

# Characterization of new *Bacillus thuringiensis* strains from Iran, based on cytotoxic and insecticidal activity, proteomic analysis and gene content

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**Abstract** Characterization of new *Bacillus thuringiensis* strains is a valuable tool to discover novel insecticidal toxins and to manage resistance problems. In this study, seven Iranian Bt strains were selected according to their toxicity against *Plodia interpunctella*, to be thoroughly characterized based on their toxicity, protein profiling, proteomic analysis, gene content and  $\beta$ -exotoxin production. The toxicity was assessed by insect bioassays and cell viability assays (a less cost, time and material consuming technique), using four lepidopteran pests and four lepidopteran cell lines from *Trichoplusia ni* (Hi5), *Helicoverpa zea* (HzGUT), *Spodoptera exigua* (UCR-SE) and *Spodoptera frugiperda* (Sf21). The selected

Bt strains showed similar protein electrophoretic profiles, but differed in toxicity. LC–MS/MS analysis of solubilized crystal proteins and gene content analyses (PCR screening) were compared and correlated with the toxicity results. Based on our data, three Bt strains could be considered as candidates for development of future bioinsecticides.

**Keywords** *Bacillus thuringiensis* screening · LC–MS/MS · Cell viability assay · Lepidopteran cell line ·  $\beta$ -exotoxin · PCR

## Introduction

*Bacillus thuringiensis* (Bt), a gram positive, rod-shaped and spore forming bacterium has proven to be a useful entomopathogen for microbial control of pests. Nowadays, Bt-based insecticides or transgenic plants expressing Bt insecticidal proteins are extensively used worldwide as a powerful alternative to chemical treatments (Bravo et al. 2011).

Bt synthesizes parasporal crystal proteins that are toxic to many insects, nematodes, mites, protozoa and human cancer cells (Schnepf et al. 1998; van Frankenhuyzen 2009; Ohba et al. 2009). Due to the high specificity, safety and specific insecticidal activity of the Bt toxins, several studies have been focused on Bt isolation from diverse sources (Bel et al. 1997; Uribe et al. 2003; Berón and Salerno 2006; Seifinejad et al.

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2008; Djenane et al. 2017). But the extensive application of Bt-based products has raised some concerns about the appearance of insect resistance, which has been reported in some species (Ferré et al. 1995). Thus, characterization of new Bt strains is necessary to discover novel toxins with new specificities or with a broader range of insecticidal activity, and to better manage insect resistance problems (Ferré et al. 1995; Juárez-Pérez et al. 1997).

In many Bt screening research works, it has been shown that PCR-based characterization is helpful to assess gene content and therefore to predict the toxicity of the new Bt isolates (Juárez-Pérez et al. 1997; Ferrandis et al. 1999). However, this quick and accurate technique lacks the ability to indicate the expression level of the detected genes. Further, the relative proportion of each protein in parasporal crystals can play an important role in the insecticidal potency of the Bt strain. Therefore, liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis could be considered as a good method to detect protein composition of the newly analyzed Bt strains and to predict their toxicity. By considering the interaction between different toxins produced by a strain in insect midgut, bioassays provide complementary and necessary characterization information. Due to the money, time and material costs associated to insect rearing and time-consuming characteristics of insect bioassays, cell-based assays have been employed for toxicity characterization of Bt strains or toxins (Kwa et al. 1998; Gringorten et al. 1999; Willcoxon et al. 2016). Moreover, the use of insect cell lines allows researchers to work with insect species without considering regulatory restrictions or prohibitions.

The aim of this study was to characterize new Bt strains isolated from soil and lepidopteran infected larvae to assess their possible potential as biopesticides against lepidopteran pests. For this purpose, strains with diverse larvicidal activity against Indian meal moth, *Plodia interpunctella* (Hubner) (Lep.: Pyralidae), were characterized according to their protein profile and gene content (*cry*, *cyt*, *vip* and *ps* genes). Afterwards the *cry* gene expression was verified by LC-MS/MS analysis of solubilized crystal proteins. The spectrum of lepidopteran toxic activity was assessed against *Spodoptera exigua* (Hubner) (Lep.: Noctuidae), *Mamestra brassicae* (L.) (Lep.: Noctuidae), *Grapholita molesta* (Busck) (Lep.:

Tortricidae) and *Ostrinia nubilalis* (Hubner) (Lep.: Crambidae) first instar larvae. Then, to complete the analysis of the insecticidal activity, in vitro toxicity assays with lepidopteran cell lines (cytotoxic activity) were performed. As a final step in characterization, the production of  $\beta$ -exotoxin was investigated as a necessary requirement to propose, at least, a new Bt strain as a candidate for commercial use.

## Materials and methods

### Bacterial strains

Seven Iranian Bt strains from the Bt stock of Insect Biological Control laboratory at the University of Tehran, were used in the study. The selected Bt strains were isolated from soil and diseased and dead larvae. Phase contrast microscopy was used to confirm the presence of the parasporal crystals and to determine their shape. The *B. thuringiensis* HD1 strain HD1S2005 (Bt HD1) (kindly provided by Valent Bioscience, Inc., Libertyville, IL, USA) and the *B. thuringiensis* subsp. *thuringiensis* strain HD2 (Berliner) (Bt HD2) (obtained from the Bacillus Genetic Stock Center, BGSC, Ohio State University, Ohio, USA), were used as reference standard strains.

