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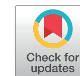
Research article

Bacillus thuringiensis strains isolated from Qatari soil, synthesizing δ-endotoxins highly active against the disease vector insect *Aedes aegypti* Bora Bora



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ABSTRACT

Bacillus thuringiensis (Bt) is a Gram-positive soil bacterium that has been recognized as an effective bioinsecticide active against plant, animal and human pathogenic and disease vector insects. During its sporulation phase, *Bt* produces crystals consisting of δ-endotoxins, which upon ingestion kill specifically insect larvae. *Bt* subsp. *israelensis* (*Bti*) is very active against dipteran insects. *Bti* based bioinsecticides are considered as a sustainable solution to control the Dipteran insects responsible of plant, animal and human diseases. In this study, *Bti* strains isolated from Qatar soil were analyzed for their insecticidal activities against the dipteran insect *Aedes aegypti Bora Bora* (*Culicidae, Diptera*) and for their δ-endotoxins yields per cell. Among the local *Bti* strains, four exceptional strains producing spherical crystals, were found to be more insecticidal than the reference strain *Bti* H14. When tested for their δ-endotoxin yield, the *Bti* QBT217 strain, producing typical spherical crystals and having the best insecticidal activity, was recognized as the best candidate strain for potential bioinsecticide production and biological control of dipteran insects, particularly the disease vector insect *A. aegypti*.

1. Introduction

Mosquitoes are considered as one of the most dangerous threats to plant, animal and human health and acting also as vectors of many deadly diseases (Shu et al., 2013). Chemical insecticides such as those containing carbamates and organophosphates have been used lavishly worldwide for the control of these mosquitoes, although having detrimental effects on humans and food safety and on other non-target organisms and environment in general (Weill et al., 2003; Poopathi and Archana, 2012). The prevalent use of these insecticides in the field has pushed some of the vectors to develop resistance to chemical insecticides available today (Weill et al., 2003; Poopathi and Archana, 2012). To overcome these shortcomings, the biocontrol methods are preferred nowadays, where *Bacillus thuringiensis (Bt)* occupies the first place (Lacey, 2007). *Bt* is a Gram-positive, spore forming, aerobic, soil bacterium that has larvicidal properties due to its parasporal crystals that might have different forms depending on the *Bt* subspecies and the coding *cry* genes. As example, *Bt* subsp *israelensis* (*Bti*) produces spherical crystals, encoded by *cry4Aa/cry4Ba* genes, active against dipteran insects and particularly mosquitos (Mittal, 2003; Jacups et al., 2013). The insecticidal crystal proteins, called δ-endotoxins, target specifically pathogenic insects with no harmful effect on vertebrates (Lacey, 2007; Merritt et al., 1989; Lee and Scott, 1989). Due to these advantages, various *Bt* subspecies have been studied and adopted for the commercial production bioinsecticides. Already since 2005, *Bt* bioinsecticides occupy 97% of the biopesticides available in the market today (Brar et al., 2005). The toxicity of the *Bti* towards dipteran insects is due to the spherical crystal proteins (Palma et al., 2014; Ben-Dov, 2014; Cohen et al., 2011). The activated Cry toxins bind to specific cell receptors to initiate cell lysis and consequent larval death (Zhang et al., 2018). In *Bti*, the insecticidal δ-endotoxins are encoded by genes present on a large 128 kb plasmid called pBtoxis (Wirth et al., 1997). These *cry* genes include *cry4A*, *cry4B*, *cry10A* and *cry11A* (Wirth et al., 1997, 2005; Berry et al., 2002). The plasmid also carries three main *cyt* genes: *cyt1A*, *cyt1C* and *cyt2A*. These genes are controlled by sporulation dependant promoters and are hence expressed only during the sporulation stage (Baum and Malvar, 1995). Along with the

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δ-endotoxins, the pBtoxis carries three other accessory non-toxin genes that are important for the structural integrity of the protein crystals: *p19*, *p20* and *bt152* (Manasherob et al., 2006; Diaz-Mendoza et al., 2020). Important efforts are being made worldwide by many countries to isolate and explore new *Bt* strains from different environments such as soil, grain dusts, mills, insect cadavers and others and find diverse insecticidal delta-endotoxins such as Cry1, Cry2, Cry4, Cry5 and competitive insecticidal activities against not only diptera by also coleoptera and lepidoptera (Hernández-Fernández, 2016). In the present study, we report for the first-time strains of *Bti* isolated from Qatar soil having very high insecticidal activities against the dipteran insect *A. aegypti* and high δ-endotoxin production yield per cell.

