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Expression of *cry1Ab* gene from a novel *Bacillus thuringiensis* strain SY49-1 active on pest insects

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ABSTRACT

In this study, the *cry1Ab* gene of previously characterized and Lepidoptera-, Diptera-, and Coleoptera-active *Bacillus thuringiensis* SY49-1 strain was cloned, expressed and individually tested on *Ephestia kuehniella* (Lepidoptera: Pyralidae) and *Plodia interpunctella* (Lepidoptera: Pyralidae) larvae. pET-*cry1Ab* plasmids were constructed by ligating the *cry1Ab* into pET28a (+) expression vector. Constructed plasmids were transferred to an *Escherichia coli* BL21 (DE3) strain rendered competent with $CaCl_2$. Isopropyl β-D-1-thiogalactopyranoside was used to induce the expression of *cry1Ab* in *E. coli* BL21(DE3), and consequently, ~130 kDa of Cry1Ab was obtained. Bioassay results indicated that recombinant Cry1Ab at a dose of $1000\,\mu g\,g^{-1}$ caused 40% and 64% mortality on *P. interpunctella* and *E. kuehniella* larvae, respectively. However, the mortality rates of *Bt* SY49-1 strains' spore–crystal mixture at the same dose were observed to be 70% on *P. interpunctella* and 90% on *E. kuehniella* larvae. The results indicated that *cry1Ab* may be considered as a good candidate in transgenic crop production and as an alternative biocontrol agent in controlling stored product moths.

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Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive aerobic or facultative aerobic spore-forming entomopathogenic bacterium that can easily be isolated from a variety of environmental sources.[1] It has specific toxicity against target insects and is safe to non-target organisms. Cry1 toxins are the most common crystal proteins characterized so far in *Bt* strains and have specific insecticidal activity against lepidopteran insects.[2] They form typical bipyramidal parasporal inclusions with 130 kDa molecular weight.[3] Biotechnological developments in agriculture have caused scientists to seek new solutions to insect pest problems. Transgenic technology, involving a wide range of pesticidal genes from *Bt*, dominates the scenario of agricultural biotechnology. The improvement of broader spectrum biopesticides using novel *Bt* strains against target insects is an important aspect for improving their persistence

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on plants. The pyralid moths *Ephestia kuehniella* (Lepidoptera: Pyralidae) and *Plodia interpunctella* (Lepidoptera: Pyralidae) are global pests, particularly of stored grains, legumes, dried fruits, nuts, dates, and cocoa beans, and they are the most commonly reported pests of stored grains. Larvae can cause extensive damage to crops and a variety of processed foods. The presence of live insects and insect parts can result in the depreciation of the grain when sold. Chemical insecticides are commonly used in controlling these important pest insects worldwide. However, due to the risks of chemical pest control to the environment and human health, the cloning and expression of *cry* genes with activity to lepidopteran pests is an important issue concerning species-specific control. In the current study, we amplified and cloned the *cry1Ab* gene of a novel *Bt* SY49-1 strain.[4] The strain had insecticidal activity to a variety of lepidopteran, dipteran and coleopteran pests, and we desired to improve the efficiency through transforming the *cry1Ab* gene into *E. coli* BL21 (DE3). Expression of the *cry1Ab* gene and its insecticidal activity against the serious stored product pests *E. kuehniella* and *P. interpunctella* were investigated. The results will reveal useful information for stored product pest control by providing Cry proteins from novel strains.

Materials and methods

Strains and plasmids

The *Bt* SY49-1 was isolated from a soil sample collected from Adana, Turkey in 2008 using sodium acetate enriched medium.[5] The strains *B. thuringiensis* SY49-1, *Escherichia coli* DH5α (kindly supplied by Middle East Technical University, METU, Molecular Biology Laboratory), cloning vector pGEMT-Easy, expression vector pET28a (+) and *E. coli* BL21(DE3) were used in experimental procedures. *Bt* and *E. coli* were cultured in Luria Bertani (LB, 10 g/L Triptone, 5 g/L yeast extract, 5 g/L NaCl) medium at 30 °C and 37 °C, respectively.

