Differing Susceptibility to *Bacillus thuringiensis* Formulations of *Thaumetopoea wilkinsoni* Populations Between Forests with Different *Bt* Management in Israel

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The susceptibility of *Thaumetopoea wilkinsoni* larvae to *Bacillus thuringiensis* (*Bt*) formulations was screened in 2003 and 2004. Eggs and larvae were collected from pine forests in 11 geographical locations in Israel. Larval mortality bioassays were conducted with commercial formulations (Delfin WG, Dipel DF and Foray 48B) at concentrations ranging from 0.001% to 0.1%. Significant differences in susceptibility to *Bt* were recorded among populations that were treated with *Bt* intensively, frequently, or never. The mortality recorded in a population that was never treated with *Bt* was twice that in an intensively *Bt*-treated population. The correlation between susceptibility to *Bt* and the possible resistance to the microbe is discussed.

KEY WORDS: *Thaumetopoea wilkinsoni*; larval bioassay; susceptibility; *Bacillus thuringiensis;* geographical locations; pine forests.

INTRODUCTION

The pine processionary moth (PPM) *Thaumetopoea wilkinsoni* is the major defoliator of pine in the Middle East, whereas the sister species *T. pityocampa* occurs in southern Europe and North Africa. The life and seasonal histories of PPM have been thoroughly studied (*e.g.* 7). Adults emerge in August – October after aestivation as pupae. The larvae feed on needles during the winter and pupate in the ground in the spring (March – May). The damage is caused mainly to young stands, open plantations and woodland in semi-arid areas (6,10). The insect is a health threat to foresters, visitors and camping travelers. Contact with the caterpillars' poisonous hairs elicits allergic reactions. Urticaria and conjunctivitis are the main health problems, which in severe cases may require hospitalization (15).

Large forest areas in the Mediterranean Basin are treated annually with *Bacillus thuringiensis* (*Bt*) (1,2). The conventional integrated pest management procedure against *T. wilkinsoni* in pine forests in Israel is based on the aerial and ground application of commercial *Bt* products (Z. Madar and N. Saphir, unpublished report). *Bt* has been used for more than four decades in Israel. Observations here during recent years have indicated that the efficacy of *Bt* commercial formulations against the PPM population in several geographical locations has been less than it used to be (JNF, Forest Department, unpublished report). It is not clear whether these differences in susceptibility derived from a selection pressure for insect resistance to *Bt* were induced as a result of intensive application

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of the microbe, or whether they resulted from interactions of genetic, environmental and management factors (3).

The high toxicity of commercial preparations of *B. thuringiensis* subsp. *kurstaki* to *T. wilkinsoni* larvae had already been recorded at the beginning of the 1960s (11). Lately, Shevelev *et al.* (14) observed a considerable intra-population variation in susceptibility of the 1^{st} instar larvae of *T. pityocampa* to several *Bt* toxins, which suggests that there may be potential in the population for the development of resistance to *Bt*. However, the variations in the susceptibility of the insect to *Bt* have never been studied in depth. According to Rausell *et al.* (12), Cry1Aa, Cry1Ab and Cry1Ac were highly toxic to 1^{st} instar *T. pityocampa* larvae. However, during larval development, the loss of one of the two Cry1Ab high-affinity binding sites was detected to occur toward the last-instar larvae.

The overall objective of the study was to screen for differences in the susceptibility of PPM to *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*). In this screening, larval bioassays for recording mortality were optimized in terms of larval age and source of the needles. The idea was to screen geographically separated populations and to determine whether differences in susceptibility could result from selection pressure for resistance to the microbe based on the intensity of *Bt* treatment during previous years.

Two objectives were designated. The first was to compare the susceptibilities to *Btk* in populations of PPM that were rated according to the intensity of *Bt* application under a multiyear regime: (*a*) regularly treated populations; (*b*) less frequently treated populations; and (*c*) populations that had never been exposed to *Bt*. The *Bt*-treated populations included both core and expanding populations (*viz.*, contributing population *vs* population which was recently established in forest previously not infested by the moth). The second objective was to compare the effectiveness of *Btk* products and formulations against the insect by means of optimized bioassay procedures.

This work constitutes part of the efforts to improve the efficacy of *Bt*-based IPM of *T. wilkinsoni*.

MATERIALS AND METHODS

Geographical locations of insect sources Table 1 details the frequencies of *Bt* application, the geographical locations of *T. wilkinsoni* populations in Israel that were used for screening differential susceptibilities to *Bt* in 2003, and pine species and ages of forests. 'Intensive exposure' refers to forests in which the study plots and their surroundings had been subjected to annual *Bt* applications; 'frequent exposure' refers to forest areas in which *Bt* is applied on approximately 25% of the susceptible stands, with the entire area covered with *Bt* spray in rotation over 4–5 years; 'never' refers to forest areas that have never been treated with *Bt*.

