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Characterization of Local *Bacillus thuringiensis* Isolates and Their Toxicity to *Ephestia kuehniella* (Zeller) and *Plodia interpunctella* (Hübner) Larvae

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ABSTRACT

Putative *Bacillus thuringiensis* isolates were obtained from soils of various agricultural fields. Of all the tested isolates U14.1, U14.2 and U14.5 were found to be positive for *cry1C*. Besides, U6.6, U14.1, U14.4 and U14.5 were determined to contain *cry1B*. These local isolates produced bi-pyramidal, spherical and cubical crystal proteins. U14.1, U14.4 and U14.5 isolates exhibited similar protein banding patterns with *Btk* producing around 45, 70 and 130 kDa proteins. However, U14.2 and U6.6 produced only 60 kDa major protein band. Some of the isolates also produced bands less than 45 kDa showing the presence of some other low molecular weight proteins. The results of this study demonstrated that isolates of *Bt* obtained from various agricultural fields may display toxicity against *Ephestia kuehniella* (Zeller) and *Plodia interpunctella* (Hübner) larvae. As a result, spore-crystal mixture of these isolates may play an important role in reducing the damage to stored products caused by these important pest insects.

Key words: *Bacillus thuringiensis*, Cry protein, lethal concentration, toxicity, *Ephestia kuehniella* (Zeller), *Plodia interpunctella* (Hübner).

INTRODUCTION

Mediterranean flour moth, *Ephestia kuehniella* (Zeller 1879, Lepidoptera: Pyralidae) and Indian meal moth *Plodia interpunctella* (Hübner 1813, Lepidoptera: Pyralidae) are important insect pests that infest a wide range of stored products (Rees, 2003; Simmons and Nelson, 1975). Larvae of these pests decrease both quality and quantity of stored products through feeding, webbing, and fecal matter (Hansen and Jensen, 2002; Johnson *et al.*, 1997).

The control of these pests in storage systems mainly depends on fumigants such as methyl bromide or phosphine. However, methyl bromide has been banned in many countries since 2004 because of its ozone depleting properties (Hansen and Jensen, 2002). Phosphine also causes serious problems and in some countries insect control failures have been reported in field situations (Taylor, 1989; Collins *et al.*, 2002). Furthermore, the use of chemical pesticides has led to many other problems, including environmental pollution and human health hazards, such as cancer and several immune system disorders (Bravo *et al.*, 2011). Many alternatives have been tested to replace these fumigants for stored product and quarantine uses. There is an urgent need to develop safe alternatives that have the potential to replace the toxic fumigants, yet are effective, economical and convenient to use (Ayvaz and Karabörklü, 2008).

Bacillus thuringiensis (Bt) is known to be an important microbial entomopathogen for the biological control of many agricultural insect pests and disease vectors (Santos *et al.*, 2010). *Bt* is characterized by its production of different insecticidal toxic proteins in para-sporal crystals during sporulation (Rowe and Margaritis, 1987; Crickmore *et al.*, 1998; Valicente *et al.*, 2010). It is mainly a soil-dwelling organism, but one that can act as an opportunistic pathogen under appropriate conditions (Raymond *et al.*, 2010). Crystal proteins of *Bt* are toxic to a wide variety of lepidopteran, dipteran and coleopteran insects that causing serious damage to economically important crops (Vidyarthi *et al.*, 2002). In fact, each habitat may contain a novel *B. thuringiensis* strain awaiting discovery which has a toxic effect on a target insect group (Baig and Mehnaz, 2010). After ingestion of *Bt*-treated diet, the crystal protein split up into smaller toxic peptide fractions by certain proteolytic enzymes in the mid-gut juice of susceptible larvae, which processed to active toxins binding to the brush border membrane receptors before insertion into the membrane. This proteolytic activation is a crucial step in the mode of action of the Cry proteins (Dammak *et al.*, 2010).

In the current study, toxicity of several local *Bt* isolates against the Mediterranean flour moth, *E. kuehniella* and the Indian meal moth, *P. interpunctella* larvae were evaluated under laboratory conditions.