Electron microscopy

For electron microscopy, a *Bt* SY49-1 spore–crystal mixture was suspended in dH_2O on a microscope slide and fixed after air drying at room temperature. The sample was sputter-coated with 10 nm Au/Pd using a SC7620 Mini-sputter coater and viewed using a scanning electron microscope (LEO440) at 20 kV beam current.

Insect cultures

The larvae of *E. kuehniella* were reared on a diet containing a mixture of wheat flour, wheat bran and glycerol, and the Indian meal moth *P. interpunctella* larvae were obtained from naturally infested dried apricot in Kayseri province. The larvae of *P. interpunctella* were maintained continuously on a diet containing 10% glycerol, 50% dried apricot and 40% wheat flour–wheat bran mixture. Throughout the experiments, insect cultures were maintained at constant temperature (27 ± 1 °C), photoperiod (14L:10D) and relative humidity (60 ± 5%).[6,7]

Amplification of cry1Ab gene from Bt SY49-1

Total DNA of *Bt* SY49-1 was extracted according to the method of Bravo et al.[8] and used as template for polymerase chain reactions (PCR). The *cry1Ab* gene was amplified using the primer pairs F-5′-GGA TCC ATG GAT AAC AAT CCG AAC ATC-‘3; R- 5′-GTC GAC TTA TTC CTC CAT AAG AAG TAA-3′.[9] *Bam*HI and *Sal*I restriction enzyme recognition sequences were added to the 5′ end of the forward and reverse primers, respectively. PCR mixes contained the reagents at a final concentration of 2.3 mM $MgCl_2$, 1× Taq buffer, 0.2 mM dNTP mix, 0.3 pmol of each primer, 0.5 U MaxTaq DNA polymerase (Vivantis, PL2201), and 30–100 ng template DNA. The PCR amplification was performed under the following conditions: Initial denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 52 °C for 1 min, 72 °C for 3.5 min, and a final extension step at 72 °C for 10 min.[9] Adenine was added to the 3′ end of the PCR products after amplification for the TA cloning process.

Transfer of cry1Ab gene into pGEM-T Easy cloning vector

To construct the pGEM-*cry1Ab*, PCR products (~3.5 kb) were purified and ligated into pGEM-T Easy vector. The ligation procedure was conducted according to the manufacturer's protocol (Promega, vector system I), and then ligate was transformed into $CaCl_2$-rendered competent *E. coli* DH5α.

Expression of cry1Ab gene in E. coli

*Bam*HI and *Sal*I-treated *cry1Ab* PCR amplicon and pET28a (+) vector were ligated according to the manufacturer's protocol (pET manual system). The pET-*cry1Ab* was transferred into *E. coli* BL21(DE3) cells rendered competent with $CaCl_2$. The positive clones were selected using the PCR method and incubated at 37 °C until reaching $OD_{600} = 0.5–1$ in 100 mL of LB medium containing 30 μg/mL kanamycin. The 50 mL of culture was induced by 1 mM IPTG for 4 h and followed by centrifuging at 4 °C and 5000 rpm for 5 min. The pellet was solubilized in 12 mL of 20 mM Tris–HCl (pH 7.5) and centrifuged as described above. The remaining pellet was solubilized in 5 mL of 20 mM Tris–Cl (pH 7.5) containing 50 μL lysozyme (10 mg/mL) and incubated for 15 min at 30 °C. The cells were then sonicated for 1 min to release proteins from lysate. Subsequently, it was centrifuged at 4 °C and 14,000 rpm for 10 min, and the pellet was resuspended in 2 mL of 20 mM Tris–HCl (pH 7.5) for SDS-PAGE analysis. The same procedure was applied for isolating the proteins from *E. coli* BL21(DE3) and *Bt* SY49-1. Total protein quantitation was determined according to the Bradford[10] method.

Cry1Ab protein quantification

The SDS-PAGE image of the approximately 130 kDa Cry1Ab band was used for determining the quantities. Cry protein concentrations were calculated by the following formula: Cry protein concentration (μg/mL) = (μg/mL total protein) × (proportion of Cry protein to total protein).[11] The proportion of Cry protein to total protein was determined

using the Biorad Chemi Doc MP Imaging System Image Lab version 5.1 (Biorad).

