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Pathogenicity of Syrian Isolates of *Bacillus thuringiensis* (Berliner) Against the Cereal Leafminer *Syringopais temperatella* Led. (Lep., Scythrididae) Under Laboratory Conditions

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Abstract

This study investigates the efficacy of 22 Syrian isolates of *Bacillus thuringiensis* (Berliner) (*Bt*) against *Syringopais temperatella* under laboratory conditions by feeding larvae on bacteria-contaminated barley leaves.The results indicated that all *Bt* isolates caused concentration and time related mortality, in which the highest mortalities were recorded at the highest concentration (original concentration), and at the latest time after *Bt* application(72 h).Mortality caused by all *Bt* isolates was significantly higher than mortality in the control. Isolate no. 1 gave significantly (P<0.05) the highest mortality percentage (73.3%) against the early larval instars. For the late larval instars, the isolates no. 21 and 22 were at bar with each other and gave significantly (P<0.05) the highest percentage of mortality (53.3%). Early larval instars were significantly (P<0.05) more susceptible to all concentrations of *Bt* isolates used than the late larval ones.In conclusion, the study showed that some of *B. thuringiensis* isolates tested in this study were pathogenic to *S. temperatella*.

Keywords: *Bacillus thuringiensis*, Cereal leafminer, Bio-insecticide, Biological control, Syrian *Bt* isolates, *Syringopais temperatella*.

1. Introduction

Wheat and barley are the main cereal crops grown in Jordan (Jordan Statistical Yearbook, 2011). One of the major constraints to the production of these crops in Jordan is the cereal leafminer, *Syringopais temperatella* Led. (Al-Zyoud *et al*., 2009). The pest causes serious annual forage and yield reduction. Outbreaks of this pest have mostly occurred in southern Jordan for the last 12 years (Al-Zyoud, 2013) with a crop infestation estimated at 25% to 75% (Al-Zyoud, 2012).

The use of synthetic insecticides is currently the predominant method of controlling *S. temperatella* in Jordan (Al-Zyoud, 2007; Al-Zyoud, 2008). In spite of the intensive use of insecticides to suppress the pest, the infested areas are continuously increasing(Al-Zyoud *et al*., 2011). In addition, the massive use of pesticides in modern agriculture caused their widespread diffusion to all environmental compartments including humans (Samiee *et al*., 2009). Increased public concerns about adverse effects of pesticides prompted search of alternative methods (i.e.,biopesticides) for pest control.

The most widely used biopesticide worldwide is the bacterium, *Bacillus thuringiensis* (Berliner) (*Bt*) (Lacey *et al*., 2001). *Bt* occurs naturally in soil, dead insects, water and grain dust (Bernhard *et al*., 1997; Schnepf *et al*., 1998). During the sporulation process, the bacteria produce large crystal proteins that are toxic to many insect pests (Daly and Buntin, 2005). When orally ingested by insects, the crystal δ-endotoxins proteins of *Bt* formed, which are highly toxic to insects (Candas *et al*., 2003; Balaraman, 2005; Roh *et al*., 2007).The *Bt* preparations are the most successful bio-control products worldwide (Kaur, 2002). *Bt* has been used to control lepidopteran, coleopteran and dipteran pests on food crops, ornamentals, forest trees and stored grains (Iriarte *et al*., 1998; Theunis and Aloali'i, 1999; Balaraman, 2005). Ammouneh *et al.* (2011) reported that *Bt* isolates are very toxic to *Ephestia kuhniella*, *Phthorimaea operculella* and *Cydia pomonella* larvae (Lepidoptera). In addition, Giustolin *et al.* (2001) and Gonzalez-Cabrera *et al.* (2011) found that *Bt* is highly efficient in controlling the tomato borer, *Tuta absoluta* (Meyrick). In spite of *Bt* was used in spray formulations for more than 40 years against nearly 3,000 species (Huang *et al*., 2004), only

