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Application of scanning electron microscopy and MALDI-TOF MS in the exploration of the parasporal insecticidal crystals of *Bacillus thuringiensis* strains isolated from Qatar soil

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ABSTRACT

Bacillus thuringiensis (*Bt*) plays an important role in health and agriculture due to its insecticidal activities. In this study, six *Bt* strains isolated from Qatar soil were used to explore their crystals morphologies and δ-endotoxin profiles. Bipyramidal crystal producing strains exhibited the genes *vip3Aa58*, *cry1Aa/Ac*, *cry2Ab*, *cry1Ba* and *cry1Ia*, predicting toxicity against lepidopteran, dipteran and hemipteran insects. A local strain QBT474 showed a high δ-endotoxin production of $647.32 \pm 8.49 \times 10^{-6}$ ng/cell compared to $359.259 \pm 15.02 \times 10^{-6}$ ng/cell in reference HD1 strain. The scanning electron microscopy showed bipyramidal, cuboidal and spherical crystals among the strains. MALDI-TOF-MS crystal protein profile data allowed to evidence differences in *Bt* crystal proteins. This is a first report that MALDI-TOF could be used in differentiating *Bt* δ-endotoxins in the strains forming same crystal forms. This study also demonstrates that local *Bt* strains might be considered for the industrial production of biological insecticides.

1. Introduction

Bacillus thuringiensis (*Bt*) is among the most known micro-organisms in agriculture for its biocontrol abilities (Jouzani et al., 2017; Palma et al., 2014). *Bt* has been used for many decades now, against many invertebrates pests for its diverse insecticidal activity and high specificity, replacing many chemical pesticides and insecticides due to their harmful effect on the environment (Jallouli et al., 2020; Kumar et al., 2021) despite the fact that these chemicals have a wide range of application and long-term effects on the environment and exposed human population (Nicolopoulou-Stamati et al., 2016).

During sporulation, *Bt* synthesizes insecticidal δ-endotoxins forming parasporal crystals that are highly specific towards certain orders of insects (Eski et al., 2018; Akhtar et al., 2021). When ingested by insects, these crystals are solubilized, and converted to protoxins that are activated by the mid-guts proteases giving activated toxins responsible for creating pores in the gut's wall leading to severe damage and death of the target species (Jacups et al., 2013). The specificity of *Bt* is associated with the type and form of δ-endotoxin produced during the sporulation phase. Crystalized δ-endotoxin (*cry* protein) and cytolytic (*cyt*) proteins are two important types of *Bt* protein. On the basis of morphology, crystalized δ-endotoxin may be spherical, cuboidal, bipyramidal and others (Mukhija and Khanna, 2018; Nair et al., 2018). For instance, *Bt kurstaki* (Bt_k) are toxic towards coleopteran and lepidopteran insects as a result of producing a mixture of different *Cry* proteins that crystalize into cuboidal and bipyramidal forms (Castro et al., 2019). Whereas in the case of *Bt israelensis* (Bt_i), which as result of producing both Cry4 and *Cyt* proteins forming spherical crystals is toxic towards the insects of dipteran order (Nair et al., 2018). Other than Cry and Cyt proteins, some *Bt* strains during their vegetative growth phase can synthesize "vegetative insecticidal proteins" (VIP) that are soluble in the medium and have insecticidal activities against lepidopteran insects (Syed et al., 2020; Abdelmalek et al., 2016). All reported *Bt* insecticidal proteins do not have any effects on animal, humans, plants, and other beneficial bacteria, which can be explained by the *Bt*'s mode of action (Liu et al., 2021). In fact, there are many synthetic pesticides available in the market with efficient activities against the agricultural pests. However, there are associated risks with the application of these formulations on

Abbreviations: MALDI-TOF-MS, Matrix-assisted laser desorption/ionization-time of flight mass spectrometry; Bt, *Bacillus thuringiensis*; SEM, scanning electron microscopy; QBT, Qatari *Bacillus thuringiensis*; VIP, vegetative insecticidal proteins; Btk, *Bacillus thuringiensis* kurstaki; CFU, colony forming units; LB, Luria Bertini; PCA, principal component analysis; PCR, polymerase chain reaction.

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the environment, human health and non-targets species. The residues of the compounds can be detected in the food chain and their adverse effects are reported on the human health. Moreover, there are cases of developing resistance in the pests against these compounds due to their persistent and uncontrolled application. The most important concern with the application of these compounds is their ecological effects, by killing non-targets species. In keeping all these limitations, it is always needed to explore safe biological resources such as *Bt* strains for their potential to control the important pests of agricultural crops with their minimal effect on the environment. It is a fact that the biological control approaches, particularly based on *Bt* crystals can't be as effective as synthetic pesticides, however several limitations of pesticides could be overcome by the bacterial compounds. In the current study, efforts were made to explore the potentialities of *Bt* strains isolated from previously unexplored extreme climatic conditions such as those isolated from Qatar soil. In our preliminary studies a huge diversity of *Bt* strains was evidenced in Qatar (Nair et al., 2018). Hence, the present study was designed to explore parasporal crystals of important local Qatari *B. thuringiensis* strains and their crystal δ-endotoxins. Scanning electron microscopy was used to explore the strains crystal morphologies and molecular investigations by PCR were performed to find out the encoding genes and predict the insecticidal activities. The investigation of the crystal protein content using for the first time MALDI-TOF MS (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry), allowed to evidence differences in the δ-endotoxin contents even among strains having similar crystal forms. The application of MALDI-TOF MS techniques will create new insights for studying *Bt* parasporal crystal δ-endotoxins and their applications in the biological control of important pests.