Insecticidal activity of Cry1Ab

Lyophilized samples of *Bt* SY49-1, *E. coli* carrying pET-*cry1Ab*, and plasmid-free *E. coli* BL21(DE3) were applied to 10 third-instar larvae of *P. interpunctella* and *E. kuehniella* at doses of 10, 25, 50, 100, 250, 500 and 1000 $\mu g\,g^{-1}$ supplied in the diet. Bioassay experiments on *P. interpunctella* and *E. kuehniella* were performed according to the method of Obeidat et al.[12] Experiments were carried out as three replicates. The data from the experiments were subjected to analysis of variance (ANOVA), and means were separated at the 5% significance level by using the Tukey HSD post hoc test. LC_{50} were estimated by probit analysis.[13]

Results

Amplification of cry1Ab gene

The total DNA of the *Bt* SY49-1 strain was screened by the PCR method using the *cry1Ab*-specific primer pairs for amplifying the full-length gene region (Fig. 1).

Spore–crystal mixture and electron microscopy

Bt SY49-1 was incubated in T3 sporulation medium at 30 °C for 4 days to obtain a spore–crystal mixture. Investigations of the mixture indicated that bipyramidal crystals were compatible with the presence of *cry1* gene products. Spherical, cubic and irregularly shaped crystals were also observed via electron micrograph (Fig. 2).

Cry1Ab gene TA cloning

PCR product corresponding to the open reading frame of *cry1Ab* gene (~3.5 kb) was amplified and inserted into the pGEM-T Easy vector system to preserve the gene for further use. The resulting combination was transferred into *E. coli* DH5α. Subsequently, pGEM-*cry1Ab* from a positive clone of *E. coli* DH5α was digested with *Eco*RI to validate the ligation (Fig. 3).

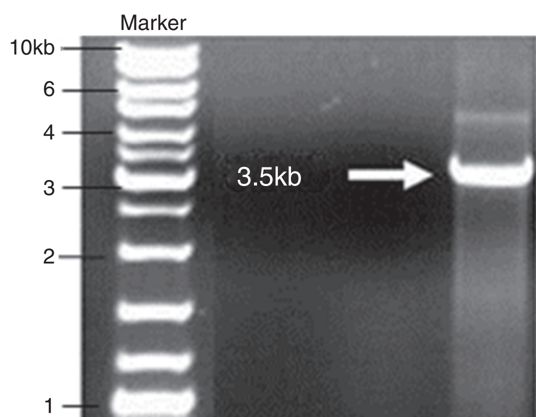

Fig. 1 – *cry1Ab* gene amplicon, Marker (Fermentas, SM 0331).

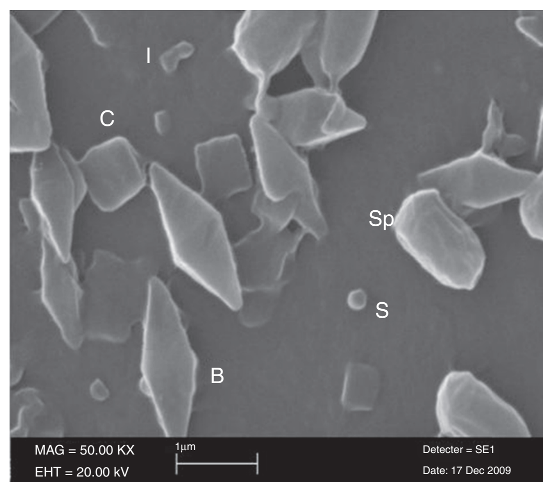

Fig. 2 – Spore–crystal morphology of *Bt* SY49-1 strain. B, bipyramidal; C, cubic; S, spherical; I, irregularly shaped spherical; Sp, spore.