2. Materials and methods

2.1. Bt strains, δ-endotoxin proteins and culture conditions

The study focuses on 19 *Bti* strains QBT205, QBT213, QBT214, QBT215, QBT216, QBT217, QBT218, QBT220, QBT221, QBT222, QBT223, QBT224, QBT225, QBT226, QBT227, QBT228, QBT229, QBT230 and QBT608 isolated from Qatari soil. All these strains were identified as *Bti* strains harbouring the genes *cry4B*, *cry11* and *cyt1A* (Supplementary file). These strains produce smooth spherical parasporal crystals during sporulation stage along with its spore. These crystals resemble that of the *B. thuringiensis* subsp. *israelensis*. The reference used in this study as positive control was the *Bti* strain H14. The negative control used in the study was the strain *Bti* 4Q7, which is Bti H14 that has lost all its plasmids and hence does not produce any parasporal crystals during sporulation (Nair et al., 2018). All the strains were grown in rich media Luria Bertini and incubated at 30 °C overnight (Sambrook et al., 1989). A single isolated colony was transferred every time from an overnight LB plate of each strain to liquid sporulation media called T3 broth (Nair et al., 2018). The cultures were then incubated at 30 °C for 96 h until complete sporulation was achieved.

2.2. Isolation and purification of spore-crystal mixture

After complete sporulation of each strain in T3 broth, the spore-crystal mixture was collected as pellet by centrifugation. The pellet was then washed with sterile cold 1M NaCl solution thrice and then thrice with sterile cold distilled water (Zouari et al., 1998). The clean spore-crystal mixture was then re-suspended in sterile cold distilled water and stored at 4 °C.

2.3. Estimation of protein concentration

Protein concentration of spore-crystal mixture of each strain was estimated by Bradford's method (Bradford, 1976) as follows. 100 μl of spore crystal mixture was incubated in sterile NaOH solution with the final concentration of 50 mM for 2 h. Once the crystals were completely dissolved, the spores were separated from the solution by centrifugation. The solubilized protein for each sample was then used for calculating the optical density (O.D) by spectrophotometer. The protein concentration was then extrapolated on a standard graph of O.D versus protein concentration of Bovine serum albumin (BSA) (Zouari et al., 1998). The non-crystal forming strain 4Q7 was used as a negative control for all the proteins that could be present corresponding to contaminating proteins other than crystal δ-endotoxins. The protein concentration obtained from 4Q7 was subtracted from the concentrations of all strains crystal proteins in order to obtain the actual δ-endotoxin concentrations.

2.4. Dipteran insects and raring conditions

In this study, *A. aegypti* was used for the insecticidal bioassay. The eggs of *A.aegypti* were obtained from *Laboratoire de Lutte contre les Insectes Nuisibles* (*LIN*), *Montpellier*, *France* on filter papers. These eggs were then transferred to water and allowed to develop into 3rd instar larvae by incubation at 26 (±2) ^{o} C with a 12:12 h light & dark photoperiod.

2.5. Quantitative insecticidal bioassay and statistical analysis

The quantitative bioassays were performed by the standard protocols of WHO (WHO, 2019). Third-instar larvae (n = 10) of *A. aegypti* were added to 100 ml water in plastic cups. Each concentration was triplicated, and the larvae were exposed to the spore-crystal mixture at 27 (±2)^{o}C with 12:12 light and dark photoperiod. As negative control, three replicates were left untreated. The number of dead and live larvae were counted after 24 h. The bioassay was first performed with different concentrations of δ-endotoxins from reference H14 and 4Q7. The LC_{50} concentration for H14 strain was estimated. The 19 *Bti* strains were tested at this concentration to investigate their efficiency against larvae of *A. aegypti*. The *Bti* strains were then grouped into three classes based on the following categories: efficiency lower than H14, efficiency resembling H14 and efficiency higher than H14. The third group with higher efficiency were tested for more concentrations to calculate their actual LC_{50} values. The LC50 values were calculated using Probit analysis software. The 95% fiducial limit range was also calculated for each strain. This range was used to draw graphs to compare the efficiency of local strains with each other and the reference (Finney, 1971).