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one study has evaluated its efficacy on *S. temperatella* using the *israelensis* strain (Al-Zyoud *et al.*, 2011). However, since some *Bt* isolates produce diverse insecticidal proteins that show differential insecticidal activities (Meihiar *et al*., 2012), there is a high possibility to find some isolates which may be used to effectively control *S. temperatella*. Nevertheless, the continued damage to cereal crops by the pest is a daunting challenge and requires the use of new control methods. Therefore, this study aimed at evaluating the toxicity of 22 Syrian *Bt* isolates against *S. temperatella*. It is hoped that the application of *Bt* will play a central role in protecting cereal crops; thereby, it will drastically replace, at least in part, some of the most dangerous insecticides currently used against *S. temperatella*.

2. Materials and Methods

2.1. Rearing of Syringopais temperatella

The rearing of *S. temperatella* was initiated from hundreds of first larval instarscollected from a barley field in Al-Rabbeh/ Karak-Jordan (Latitude of 31°11" N and Longitude of 35°42" E, altitude of 980 m). The larvae were maintained on potted (12x12 cm) barley plants in a rearing room. The infested plants were kept in meshed cages of 50x50x50 cm under laboratory conditions of 20±5°C temperature, 60±10% relative humidity and 12: 12 (L: D) h photoperiod. Barley of Mu'tah cultivar was used for rearing the pest and conducting the experiments. The plants were frequently replaced whenever needed to maintain adequate host-plant supply. Two groups of larvae were used in the experiments, which were randomly selected from the culture. The first group consisted of early larval instars ($L_1$and L_2), while the second group consisted of late larval instars (L_4 and L_5).

2.2. Culturing, Preparation and Characterization of Bacillus thuringiensis Isolates

The *Bt* isolates were obtained from Dr. Maysa Meihiar as 22 biochemical types. *Bt* was isolated from Syrian soils of different plant communities (Meihiar *et al.*, 2012) following the method described by Ohba and Aizawa (1986). *Bt* subsp. *Kurstaki* HD1 (Abbott Laboratories, Chicago, USA) was used as a reference for comparison with the previous results of the nearest insect species (*Ephestia kuehniella* Zeller) and also as a positive control for results of the present study. One ml of each *Bt* isolate-suspension was added into 10 ml of T_3 medium (g per liter: 3 tryptone, 2 tryptose, 1.5 yeast extract, 0.05 M sodium phosphate at pH 6.8 and 0.005 of $MnCl_2$). The suspension was incubated at 30ºC for 7 days until the bacterial cells have sporulated for crystal production (Travers *et al*., 1987; Martin and Travers, 1989). In order to know the number of viable cells at the time of bioassay and after 7 days of incubation,1 ml of each of the 22 cultures was separately poured in 9 ml of sterile distilled water, and hereafter 9-fold serial dilutions were made. Aliquots of 1 ml of 7-9 fold dilutions were plated in duplicate by pour-plate technique using nutrient agar, and incubated at 30ºC for 2 days. The colony forming units (CFU) were visually counted for the 22 different bio-assayed isolates (Kango, 2010) (Table 1).

Table 1. Colony forming units (CFU) and shape of crystals of the twenty two bio-assayed *Bacillus thuringiensis* soil isolates.