2. Materials and methods

2.1. Bt strains and culture conditions

In this study, 6 Qatari soil *Bacillus thuringiensis* strains (QBT282, QBT376, QBT474, QBT556, QBT671, QBT674) were used. The *Btk* strain HD1 (Monroy et al., 2021) was used as reference. Luria Bertini medium (Park et al., 2017) was used as growth media. The sporulation and parasporal synthesis were carried out using T3 medium (Nair et al., 2018) by incubating triplicate bacterial culture plates at 30 °C for 4 days until complete sporulation (Nair et al., 2021).

2.2. Evidence of Bt insecticidal protein coding genes by polymerase chain reactions (PCR)

For the *Bt* insecticidal protein coding genes analysis, plasmid DNA was extracted from all six strains as well as reference strain HD1 strain using the alkaline method (Rashki et al., 2021). The polymerase chain reactions (PCR) were carried out using the primers for different genes listed in the Supplementary Table 1. The PCR reactions were performed in 25 μL tubes using Veriti Thermal Cycler, Applied Biosystems (Nair et al., 2021).

2.3. Estimation of the δ-endotoxins concentration

The δ-endotoxins concentration of *Bt* strains was determined by culturing bacterial cell on T3 media for 96 h. The crystal/spore were separated by centrifugation of bacterial cultures at 4000 rpm for 5 min. The obtained pellets were washed with sterile distilled water (chilled) for two times. The crystals were then subjected to solubilization by adding NaOH (50 mM). Bradford's assay was used to determine the δ-endotoxin concentration (Nair et al., 2021). Appearance dilutions were plated on LB media to calculate the CFU (colony forming unit) per mL. The δ-endotoxin production per spore of bacteria was calculated by dividing δ-endotoxin concentration with CFU per mL.

2.4. Scanning electron microscope (SEM)

Bacterial cells of all six strains as well as *Bacillus thuringiensis kurstaki* (*Btk*) were cultured for three days in T3 medium. Spores and crystals were observed under compound light microscope for primarily morphological investigation. For the scanning electron microscopy (FEI Nova NanoSem 450, USA) procedure described by Loutfi et al. (2020) was followed. Briefly, to a 100 μL of mixture of spores and crystals, 1.9 mL sterile distilled water was added. After mixing, 50 μL was placed on SEM sample holder. Samples were dried at 37 °C in oven, metalized and placed in vacuum chamber of SEM to capture images. Crystals and spores' morphologies of each strain were analyzed individually and then compared with each other and with *Bacillus thuringiensis kurstaki* (*Btk*) used as reference strain (Monroy et al., 2021). This strain produces bipyramidal and cuboidal parasporal crystals, very useful in the screening of the new Bt isolates through the observation of their crystal forms.

2.5. MALDI-TOF MS protein profiling

To investigate the crystal protein content of different *Bt* strains as well as their relation to the type of crystals produced, MALDI-TOF MS technique was utilized. The crystal solubilization was done as explained above followed by pipetting 1 μL of the solubilized sample onto the MALDI biotarget plate (48-sample spots from Bruker Daltonics/Germany). For crystallization of proteins, the sample once dried, was overlaid with 1 μL of CHCA matrix (α-cyanohydroxycinnamic acid). After installing the biotarget plate in the MALDI-TOF MS instrument (Bruker Daltonics/Germany), the Biotyper Real-Time Classification (RTC) software was used to label the sample spots before initiating the protein profiling of the samples.

2.6. Acquisition and pre-processing of protein profiles

To acquire the proteins' mass spectral profiles, Bruker Flex Control software was utilized. The software settings were adjusted i.e., laser frequency (60 Hz), intensity (35 %), linear and positive mode, the source voltage was 18.7 kV, while the acceleration voltage was 20 kV. A total of 240 laser shots in 40-shot steps for each spectrum were used to generate the protein profile from each sample spot. The mass range was set from 0 to 10,000 Da.

To analyze and pre-process the protein profiles, Flex Analysis (V3.4) software was used. The pre-processing was performed through smoothing, baseline subtraction, and peak detection using default algorithms. The profiles were then visualized in stack mode for a better comparison of mass spectra acquired from different samples.

2.7. Statistical analysis

Principal component analysis (PCA) was performed as a statistical tool for the comparison of multi-variate data. PCA helps to categorize similar spectra together resulting in the formation of groups (of closely related spectra) that are dissimilar to each other. The PCA results are visualized in 2D or 3D graphs depending upon the variability in the data set.