MATERIALS AND METHODS

Isolation of *Bacillus thuringiensis*

Bt isolates were obtained from soil samples of various agricultural fields (Table 1), by the method of Travers *et al.,* (1987). One g of soil sample was added to 20 ml of Luria Bertani Broth buffered with 0.25 M sodium acetate (pH 6.8), and incubated for 4 h at 30 °C then, centrifuged at 200 rpm. One ml of sample was then heated at 80 °C for 5-10 min. Then 50 µl aliquot was spread on nutrient agar in each Petri dish and incubated overnight at 30 °C. *Bt* subsp. *kurstaki* (Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico) was used as reference strain.

Table (1): *Bt* isolates and their GPS Location

Bt isolates	GPS Location	Agric. Field
U6.6	38°51'15.37"K; 35°19'17.47"D, 1082m	Squash
U14.1	38°30'53.82"K; 36°29'59.12"D, 1708m	Wheat
U14.2	38°30'40.93"K; 36°30'50.12"D, 1611m	Beet
U14.4	38°30'49.22"K; 36°30'43.76"D, 1626m	Apple
U14.5	38°30'11.43"K; 36°31'28.88"D, 1584m	Potato

Polymerase chain reaction (PCR)

Molecular characterization of the isolates was performed by PCR analysis using the primers:

- *cry2* (5'-TAAAGAAAGTGGGGAGTCTT-'3, 5'-AACTCCATCGTTATTTGTAG-'3)
- *cry1C* (5'-AAAGATCTGGAACACCTTT-'3 5'-CAAACTCTAAATCCTTTCAC-3'),
- *cry1B* (5'-CTTCATCACGATGGAGTAA-3', 5'-CATAATTTGGTCGTTCTGTT-3')
- *cry1Aa/Ad* 5'-TTATACTTGGTTTCAGGCCC-3', 5'-TTGGAGCTCTCAAGGTGTAA-3')

Each reaction contained the reagents at a final concentration as 2.3 mM $MgCl_2$, 1X taq buffer, 0.2 mM dNTP mix, 0.3 pmol primers (each), 0.5 U taq DNA polymerase, and 30-100 ng template DNA. The PCR amplification was performed under the following conditions: Initial denaturation at 95 °C for 2min, followed by 34 cycles at 95 °C for 1 min, 48-50 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min and the conditions with the primers used were similar (Bravo *et al.,* 1998).

Spore-crystal mixture, freeze drying and electron microscopy

Bt isolates were grown in 150 ml T3 medium (3 g triptone, 2 g triptose, 1.5 g yeast extract, 0.005 g $MnCl_2$, 6 g NaH_2PO_4, 7.1 g Na_2HPO_4) and incubated for 7 days at 30 °C to induce spore formation (Travers *et al.,* 1987). Suspensions were centrifuged at 4 °C and 15.000xg for 10 min to harvest spore-crystal mixtures. Pellets were washed twice and centrifuged at 15.000xg for 10 min in 20 ml sterile dH_2O.

Bt spore-crystal mixtures were freeze dried using Labconco-Welch freeze-drier according to the manufacturer's instruction and were stored at 4 °C until further use. Spore-crystal samples were spread on a microscope slide and fixed after air dry at room temperature. Samples were sputter coated with 10nm Au/Pd using a SC7620 Mini-sputter coater and viewed using a LEO440 scanning electron microscope at 20kV beam current.

Plasmid DNA extraction

Plasmid DNA isolation was performed using the methods of Jensen *et al.,* (1995) and Porcar *et al.,* (1999) with some modifications. The bacteria were grown in 5 ml LB broth for 14h with continuous shaking at 30 °C and 200 rpm. 4 ml of cells was pelleted and resuspended, using 100 µl of TE buffer (40 mM Tris-HCl, 2 mM EDTA, pH 7.9). Cells were then lysed in 200 µl of lysing solution (3% SDS, 15% sucrose, 50 mM Tris-hydroxide, pH 12.5). The lysate was incubated at 60 °C for 30 min, and 2µl of proteinase K (20 mg/ml) was added. The solution was inverted several times and incubated at 41°C for 90 min. Thereafter, 1 ml of phenol: chloroform:isoamyl alcohol (25:24:1) were added to the solution and the tube was inverted 40 times. After centrifugation at 6.000xg for 7 min, the upper aqueous layer was transferred to a clean tube and 500 µl of chloroform: isoamyl alcohol (24:1) was added and centrifuged again at 6000xg for 7 min. Upper aqueous layer was subjected to electrophoresis in 0.5% agarose gel (with 1X TBE buffer and 1mg/ml ethidium bromide) at 65V and 5 °C for 8 h.