Isolate no.	Isolate subsp.	CFU/ml (mean)	Crystal morphology
Bt 1	Untypable	9.00 E + 08	Bipyramidal + Cuboidal
Bt 2	Untypable	3.50 E + 09	Bipyramidal + Cuboidal
***Bt*3**	***Kurstaki* HD1**	**6.00 E + 09**	**Bipyramidal + Cuboidal**
Bt 4	*kurstaki*	5.20 E + 09	Bipyramidal + Cuboidal
Bt 5	Untypable	8.25 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 6	*Sotto*	1.50 E + 11	Bipyramidal
Bt 7	Untypable	1.44 E + 11	Rectangular + Bipyramidal + Cuboidal
Bt 8	Untypable	1.05 E + 11	Irregular + Bipyramidal + Cuboidal
Bt 9	Untypable	1.57 E + 11	Bipyramidal + Cuboidal
Bt 10	Untypable	1.95 E + 09	Bipyramidal + Cuboidal
Bt 11	Untypable	9.50 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 12	*kurstaki*	2.50 E + 08	Bipyramidal + Cuboidal
Bt 13	*kurstaki*	8.50 E + 08	Bipyramidal + Cuboidal
Bt 14	Untypable	1.65 E + 10	Rectangular + Bipyramidal + Cuboidal
Bt 15	Untypable	8.50 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 16	Untypable	9.00 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 17	Untypable	2.15 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 18	Untypable	9.50 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 19	Untypable	1.45 E + 09	Rectangular + Bipyramidal + Cuboidal
Bt 20	Untypable	1.60 E + 10	Rectangular + Bipyramidal + Cuboidal
Bt 21	Untypable	1.00 E + 09	Like*Bt*subsp. *Finitimus* (rhomboid in shape)
Bt 22	Untypable	1.00 E + 09	Bipyramidal + Cuboidal

**Bt* subsp. *Kurstaki* HD1 (Dipel®- Abbott Laboratories, Chicago, USA) used as a reference strain

2.3. Bioassay

The bioassay was set up in Petri-dishes of 5.5 cm in diameter that were partially filled with 0.5 cm thick layer of wetted cotton pad. The lid of each Petri-dish had a hole closed with organdie fabric for ventilation. Barley leaf strips of10 cm^2area, which were cut from un-infested barley plants, were placed in the Petri-dishes. Either *S. temperatella* early or late larval instars were gently transferred using a Camel hairbrush into the Petri-dishes in groups of ten larvae/Petri-dish. Larvae in the control treatments (n=10) were provided with leaf strips dipped in sterilized distilled water for 30 sec, while larvae in the *Bt* treatments (n=10) were provided with leaf strips dipped in an aqueous solution of the required *Bt* isolate and concentration (original and twofold serial dilutions) for 30 sec. The Petri-dishes were kept under the above-mentioned laboratory conditions. Larval mortality was recorded 1, 2 and 3 days post-treatment with *Bt*. Larvae were considered dead if they do not move when gently prodded with fine brush. Data obtained for mortality percentages of early and late larval instars of *S.*

temperatella were plotted as a linear correlation function versus the CFU.

2.4. Statistical Analysis

The statistical analysis was performed using the proc GLM of the statistical package Sigma Stat version 16.0 (SPSS, 1997). The experimental design was a complete randomized design (CRD) with three replications for each isolate and concentration. The data were analyzed using one way ANOVA to detect any differences in the pest mortality (Zar, 1999). When significant differences were detected, means were compared using LSD at 0.05 probability level (Abacus Concepts, 1991). The *t-test* was used for comparison between only two means (Anonymous, 1996). In addition, the correlation between mortality and progress of time after spraying as well as mortality and concentration of *Bt* was calculated by Spearman's correlation method (Zar, 1999).

3. Results

The results presented herein are for *Bt* isolates as an original concentration (named conc. 1), first-fold dilution (named conc. 2) and second-fold dilution (named conc. 3). However, the results revealed that the original concentration of *Bt* (ranged between CFU = 2.5×10^{8} for isolate *Bt* 12 (*Bt kurstaki*) to 1.57×10^{11} for isolate *Bt* 9) gave the highest percentage of mortality for both *S. temperatella* early (F=4.250; 2, 195 df; P=0.016) and late (F=6.655; 2, 195 df; P=0.002) larval instars tested, but it was at bar with the first-fold dilution, while it differed significantly from the second-fold dilution (Figure 1). The mortality percentages resulted from the original and the second dilutions were 20.8% and 12.5% (early instars), and 16.5% and 8.6% (late instars), respectively. Furthermore, early larval instars were significantly more susceptible to all *Bt* isolates and concentrations used than the late larval instars (Figure 1).