### Insect rearing

*Plodia interpunctella* was obtained from the Insect Physiology laboratory, Department of Plant Protection, University of Tehran (Iran), and reared for at least five years in Insect Biological Control laboratory at the same University. *S. exigua* and *M. brassicae* were obtained from the laboratory of Dr. Primitivo Caballero (Universidad Pública de Navarra, Spain). *Grapholita molesta*, was obtained from Entomos AG (Switzerland). *Ostrinia nubilalis* was obtained from the INRA (France). These insect colonies have been reared for at least three years in the laboratory of the University of Valencia (Spain) on artificial diet (McGaughey and Beeman 1988; Hernández-Martínez et al. 2008; Gomis-Cebolla et al. 2017; Bel et al., 2011), and kept at  $25 \pm 1$  °C with  $70 \pm 5\%$  RH with a 16:8 h L:D photoperiod.

## Toxicity bioassays

The insecticidal activity of seven Bt strains was assessed against *P. interpunctella* second instar larvae and first instar larvae of *S. exigua*, *G. molesta*, *O. nubilalis* and *M. brassicae*. The susceptibility of *P. interpunctella* larvae towards selected Bt strains was investigated in a single discriminating dose of  $10^8$  spores  $\text{ml}^{-1}$  based on previous analyses (data not shown) by food incorporation method. The assays with *P. interpunctella* were carried out in triplicate using 20 second instar larvae in each replicate and the mortality was recorded 72 h after treatment.

The susceptibility of *S. exigua*, *G. molesta*, *O. nubilalis* and *M. brassicae* first instar larvae to solubilized protoxins or to activated toxins (see next paragraph for solubilization and activation of the Cry proteins) at a single concentration of  $1000 \text{ ng cm}^{-2}$ , was assessed by surface contamination method (Hernández-Martínez et al. 2008). The bioassays were repeated three times (48 larvae in each replicate). The buffer used for Bt crystal proteins solubilization (carbonate buffer 50 mM, 0.1 M NaCl, 10 mM dithiothreitol, pH 10.5) was used for negative controls. In order to compare the toxicity of the tested strains with previous studies, we selected Bt HD1 as reference strain. Bioassays were conducted at  $25 \pm 1^\circ\text{C}$ ,  $70 \pm 5\%$  RH and a 16:8 h L:D photoperiod. Mortality was scored after seven days. The mean of mortality percentages and the SE were calculated using Graphpad Prism 7.0 software (La Jolla California, USA). The same software was used to compare the mortalities with a one-way analysis of variance (ANOVA). If the one-way ANOVA was significant ( $P < 0.05$ ), mortality means were analyzed using the Tukey test, considering them significantly different when  $P < 0.05$ .

## Production, solubilization and activation of Cry proteins

The production and further solubilization of the Bt crystal proteins was performed as described by Estela et al. (2004). The solubilized protoxins obtained were mixtures of different non-activated, partially-activated, and activated forms. The full protein activation was carried out with 10% trypsin (w/w) for 2 h at  $37^\circ\text{C}$ . The solubilized protoxins and trypsin activated proteins were analyzed by sodium dodecyl sulphate

12% polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Protein concentrations were determined by Bradford assay (Bradford 1976).

## Insect cells lines and cell viability assays

Four insect cell lines were used in this study: BTI-Tn-5B1-4 (Hi5) from ovarian cells of cabbage looper, *Trichoplusia ni* (Granados et al. 1994), RP-HzGUT-AW1 (HzGUT) from gut cells of *Helicoverpa zea* (Goodman et al. 2004), UCR-SE from beet armyworm, *S. exigua* (Gelernter and Federici 1986) and Sf21 cells from ovaries of the fall armyworm, *S. frugiperda* (Vaughn et al. 1977). HzGUT and UCR-SE cells were grown in Excell insect cell culture medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS). Hi5 and Sf21 were maintained in TNM-FH medium (Sigma-Aldrich) with 5% FBS and Grace's medium (Gibco® Life technologies) with 10% FBS, respectively. Insect cell lines were maintained under the conditions recommended by the supplier (Laboratory of Virology, Wageningen University, The Netherlands).

The cytotoxic activity was assessed by microscopy and cell viability assays. Cells with clear morphological changes induced by toxins (swollen cells, balloon-shaped cells and lysed cells), were recognized with an inverted microscope (Leica DMI 3000B) and recorded as affected cells. For the assays, the number of cells was determined with a Countess Automated Cell counter (Invitrogen). Aliquots of  $100 \mu\text{l}$  of cell suspension at a concentration of  $2 \times 10^6$  cells  $\text{ml}^{-1}$  were seeded into 96-well plates and incubated at  $25^\circ\text{C}$  for at least 2 h. For the single-dose assays,  $10 \mu\text{l}$  of either solubilized protein ( $1 \text{ mg ml}^{-1}$ ) or trypsin activated toxin ( $0.7 \text{ mg ml}^{-1}$ ), were added into each well and cell viability was measured after 16 h. For dose-response assay, cells were treated with  $10 \mu\text{l}$  of trypsinized protein at five different concentrations (from 0.64, 3.2, 16, 80 to  $400 \mu\text{g ml}^{-1}$ ) and cell viability was measured after 6 h. After adding CellTiter 96® AQueous One Solution Reagent (Promega Co., Madison, WI, USA), cell viability was quantified by measuring the absorbance at 490 nm in a spectrophotometry plate reader (Tecan infinite Pro200). Negative controls were performed with protein buffer (50 mM  $\text{Na}_2\text{CO}_3$  buffer, pH 10.5) and positive controls were performed with 0.2% Triton X-100. The percentages of viability were calculated

as:  $[(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{positive control}}) / (\text{Absorbance}_{\text{negative control}} - \text{Absorbance}_{\text{positive control}})] \times 100$ . Three different experiments (each one with replicated points) were carried out for each protein and insect cell line. Taking into account the number of cells seeded on the plates and the percentages of viability obtained, the number of viable cells were calculated. The mean of the viable cells in each cell line tested with activated and non-activated crystal proteins were compared using an unpaired *t* test. Values were considered statistically different when P values were lower than 0.05. The assays performed with different protein concentrations were analyzed with non-linear regression (sigmoidal curve fitting) using Graphpad Prism 7.0 software. This analysis provides the parameters of the half-maximal effective concentration ( $EC_{50}$ ), with 95% confidence interval and the slope of the sigmoidal curve.  $EC_{50}$  represents the concentration of a compound where 50% of its maximal effect is observed. The slope describes the steepness of the dose–response curve.  $EC_{50}$  values were considered similar when overlapped 95% confidence intervals, and different when there was no overlapping.