Protein electrophoresis

SDS-PAGE was conducted as described by Valicente *et al.,* (2010) with some modifications. The lyophilized spore crystal mixtures were resuspended in 1 ml 0.01% Triton X-100 solution. This step was repeated three times. Pellets composed of a mixture of spore-crystal were solubilized in 500 µl buffer solution (0.01% Triton.10 mM NaCl and 50 mM Tris–HCl, pH 8.0), and one aliquot of 100 µl was withdrawn after this step. The mixture was centrifuged at 14.000 rpm for 5 min and the pellet was resuspended in 500 µl of sodium bicarbonate buffer (50 mM sodium bicarbonate and 10 mM b-mercaptoethanol, pH 10.5) and maintained at 37 °C for 3 h under continuous shaking. Samples were then centrifuged at 14.000 rpm for 10 min, and the supernatant were transferred to a new tube. The remaining pellets were resuspended in 250 µl. of 0.1 M Tris, pH 8.0. Equal amounts of supernatant

and resuspended pellet were sampled and equal volume of sample buffer (0.0625 M Tris, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue, pH 6.8) was added. The mixture was maintained for 5-10 min in boiling water. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12% running and 5% stacking gels. The molecular mass of proteins was determined with SM0431 protein molecular weight marker (Fermentas) and *Btk* HD1 strain was used as a reference. The gel was stained with 0.4% comassie brilliant blue R250 described by Temizkan and Arda (2004).

Bioassay

E. kuehniella and *P. interpunctella* larvae were reared on their artificial diet at 27±1 °C with a photoperiod of 14:10 (L: D) h and 60±5% RH in a rearing cabinet (Ayvaz *et al.,* 2010). Freeze-dried spore-crystal mixture was suspended in sterile distilled water at 250, 1000 and 1500 μg ml^{-1} concentrations. One gram of nut was ground and soaked into 1 ml of spore-crystal mixture solution, left to absorb the toxin for 20 minutes and allowed to dry at room temperature. The mixture was then transferred into Petri dishes (90x15 mm) together with 10 larvae (25 days old) per each and left in an acclimatized chamber at 27±1 °C and 60±5 RH with a photoperiod of 14:10 (L:D) h for 10 days. Sterile dH_2O was used as a control instead of spor-crystal mixture and three replicates were set up for each treatment.

Statistical analysis

Data from the bioassay experiments were subjected to the analysis of variance (ANOVA) using SPSS for Windows (SPSS, 2001) and means were separated at the 5% significance level by the least significant difference (LSD) test. The data were subjected to probit analyses using the same statistical program to estimate LC_{50} and LC_{95} values for *E. kuehniella* and *P. interpunctella* larvae.

RESULTS AND DISCUSSION

***Bt* isolation from soil samples**

Soil samples from different agricultural fields were screened for the presence of *Bt* isolates. Totally sixty different bacterial colonies were analyzed with PCR method using *cry1C, cry1B, cry1Ab/Ad* and *cry2* primer pairs. Of all these isolates U14.2 were positive for *cry1C*, U14.4 and U6.6 were positive for *cry1B*, U14.1 and U14.5 were positive for both *cry1C* and *cry1B* (Fig.1). Neither of the isolates produced expected PCR product with *cry1Ab/Ad* and *cry2* primer pairs.

Scanning electron micrograph of spore-crystal mixture

Spore-crystal samples were examined under scanning electron microscope to show detailed view of *Bt kurstaki* and, products of U6.6, U14.1, U14.2, U14.4 and U14.5 isolates. It was evident that local isolates produced bipyramidal, spherical and cubic crystal proteins with different sizes (Fig. 2).

Plasmid profile of isolates and reference strain

Plasmids were resolved by agarose (0.5%) gel electrophoresis and visualized under UV luminescence (Fig. 3). U14.1, U14.4 and U14.5 isolates had similar banding patterns with *Btk.* All strains produced plasmid bands larger than 19.3 kb except U14.2. The isolate U.6.6 did not contain smaller plasmid bands under 19.3kb.