PCA analysis was done within the MALDI-TOF MS instrument using Biotyper RTC 3 software. All the mass spectra were pre-processed through baseline subtraction and smoothing. The processed spectra were then analyzed using PCA tools of the software following the standard operating procedure.

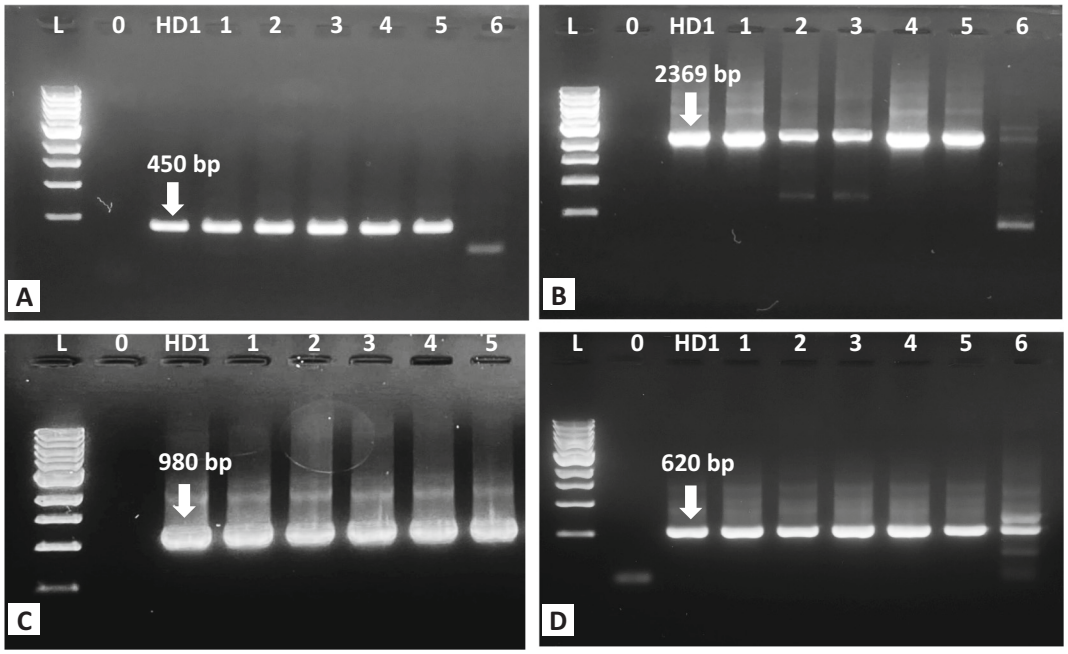

Fig. 1. Agarose (1 %) gel electrophoresis of PCR products amplified for (A) *vip3Aa58* gene using Vip1 and Vip2 primers; (B) *vip3Aa58* full gene using Vip1 and Vip3 primers; (C) *cry1Aa/cry1Ac* gene using Lep2A and Lep2B primers, and (D) *cry2Ab* gene using Cry2A and Cry2B primers for both reference and native isolated strains of *B. thuringiensis*. Lanes: L: 1-kb ladder (500-bp, 1-kb, 1.5-kb, 2-kb, 3-kb, 4-kb, 6-kb, 8-kb and 10-kb), 0: negative control, HD1: *Bt kurstaki* "control", 1: QBT282, 2: QBT376, 3: QBT474, 4:QBT556, 5: QBT671, 6: QBT674. The mentioned arrows show the expected PCR fragment size for each primer pair.

3. Results and discussion

3.1. Investigation of the insecticidal protein coding genes of the Qatari B. thuringiensis strains

In this study, PCR amplifications using 11 different pairs of primers allowed the investigation of the *cry* gene content of the 6 different strains of *Bt* isolated from Qatar soil, including the reference strain HD1. A positive amplification for part of *vip3Aa58* gene was observed in the strains HD1, QBT282, QBT376, QBT474, QBT556, and QBT671 while using vip1 and vip2 primers, with a fragment having the expected size of 450 bp (Fig. 1A), indicating the presence of *vip3Aa58* gene in these strains.