### Proteomic analysis

LC–MS/MS analysis were performed at the proteomics facility of the SCSIE (Servei Central de Suport a la Investigació Experimental), at the University of Valencia, Valencia, Spain. Briefly, samples were subjected to trypsin digestion and loaded onto a trap column (NanoLC Column, 3  $\mu\text{m}$  C18-CL, 75  $\mu\text{m} \times 15$  cm; Eksigent). The peptides obtained were loaded onto an analytical column (LC Column, 3  $\mu\text{m}$  C18-CL, 75  $\mu\text{m} \times 12$  cm, Nikkyo) and analyzed in a mass spectrometer nanoESI qTOF (5600 TripleTOF, ABSCIEX). ProteinPilot v5.0. search engine (ABSciex) with default parameters was used to generate peak list. The Paragon algorithm of ProteinPilot was used to search NCBI and ExPasy protein database. The LC–MS/MS results with the confidence of identification of at least 95% were considered significant. Redundant proteins obtained in the analysis were omitted, but proteins with high ProtScore values and proteins with several discriminating, distinct and specific peptides, were recorded.

### $\beta$ -exotoxin detection

The presence of  $\beta$ -exotoxin in seven Bt strains was also determined by LC–MS/MS. Sample preparation was done as described by Hernández et al. (2003). The standard  $\beta$ -exotoxin (type I) from Bt HD2 was kindly supplied by I. Thiéry (Institut Pasteur, France) and was used to obtain the calibration curve. It has been reported that  $\beta$ -exotoxin can be detected in Bt HD2 and *B. thuringiensis* subsp. *thuringiensis* VMA10.30 strains, but is absent in Bt HD1 strain (Hernández et al. 2003). Samples with absence of  $\beta$ -exotoxin prepared from Bt HD1 strain were used as negative controls and samples with  $\beta$ -exotoxin, prepared from Bt HD2 and VMA10.30 strains, were used as positive controls.

### Gene content identification

The total DNA from the selected Bt strains was extracted and purified following Ferrandis et al. (1999). Bt strains were characterized by using primers designed in this work or previously described in the literature (Supplementary Table S1). PCR reactions were conducted in 25  $\mu\text{l}$  reaction mixtures containing 1  $\mu\text{l}$  of 100  $\text{ng } \mu\text{l}^{-1}$  of DNA template, 1 U of *taq* DNA polymerase (Biotools B&M labs, S.A. Madrid, Spain), 2.5  $\mu\text{l}$  of 10 $\times$  standard reaction buffer with  $\text{MgCl}_2$  (50 mM  $\text{MgCl}_2$  solution), 0.4  $\mu\text{M}$  of each primer and 0.2 mM of dNTPs, in an Eppendorf Mastercycler thermal cycler (Eppendorf AG, Barkhuasenweg, Germany). PCR conditions were: 5 min of initial denaturation at 94  $^{\circ}\text{C}$  followed by 30 cycles of amplification with 1 min of denaturation at 94  $^{\circ}\text{C}$ , 45–60 s of annealing at 45–67  $^{\circ}\text{C}$  (depending on each pair of primers), 1–2 min of extension at 72  $^{\circ}\text{C}$  and an extra extension step of 10 min at 72  $^{\circ}\text{C}$ . PCR products were analyzed by 1% agarose gel electrophoresis. The amplified DNA was purified using NucleoSpin Gel and PCR Clean-up kit (MACHEREY–NAGEL, Germany) and sent to Stab Vida (Investigação e Serviços em Ciências Biológicas Lda, Portugal) for sequencing. The sequences were edited and analyzed using Geneious software (version 10.0.9). The DNA sequence analyses including alignment and Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov>) were performed using the NCBI database and Geneious software.

## Results

### *B. thuringiensis* strains characterization

The selection of Iranian Bt strains was performed according to the toxicity of 130 Bt strains against Indian meal moth, *P. interpunctella*, second instar larvae at a single discriminating concentration of  $10^8$  spores  $\text{ml}^{-1}$  (data not shown). Five Bt strains, IE-1, AzLp, IE-2, IP-2 and IEp, isolated from infected larvae and showing bipyramidal crystals, were highly toxic *P. interpunctella* (Table 1). Besides, KhF (spherical crystals) and RM (irregular crystals) strains respectively with low and non-toxic activity towards *P. interpunctella* (Table 1), were chosen to determine the specific properties of the five highly toxic ones.

A bioassay screening at a single concentration of protoxins and trypsin activated toxins was performed for the seven selected strains and for the Bt HD1 reference strain, using first instar larvae of *S. exigua*, *G. molesta*, *M. brassicae* and *O. nubilalis* (Table 1). As a result, *G. molesta* and *O. nubilalis* were the most susceptible insects. Various degrees of mortality were found in *S. exigua* and *M. brassicae* first instar larvae treated with the highly toxic strains. Among those, AzLp, IE-2 and IP-2 protoxins and activated toxins, exhibited higher levels of toxicity for *S. exigua* and *M. brassicae* compared to that of the reference strain, Bt HD1. Neither toxic activity nor growth inhibition was observed for KhF protoxins. The toxicity of the trypsinized proteins of KhF was not analyzed since it was not possible to solubilize the KhF crystals not even after trying different buffer compositions and pH conditions (data not shown).