Expression of cry1Ab in E. coli BL21(DE3)

The *cry1Ab* gene excised with *Bam*HI and *Sal*I was inserted into expression vector pET28a (+) for obtaining pET-*cry1Ab*. The resulting pET-*cry1Ab* was transformed into the *E. coli* BL21(DE3) strain, and positive clones were validated by colony PCR (Fig. 4). A positive clone, *E. coli* BL21 (DE3) pET-*cry1Ab*, was cultured in kanamycin containing LB medium until OD_{600} = 0.5–1 was achieved and subsequently induced with IPTG. The expression of recombinant products was analyzed by SDS-PAGE, verifying the presence of a 130 kDa protein band (Fig. 5).

Cry1Ab quantification

The protein concentration was calculated according to the following formula: Cry1Ab concentration (μg/mL) = (μg/mL total protein) × (% proportion of Cry1Ab to total protein). Here, the total protein was 7.63 μg/mL, the proportion of Cry1Ab to total

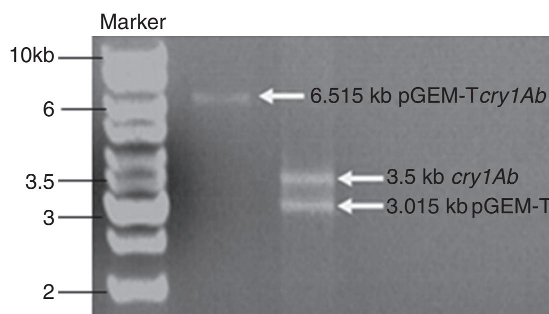

Fig. 3 – Validation of recombinant pGEM-*cry1Ab* by restriction digestion with *Eco*RI, Marker (Fermentas, SM 0331).

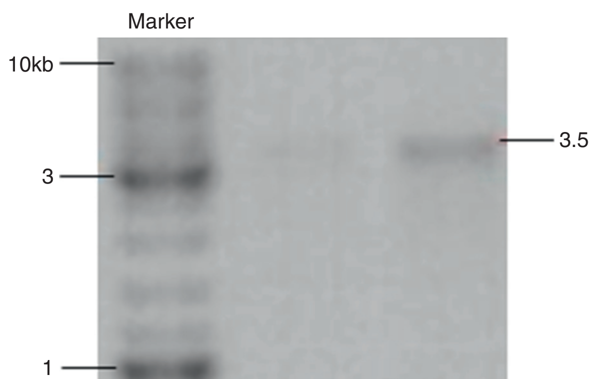

Fig. 4 – Validation of *cry1Ab* gene in positive clones using colony PCR, Marker (Fermentas, SM 0331).

protein was 2.58 μg/mL, and the proportion of Cry1Ab to total protein was 33.82%.

Toxicity of pET-cry1Ab, Bt SY49-1 and plasmid-free E. coli BL21(DE3)

The LC_{50} values of recombinant Cry1Ab on third-instar larvae of *E. kuehniella* and *P. interpunctella* were found to be 685.67 and 1320.84 $\mu g\, g^{-1}$, respectively. The toxicity of the source strain SY49-1 was higher compared with recombinant protein ($LC_{50} = 365.17\, \mu g\, g^{-1}$ for *E. kuehniella* and 582.179 $\mu g\, g^{-1}$ for *P. interpunctella*). Plasmid-free *E. coli* BL21(DE3) did not exert significant activity on both pest larvae. The mortality rates are supplied in Figs. 6 and 7. (Fig. 6; (A) $F = 7.710$; $df = 7$; $P \leq 0.0001$; (B) $F = 22.136$; $df = 7$; $P \leq 0.0001$; (C) $F = 0.635$; $df = 7$; $P = 0.721$; Fig. 7; (A) $F = 4.082$; $df = 4$; $P \leq 0.037$; (B) $F = 8.799$; $df = 4$; $P \leq 0.003$; (C) $F = 0.474$; $df = 4$; $P = 0.754$).