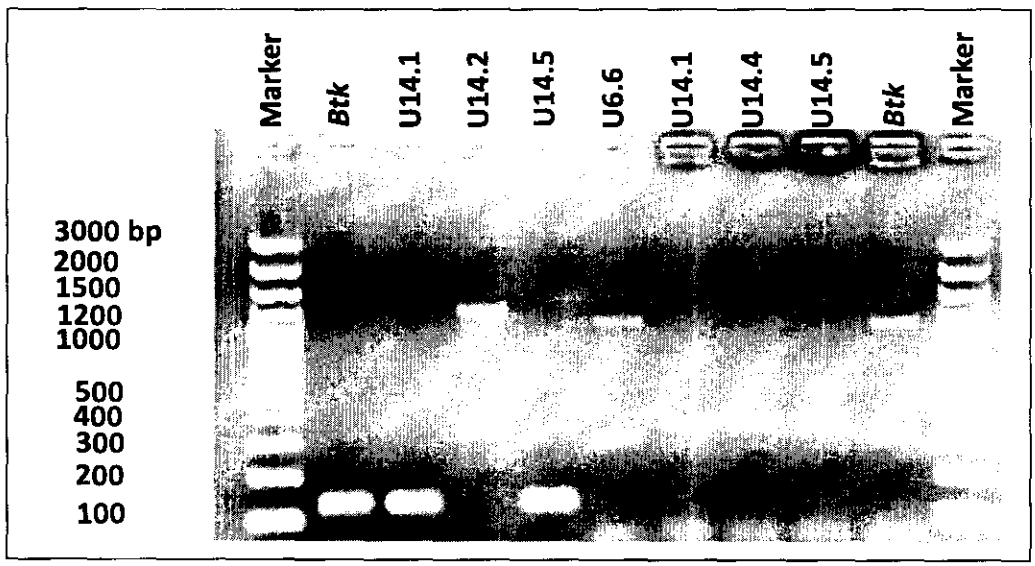

Fig. (1): Agarose gel (0.8%) electrophoresis of the PCR products amplified by using *cryl* primers (*cry1C* 130 and *cry1B* 367).