In 2004, the comparison of susceptibilities to *Bt* was focused on the Segev population that was exposed to 'intensive' application of *Bt*, and the Hafurit population that had never been exposed to it.

Insects Egg masses, the hatching neonates, as well as 1^{st} to 3^{rd} instar larvae of *T. wilkinsoni* were collected in pine forests and placed in petri dishes containing moistened filter paper that were kept at room temperature (22–25°C) and under a natural photoperiod. The hatching larvae were offered pine (*Pinus halepensis*) branch tips cut from saplings grown in a greenhouse in the experimental plot of the Department of Entomology, ARO, at Bet Dagan. Since high natural larval mortality in the control was recorded in our early

trials when 1–3-day-old larvae were used, we used 4–6-day-old larvae in the bioassays. In the 2003 season the live insect material was collected in all 11 forests (Table 1). From each population, 10–20 egg batches and 5–20 nests were collected. The nests of 2^{nd} and 3^{rd} instar caterpillars were collected in November–December.

Products and formulations The following *Bt* products were used in this work:

- Delfin WG, water dispersible granule, 32,000 IU/mg. Certis USA, Columbia, MD, USA.
- Dipel DF, wettable powder, 32,000 IU/mg. Valent BioSciences Corp., Libertyville, IL, USA.
- Foray 48B, concentrated liquid, 10,600 IU/mg, Valent BioSciences Corp.

In 2003, Delfin was bioassayed at 0.1%, 0.01% and 0.001%, and the aqueous mixture included 0.05% (w/v) of Colfix (an adhesive containing 40% polyvinyl resins and 60% adjuvants; Rimi, Petah Tiqwa, Israel). The formulation was tested on *T. wilkinsoni* populations from 11 different geographical locations.

In 2004 the three *Bt* products were applied to compare their efficacy on what seems to be the most susceptible population (Hafurit) with what seems to be the most resistant population (Segev). Delfin and Dipel DF were tested at 0.01% and Foray 48B at 0.03%, so that the three products were used at equal potency.

Sources of pine needles Mature 7–9-cm-long needles were taken from 18-month-old *P. halepensis* potted saplings grown in a screen greenhouse. The larvae were fed with true needles removed from the upper part of the seedlings.

For the bioassay, only previous-year needles (usually the last three cohorts) were used. The terminal ends of needles that were longer than 9 cm were cut off to achieve a standard length. In order to preserve the vitality of the needles, they were removed together with the sheet. The needles were washed with water, dried with blotting paper, dipped in the *Bt* mixtures for 10 s, and air-dried at room temperature in a microbiological hood.

***Bt* bioassays** Small glass test tubes (95 × 10 mm, length × internal diameter) each containing three brachyblasts (a total of six needles) and ten larvae, were used. Each tube constituted a replicate and there were five replicates per treatment. The larvae were of the same age and were randomly removed from two or three colonies with a soft camel-hair brush; the same group of egg masses was never used in a different measurement.

The tubes were closed with cling-plastic adhesive wrap, and incubated at 23°C, 60–80% r.h. and 12:12 L:D photoperiod. The adhesive plastic sheet allowed the passage of ventilating air and humidity. The bioassay period was 144 h and mortality counting started after 72 h. The experiments were conducted from November until mid-December 2003.

In 2004 the experimental season extended from mid-November until the beginning of January. During the 2004 insect season the bioassay technique was further optimized by meticulousness of a uniform insect age with the tested larva instar and needle sources which proved to cause minimum natural mortality. Needle consumption by the larvae was estimated visually.

Statistical analysis The Lifetest procedure from the SAS software package (13) was used for the analysis of larval mortality. The proportion (P) of mortality was transformed into arc sin $\sqrt{}$ P.