Discussion

Bt SY49-1 is a novel strain, and the toxicity of its wettable spore–crystal powder was previously determined against insect pests from different orders.[4] *E. kuehniella* and *P. interpunctella* are two troublesome pests posing serious problems in stored products worldwide. In the present study, the *cry1Ab* gene (~3.5 kb) of a previously characterized *Bt* SY49-1 strain was used thorough cloning and expression in *E. coli* BL21(DE3). It is well known that *cry1A* genes have specific insecticidal activity against lepidopteran pests.[14,15] *Bt* SY49-1 produces a 130–140 kDa Cry1 band corresponding to the bipyramidal crystal structure (Fig. 2). Cry1Ab (~130 kDa) was overexpressed in IPTG-induced *E. coli* BL21 (DE3), and its bioactivity was tested on *E. kuehniella* and *P. interpunctella*. The results indicated that recombinant Cry1Ab exhibited considerable mortality on pest larvae. However, increasing doses of Cry1Ab from 50 to 500 $\mu g\, g^{-1}$ had little additional toxicity on the larvae when compared with the spore–crystal mixture of source organism. Similar trends were reported with the recombinant Cry3Aa on *Hypothenemus hampei*.[16] Zhang et al.[17] also reported that the activity of recombinant *cry8Ab1* has lower toxicity compared with the source organism, suggesting that this phenomenon may originate from less Cry8Ab1 expression in the host organism and more than one type of differently sized crystals being expressed in wild-type *Bt*. Comparatively lower toxicity was

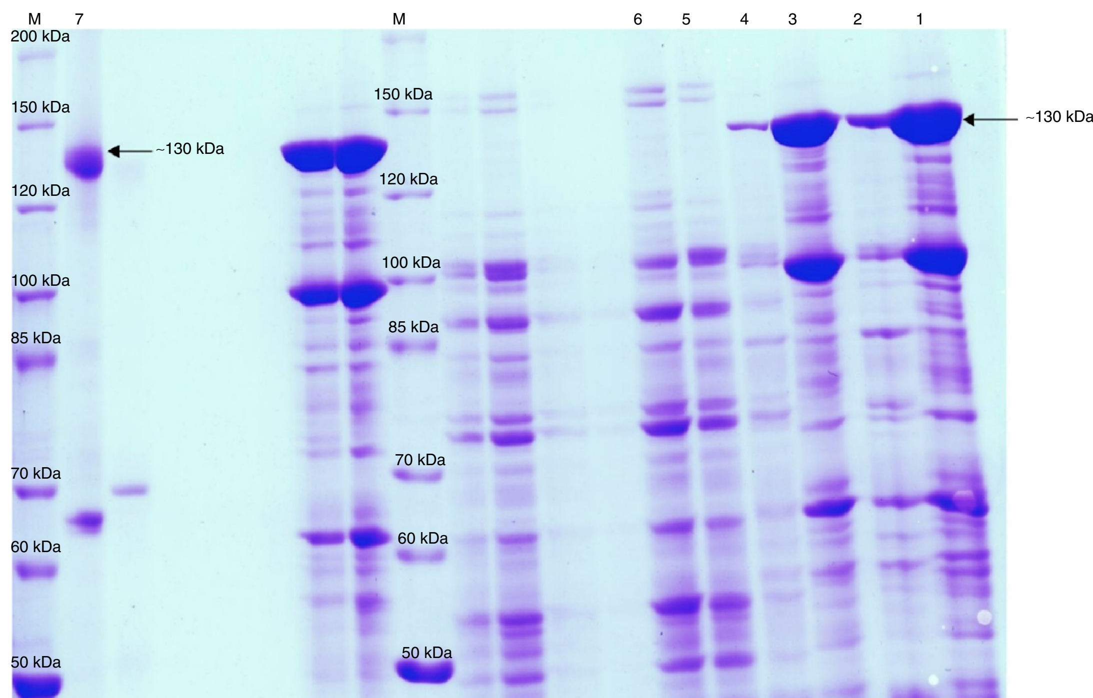

Fig. 5 – SDS-PAGE analysis of *cry1Ab* gene expressed in *E. coli* BL21(DE3). M; Marker (Fermentas SM0661), Lane 1: IPTG-induced *E. coli* BL21(DE3) pET-*cry1Ab*; lane 2: *E. coli* BL21(DE3) pET-*cry1Ab*; lane 3: IPTG-induced *E. coli* BL21(DE3) pET-*cry1Ab*; lane 4: *E. coli* BL21(DE3) pET-*cry1Ab*; lane 5: *E. coli* BL21(DE3); lane 6: IPTG-induced *E. coli* BL21(DE3) IPTG; lane 7: *B. thuringiensis* SY49-1.