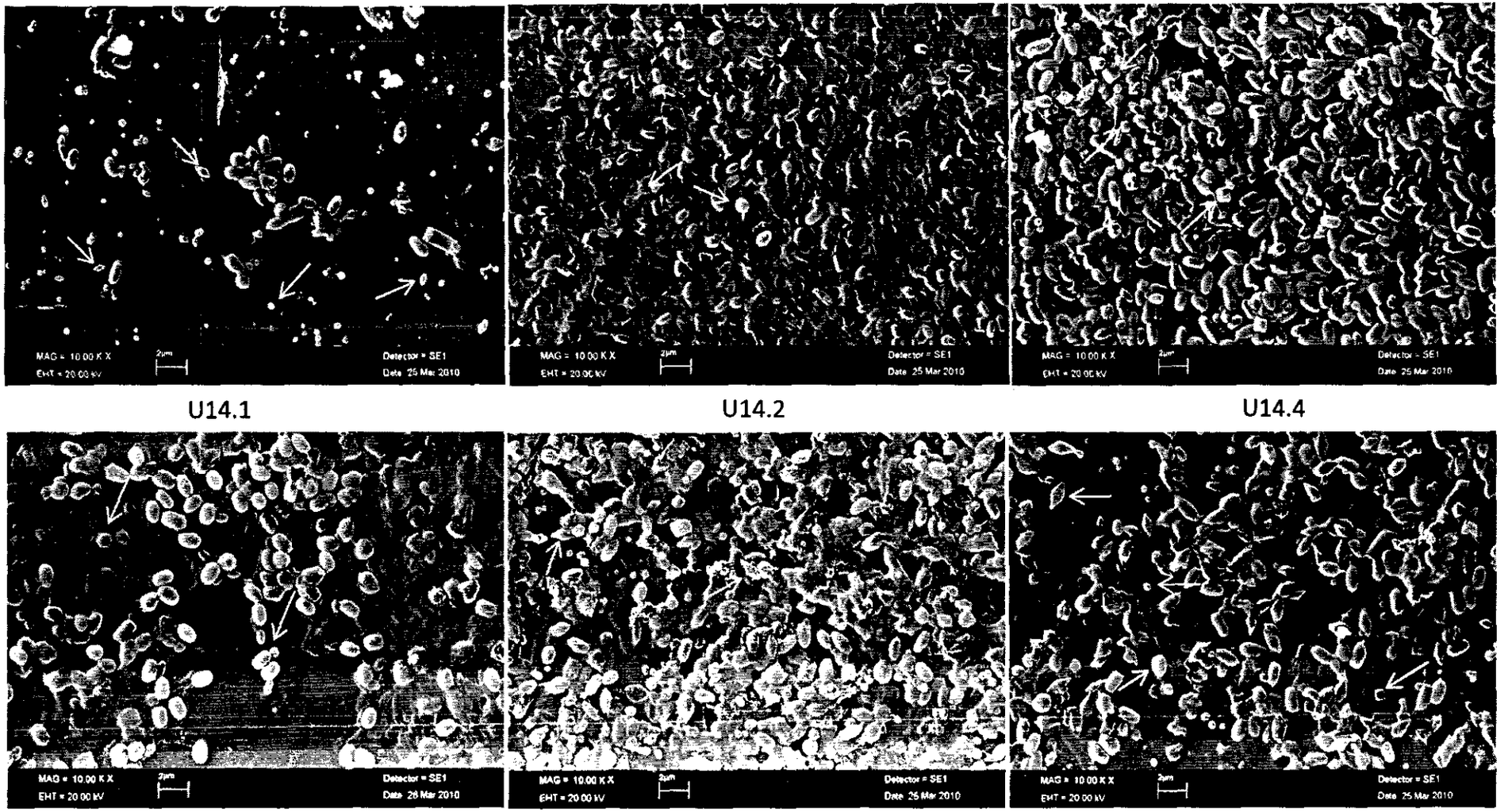

Fig. (2): Electron micrograph of isolates spore-crystal mixture.

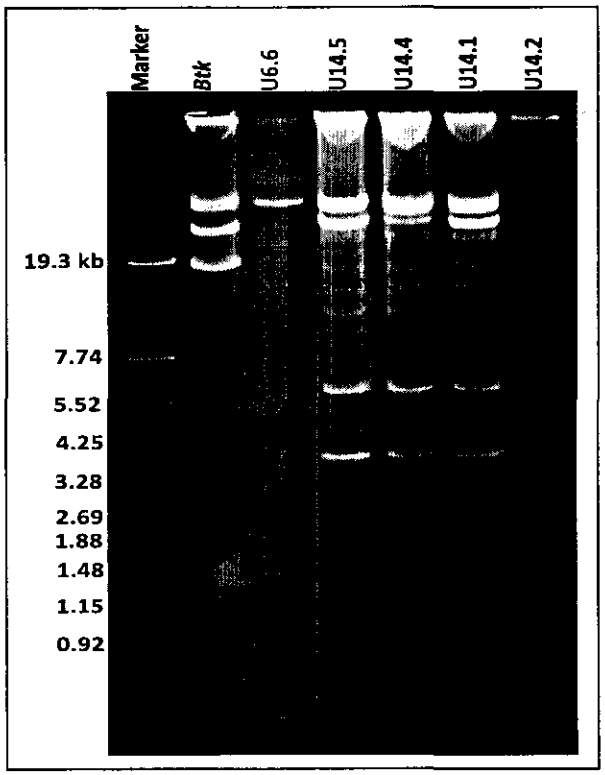

Fig. (3): Plasmid profile of local *Bt* isolates.