In order to analyze the effect of various treatments at different times, the GLM

TABLE 1. Sites in Israel where pine processionary moth egg-masses were collected for use in screening differential susceptibilities to *Bacillus thuringiensis (Bt)* (sites grouped according to frequency of *Bt* application)

Location and exposure to *Bt*	Longitude	Latitude	*Pinus* species	Years on site[z]
Intensive				
Segev (SEG)	35°14'E	32°52'N	*P. brutia*	8
Yatir (YA)	35°03'E	31°20'N	*P. halepensis*	28
Qiryat Shemona (KH)	35°33'E	33°11'N	*P. brutia*	30
Dishon (DI)	35°31'E	33°04'N	*P. brutia*	5
Frequent				
Haruvit (HA)	34°50'E	31°45'N	*P. halepensis*	50
Eshta'ol (ES)	35°00'E	31°47'N	*P. halepensis*	53
Kissufim (QIY, QIM)[y]	34°24'E	31°22'N	*P. halepensis*	30
Never				
Hafurit (HP)	35°00'E	31°40'N	*P. halepensis*	35
Moran (MOR)	35°23'E	32°56'N	*P. halepensis*	35
Hadera (HDR)	34°56'E	32°29'N	*P. canariensis*	30

[z] Years after the colonization of the moth in the area.
[y] QIY, young stand; QIM, mature stand.

procedure was used. When differences among means of the treatments were significant, a Student-Newman-Keuls test was used for multiple comparisons among means. In order to compare the various formulations, Wilcoxon's χ-square test was used.

RESULTS

Effects of location and *Bt* concentration (Delfin) on mortality in 2003 As shown in Table 2, all tested concentrations of Delfin produced mortality of 1^{st} and 2^{nd} instars significantly higher than in control already at 72 h exposure. For the 3^{rd} instar, similar significant differences in efficacy of Delfin concentrations and control were obtained only after 144 h. Significant differences in susceptibility were obtained among populations for the 1^{st} instar both after 72 h and after 144 h exposure. Similar results were obtained for the 2^{nd} instar after 72 h exposure and after 144 h exposure. For the 3^{rd} instar, the significant differences in susceptibility of populations to Delfin were detected after 144 h exposure of larvae on treated needles.

TABLE 2. Statistical analysis of differential susceptibility to *Bacillus thuringiensis* in *Thaumetopoea wilkinsoni* from different geographical populations (GLM and Student-Newman-Keuls test)

	Concentrations					
	1st instar (df=35, 3)		2nd instar (df=27, 3)		3rd instar (df=15, 3)	
Feeding time (h)	72	144	72	144	72	144
F value	21.29	88.81	14.75	75.17	0.72	32.29
P>F	<0.0001	<0.0001	<0.0001	<0.0001	0.5452	<0.0001
	Locations					
	1st instar (df=35, 8)		2nd instar (df=27, 6)		3rd instar (df=15, 3)	
Feeding time (h)	72	144	72	144	72	144
F value	8.37	9.50	29.48	41.10	4.93	15.85
P>F	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001

In Figures 1, 2 and 3, populations are grouped by frequency of *Bt* treatments. The larvae hatched from egg masses collected from never-sprayed or frequently sprayed forests

TABLE 3. Comparison (based on LT_{50}) of population that was never treated by *Bacillus thuringiensis (Bt)* (Hafurit) and population treated intensively by *Bt* (Segev), with three *Bt* formulations

Formulation	LT_{50}, h (95% CI)		Mean±SE		Chi-square (Wilcoxon)	P^z df = 1
	Never treated	Treated intensively	Never treated	Treated intensively		
Delfin	108 (96–144)	132 (120–.[y])	121±5.3	136±4.5	4.9444	0.0262
Dipel	126 (96–144)	>168 (168–.)	123±5.4	150±3.9	16.6943	<0.0001
Foray	128 (120–168)	156 (144–.)	130±5.2	145±4.4	4.9228	0.0265
Control	–	–	165±2.1	160±3.4	–	–

[z] Significant differences between Hafurit and Segev populations at $P<0.05$.

[y] Upper confidence interval was not estimated because time of observation was limited (mortality was censored beyond the sampling period).

exhibited similar mortalities: the median lethal time calculated for 0.01% Delfin acting on these groups ranged from 84 h for the Hafurit and Kissufim populations to 108 h for the Haruvit population. However, larvae hatched from egg masses collected from sites with annual exposure to *Bt* formulations were less susceptible, with median lethal times ranging from 124 h for the Yatir populations to more than 144 h for the Qiryat Shemona and Segev populations, which indicates a potential for resistance development.

Differential susceptibility to *Bt* in PPM populations from Segev and Hafurit - 2004 Mortality among the larvae of the two populations was significantly higher than that in the control ($\chi^2 = 49.4990$, df = 3, $P<0.0001$ for the Hafurit population and $\chi^2 = 21.7889$, df = 3, $P<0.0001$ for the Segev population). The median lethal times of 1^{st} instar larvae hatched from the Hafurit populations were significantly less than those of the Segev population under exposure to Delfin, Dipel and Foray 48B products (Fig. 4, Table 3). The variability in susceptibility of the Hafurit population exposed to the various *Bt* formulations was not significant ($\chi^2 = 1.1400$, df = 2, $P= 0.5655$), in contrast to the Segev population – which showed small but significant differences in susceptibility to the *Bt* formulations ($\chi^2 = 6.5298$, df = 2, $P=0.0382$). Mortality among Segev larvae exposed to Delfin occurred sooner than among those exposed to Dipel or Foray (Table 3). Mortality of the Hafurit larvae at 144 h was 62–70% for the three *Bt* formulations but in the Segev population it was 36–56%.

