entomocidus*.

A: shows the negative control where there was no toxin added.

B: shows that the parent isolate NMO10 had no toxicity at 2000 ppm where it looked like the negative control.

C: shows the toxicity of tNMO10 and how the mortality reached to 100 % at 100 ppm concentration (the red arrows refer to the dead larvae).

D: shows the toxicity of *Bt entomocidus* where there were some larvae still alive at 100 ppm concentration (the black arrows refer to the alive larvae)

Table 1. The LC_{50} values in ppm of toxins used against the cotton leaf worm *S. littoralis*

Strain / toxin	LC_{50} (ppm)	95% confidential limits	Slope/SE
tNMO10 Cry1C	103.0655	(66.12 – 158.901)	1.945 ± 0.498
Bt Entomocidus Cry1C	202.95	(130.355-333.309)	1.480 ± 0.515

*Bioassays were performed on spore-crystal preparations from T_3 liquid cultures.

*LC_{50} is a concentration of toxin required to kill 50% of 1st instar larvae

*LC_{50}s were calculated by probit analysis.

*Probit model is $Y = a + b * x$

where Y = probit value

a = intercept, probit value for x =0

b = slope, regression coefficient of y on x

x = log (dose)

*SE is the standard error