Baranek et al. (2015) reviewed the activities of *Bt* vip3Aa58 proteins against the larvae of Lepidopteran insect such as fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), cotton bollworm (*Helicoverpa armigera*), black cutworm (*Agrotis ipsilon*), tobacco budworm (*Heliothis virescens*), and corn earworm (*Helicoverpa zea*). Hence, it is predicted that these local Qatari strains carry out these insecticidal activities. Similarly, the results presented in Fig. 1B displayed a positive amplification for the entire gene of *vip3Aa58* having a fragment size of 2369 bp using the primers vip1 and vip3 for the strains HD1, QBT282, QBT376, QBT474, QBT556, and QBT671. The results confirm the previous PCR results of Fig. 1A where internal *vip3Aa58* gene primers were used. Hence the entire *vip3Aa58* was amplified and allowed the prediction of the VIP insecticidal activities and therefore, holds a great economic importance. Using of Lep2A and Lep2B as primers resulted in a positive amplification of part of *cry1Aa/cry1Ac* genes giving a fragment of 980-bp in 6 different *B. thuringiensis* strains, i.e., HD1, QBT282, QBT376, QBT474, QBT556 and QBT671 as seen in Fig. 1C. The strains that contain *cry1Aa/cry1Ac* genes tend to exhibit potential toxicity against lepidopteran orders (Hernández-Rodríguez et al., 2013), hence it is predicted that the strains amplifying this gene are likely to exhibit the insecticidal activity. The amplification of the *cry1Aa/cry1Ac* genes confirms the crystals morphologies observed through the SEM, as this gene encodes for the bipyramidal crystal in the *Bt* strains (Evdokimov et al., 2014). This also explains the reasons for no amplification in the strain QBT674 as it produces only spherical crystals.

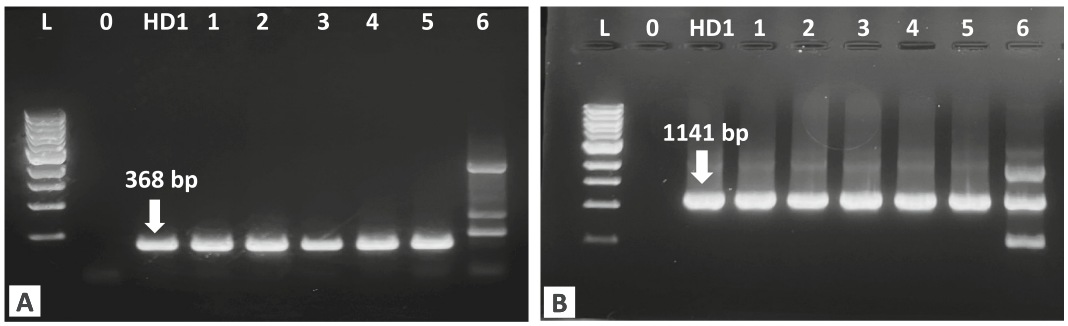

Fig. 2. Agarose (1 %) gel electrophoresis of PCR products amplified for (A) *cry1Ba* gene using cry1-B1 and cry1-B2 primers, and (B) *cry1Ia* gene using the pair of primers Cry1-I1 and Cry1-I2 for both reference and native isolates of *B. thuringiensis*. Lanes: L: 1-kb ladder (500-bp, 1-kb, 1.5-kb, 2-kb, 3-kb, 4-kb, 6-kb, 8-kb and 10-kb), 0: negative control, HD1: *Bt kurstaki* "control", 1: QBT282, 2: QBT376, 3: QBT474, 4:QBT556, 5: QBT671, 6: QBT674. The mentioned arrows show the expected PCR fragment size for each primer pair.

Table 1
Determination and statistical analysis of δ-endotoxin yield for the explored Qatari *Bt* strains (mean ± SD).

Strains	δ-Endotoxin (μg/mL)	Cell biomass (10^6) cfu/mL	δ-Endotoxin content (ng/10^6cfu)
HD1	48.50 ± 1.48	135 ± 3.86	359.259 ± 15.02
QBT474	161.83 ± 1.90	250 ± 1.46	647.32 ± 8.49
QBT556	40.17 ± 2.15	173 ± 2.56	232.19 ± 12.88

A positive amplification (band size 620 bp) was observed for *cry2Ab* gene in the strains HD1, QBT282, QBT376, QBT474, QBT556, and QBT671 using the primers Cry2A and Cry2B (Fig. 1D). This augment the findings of SEM, where the cuboidal crystals were observed in these strains, as the cuboidal form of crystals is encoded by the gene Cry2. The presence of *cry2Ab* gene in the strain tends to have insecticidal activities against insects of orders Lepidoptera and Diptera by encoding δ-endotoxins forming cuboidal crystals (Pan et al., 2019). Therefore, it can be predicted that these strains harbor the same insecticidal activities.

Using primer pair Cry1-B1 and Cry1-B2, amplifications were observed in strains HD1, QBT282, QBT376, QBT474, QBT556, and QBT671 for the gene *cry1Ba* having a fragment size of 368 pb as shown in Fig. 2A. This gene is responsible for encoding bipyramidal crystals (Jain et al., 2017).