Optimization of the bioassay procedure Preliminary tests in 2003 revealed that the petri dish bioassay was unacceptable because the larvae did not consume the needles and they died rapidly. The alternative test, using small glass tubes sealed with plastic wrap, was used throughout all the experiments. Two aspects of the bioassay were evaluated further during the experiments conducted in 2004.

Effects of pattern of egg hatching on larval survival Figure 5 shows that in bioassays with larvae that hatched on the same day (synchronized hatching), the difference between the Segev and Hafurit populations in susceptibility to *Bt* was highly significant ($\chi^2 = 16.6943$, df = 1, $P<0.0001$); the population from Hafurit was twice as susceptible to *Bt* (80% mortality) as that from Segev (40% mortality). These differences were not significant for larvae that hatched over a period of 3–4 days instead of on the same day.

Effect of larval age within 1^{st} instar on natural mortality Figure 6 shows that natural mortality differed depending on age of larvae which were used for bioassay. When neonates or 1–2-day-old larvae were used for the bioassay, the natural mortality was close to 100% after 72 and 144 h, whereas mortality of larvae aged 3–4 days was approximately 50%

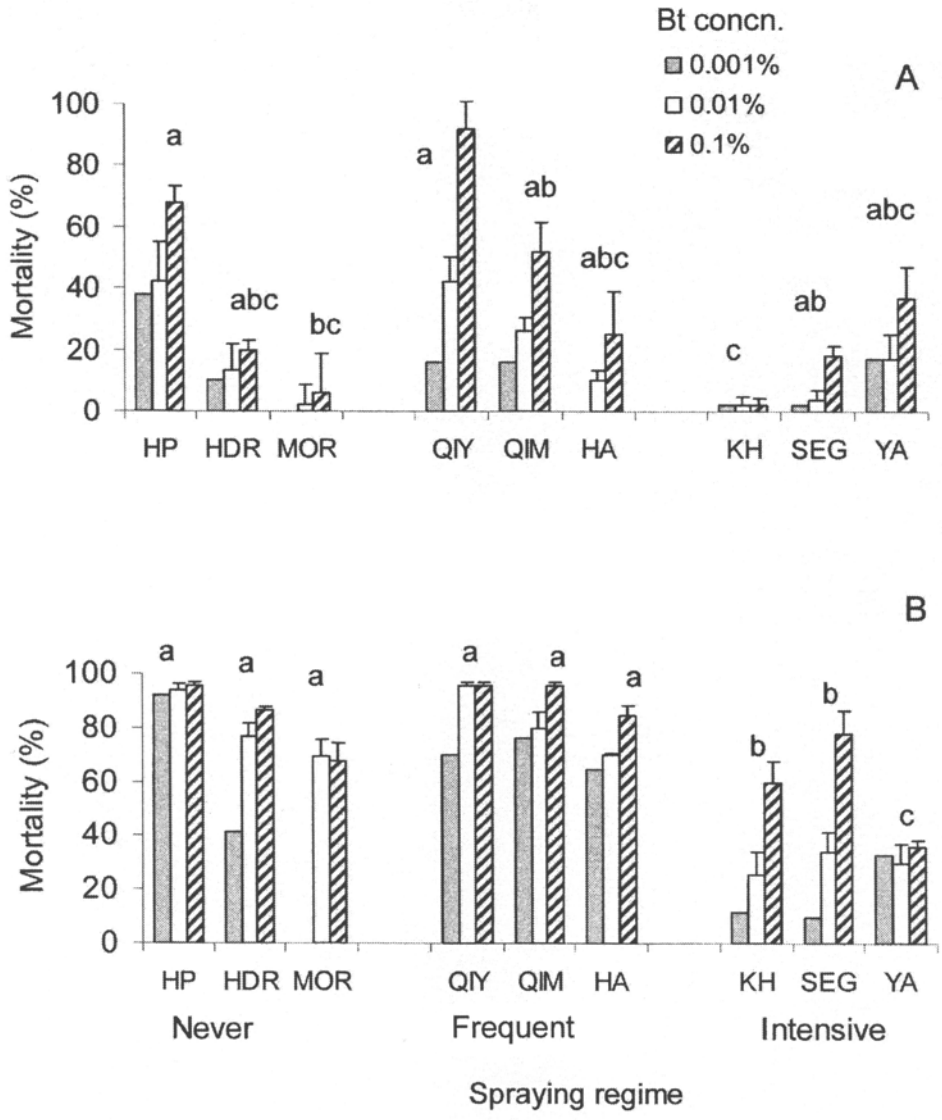

Fig. 1. Mortality (mean ±SE) of 1^{st} instar of *Thaumetopoea wilkinsoni* caused by three concentrations of *Bt* (Delfin) during 72-h (A) and 144-h (B) bioassays. Populations were from different geographical locations as related to the frequencies of *Bt* application (see Table 1). Populations with a common letter do not differ significantly (P=0.05) in their overall mortality from the test *Bt* formulation.

and in 5- to 6-day-old larvae it was not more than 10%. Moreover, it was observed that the 1-day-old larvae used for the bioassay ate very little. In contrast to this, when the entire family brood was kept together for a longer time after hatching, and the larvae were able to maintain their natural aggregative behavior for 3–4 days, their feeding activity in

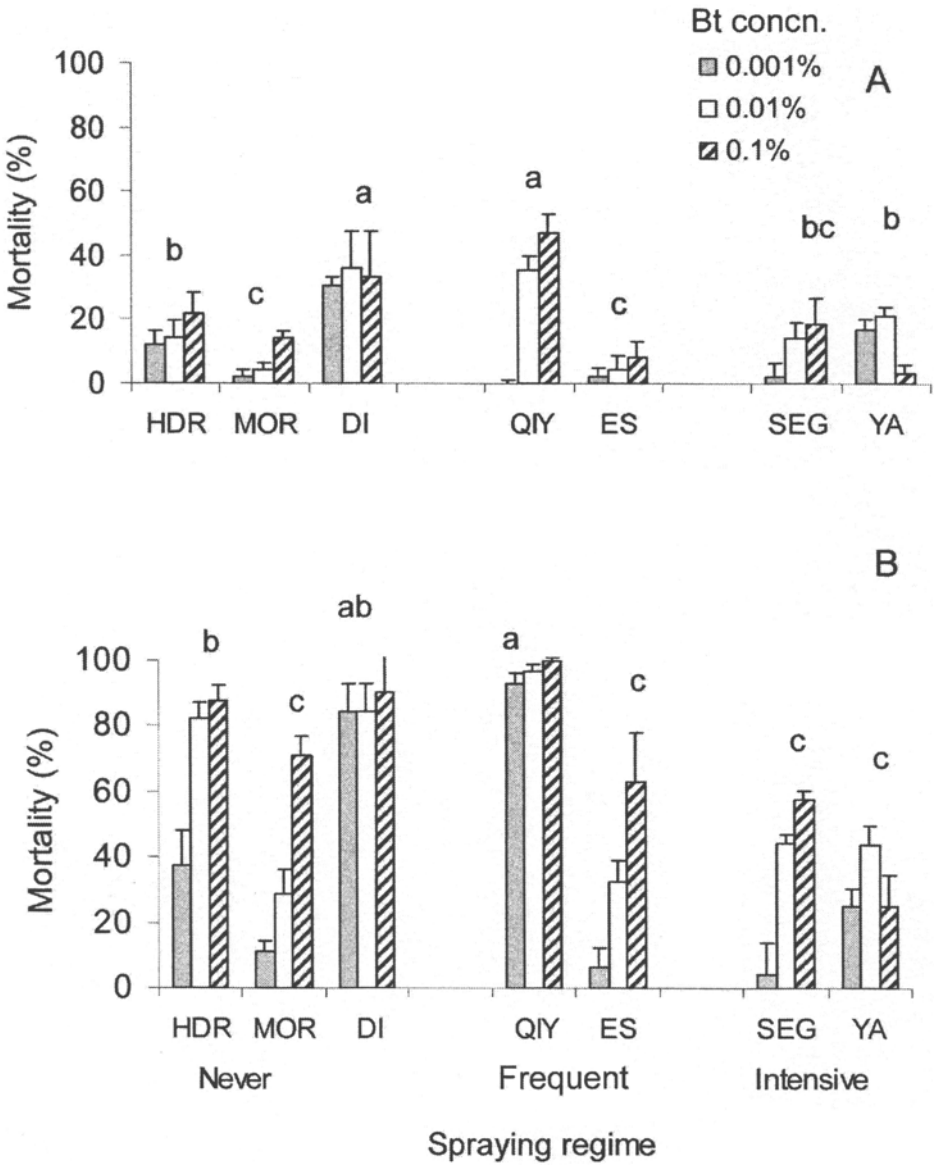

Fig. 2. Mortality (mean ±SE) of 2^{nd} instar of *Thaumetopoea wilkinsoni* caused by three concentrations of *Bt* (Delfin) during 72-h (A) and 144-h (B) bioassays. Populations were from different geographical locations as related to the frequencies of *Bt* application (see Table 1). Populations with a common letter do not differ significantly (P=0.05) in their overall mortality from the test *Bt* formulation.