The highest larval mortality was recoded after 72 hr of *Bt* application, and it was significantly different from those at24 and 48 hr post application for both early (F=6.990; 2, 63 df; P=0.002) and late larval instars (F=22.987; 2, 63 df; P=0.000) (Figure 2). Mortalities of 26.8% and 21.0% for early and late larval instars' were reported 72 hr post *Bt* application, respectively, while the mortalities were only 9.6% and 3.6% after 24 hr of *Bt* application, respectively. Early larval instars were more susceptible to *Bt* than the late instars at all times after application (24, 48 and 72 hr) (Figure 2).

Overall mortality percentage of *S. temperatella* as a result of leaf-dipping in the three different concentrations of *Bt* isolates for both early and late larval instars are presented in Figure 3. Mortality caused by all *Bt* isolates was significantly higher than that of the control. Moreover, the isolates; *Bt*1, *Bt* 21 and *Bt*13 (*Bt kurstaki*) caused significantly the highest mortality percentages with 28.2%, 26.5% and 25.7%, respectively (F=4.884; 22, 391 df; P=0.000). In contrast, the isolates; *Bt*8, *Bt*17 and *Bt*4 (*Bt kurstaki*) caused significantly the lowest mortality with 4.3%, 5.8% and 5.9%, respectively.

Since the results in Figure 1 and 2 showed that the highest efficacy of *Bt* was obtained 72 hr post *Bt* application at the original concentration, further statistical analysis was made at these time and concentration to check the efficacy of all *Bt* isolates tested as shown in Figure 4. The results indicated, for early larval instars, that the isolate *Bt* 1 gave significantly the highest mortality percentage (73.3%), followed by the isolates; *Bt* 13 (*Bt kurstaki*) (66.7%), 19 (63.3%) and 16 (63.3%), while the isolates; *Bt* 8, *Bt*9, *Bt* 17, *Bt*14 and *Bt* 12 (*Bt kurstaki*) were at bar with the control treatment (mortality: 0.0-6.7%) (F=23.040; 22, 46 df; P=0.000). For late larval instars, the isolates; *Bt* 21 and *Bt*22 were at bar with each other and gave significantly the highest percentage of mortality (53.3%). Moreover, all isolates gave significantly higher mortalities to the late larval instars than control (F=20.996; 22, 46 df; P=0.000). Out of 22 isolates, three isolates (*Bt*20, *Bt*21 and *Bt*22) caused higher mortality for both early and late larval instars.

There was a positive significant correlation between time after *Bt* application and mortality percentage as well as between *Bt* isolate's concentration and mortality percentage (Table 2). This means that with increasing time after *Bt* application and *Bt* concentration there was an increase in the mortality of *S. temperatella*. No clear relationship was noticed between the efficacy of *Bt* isolates and shape of crystal proteins they have; however, it is to be mentioned that most of the effective and ineffective isolates have bipyramidal and cuboidal parasporal crystal protein shapes. The correlation between CFU and larval mortality was plotted for the promising isolates (that gave mortality higher than 40%) against early (Figure 5A) and late (Figure 5B) larval instars. The results indicated that the relationship isof increasing function; higher mortalities were obtained from effective isolates of higher CFU.