To assess the possible toxicity of the strains due to  $\beta$ -exotoxin production, LC-MS/MS was also used to check whether the selected strains produce this metabolite. The results showed that none of the seven selected Bt strains produced type I  $\beta$ -exotoxin, similarly to what was found for the negative control strain Bt HD1.

### Cell viability

The cytotoxic spectrum of the selected Bt strains and the reference strain Bt HD1 was studied against four lepidopteran cell lines in terms of loss of cell viability. Figure 1 shows the results of cytotoxic activities (% viability) of activated vs. non-activated proteins. In

HzGUT cells, the cytotoxic activities of activated and non-activated proteins were significantly different for all the Bt strains (P values from  $< 0.0001$  to 0.0268), whereas in the Sf21 cell line no significant differences were found. In UCR-SE and Hi5 cell lines, significant differences in viability were observed when the cells were treated with protoxins or activated toxins of IE-2 (P values 0.0350 and 0.0004, respectively), and IEp (P values 0.0057 and  $< 0.001$ , respectively) strains, and IP-2 treatments also induced differences in UCR-SE (P-value = 0.0044). Differences in membrane associated proteases involved in Cry proteins activation could explain the results obtained from the analyzed cell lines.

In general, proteins from Bt HD1 showed similar toxicity to all cell lines (94% mean value of cells viability). Proteins from AzLp, IE-2 and IP-2 Bt strains were highly cytotoxic for the four tested cell lines with 60–100% cell viability reduction (Fig. 1). Indeed, after 16 h cell exposure, no Sf21 viable cells could be observed. The least toxic for all the tested cell lines were proteins from IE-1 and RM strains (92% mean value of cells viability). The toxicity of IEp protoxins to all cell lines was similar (92% mean value of cells viability), while the toxicity of IEp trypsinized toxins ranged from 24 to 93%.

Sf21 cell line was employed for further investigation due to its high susceptibility to Bt toxins. The results of the dose–response assays with trypsin activated proteins of AzLp, IE-2 and IP-2 strains, revealed that the viability of Sf21 cells decreased with the increase of trypsin activated toxin concentrations. Osmotic swelling and balloon-shaped cells were observed after 3 h exposure of the cells to a mid-protein concentration ( $16 \mu\text{g ml}^{-1}$ ) and lysis was observed when cells were exposed to highest concentrations (Fig. 2). The statistical analyses showed that  $\text{EC}_{50}$  values of AzLp and IE-2 were different (Table 2).

### Protein analyses

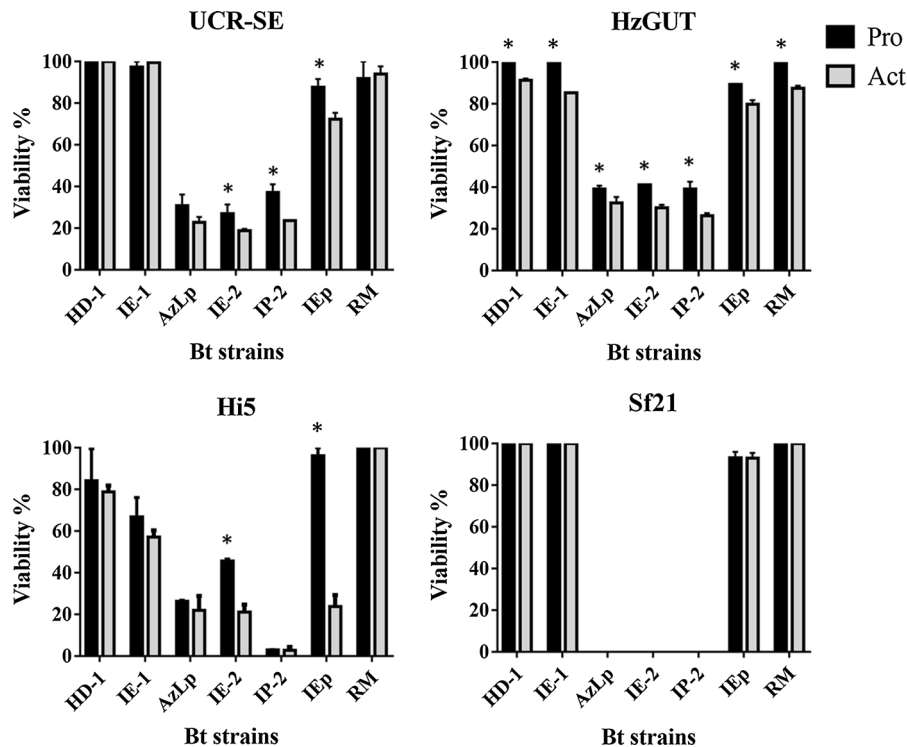
The protein patterns of the parasporal crystals of the seven selected Bt strains were obtained by SDS-PAGE electrophoreses. The SDS-PAGE profiles of the standard strain Bt HD1 and the selected strains were similar, with protein bands of molecular weights between 60 and 130 kDa (Fig. 3a). As it is known, Bt HD1 produces Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa

**Table 1** Insect toxicities of the seven selected Iranian Bt strains, and the reference strain Bt HD1

Strain	Origin	$10^8$ spores $ml^{-1}$				Trypsin activated ( $1000\text{ ng cm}^{-2}$ )				
		<i>P. interpunctella</i>	<i>S. exigua</i>	<i>G. molesta</i>	<i>O. nubilalis</i>	<i>M. brassicae</i>	<i>S. exigua</i>	<i>G. molesta</i>	<i>O. nubilalis</i>	<i>M. brassicae</i>
Bt HD1	Valent bioscience	–	$6 \pm 4^c$	$99 \pm 1^a$	$100 \pm 0^a$	$38 \pm 13^b$	$10 \pm 5^c$	$100 \pm 0$	$100 \pm 0^a$	$60 \pm 11^{ab}$
IE-1	<i>Ectomyeolis ceratonia</i> larvae	$67 \pm 3^c$	$56 \pm 7^b$	$99 \pm 1^a$	$94 \pm 6^a$	$8 \pm 2^c$	$48 \pm 8^b$	$100 \pm 0$	$100 \pm 0^a$	$41 \pm 17^{bc}$
AzLp	<i>P. interpunctella</i> larvae	$68 \pm 6^{bc}$	$99 \pm 1^a$	$99 \pm 1^a$	$98 \pm 2^a$	$94 \pm 4^a$	$97 \pm 3^a$	$100 \pm 0$	$100 \pm 0^a$	$100 \pm 0^a$
IE-2	<i>E. ceratonia</i> larvae	$88 \pm 4^{ab}$	$95 \pm 4^a$	$100 \pm 0^a$	$98 \pm 2^a$	$79 \pm 13^a$	$96 \pm 4^a$	$100 \pm 0$	$86 \pm 7^a$	$82 \pm 17^{ab}$
IP-2	<i>P. interpunctella</i> larvae	$100 \pm 0^a$	$100 \pm 0^a$	$100 \pm 0^a$	$98 \pm 2^a$	$97 \pm 2^a$	$98 \pm 0^a$	$100 \pm 0$	$98 \pm 2^a$	$100 \pm 0^a$
IEp	<i>Ephesia kuehniella</i> larvae	$100 \pm 0^a$	$41 \pm 11^b$	$100 \pm 0^a$	$92 \pm 2^a$	$22 \pm 7^{bc}$	$55 \pm 3^b$	$100 \pm 0$	$100 \pm 0^a$	$24 \pm 14^{bc}$
RM	Forest (flowering plant)	$0 \pm 0^c$	$20 \pm 9^{bc}$	$100 \pm 0^a$	$98 \pm 2^a$	$8 \pm 6^c$	$42 \pm 10^b$	$100 \pm 0$	$100 \pm 0^a$	$29 \pm 9^{bc}$
KhF	Forest (grass)	$42 \pm 2^d$	$2 \pm 2^c$	$25 \pm 3.5^b$	$2 \pm 2^b$	$4 \pm 2^c$	–	–	–	–
ANOVA statistics										
	F-value	88.4	47.8	383.8	139.4	44.1	33.3	–	3.1	7.6
	Degrees of freedom numerator	6	7	7	7	7	6	–	6	6
	Degrees of freedom denominator	14	16	16	16	16	14	–	14	14
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.038	0.0009

Values represent the mean of the percentages of mortality  $\pm$  SE. The bioassays were performed against second instar larvae of *P. interpunctella*, or first instar larvae of the other lepidopteran species. Statistics of one-way ANOVA analysis for each column data are included, and the analyses which rendered P values lower than 0.05 were followed by Tukey test. Different letters within each column represent statistical significant differences (Tukey test P-value < 0.05) among the Bt strains used. – not analyzed