2.6. Obtaining completely sporulated culture for protein estimation

The four efficient strains from the Group 3 (Figure 2) were inoculated on LB plates and incubated overnight. Single isolated colony was transferred to a pre-culture of 3 ml LB broth and incubated overnight at 30 °C at 150 rpm (rounds per min). After 16 h, 500 ul of preculture were transferred to a second pre-culture of 50 ml LB broth and incubated overnight at the same conditions as above. The OD of the second preculture was taken at 600 nm of the light spectrometer. Accordingly, the amount of pre-culture to be added to sporulation media was calculated such that the sporulation media starts at an OD of 0.1. The sporulation media used in this study was the glucose-based media adopted from Ghribi et al. (2007). The sporulation culture was incubated at the same conditions as above for 120 h, until complete sporulation. 1 ml of this sporulated culture was transferred to each of 4 Eppendorf tubes that are used for the estimation of the δ-endotoxin concentration and the CFU (colony forming units), as described in the following paragraphs.

2.7. Estimation of the produced δ-endotoxin concentration

Three Eppendorf tubes with one millilitre from sporulated culture were centrifuged to obtain the spore crystal mixture. The spore-crystals were washed thrice in 1M NaCl and then thrice in distilled water. The spore-crystal mixture was then resuspended in 50 mM NaOH to solubilize the crystal for two hours. The solubilized proteins are separated from the spores by centrifugation. The amount of protein content per millilitre was calculated by Bradford's method, using different concentrations of Bovine Serum Albumin (BSA) for standard graph (Ghribi et al., 2007).

2.8. Estimation of the Bti strains sporulated cultures colony forming units

One millilitre from the sporulated culture was serially diluted till 10^{-7} and the last four concentrations of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were spread on LB plates in triplicate. The plates were then incubated at 30 °C overnight and the colony forming units per millilitre was calculated from each replicate for each dilution.

2.9. Estimation of δ-endotoxin production yield

The protein production yield per cell for each isolate was calculated by dividing the mean protein production values by the mean CFU values. The calculated protein production capacity in the form of nanogram per

CFU for the negative control *Bti* 4Q7 was subtracted from other test strains. This was done in order to eliminate the readings from the extra proteins other than the δ-endotoxins. The calculated values were then plotted on graph to represent the efficiency of the local strains compared to the reference strains.

2.10. Observation of the crystal morphology of the strain QBT217

QBT217 strain was plated on T3 medium and incubated for 3 days at 30 °C. The spores and crystals were observed using FEI Nova NanoSem 450 Scanning Electron Microscope (SEM), USA.

2.11. Determination of the δ-endotoxin gene content by PCR

The PCR amplifications were performed, as described by Nair et al. (2018), using DNA and pairs of primers (Table 1) amplifying specifically parts of the genes *cry4*, *cry11*, *cyt1A*. The PCR screenings were performed with the 19 *Bacillus thuringiensis* strains used in this study that are: QBT205, QBT213, QBT214, QBT215, QBT216, QBT217, QBT218, QBT220, QBT221, QBT222, QBT223, QBT224, QBT225, QBT226, QBT227, QBT228, QBT229, QBT230 and QBT608.

3. Results

3.1. Bti strains cry genes profiles

The 19 *Bti* strains were screened for the presence of dipteran specific δ-endotoxin genes *cry4*, *cry11* and *cyt1A*. As controls, the crystalliferous *Bti* strain H14 and the acrystalliferous strains *4Q7* (Nair et al., 2018) were used as positive and negative controls, respectively. Table 2 illustrates the gene content of the 19 strains. The PCR amplifications showed that all the 19 *Bti* strains harbour the 3 genes *cry4*, *cry11* and *cyt1A*.