It is reported that strains containing the gene *cry1Ba* have insecticidal activities against potato tuber moth and European corn borer, as well as having a relatively low potential toxicity against coleopterans and Colorado Potato Beetles (Salehian et al., 2021). The gene *cry1Ia* was explored in the present work using the corresponding primers pair Cry1-I1/Cry1-I2. The gene *cry1Ia* was amplified in the DNA of the strains HD1, QBT282, QBT376, QBT474, QBT556, and QBT671 with a single band of 1141 bp (Fig. 2B). However, QBT674 did not give the expected size. Strains carrying *Cry1Ia* gene tend to display insecticidal activities against lepidopterans (*Ostrinia nubilalis*), Fall armyworm (*Spodoptera frugiperda*), and coleopterans (*Leptinotarsa decimlineata*) as reported by Khorramnejad et al. (2020). PCR using other primer pairs amplifying genes *cry7–8*, *cry9*, *cry19*, *cry1D*, *cry1E* and *cry13* revealed the absence of these genes in the studied strains (data not shown). In fact, PCR using other primer pairs amplifying the genes *cry7–8*, encoding insecticidal activities against coleoptera; *cry9* and *cry19* encoding insecticidal activities against Diptera; *cry 1D* encoding insecticidal activity against specifically *Spodoptora littoralis*; *cry1E* encoding insecticidal activity against specifically *Spodoptora littoralis*; and *cry13* gene encoding potential toxicity against nematodes; revealed the absence of all these genes in the studied strains (data not shown). The strains that exhibited the same crystal forms displayed similar amplification patterns. The presence of the following genes was confirmed in the studied strains similar to HD1; *vip3Aa58*, *cry1Aa/cry1Ac*, *cry2Ab*, *cry1Ba* and *cry1Ia*, which exhibits potential toxicity against the orders lepidopteran, dipteran, and hemipteran, respectively.

3.2. Study of the synthesis of δ-endotoxins by Bt strains

The exploration of the synthesis of the δ-endotoxins revealed differences in the production per mL and per cell. The strain QBT474 produced the highest concentration of δ-endotoxins (161.83 ± 1.90 μg/mL), and the strain QBT556 the lowest (40.17 ± 2.15 μg/mL) (Table 1). In term of CFU, QBT474 reached the highest value (250 ± 1.46 × 10^6 cfu/mL). Therefore, in term of δ-endotoxin synthesis per cell, QBT474 has the highest rate of 647.32 ± 8.49 10^{-6} ng/cell (Table 1). Along with the nature of *cry* genes and growth conditions, the production of