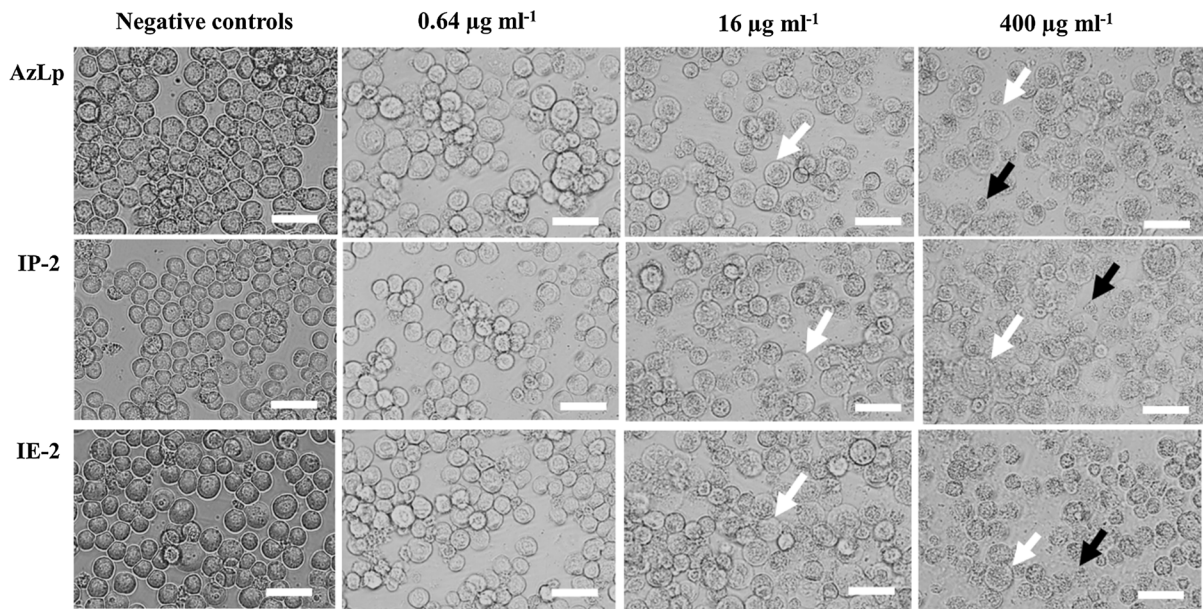


**Fig. 1** Susceptibility of UCR-SE, HZGUT, Hi5 and Sf21 cells to protoxins (Pro) and trypsin activated proteins (Act) from the different Bt strains. Bars represent percentages of viability after 16 h of exposure to 1 mg ml<sup>-1</sup> of protoxins or 0.7 mg ml<sup>-1</sup> of activated toxins. SE of the results from three different experiments were represented in each bar. (\*) Statistically significant differences between protoxin and activated protein treatments (*t* test): in HZGUT cell line; HD-1 (*t* = 18.6, *df* = 4, *P* < 0.0001), IE-1 (*t* = 254.6, *df* = 4, *P* < 0.0001), AzLp

(*t* = 3.4, *df* = 4, *P*-value = 0.0268), IE-2 (*t* = 12.7, *df* = 4, *P*-value = 0.0002), IP-2 (*t* = 6.0, *df* = 4, *P*-value = 0.0039), IEp (*t* = 8.6, *df* = 4, *P*-value = 0.0010) and RM (*t* = 18.0, *df* = 4, *P* < 0.0001). In UCR-SE cell line; IE-2 (*t* = 3.1, *df* = 4, *P*-value = 0.0350), IP-2 (*t* = 5.8, *df* = 4, *P*-value = 0.0044) and IEp (*t* = 5.4, *df* = 4, *P*-value = 0.0057). In Hi5 cell line; IE-2 (*t* = 11.2, *df* = 4, *P*-value = 0.0004) and IEp (*t* = 18.7, *df* = 4, *P*-value < 0.0001)

proteins (Schnepf et al. 1998). This composition fits with the occurrence of two main bands of about 130 kDa (which would correspond to Cry1 protoxins) and 65 kDa (which would correspond to Cry2 protoxins). The presence of other bands between 130 and 60 kDa can be due to partial processing of the main proteins. Similarly to Bt HD1, our strains contained a protein band of around 130 kDa in size, which could correspond to Cry1 but also to Cry7, Cry8 or Cry9-type proteins (Arrieta et al. 2004). Parasporal inclusions from strains Bt HD1, IE-1, IP-2 and IEp showed a distinct additional band of 65–75 kDa that could correspond to Cry2, Cry3 or Cry22-type proteins. After solubilization and trypsin activation, the protein profiles were almost identical for all strains and showed bands of about 60 kDa (Fig. 3b), a common molecular weight of activated Cry toxins.

The insecticidal crystal proteins produced by each one of the Bt strains were analyzed by LC–MS/MS. The proteins were retrieved from the raw data following the criteria specified in Materials and Methods and were further validated by using BLAST. The detected proteins for each Bt strains were sorted according to their relative abundance based on the numbers of distinct peptides found (having at least 95% confidence) for a specific protein (Supplementary Table S2). The final analysis showed that the proteins present in the solubilized crystal protein mixtures of the selected Bt strains were Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Da and Cry2Aa (Table 3). The proteins from the Cry1A family were the most abundant ones according to quantitative values. Cry1Ac was the only protein assigned to RM strain. For KhF strain, no protein similar to the known Bt insecticidal crystal



**Fig. 2** Sf21 cells after treatment with AzLp, IE-2 and IP-2 activated toxins. Morphological changes recorded after 3 h of exposure to 0.64, 16 and 400  $\mu\text{g ml}^{-1}$  of protein. Bars in the

figure represent 50  $\mu\text{m}$ . White arrows point to swollen cells. Black arrows indicate lysed cells

**Table 2** Toxicity parameters of trypsin activated proteins of AzLp, IE-2 and IP-2 Bt strains vs. Sf21 cells

Bt strain	$\text{EC}_{50}$ ( $\mu\text{g ml}^{-1}$ )	95% confidence interval	Slope $\pm$ SE
AzLp	1.89	1.50–2.36	$-1.67 \pm 0.22$
IE-2	1.03	0.84–1.27	$-1.53 \pm 0.18$
IP-2	1.60	1.14–2.23	$-1.46 \pm 0.24$

The table include the half-maximal effective concentration ( $\text{EC}_{50}$ ) values, with 95% confidence interval and the slope of the dose-response curve

proteins were identified. Probably, either the insecticidal crystal proteins in KhF strain were under the detectable level of Paragon algorithms employed, or the KhF parasporal crystals contain new insecticidal crystal proteins.

#### PCR screening of insecticidal Bt coding genes

PCR analysis was carried out to identify genes encoding proteins secreted during vegetative phase of Bt growth (such as *cryII*, *vip1*, *vip2*, *vip3* and *sip1*), as well as genes encoding proteins that accumulate in crystals produced during the sporulating phase, of the *cry*, *cyt* and *ps* gene families. The amplification of *cry2* gene was observed in all tested strains. Conversely, none of the strains showed amplification products for