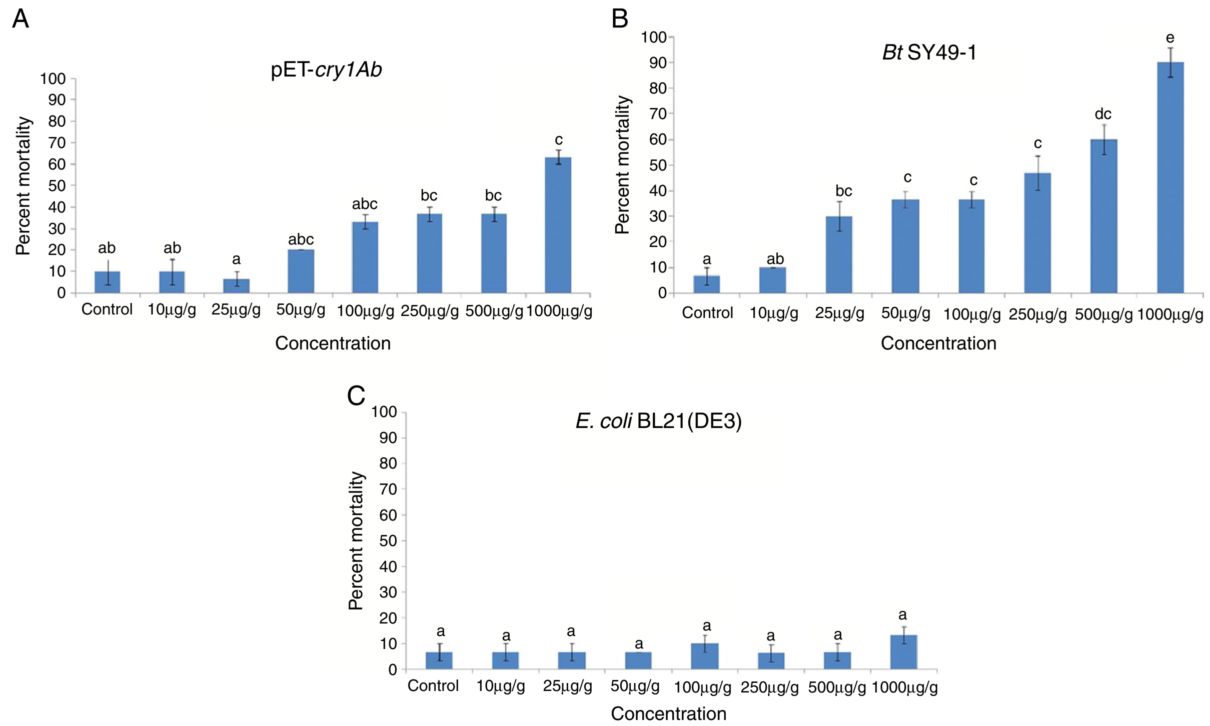

Fig. 6 – Toxicity on *E. kuehniella* larvae. (A) T pET-*cry1Ab*; (B) *Bt* SY49-1 spore–crystal mixture; (C) *E. coli* BL21(DE3) total protein.