3.2. Investigation and comparison of the insecticidal activities of local Bti strains

Among the different concentrations of δ-endotoxins tested for the *Bti* H14, all concentrations below 10 ng/l could not kill any *A. aegypti* larvae and all concentrations above 250 ng/l killed all the larvae (data not shown). The concentration of 100 ng/l of δ-endotoxins from H14 could kill about 50% of the larvae (data not shown). Therefore, the concentration of 100 ng/l was considered as the estimated LC_{50} value for the reference H14, that was used to compare the insecticidal activities of the local *Bti* strains. When tested at a δ-endotoxin concentration of 100 ng/l, local Qatari *Bti* strains showed varied insecticidal activities against *A. aegypti* larvae depending on the tested strain. Based on their efficiency, the local *Bti* strains were divided in to three groups. The first group consisted of QBT205, QBT222, QBT224, QBT227, QBT226, QBT213 and QBT214. These strains could kill only less than 40% larvae. The second group consisted of QBT223, QBT230, QBT215, QBT229, QBT216, QBT225, QBT228 and QBT608. Their activities resembled that of the reference H14; they killed about 50% of the larvae. The third group consisted of QBT221, QBT217, QBT218 and QBT220. They were able to kill more than 60% of the larvae of *A. aegypti*. Thus, the three formed groups are shown in Figure 1.

3.3. Quantified insecticidal activity of the efficient local Bti strains

When tested at more concentrations of δ-endotoxins, the group 3 strains could not kill any larvae of *A. aegypti* at or below 5 ng/l and killed all the larvae at or above 250 ng/l. These strains killed about 50% of larvae at a concentration of 75 ng/l of δ-endotoxins. At other concentrations of 30 ng/l, 50 ng/l, 100 ng/l and 125 ng/l, they killed 19%, 38%, 66% and 86%, respectively. Among these Group 3 strains, the percentage of larvae killed by QBT220 was slightly higher as shown in Figure 2. Probit analysis software showed that the calculated LC_{50} for the reference strain H14 was 95 ng/l. On the other hand, the calculated LC_{50} for QBT217, QBT218, QBT220 and QBT221 were 65 ng/l, 66 ng/l, 60 ng/l and 68 ng/l with an error of +/- 20 ng/l, respectively. According to the 95% fiducial limit, the LC_{50} values do overlap. But, the most efficient among the strains was found to be QBT220.

3.4. Estimation of the produced δ-endotoxin concentration of the highly insecticidal Bt strains

The investigation of the δ-endotoxin synthesis by the candidate highly insecticidal *Bt* strains, using as positive control the reference strain H14, revealed that the latter strain H14 produced about 36 +/- 2 μg/ml. This was the highest amount of δ-endotoxins production seen among the strains tested. The Group 3 strains produced lesser δ-endotoxins. In fact, QBT217 produced 26.8 +/- 2 μg/ml, QBT218 produced 23.4 +/- 2 μg/ml, QBT220 produced 20.6 +/- 2 μg/ml and QBT221 produced 19.5 +/- 1 μg/ml of proteins. The negative control strain 4Q7 (Cry^-) produced about 3.2 μg/ml of proteins (Figure 3). Contrary to the δ-endotoxins production per ml, the same strains showed a different trend when we looked to the δ-endotoxins synthesized per cell, using the cfu/ml values. The reference strain H14 had 1.7+/-0.1 × 10^7 cfu/ml and the negative control 4Q7 had 5.4 +/-0.1 × 10^7 cfu/ml. Among the local *Bti* strains, QBT217 had the lowest growth rate of 1.0 +/-0.1 × 10^7 cfu/ml, followed by QBT220 with 1.8+/- 0.1 × 10^7 cfu/ml, QBT218 with 6.4 +/- 0.1 × 10^7 cfu/ml and QBT221 with 8.6 +/- 0.1 × 10^7 cfu/ml (Figure 4). The highest yield of δ-endotoxins was seen with QBT217 producing 265 ng +/- 33 ng δ-endotoxins per 10^5 cells. Comparatively, the reference strain H14 had a lower δ-endotoxins yield of 209 +/- 17 ng δ-endotoxins per 10^5 cells. The two other candidate strains QBT218 and QBT221 gave very low δ-endotoxins yields of respectively 37 +/- 3 ng per 10^5 cells and 23 +/- 1 ng per 10^5 cells. Even though not as good as QBT217, QBT220 had comparatively good yield with 111 +/- 12 ng δ-endotoxins per 10^5 cells (Figure 5).

3.5. Exploration of the morphology of the crystals produced by the strain QBT217

The strain QBT217 that produces the highest concentration δ-endotoxins per cell and that, in term of insecticidal activity against *A. aegypti*, was ranked among the best 4 QBT strains of the 19 strains with a LC_{50} of 65 +/- 20 ng/l, was chosen for the observation by SEM of it spores and

Table 1. Primers used in PCR experiments.