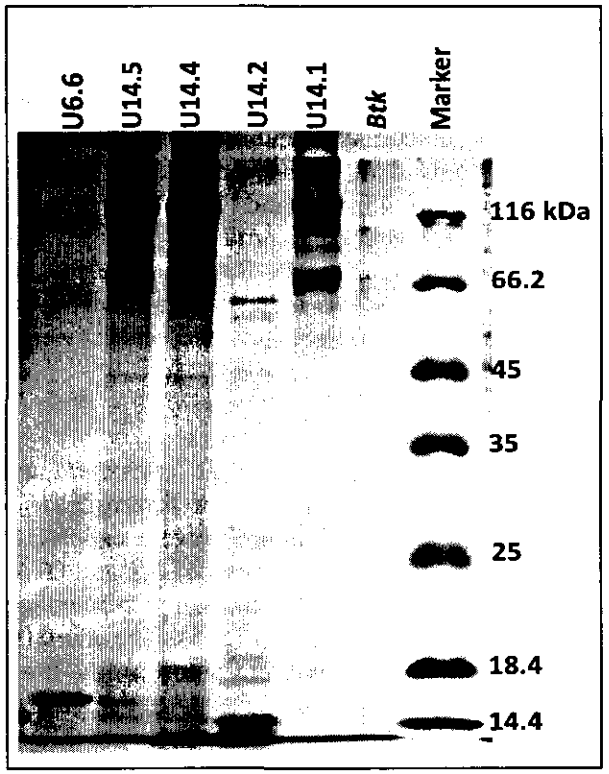

Fig.(4): SDS-PAGE (12%) profile of local *Bt* isolates.

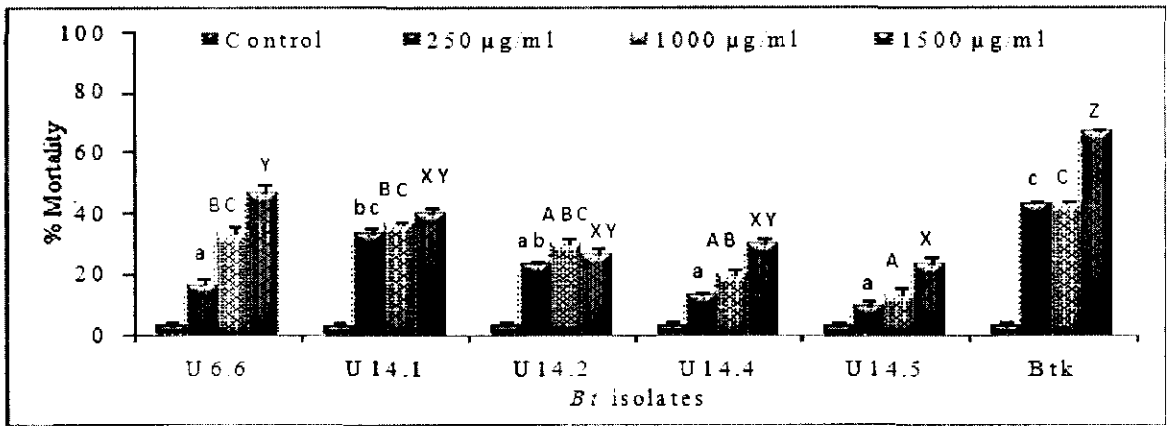

Fig. (5): Percent mortality of *E. kuehniella* larvae after exposure to spore-crystal mixture of local *Bt* isolates. Bars with the same letter are not significantly different for each dose. Error bars indicate standard errors of means.