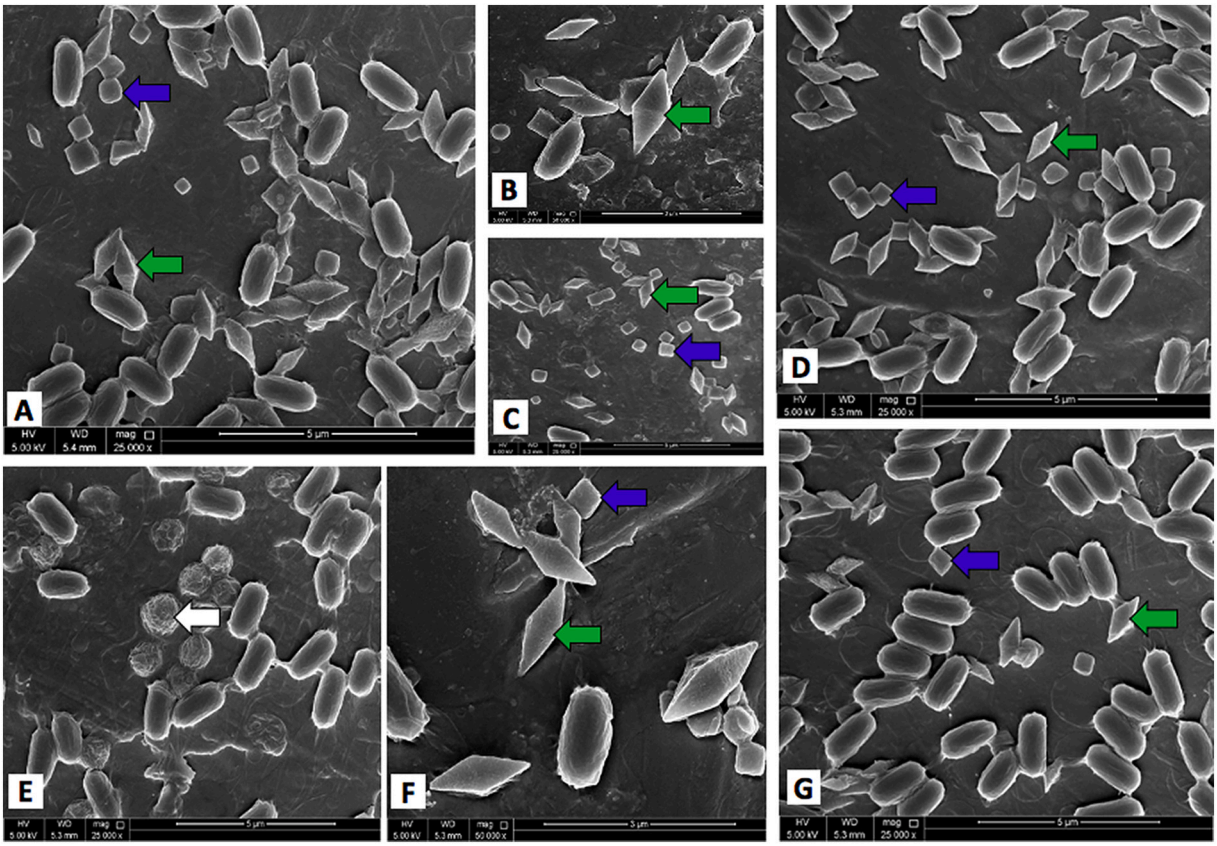

Fig. 3. Scanning electron microscopy (SEM) observation of *B. thuringiensis strains* spores and crystals. A, *Bt kurstaki* HD1 (reference strain); B, *Bt* strain QBT282; C, *Bt* strain QBT474; D, *Bt* strain QBT376; E, *Bt* strain QBT674; F, *Bt* strain QBT556; G, *Bt* strain QBT671. Green arrows are pointing at bipyramidal, blue at cuboidal and white at spherical crystals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

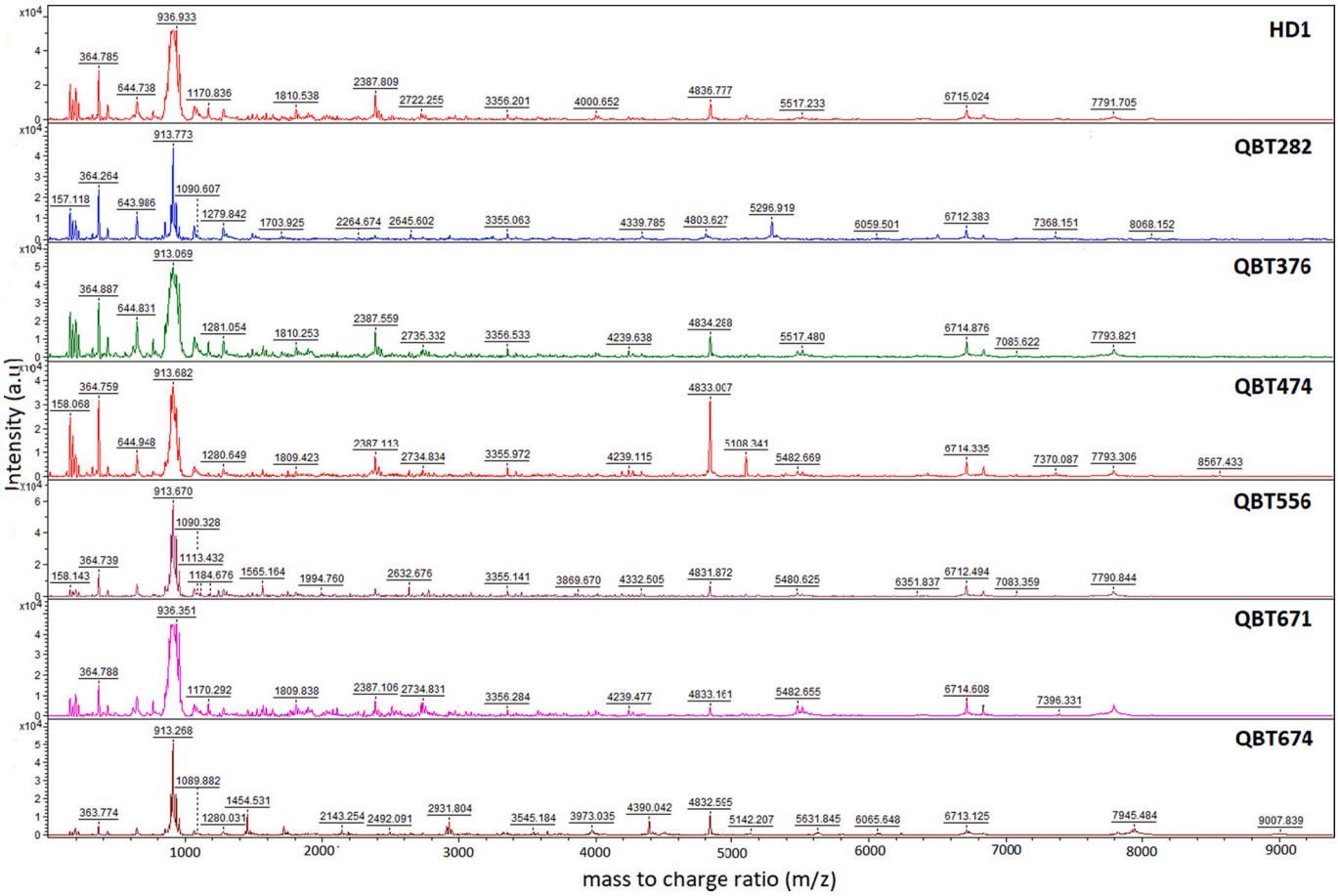

Fig. 4. Crystal protein spectra of *B. thuringiensis* strains through MALDI-TOF MS.