Gene	Primer pairs	Sequences	Expected PCR product size (bp)
cry4B	Dip2A	5' GGTGCTTCCTATTCTTTGG3'	1293
	Dip2B	5' TGACCAGGTCCCTTGATTAC3'	
cyt1A	Cyt1A1	5' GTTGTAAGCTTATGGAAAAT3'	701
	Cyt1A2	5' TTAGAAGCTTCCATTAATA3'	
cry11	Cry11-1	5' TTAGAAGATACGCCAGATCAAGC3'	304
	Cry11-2	5' CATTTGTACTTGAAGTTGTAATCCC3'	

Table 2. Gene content of the explored 19 *Bti* strains.

Genes Strains	*cry4B*	*cry11*	*cyt1A*
H14	✓	✓	✓
4Q7	×	×	×
QBT205	✓	✓	✓
QBT213	✓	✓	✓
QBT214	✓	✓	✓
QBT215	✓	✓	✓
QBT216	✓	✓	✓
QBT217	✓	✓	✓
QBT218	✓	✓	✓
QBT220	✓	✓	✓
QBT221	✓	✓	✓
QBT222	✓	✓	✓
QBT223	✓	✓	✓
QBT224	✓	✓	✓
QBT225	✓	✓	✓
QBT226	✓	✓	✓
QBT227	✓	✓	✓
QBT228	✓	✓	✓
QBT229	✓	✓	✓
QBT230	✓	✓	✓
QBT608	✓	✓	✓

As controls, the crystalliferous *Bti* strain H14 and the acrystalliferous strains *4Q7* (Nair et al., 2018) were used as positive (✓) and negative (×) controls, respectively.

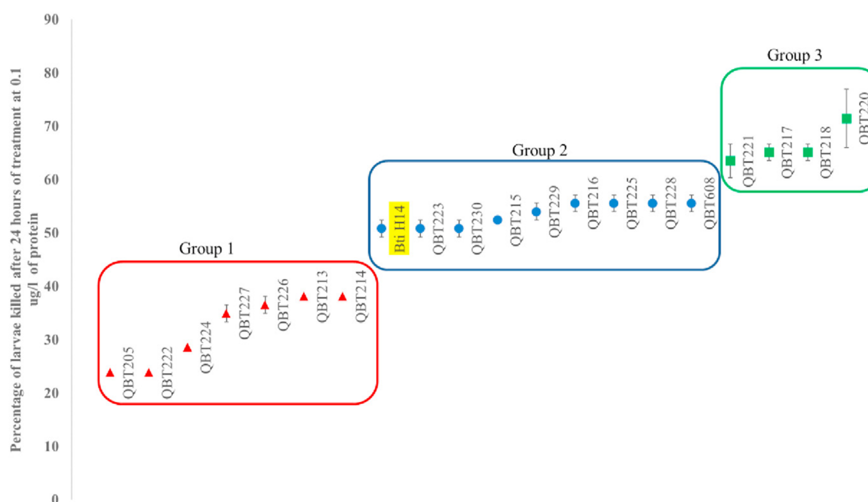

Figure 1. Distribution of *Bti* strains depending on their insecticidal activities against *A. aegypti*. The graph represents the percentages of larvae killed by the reference strain and the local *Bti* strains at the δ-endotoxin protein concentration of 0.1 μg/l; error bars are plotted with the standard errors calculated based on three readings for each strain; Group 1 (red box) consists of *Bti* strains that could kill only 40% or less larvae; Group 2 (blue box) consists of *Bti* strains and reference *Bti* H14 (highlighted in yellow) that could kill 50% of larvae; Group 3 (green box) consists of *Bti* strains that could kill more than 60% of larvae.

crystals. QBT217 showed (Figure 6) typical spherical crystals of the common *Bti* strains.