the bioassay was more normal, and the mortality of those transferred to test tubes did not usually exceed 20% by day 6 of the bioassay. In another experiment it was shown that

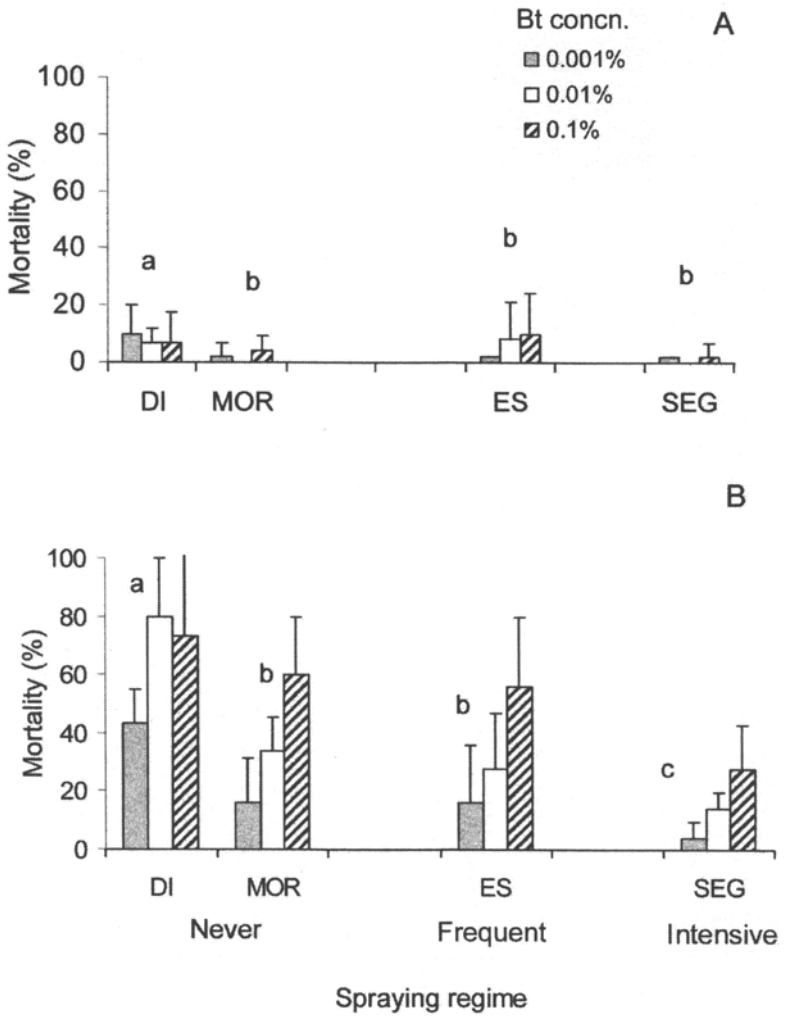

Fig. 3. Mortality (mean ±SE) of 3^{rd} instar of *Thaumetopoea wilkinsoni* caused by three concentrations of *Bt* (Delfin) during 72-h (A) and 144-h (B) bioassays. Populations were from different geographical locations as related to the frequencies of *Bt* application (see Table 1). Populations with the same letter do not differ significantly (P=0.05) in their overall mortality from the test *Bt* formulation.

the natural mortality of the larvae fed needles of the current year selected from the upper pine canopy did not differ significantly according to whether they were fed needles from 1.5-year-old or 3-year-old saplings.