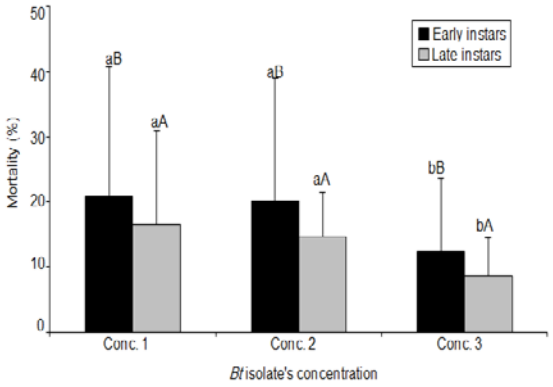

Figure 1. Mortality percentage (±SD) of *Syringopais temperatella* in relation to larval instars resulted from application of different *Bacillus thuringiensis* isolates in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions. [Different small letters above bars indicate significant differences among the different *Bt* concentrations within the same instar, while capital letters above bars indicate significant differences between the different instars within the same concentration at p<0.05 (one-factor analysis of variance)].

Table 2. Correlation analysis of mortality percentage of *Syringopais temperatella* versus time and concentration of *Bt* isolates in a feeding larval test on bacteria-contaminated barley leaves under laboratory conditions.

Correlated variables	Instar	R value	Significance
Time vs. Mortality percentage	Early instars	0.411**	0.001
	Late instars	0.707**	0.000
	Both (early and late)	0.682**	0.000
Bt isolate's concentration vs. Mortality Percentage	Early instars	0.233*	0.050
	Late instars	0.385**	0.001
	Both (early and late)	0.365**	0.003

*Correlation is significant at the 0.05 probability level.
**Correlation is significant at the 0.01 probability level

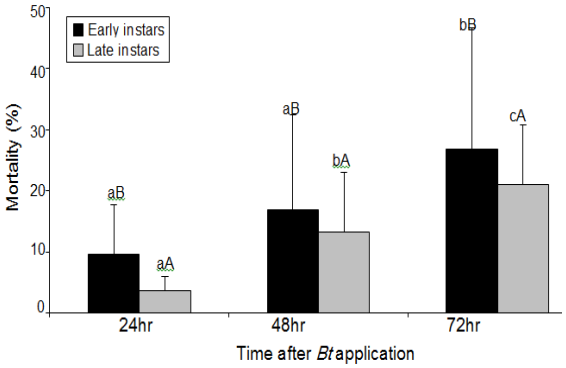

Figure 2. Mortality percentage (±SD) of *Syringopais temperatella* in relation to time after *Bt* application resulted from different isolates in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions. [Different small letters above bars indicate significant differences among the different times within the same instar, while capital letters above bars indicate significant differences between the different instars within the same time at $p<0.05$ (one-factor analysis of variance)].

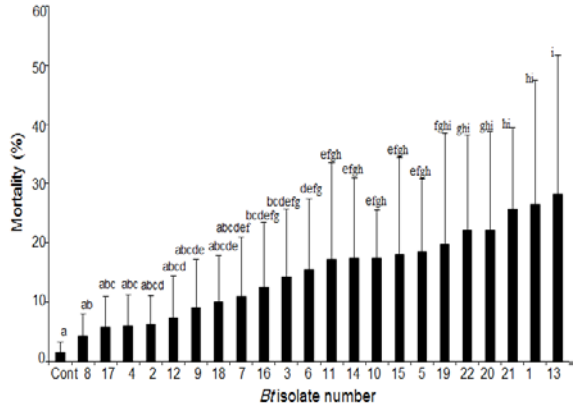

Figure 3. Overall effect of *Bacillus thuringiensis* different isolates (all concentrations) on *Syringopais temperatella* mortality percentage (±SD) in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions.Isolate no. 3 (*Bt* subsp. *kurstaki* HD1) is the reference strain.[Different small letters above bars indicate significant differences among the different *Bt* isolates at $p<0.05$ (one-factor analysis of variance)].