4. Discussion

Bti based bioinsecticides are very efficient and environmentally very safe for the control of dipteran insects, particularly disease vectors (Mittal, 2003; WHO, 2019). This has pushed world-wide the set-up of isolation and screening programs of *Bti* isolates from different ecologies in order to find competitive strains and *Bti* formulations useful for the industrial production of efficient bioinsecticides. In this study, the insecticidal activities of an existing important collection of *Bti* strains, isolated from Qatar soil, against the dipteran insect *A. aegypti* and their bio-insecticides (δ-endotoxins) production capacity were investigated for the 1st time in the region and showed differences among the strains in term of δ-endotoxin production/cell and insecticidal activities. This comparative study was feasible since we have enough *Bti* strains, abundant in the region as reported in our earlier study (Nair et al., 2018), contrarily to other regions in the world like in North Africa as reported earlier (Zribi et al., 2006). The straight forward strategy adopted to assess the insecticidal activities of these 19 strains and to determine the LC_{50} of their δ-endotoxins was very successful and allowed the accurate and quick classification of the local strains into groups that are less, more or as efficient as the reference strain H14 (Figure 1). Group 3 (Figure 2) includes the most insecticidal strains (QBT217, QBT218, QBT220 and QBT221) against the 3rd instar larvae of *A. aegypti*, when compared to the reference *Bti* strain. In fact, the LC_{50} value for H14 was 95 ng/l +/- 20 ng/l and those for Group 3 strains were between 60 ng/l+/- 20 ng/l to 68 ng/l +/- 20 ng/l. These differences might be due to the differences in the δ-endotoxin sequences, gene expression levels or in the metabolisms of these strains, therefore affecting the amounts of the synthesized δ-endotoxins forming the insecticidal crystals (Elleuch et al., 2015). The

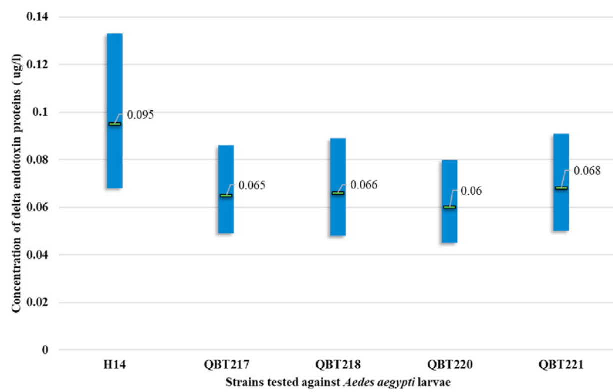

Figure 2. The LC_{50} values of the Group 3 local *Bti* strains and reference H14 against larvae of *A. aegypti*; The bars represent the range of LC_{50}values for each strain, the black line and the associated values show the calculated LC_{50} values for each strain.

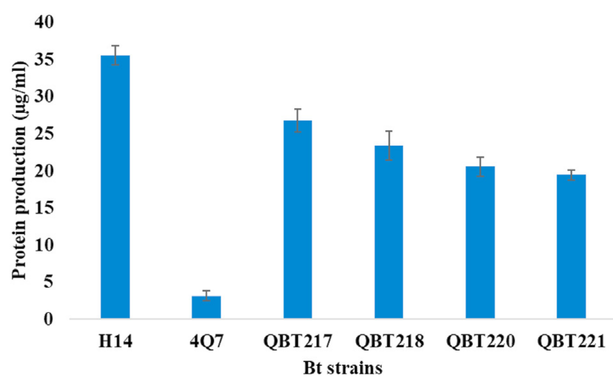

Figure 3. Comparing the protein (δ-endotoxins) production capacity (μg/ml) of each of the local *Bti* strains with that of the reference H14 in glucose-based media.

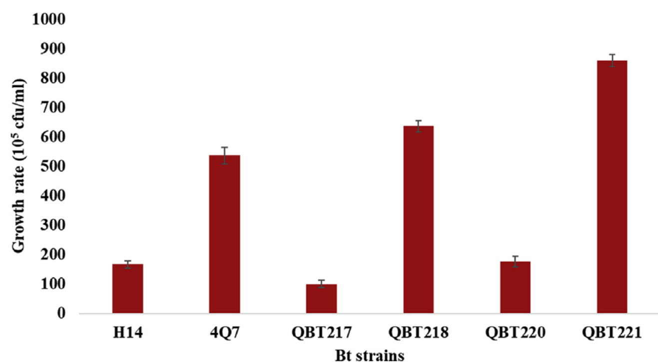

Figure 4. Comparing the growth rate of each of the local *Bti* strains with that of the reference H14 in glucose-based media.