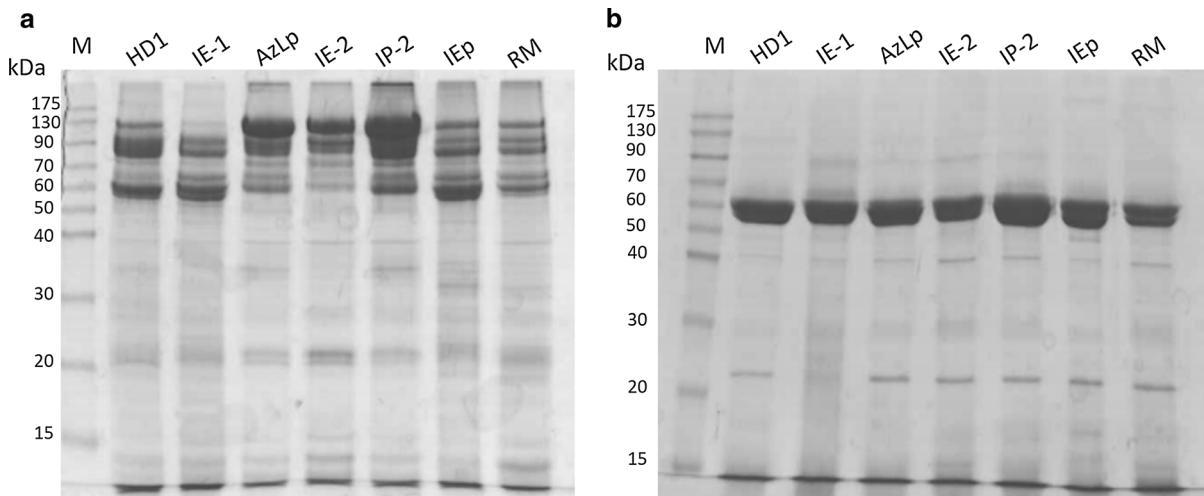
*cryIAd*, *cryIAg*, *cryIG*, *cryIH*, *cyt1*, *cyt2*, *vip1*, *vip2*, *sip1*, *ps1*, *ps2*, *ps3* and *ps4* genes.

Strains IE-1, AzLp, IE-2, IP-2 and IEp showed positive amplification of the predicted band size for *cryIAC*, *cryII*, *vip3* genes. The *cryIAa* gene was present in IE-1, AzLp, IE-2 and IP-2. The *cryIC* and *cryID* genes were found in three strains: AzLp, IE-2 and IP-2 (Table 3).

#### Discussion

In this study, the characterization of seven Iranian Bt strains has been investigated, on the basis of lepidopteran toxic activity spectrum, in vivo and in vitro toxicity assays, protein profiling, proteomics analysis,





**Fig. 3** SDS-PAGE analysis of solubilized crystal proteins. **a** Protoxins, **b** trypsin activated proteins. The names of the different Bt strains are indicated in the top of each lane. M MW markers (Prestained protein ladder, Nippon Genetics), HD1 reference strain Bt HD1

**Table 3** Genes and crystal proteins identified in the Bt strains

Strain	Crystal protein composition <sup>a</sup>	Gene content
IE-1	Cry1Ac, Cry1Aa, Cry2Aa	<i>cry1, cry1Aa, cry1Ac, cryII, cry2, vip3</i>
AzLp	Cry1Ab, Cry1Ca, Cry1Aa, Cry1Da, Cry1Ac	<i>cry1, cry1Aa, cry1Ab, cry1Ac, cry1C, cry1D, cryII, cry2, vip3</i>
IE-2	Cry1Ca, Cry1Aa, Cry1Da	<i>cry1, cry1Aa, cry1Ac, cry1C, cry1D, cryII, cry2, vip3</i>
IP-2	Cry1Ab, Cry1Ca, Cry1Da, Cry1Aa	<i>cry1, cry1Aa, cry1Ab, cry1Ac, cry1C, cry1D, cryII, cry2, vip3</i>
IEp	Cry1Ac, Cry2Aa	<i>cry1, cry1Ac, cryII, cry2, vip3</i>
RM	Cry1Ac	<i>cry1, cry1Ac, cry2</i>
KhF	–	<i>cry2</i>

The crystal proteins were identified by LC–MS/MS analysis. The PCR screening was performed to detect *cry*, *cyt*, *vip*, *sip* and *ps* gene families

<sup>a</sup>The detected proteins were ordered according to their relative abundance (from highest to lowest) in the crystals

gene content identification and β-exotoxin production. The *P. interpunctella* most toxic strains produced bipyramidal crystals. It has been described that the toxic Bt proteins for *P. interpunctella* are Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ad, Cry1B, Cry1C, Cry1D, Cry1E, Cry1F, Cry1I, Cry2A, Cry9, Cry39 and Cry40, (Gryspeirt and Gregoire 2012; Shojaaddini et al. 2012). The Cry protein contents identified by LC–MS/MS analysis in our study showed that the two strains causing 100% mortality to *P. interpunctella* (IP-2 and IEp) have completely different protein contents. The comparison of toxicity and proteomics analysis indicates that not only composition but also the relative abundance of the expressed proteins in each strain is important for toxicity. This statement is

reinforced if we compare the proteins of IEp (100% toxicity and Cry1Ac and Cry2Aa as major proteins in the crystals), IE-1 (67% mortality with Cry1Ac, Cry1Aa and Cry2Aa as major proteins in the crystals) and RM (low toxicity, producer of only Cry1Ac similarly to Bt subsp. *kurstaki* HD-73 (Ibrahim et al. 2010)).

In general, the proteomic analysis and PCR results were consistent in the sense that when a protein was detected in a strain, the corresponding gene was detected by PCR. This correspondence did not always happen in the opposite direction. Indeed, the *cry2* gene was amplified in all tested strains but the protein was only detected in IE-1 and IEp. Also the *cry1Ac* gene was detected in IE-2 and IP-2 but the protein was not

detected in the LC–MS/MS analyses. This could be explained by a low transcription rate of the gene for diverse reasons, or due to the presence of defective untranscribed sequences. In conclusion, the PCR analyses, widely used in Bt screenings, can be useful solely to predict the proteins that may compose the Bt crystal.