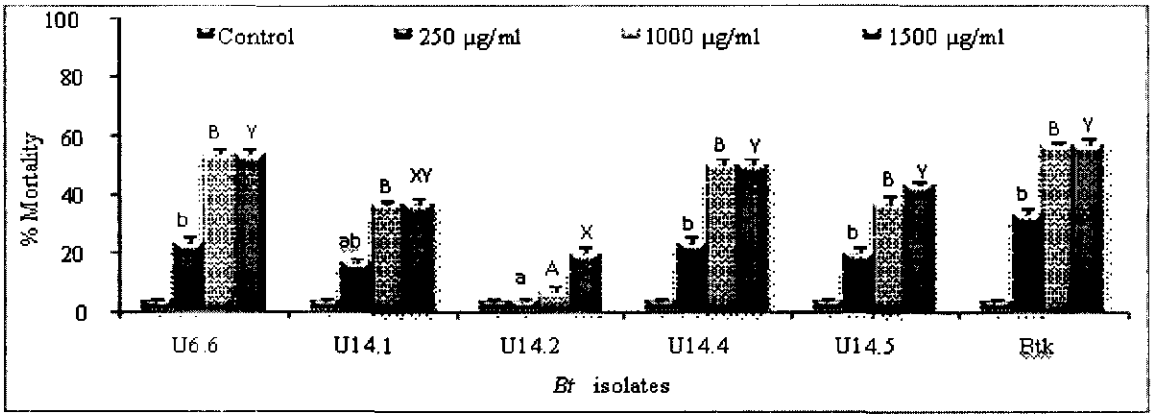

Fig. (6): Percent mortality of *P. interpunctella* larvae after exposure to spore-crystal mixture of local *Bt* isolates. Bars with the same letter are not significantly different for each dose. Error bars indicate standard errors of means

SDS-PAGE analysis

The crystal protein profile of the isolates was determined by SDS-PAGE analysis. Each isolate produced a characteristic banding pattern with some differences. U14.1, U14.4 and U14.5 isolates produced around 45, 70 and 130 kDa proteins. However, U14.2 and U6.6 produced only 60 kDa major protein band (Fig. 4). Some of the isolates also produced bands less than 45 kDa showing the presence of some other low molecular weight proteins.

Bioassay

Toxicity of isolates against *E. kuehniella* and *P. interpunctella* larvae are shown in figures 5 and 6. When the larvae of *E. kuehniella* were exposed to 250 µg ml^{-1} spore-crystal mixtures of *Btk* and U14.1 isolate, 43.34% and 33.34 mortality percentages were observed, respectively (Fig. 5). The mortality resulted by other isolates were significantly lower than that with U14.1 and *Btk*. At the highest concentration (1500 µg ml^{-1}), mortality rates were 40.00, 46.67 and 66.67% for U14.1, U6.6 and *Btk*, respectively. *Btk* showed highest insecticidal activity (33.34%) against *P. interpunctella* larvae at 250 µg ml^{-1} concentrations (Fig. 6). The insecticidal activity of U6.6, U14.1, U14.4 and U14.5 isolates was nearly 20% at the same concentration. However, U14.2 isolate did not show any toxicity against the larvae compared to other isolates ($F= 4.113$; $df = 5$; $P <0.021$). Higher larval mortality due to higher toxin concentration was obvious in most of the isolates. Mortality rates caused by U6.6, U14.4 and *Btk* at the 1500 µg ml^{-1} concentration were 53.34, 50.00 and 56.67%, respectively, but the mortality caused by U14.2 isolate was only 20% at the same concentration ($P<0.016$).

Lethal concentrations (LC_{50} and LC_{95}) of *Btk*, U6.6, U14.1, U14.2, U14.4 and U14.5 for *E. kuehniella* and *P. interpunctella* are shown in Table 2. They were 1032.63 and 2834.46 for *Btk*, and 1524.33 and 3288.34 µg ml^{-1} for U6.6, respectively. The LC_{50} and LC_{95} values required for *P. interpunctella* were similar to *E. kuehniella* for *Btk* (1084.63, 1032.63 and 2837.94, 2834.46 µg $ml^{-1}$for *P. interpunctella* and *E. kuehniella* respectively). However, corresponding values for U6.6 isolate against the *P. interpunctella* larvae were 1197.84 and 2833.78 µg ml^{-1}.

Bt products display high toxicity against a wide range of lepidopteran, coleopteran, and dipteran pests (Zi-Quan *et al.*, 2008). Our local isolates were characterized by PCR and SDS-PAGE analysis by determining the presence of *cryI* genes and Cry proteins. PCR analysis revealed that U14.1 and U14.5 were positive for *cryIB* and *cryIC*, and

Table (2): LC_{50} and LC_{95} (µg/ml) values of isolates against *E. kuehniella* and *P. interpunctella* larvae