DISCUSSION

Differences in susceptibility to *Bt* were significant mostly in 144-h bioassays with 1^{st}, 2^{nd} and 3^{rd} instars (Figs. 1, 2, 3; Table 2). In this bioassay protocol, it seems that larval susceptibility correlated well with the patterns of *Bt* application: the lowest mortality was

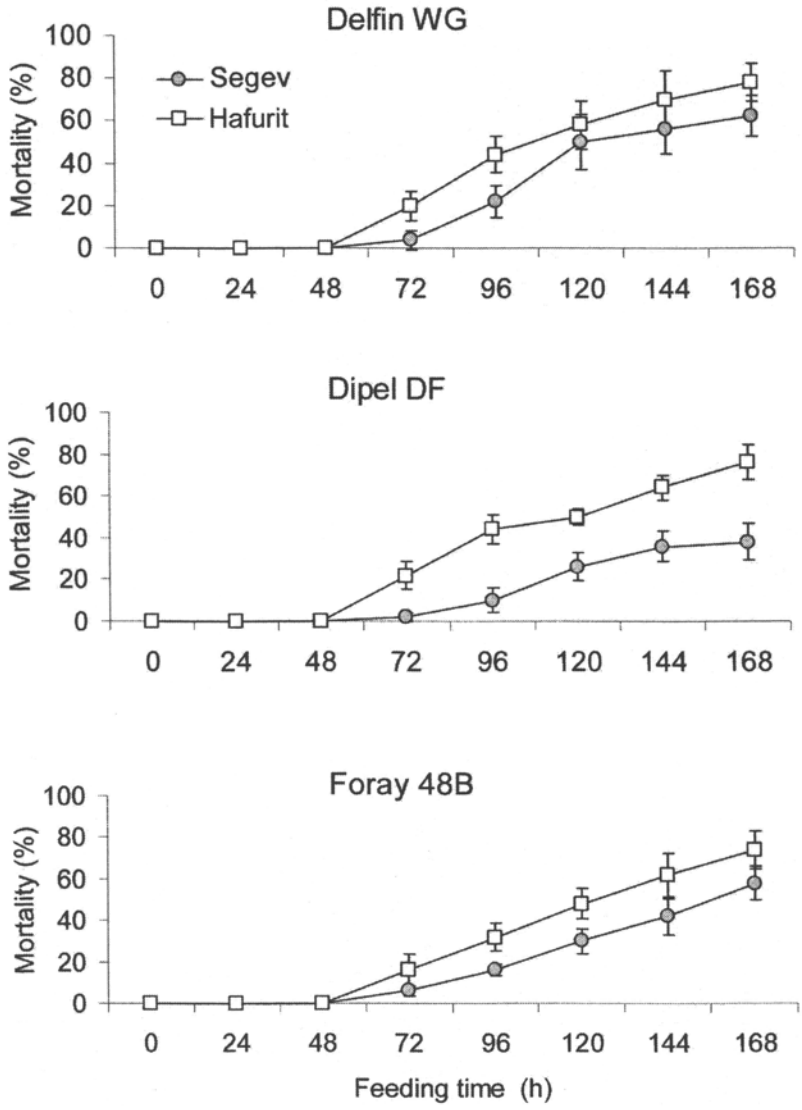

Fig. 4. Comparison of mean (±SE) percentage mortality during 168-h bioassays of 1^{st} instar of *Thaumetopoea wilkinsoni* from Segev (treated intensively with *Bt*) *vs* Hafurit (never treated with *Bt*) populations fed on pine needles treated with the three *Bt* formulations (0.01% Delfin WG, 0.01% Dipel DF, 0.03% Foray 48B).

found among larvae from forests intensively treated with *Bt*, and the highest among those from forests that had never been treated with *Bt*. This correlation was further confirmed by the comparison of susceptibility to *Bt* between populations from Segev – an intensively treated forest, and from Hafurit – a forest that had never been treated with the microbe. In this comparison, larval mortality was twice as great in the Hafurit as in the Segev populations (Fig. 4). These differences in mortality among the populations from the various