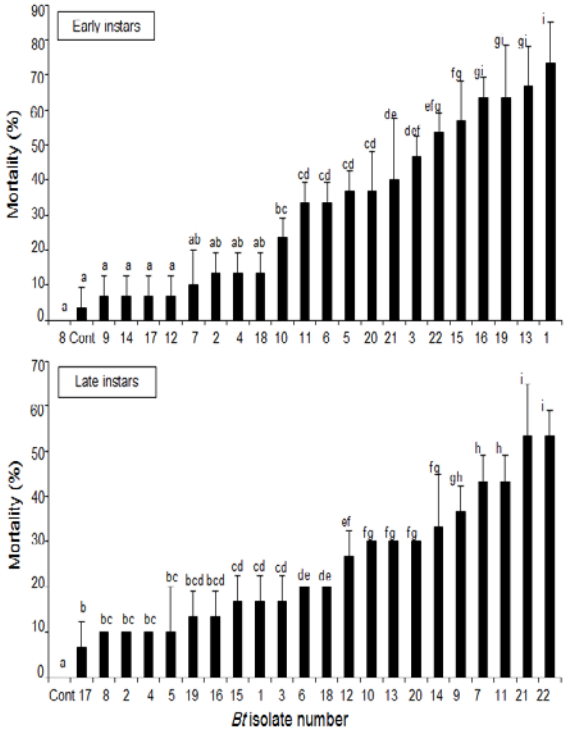

Figure 4. Mortality percentage (±SD) of *Syringopais temperatella* early and late larval instars as a result of application by the higher concentration of *Bacillus thuringiensis* different isolates after 72 hrin a feeding larval test on bacterial contaminated barley leaves under laboratory conditions.Isolate no. 3 (*Bt* subsp. *kurstaki* HD1) is the reference strain.[Different small letters above bars indicate significant differences among the different *Bt* isolates at $p<0.05$ (one-factor analysis of variance)].

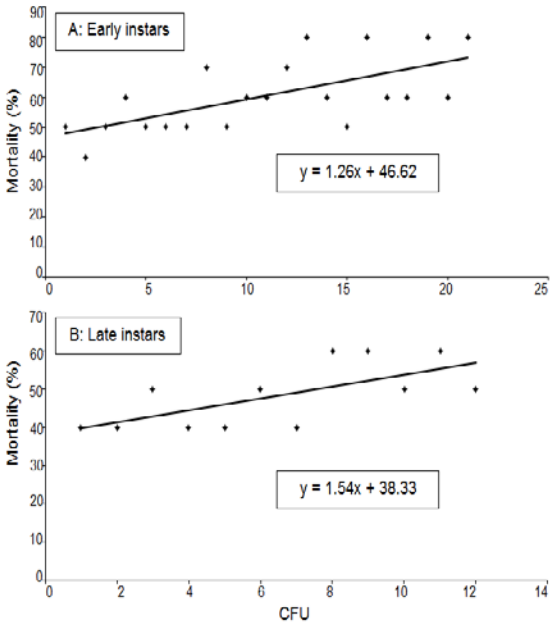

Figure 5. Scatter diagram with a line of best fit curve of obtained mortality percentages of the early (A) and late (B) larval instars of *Syringopais temperatella* after 72 hr as a function of colony forming units (CFU) of the effective *Bacillus thuringiensis* isolates.