Isolates	LC_{50}	LC_{95}	χ^2	*Df*	*P*
	E. kuehniella				
U6.6	1524.33	3288.34	05.52	2	0.063
U14.1	1726.47	4532.37	25.30	2	0.000
U14.2	2474.34	5951.75	16.47	2	0.000
U14.4	2256.60	4729.10	03.98	2	0.136
U14.5	2780.43	5602.88	02.23	2	0.312
Btk	1032.63	2834.46	36.01	2	0.000
	P. interpunctella				
U6.6	1197.84	2833.78	17.58	2	0.000
U14.1	1723.02	3827.54	10.28	2	0.006
U14.2	2822.20	5156.24	02.06	2	0.357
U14.4	1280.04	3032.87	16.94	2	0.000
U14.5	1565.36	3542.64	09.77	2	0.000
Btk	1084.63	2837.94	26.29	2	0.000

U14.2, U14.4, and U6.6 were positive for *cryIB* gene having insecticidal activity primarily against lepidopteran pests. Wang *et al.*, (2003) reported that especially Cry1, Cry2, and Cry9 group of proteins display strongest activity against lepidopteran pests.

The results of the present study show that isolates carrying *cryI* type genes show considerable mortality effect at the highest concentrations of spore crystal mixture against the larvae of *E. kuehniella* and *P. interpunctella*. We used spore-crystal mixture in bioassay studies because spores and crystals included in the suspensions produce a higher level of mortality than either crystals or spores alone (Crickmore, 2006). Mortality rates of local isolates were found to be lower than those of *Btk*. The mortality rates of U14.1 and U6.6 were 40 and 47% against *E. kuehniella* larvae at the highest concentration, respectively. Moreover, U14.4 and U6.6 showed 50 and 54% mortality against *P. interpunctella* larvae. Santoso *et al.*, (2004) reported that eight out of twelve tested toxins killed nearly 50% of *Conopomorpha cramerella* (Snellen 1904, Lepidoptera: Gracillaridae) in the 3rd-4th instar larvae. Although U14.5 harbors both *cryIC* and *cryIB* genes and corresponding Cry protein bands, it exhibited fairly low toxicity against *E. kuehniella* larvae when compared to U14.1, U14.2, U14.4, and U6.6. Nevertheless, while U14.4 and U6.6 having the same type of *cry* gene, their insecticidal activity was found to be different even against the same type of larvae. U14.1, U14.4 and U14.5 isolates exhibited highly similar plasmid profile resembling *Btk*, but U6.6 produced only one type of plasmid band bigger than 19.3 kb.

Our local isolates produced cuboidal, bi-pyramidal and spherical shaped crystal proteins in

varying sizes around 45, 60, 70 and 130 kDa. Obeidat *et al.,* (2004) stated that strains producing bi-pyramidal and cuboidal crystal proteins showed similar protein profiles. Although both U6.6 and U14.2 had spherical crystal protein and similar SDS-PAGE protein profile, their insecticidal activity was not identical. Isolates of U14.1 and U14.5 produced bipyramidal and spherical Cry proteins and showed similar SDS-PAGE profile as well. Furthermore, U14.1 and U14.5 bear both *cry1C* and *cry1B* genes, but insecticidal activity caused on the larvae was not equal. Our results are in agreement with the study of some researchers who stated that strains sharing the same *cry* genes showed significantly different insecticidal potency (Dulmage, 1981; Ceron *et al.,* 1995; Martinez *et al.,* 2005 and Bozlağan *et al.,* 2010). In another study, Hongyu *et al.,* (2001) reported that 71% of their 122 isolates belonged to different mortality groups with 60% against *Spodoptera exugia* (Hübner, Lepidoptera: Noctuidae).

Yılmaz (2010) also reported that their local isolates collected from soil samples in Adana carrying the same type of *cry* genes exhibited different insecticidal activity (20 to 80%) against the larvae of the same species. These kinds of results are attributed to environmental factors, the target insect species, concentration and distribution methods of the product of different strains of microorganisms (Bauce *et al.,*. 2002; Carisey *et al.,* 2004; Kouassi, 2001). Insect metabolism may also have a profound effect on the efficacy of the toxins.

Results of this study suggest that local *Bt* isolates exhibit toxic effects on the survival and development of the *E. kuehniella* and *P. interpunctella* larvae. Although the spore-crystal mixture of the local *Bt* isolates did not display mortality higher than 54% at the highest experimental concentration, they caused a considerable decrease in the rate of development in the treated pest larvae. It can be suggested that spore-crystal mixture of these isolates can be used to reduce the damage on stored products caused by these important pest insects.

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