strains tested for their δ-endotoxins production per cell were QBT217, QBT218, QBT220 and QBT221. It was found that the reference H14 produced the highest δ-endotoxins quantity per ml (Figure 3), followed by QBT217, QBT218, QBT220 and QBT221. On the contrary, when the growth rates of these strains were compared, the trend seen was very different. The reference H14, QBT217 and QBT220 had a much lower growth rate compared to QBT218, QBT221 and 4Q7 (Figure 4). This is important as all the strains were inoculated into the sporulation medium with the same initial cell concentration. So, after culturing for the same time at the same conditions until the end of the sporulation, the huge

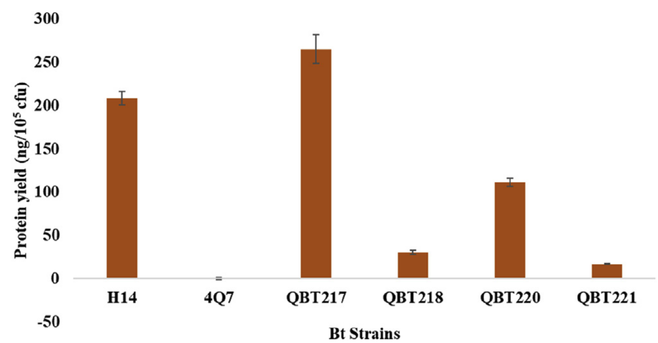

Figure 5. Comparing the δ-endotoxin yield per spore (ng/10^5 cfu) of each of the local *Bti* strains with that of the reference H14 in glucose-based media.

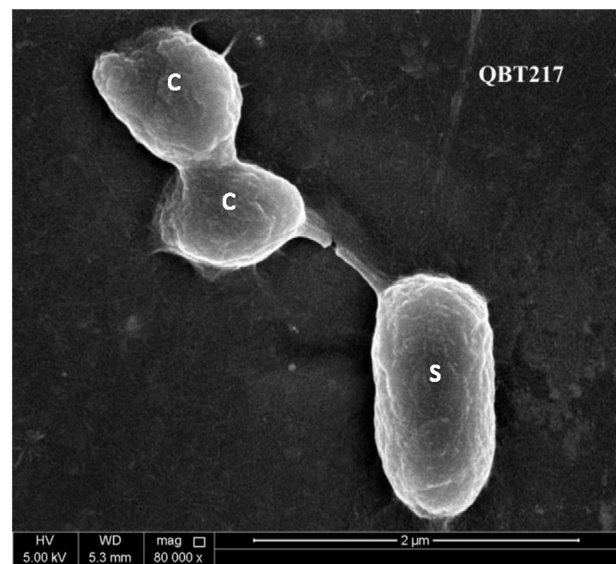

Figure 6. Observation of the *Bt* strain QBT217 crystals (C) and spores (S) by Scanning electron microscopy.

differences in their colony forming units (CFU) show the differences in their growth rate. It is even more interesting to note that the δ-endotoxins yield per spore is not proportional to the growth rate. This means that the higher amount of δ-endotoxins produced could be due to either of the two reasons: the higher number of cells/ml or the high capacity to synthesize δ-endotoxins per cell as explained above. When the δ-endotoxins protein yield per cell was calculated (Figure 5), it was observed that the strain QBT217 has the highest δ-endotoxin yield per cell. This *Bti* QBT217 strain, producing typical *Bti* spherical crystals confirmed by electronic microscopy (Figure 6), and having the best insecticidal activity, could hence be recognized as the best candidate strain for bio-insecticide production and biological control of dipteran insects and particularly the disease vector insect *A. aegypti*.

5. Conclusion

This study showed an important diversity among 19 locally isolated *B. thuringiensis* subsp. *israelensis* strains in term of δ-endotoxins yield per cell and insecticidal activities against the third-instar larvae of *A. aegypti*. Four local strains QBT217, QBT218, QBT220 and QBT221 producing typical *Bti* spherical crystals, were found to have the highest insecticidal activities than the reference strain H14. QBT217 had the highest δ-endotoxins yield per cell, and therefore can be considered as an excellent candidate strain for the industrial bio-insecticide production, and application in the biocontrol of the pathogenic disease vector insects.

Declarations

Author contribution statement

K. Nair: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

R. Al-Thani: Conceived and designed the experiments; Performed the experiments.

C. Ginibre, F. Chandre, M. Alsafran: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. Jaoua: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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