4. Discussion

In this study four variables were considered; the *Bt* isolates (22 different isolates), the target larval instar (early and late), the *Bt* concentration (original, first-fold and second-fold dilutions) and the effect of time after exposure to *Bt* (24, 48 and 72 hr). The reference strain we used (*Bt* subsp. *kurstaki* HD1) is the same used by Meihiar *et al.* (2012). We reported 48% mortality for the reference strain against *S. temperatella* while 53% mortality was reported by Meihiar *et al.* (2012) against *E. kuehniella* from the same insect order. For the 72 hr-mortality at the higher *Bt* concentration used, 6 and 2 isolates out of the 22 isolates were effective against early and late larval instars of *S. temperatella*, respectively. They reported mortality percentages more than that reported for the reference strain. We think that these promising isolates produce *cry* gene toxic against the pest larvae as reported by many researchers (Tabashnik *et al.*, 1994; Porcar *et al.*, 2000; Song *et al.*, 2003). In addition, the level of gene expression plays a role in the toxicity as proposed by Porcar *et al.* (2000). However, the *Bt* toxicity did not depend on *cry* gene content only, because factors other than *cry* proteins may contribute to the toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β–exotoxin (Porcar *et al.*, 2000). Moreover, Martinez *et al.* (2004) suggested that the biological activity of a strain cannot be fully predicted on the basis of its *cry* gene content alone. The relative proportion of the *cry* proteins produced, their interaction, and the possible presence of undetected crystal proteins are all important. The present results revealed that the isolate number 13 (*Bt kurstaki*) caused 68% mortality after 3 days of exposure to early instar larvae of *S. temperatella* which is coincide with the previously reported results for this bacteria subspecies using the same pest with 71.4%% mortality (Al-Zyoud *et al*., 2011). Furthermore, we suggest that isolates are mostly produced the toxic proteins after two days of infection. What Al-Zyoud *et al.* (2011) found is supporting our suggestion, where they found that a gradual increase in mortality with time post *Bt* spraying against *S. temperatella* larvae. Moreover, the current results indicated that all *Bt* isolates caused concentration related mortality, in which the highest mortality was recorded at the highest concentration. Therefore, the results showed that both time and concentration play an important role in the bacteria efficacy. On the contrary of Obeidat *et al.* (2004) and Meihiar *et al.* (2012) findings, the shape of the isolates' crystal proteins did not correlate with the isolate efficacy; where bipyramids and cuboids crystal proteins were found in our both effective and ineffective isolates. Obeidat *et al.* (2004) found that out of the twenty-six strains of *Bt*, serotypes *kenyae*, *kurstaki* and *kurstaki* HD1 produced bipyramid crystal proteins which were toxic to *E. kuehniella*. Moreover, Meihiar *et al.* (2012) confirmed Obeidat *et al.*(2004) finding, in which *Bt* strains producing bipyramid and cuboid crystal shapes are the most toxic to the same insect. In the current study, the remaining 11 isolates were found less effective against the *S. temperatella* larvae. The reason behind this is that such isolates might not produce *Cry* proteins or their genes were of low level of expression. In addition, the same isolates investigated in this study were previously bio-assayed against *E. kuehniella* by Meihiar *et al.*(2012), and PCR was used to examine their *cry* genes content. Their findings demonstrated that the most toxic isolates harbor different specific *cry* genes including *cryI* and *cryIV* which have an insecticidal activity to lepidopteran insects. This finding is also proposed previously by Ammouneh *et al*. (2011), in which the type of the *cry* genes was found to correlate with its insecticidal activity.

The present results revealed that the isolate *Bt* 1 was effective in controlling the early instars larvae but it showed low efficacy against late instars. This might be due to the low number of bacterium cells in the original concentration due to the low sporulation rate, and as a result low number of crystal proteins (as shown in Table 1)as compared with the isolates; *Bt* 21 and *Bt* 22, which were efficient against the late instars. Therefore, this little number of crystal proteins in the isolate could not produce enough amounts of toxins to affect the late larval instars which are much larger in size than the early ones. This justification is supported in our findings (as shown in Figure 5) as indicated by the relationship between the number of bacterial cells and the mortality obtained. This relationship is of a positive trend; the more number of bacterial cells the higher the mortality obtained. It is to be mentioned that the number of bacterial cells is positively correlated with the amount of crystals and subsequently the amount of toxins they produced to cause death to the pest larvae.

In conclusion, some of *Bt* isolates used in this study exhibit a toxic potential against the pest and, therefore, they could be adopted for future control program to suppress the pest as a part of IPM program, and thus will reduce and/or replace the most dangerous chemical insecticides currently used against the pest in Jordan and surrounding countries. Better pest control strategy can increase farm incomes and reduce the hazards in rural areas associated with insecticides' use as well as will contribute to improve food security where wheat and barley are major sources of food for human and their animals in the region.

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