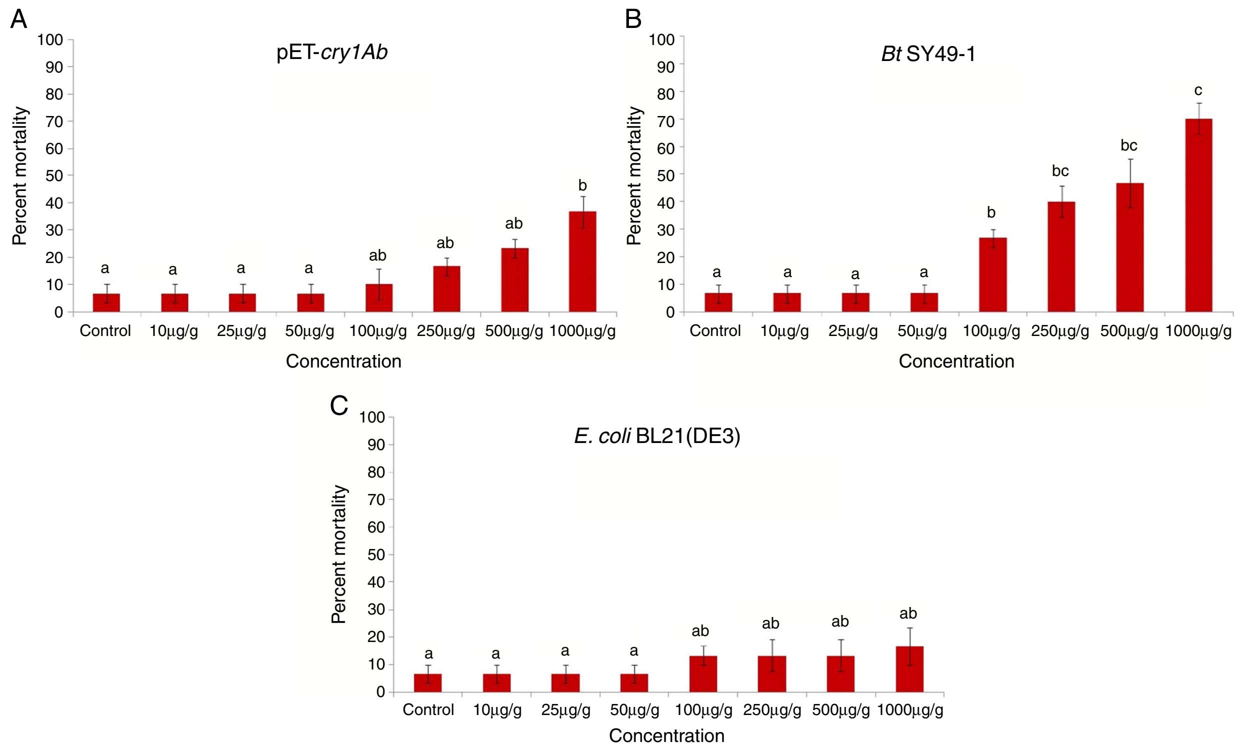

Fig. 7 – Toxicity on *P. interpunctella* larvae. (A) pET-*cry1Ab*; (B) *Bt* SY49-1 spore–crystal mixture; (C) *E. coli* BL21(DE3) total protein.

also reported by some other researchers on *E. kuehniella* using Cry1Aa, Cry1Ac, and Cry2Aa[18] and on *Chironomus tepperi* using Cry11A and Cry4B.[19] On the other hand, Park et al.[20] reported similar toxicity to the parent strain against *Culex* mosquitoes with Cry11B from *B. thuringiensis* subsp., *jegathesan*. Similarly, *Bt* SY49-1 harbors *cry1*, *cry2*, *cry4*, *cry5* and *cry9* genes, and its higher activity may possibly result from the combined activity of these gene-corresponding products. Therefore, this study was intended to determine the effectiveness of Cry1Ab, independent of other genes encoding insecticidal proteins.

In the present study, the individual toxicity of Cry1Ab (expressed in *E. coli*) was evaluated on *E. kuehniella* and *P. interpunctella* to avoid potential synergistic interactions between spores and crystals.[21] As far as we know, this is the first report evaluating the toxicity of *E. coli* BL21(DE3) pET-*cry1Ab* on *E. kuehniella* and *P. interpunctella*. Significant differences were not observed with respect to toxicity between *E. coli* BL21 (DE3) and watery control. The individual toxicity of recombinant Cry1Ab on the third-instar larvae of these two stored product pests was precisely estimated.

In conclusion, the *cry1Ab* gene of the *Bt* SY49-1 strain was successfully cloned, expressed and tested on *E. kuehniella* and *P. interpuctella* larvae. The results indicated that *cry1Ab* may be considered as a good source in transgenic crop production and as an alternative biocontrol agent in controlling stored product moths.

Ethical statement

The authors declare that the experiments complied with the current laws of the country in which they were performed.

Conflict of interest

The authors declare that they have no conflict of interest.

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