Genes of the *cryI* family were found in all strains, excluding KhF, in accordance with the presence of the major band of about 130 kDa in SDS-PAGE and with the proteomic analyses. In the previous reports (Uribe et al. 2003; Thammasittirong and Attathom 2008), *cryIG* was considered as an abundant gene in the Bt collections, contrarily to our results. On the other hand, in the Chinese (Wang et al. 2003) and Iranian (Seifinejad et al. 2008) Bt collection, *cryIG* and *cryIH* were found rarely in agreement with our results. Therefore, the presence of these genes could be related with the origin of the strains. In the present work, the *cry2* gene was detected in all strains, in agreement with several studies that report the high frequency of *cry2* genes, especially among *cryI* containing strains (Porcar and Juárez-Pérez 2003; Seifinejad et al. 2008). Regarding *vip* and *sip* genes, the frequencies found in this and other screening studies differ or not depending on the origin of the samples analyzed (Salehi Jouzani et al. 2008; Seifinejad et al. 2008; Hernandez-Rodriguez et al. 2009; Djenane et al. 2017).

The toxicity spectrum of second series of bioassays was restricted to four different lepidopteran families, namely Noctuidae, Tortricidae, Crambidae and Pyralidae. As a result, three Bt strains, AzLp, IE-2 and IP-2, showed higher toxicity against *S. exigua* and *M. brassicae* first instar larvae than the standard Bt HD1 (Table 1). This was in accordance with the loss of viability observed in the UCR-SE cells exposed to the activated toxins of AzLp, IE-2 and IP-2 strains (Fig. 1). The toxicity of these strains on *S. exigua* could be due to the presence of Cry1C and Cry1D proteins (Hernández-Martínez et al. 2008). *Mamestra brassicae* first instar larvae are susceptible to Cry1C (Gilliland et al. 2002), and accordingly, the highest mortality was recorded in the Cry1C protein producers, AzLp, IE-2 and IP-2 strains (Tables 1, 3).

*Grapholita molesta* was the most susceptible insect in this study, due to the high toxic effect of Cry1Aa, Cry1Ac and Cry1C proteins against this pest (Scaramal Ricietto et al. 2016). As reported in other studies (Hernández-Martínez et al. 2008) Cry1Ac is not toxic

for *S. exigua*, which would be in agreement with the absence of toxicity exhibited by RM, a strain that following our proteomics and PCR results only possesses and express *cryIac* gene (Table 3). There are several studies that report susceptibility of *O. nubilalis* to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, Cry1Ie2, Cry9Ca and Cry9E (van Frankenhuyzen 2009; Zhao et al. 2015). Regarding the presence of Cry1Aa, Cry1Ab and Cry1Ac in the Bt strains studied, high susceptibility of *O. nubilalis* was expected and indeed observed (Table 1).

To expand our knowledge of the insecticidal activities of the selected strains, four lepidopteran derived cell lines were used. It has been discussed that the response of cell lines to different Bt toxins is not necessarily correlated with the in vivo activity toward the insect from which the cells are derived (Witt et al. 1986). To test this, the toxicity of our Bt strains towards *S. exigua* first instar larvae and the *S. exigua* cell line UCR-Se was assessed and compared. Our results showed high correlation between bioassays and cell viability assays (Table 1 and Fig. 1), pointing to the feasibility of using insect cell lines for toxicity screening.

In this study, the cell viability assays indicated that the crystal proteins from AzLp, IE-2 and IP-2 (the three most toxic strains in bioassays) were highly toxic for *H. zea*, *T. ni*, *S. exigua* and *S. frugiperda* cells. This is in accordance to what has been described for Sf21 and Hi5 cells lines, highly susceptible to Cry1C toxins (Kwa et al. 1998; Gringorten et al. 1999). According to our cell viability assay, Sf21, UCR-SE, Hi5 and HzGUT were not susceptible to IE-1 and RM, strains that mainly produced Cry1Ac protein. This is in agreement with Gringorten et al. (1999) and Chen et al. (2015) that reported no susceptibility for Sf9, Sf21 and Hi5 cells to Cry1Aa, Cry1Ab and Cry1Ac proteins. Cry1Ac and Cry1F are toxic for heliothines (Siebert et al. 2008), in contrast to our cell viability assay results, that showed low susceptibility of HzGUT to IE-1, IEp and RM strains, in which the *cryIac* gene was expressed. These results point out that cell cultures do not always show the toxicity responses expected based on the bioassays performed on the insects from which they originally derived.

The results of the bioassay with the protoxins of the KhF strain indicated that this strain was almost not toxic for the tested insects. The PCR technique only detected *cry2* genes and proteomics did not reveal any

known Cry protein. But KhF spore and crystal mixture had been moderately toxic for second instar larvae of *P. interpunctella*. These results can be due to the presence of immune inhibitors and Bacillolysin as bioactive factors, which had been detected in KhF strain by LC–MS/MS analysis (data not shown).

In summary, to predict the insecticidal potency of newly isolated Bt strains, the LC-MS/MS analysis (which assess crystal protein composition) is a useful technique much more convenient than PCR-based characterization which can only offer a prediction of the proteins composing the crystal. But bioassays (with insects or with cell lines) are crucial and unavoidable steps to complete the strains characterization, since protein proportion and protein interaction between different toxins seem to be decisive for toxicity. As a result, due to the high toxicity towards different lepidopteran insect larvae and cell lines, the presence of the insecticidal proteins Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca and Cry1Da, and the absence of  $\beta$ -exotoxin, AzLp, IE-2 and IP-2 Bt strains could be considered as important candidates for development of future Bt based insecticides.

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