δ-endotoxins by *Bt*, largely depends on the expression of these *cry* genes encoding δ-endotoxins (Jamil et al., 2021). In line with the present study, on the basis of δ-endotoxins production, Nair et al. (2018) found at least 16 different protein profiles among the *Bt* strains isolated from Qatari soil. In another study, Hassan et al. (2021), isolated 12 *Bt* strains from the Eastern province of Saudi Arabia having potential to produce δ-endotoxins. Although they didn't quantify the levels of δ-endotoxins per bacterial cell, they found typical *Bt* crystals, *cry* genes and insecticidal activities against dipteran and lepidopteran larvae.

3.3. Exploration of the B. thuringiensis strains crystal forms by scanning electron microscopy (SEM)

B. thuringiensis produces parasporal crystals proteins that play a major role in their insecticidal activity. These parasporal crystals have different forms depending on the nature of their δ-endotoxins and corresponding genes (Wang et al., 2013). The level of crystals synthesized by the bacteria depends on the expression of encoding genes. In the present study, to characterize each strain in terms of their parasporal activity, the morphology of their crystals was studied using electron microscopy. Fig. 3 shows the SEM images of crystal morphologies of different *Bt* strains.

As shown in Fig. 3, strains QBT282 (Fig. 3B), QBT474 (Fig. 3C), QBT376 (Fig. 3D), QBT556 (Fig. 3F) and QBT671 (Fig. 3G) are displaying similar characteristic to the reference strain HD1 (Fig. 3A) by producing bipyramidal and cuboidal crystals. *cry2Ab* is a gene encoding cuboidal crystal structures (Adalat et al., 2017), and the genes *cry1Aa* and *cry1Ac* are encoding the bipyramidal crystals (Evdokimov et al., 2014). The presence of these genes in the genetic material of the strains was further investigated in this study using specific PCR primers. The majority of the Qatari *Bt* explored strains produce bipyramidal and cuboidal crystals including the HD1. Only the strain QBT674 exhibited a spherical form of crystals. Despite many strains displaying almost similar morphologies in both the light microscopic and SEM observations, the shapes and the sizes were not completely identical; the differences in their structure, width and length could be noticed from the SEM. The form of crystals presents in each strain mainly depends on the sequence of the proteins included in the crystals. This trait is considered to be unique for *B. thuringiensis*. Hassan et al. (2021) found similar bipyramidal and cuboidal crystal morphologies in 12 *Bt* strains isolated from the soil samples in Saudi Arabia. Although crystals morphologies were similar but the *cry* gene distribution in the strains ranged from 54 % to 100 %, whereas *cry1* gene was found in 100 % of the strains.

3.4. Investigation of the crystal protein content through MALDI-TOF-MS

In this work, the combination of MALDI-TOF MS and PCA was used for the 1st time, to study the protein content of different crystals for 6 different Qatari *Bt* strains along with reference HD1 strain. The spectra generated through MALDI-TOF on the bacterial crystal proteins define many of the characteristics of the crystals in each strain, as it is responsible for its structural form as well as its insecticidal activity. In Fig. 4, despite similarity in crystals morphologies, the protein spectra are quite different in different strains of bacteria. These differences might be associated with the size variation as well as the range of insecticidal activities of bacterial strains. For instance, strains HD1 (reference strain), QBT474, QBT376, QBT556, and QBT671 all produced bipyramidal and cuboidal crystals as shown in Fig. 3. But their crystals protein spectra are not similar, suggesting differences in the crystal's proteins and/or detailed morphological differences. Among all the