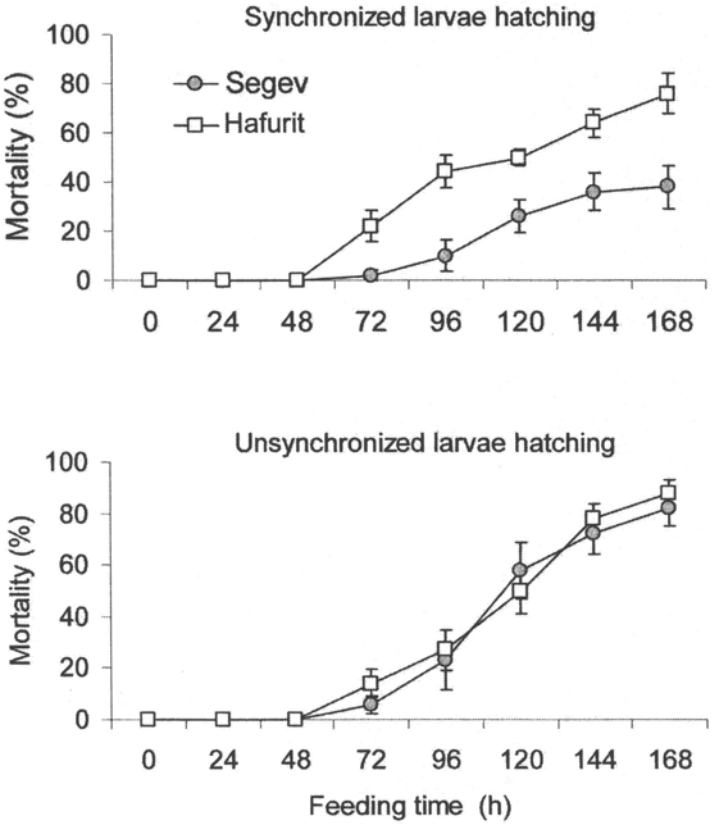

Fig. 5. Comparison of mean (±SE) percentage mortality during 168-h bioassays of 1^{st} instar of *Thaumetopoea wilkinsoni* fed on pine needles treated with Dipel DF formulation (0.01%), as affected by the pattern of larval hatching, synchronized (within one day) *vs* unsynchronized (within 3–4 days). The test was conducted with a population treated intensively with *Bt* (Segev) and a population that was never treated with *Bt* (Hafurit).

geographical locations are rather small. High levels of resistance to *Bt* were recorded in the diamondback moth *Plutella xylostella* (17), which is one of the few lepidopterous pests in which natural resistance to *Bt* was recorded in the field (8,9). The natural resistance in *P. xylostella* was studied by means of heavy treatment with Dipel. That diamondback moth population proved less susceptible to this product than populations that were exposed to lower *Bt* concentrations, and the level of resistance in the field was enhanced 30-fold (17). It seems that *P. xylostella* is an exception among many lepidopterous insects, in gaining rapid resistance to *Bt* (18). Other resistance studies were based on exposure of laboratory-bred or field-collected insects to heavy selection pressure in the laboratory for many generations (4). However, these studies are not relevant to our work, because they addressed artificial selections for resistance. The regular use of *Bt* against *T. wilkinsoni* in Israel is limited to one spray per year, but the development of a two-digit record of resistance to *Bt* in

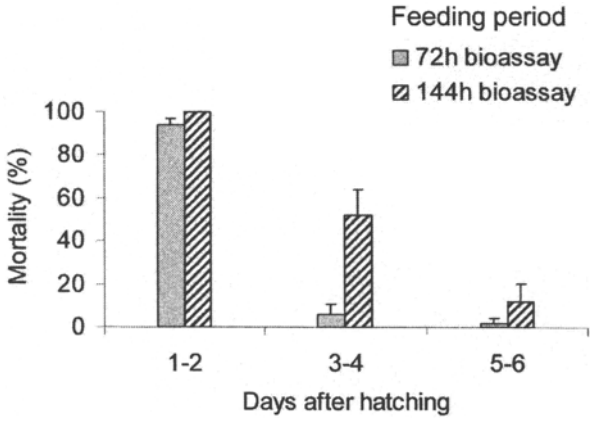

Fig. 6. Comparison of mean (±SE) percentage natural mortality of 1^{st} instar of *Thaumetopoea wilkinsoni* of the Segev population, fed on pine needles, as affected by larval age (days after hatching).

T. wilkinsoni would need a higher frequency of *Bt* application over the course of many years. Moreover, it is expected that development of resistance to *Bt* in a univoltine pest such as *T. wilkinsoni* would be much slower that in multivoltine lepidopteran insects with overlapping annual generations. It seems that a single *Bt* spray per year is marginal as a factor enhancing development of resistance, part of it due to the prolonged diapause (*e.g.* 5) Prolonged diapause is responsible for the mixing of cohorts that might have been treated or not, thus reducing the probability of fixation of the alleles conferring resistance. A large pine area where *Bt* is not applied contributes to a delay in the resistance upsurge (16). If this were to be the case, alternating *Bt* control with other biological IPM strategies would be recommended to reduce the selection pressure for resistance to *Bt* in this insect. Similar susceptibility to *Bt* was recorded in widely separated populations that had undergone the same pattern of *Bt* application, for example the Segev population is located far away from that of Yatir. Therefore, it is rather unlikely that the climate or the physical and chemical condition of the host tree could have played a role in eliciting resistance to *Bt*.

The reported decreasing susceptibility of *T. wilkinsoni* to *Bt* in intensive *Bt* IPM that was revealed in the present study must be taken into account in adopting guidelines for the use of *Bt* to control this pest. Factors to be considered include records of persistence of *Bt* in the forest during the insect season (Gindin *et al.*, accepted for publication).

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