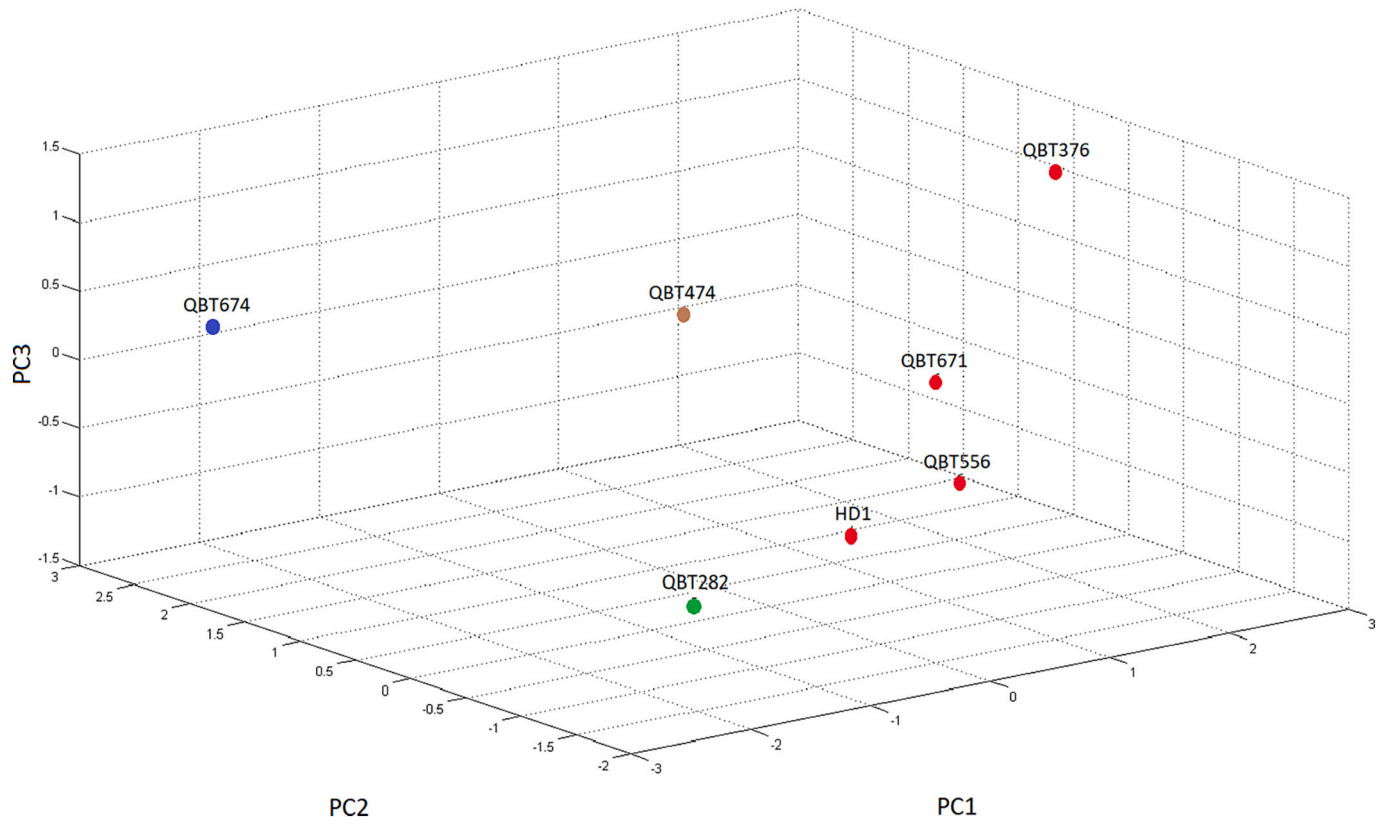

Fig. 5. 3D plot of the principal component analysis of the MALDI-TOF MS spectra obtained from analyzing the protein content of crystals of different strains of *B. thuringiensis* (PC1, PC2, and PC3 together showed 85 % of the variation in the dataset).

studied strains, QBT376 showed the most similar spectra to the reference HD1 strain, which was further confirmed by the principal component analysis (PCA) shown in Fig. 5. In the accessible published literature, there is no similar study on the protein profiling and differentiation of *Bt* crystal.

Principal component analysis (PCA) of Bt protein spectra obtained through MALDI-TOF shows a significant difference among the strains even though they produced similar crystals. Most of the strains (HD1, QBT376, QBT474, QBT556, QBT671) produced both bipyramidal as well as cuboidal crystals and are relatively closer to each other in the PCA plot. All these strains are positively correlated to PC1, which demonstrates 45 % of the variation in the dataset. Strain QBT282 produced mainly bipyramidal crystals and is much far from the reference HDI (producing both bipyramidal and cuboidal crystals) having negative correlation to both PC1 and PC2 (represents 28 % of the variation in the dataset).

Similarly, strain QBT674 produced only spherical crystals and is placed far away from the HD1 in the PCA plot demonstrating differences in their protein contents. These results indicate that even minor differences in the crystal morphologies (or their insecticidal properties) are clearly highlighted in the MALDI-TOF and PCA analysis suggesting that this is a powerful tool for studying the Bt strains. Several biocontrol agents work very efficiently under in vitro conditions. However, when these are tested in the field conditions, there efficacy is not as good as expected. This might be the case with our local Bt strains which produced high levels of cry proteins, known for their insecticidal potential.

4. Conclusions

PCR confirmed the presence of different *cry* and *vip* genes that encode for a variety of insecticides active against wide range of pests. A local strain (QBT474) had the highest δ-endotoxin production, which might be considered for the industrial production of biological insecticides. Five *Bt* strains showed to have bipyramidal and cuboidal protein crystals, while one (QBT674) showed a spherical protein crystal. MALDI-TOF protein profiling showed that QBT376, QBT556 and QBT671 have a greater similarity to HD1. For the first time it was demonstrated that application of MALDI-TOF crystal profiling allows to evidence even minor differences in *Bt* crystal proteins.

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CRediT authorship contribution statement

A.A. and S.J.: Conceptualization; A.A., M.Y.A. and S.J.: Methodology; A.A., M.Y.A., Z.U., R.A., and S.J.: Validation and analysis of results; S.J: Resources provided; A.A., Z.U., M.Y.A., R.A. and S.J.: Writing and reviewing; S.J.: Supervision.

Declaration of competing interest

There is no conflict of interest regarding this manuscript submitted for publication in Bioresource Technology Reports.

This manuscript has neither been published elsewhere, nor has been simultaneously submitted elsewhere for publication.

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