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Susceptibility of *Cydalima perspectalis* (Lepidoptera: Crambidae) larvae to some reduced-risk insecticides in laboratory bioassays

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Abstract: Box tree moth (BTM) *Cydalima perspectalis* (Walker, 1859) is the most harmful pest of different boxwood species in Europe and Asia including Caspian boxwood *Buxus hyrcana* in the Hyrcanian forests of Iran. Accessible and effective eco-friendly insecticides are required for the pest control. Thus, susceptibility of 2nd and 4th instar larvae of BTM to commercial formulation of *Bacillus thuringiensis* (Bt), two plant extract formulations, Bio1® and Matrine®, and three insect growth regulator (IGR) insecticides, chlorfluazuron, chromafenozide and diflubenzuron were investigated in laboratory bioassay. Except for diflubenzuron, significant mortality of both instar larvae was observed. At the endpoint of the experiments (96 h), 75.2–90% of second and 80–85% of fourth instar larvae had already died, as a result of feeding on leaves treated with the highest concentration of the insecticides. Furthermore, based on Probit analysis, Matrine® exhibited the highest efficiency (lethal concentration LC_{50} = 2.87 μL·L^{-1}) on 2nd instar larvae and followed by Bio1® (8.07 μL·L^{-1}), chlorfluazuron (173.3 μL·L^{-1}) and Bt (326.3 mg·L^{-1}). The LC_{50} of Matrine® and Bt for 4th instar larvae were 1.75 μL·L^{-1} and 335.8 mg·L^{-1}, respectively. Our study revealed that Matrine® and chromafenozide could be alternatively used against BTM in situations where there is a permission and need to use insecticides.

Keywords: box tree moth; *Bacillus thuringiensis*; botanical insecticide; chromafenozide; *Buxus* spp.

The box tree moth (BTM), *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae), which is native to Korea, Japan and China, was recorded in Europe for the first time in southwestern Germany and in the Netherlands in 2007 (Leuthardt, Baur 2013). The species spread rapidly and its established populations have been recorded in a number of locations across Europe (Geci et al. 2020; Kulfan et al. 2020). Also, the pest has been recently reported from the African continent (Haddad et al. 2020). BTM is a herbivorous insect that is highly monophagous and specializes on the plant genus *Buxus* (Leuthardt, Baur 2013). The life cycle of this pest occurs completely on *Buxus* plants, where oviposition, larval development, pupation and overwintering take place (Gottig 2017). The Caspian boxwood,

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Buxus hyrcana Pojark, is an endemic and evergreen species in the Hyrcanian forests. The Hyrcanian region is located at the southern stretch of the Caspian coastline in Iran (Esmaeilnezhad et al. 2020). BTM, as an invasive exotic pest, has recently been reported in Iran. It seems that the pest is rapidly expanding its distribution to new geographical areas from the west to the east of Hyrcanian forests and to neighbouring countries. As the number of its yearly generations is mainly dependent upon the climate conditions, BTM has three generations in Hyrcanian forests (Farahani et al. 2021). The larvae of BTM feed principally on the leaves but may also attack the bark of box trees. When the number of larvae is huge, they could cause total defoliation and, in the end, withering and death of plants (Leuthardt, Baur 2013).

No effective biological control method is available yet to control this pest. However, some research has been conducted to identify parasitoids, nematodes, and pathogenic viruses of BTM (Rose et al. 2013; Wan et al. 2014; Gottig 2017; Gottig, Herz 2018; Martini et al. 2019). Pheromone traps are used to detect and monitor this insect (Santi et al. 2015), but because only male insects are trapped, the traps have no control function. Recently, good results have been obtained from a mixture of methyl salicylate, phenylacetaldehyde and eugenol to attract the female insects (Molnar et al. 2019).

Chemical control of BTM by some broad-spectrum pyrethroid insecticides has been the major and rapid solution in parks, green belts, or nurseries in Japan and China. Spinosad and fipronil have also been recommended for the pest control in China (Wan et al. 2014). In Romania, the field effectiveness of 15 various insecticides (Fora et al. 2016; Somsai et al. 2019) and in France, mortality of neonate larvae as a consequence of emamectin benzoate injection into box trees (Bras et al. 2017) were assayed.

As environmental regulations are concerned, the control programs for such pests should rely on using eco-friendly methods and biorational insecticides. Some successful control measures of BTM were achieved by bioinsecticides based on Neem oil and *Bacillus thuringiensis* var. *kurstaki* in China (Wan et al. 2014). Laboratory evaluation of NeemAzal, a plant extract formulation (active ingredient Azadirachtin A) against 3rd larval instars, and Nemastar, the commercial preparation of entomoparasitic nematode, *Steinernema carpocapsae* (Weiser), against 2nd and 4th larval instars of BTM caused significant mortality, but their field assessment, in comparison with two commercially available *B. thuringiensis* preparations (containing the subspecies *aizawai* or *kurstaki*) did not result in an appropriate control (Gottig, Herz 2018). The efficiency of baculovirus *Anagrapha falcifera* (Kirby) nucleopolyhedrovirus (AnfaNPV) against BTM neonate larvae has also been investigated in laboratory bioassays (Rose et al. 2013).

Environmental considerations must be taken into account for the chemical control of forest pests. Therefore, those insecticides are permitted that do not possess any adverse effects on forests and other ecosystems attacked by this pest. Little information is available on the susceptibility of BTM to biorational insecticides, hence in this study, lethal effects of two botanical insecticides, Bio1® and Matrine®, a commercial formulation of *B. thuringiensis* and three insect growth regulators (IGR), chlorfluazuron, chromafenozide and diflubenzuron were investigated against 2nd and 4th larval instars of BTM in laboratory conditions. Bt formulations have been among the practical options, with variable results, for controlling lepidopteran forest insect pests. Plant-based commercial insecticides also meet the primary requirements of pest control in forest and urban areas. Diflubenzuron is the oldest registered IGR insecticide for lepidopteran control in forest ecosystems but the introduction of new and effective IGR insecticides, provided they are safe in these situations, is an acceptable research field.

MATERIAL AND METHODS

Insects rearing. The laboratory colony of BTM originated from egg masses collected from infested Caspian boxwood, *B. hyrcana*, trees in Hyrcanian forests. For the establishment of an initial larval colony, the leaves with deposited eggs were placed in transparent cubic containers (length 25 cm × width 15 cm × height 15 cm) lined with wet cotton and for the cut twigs, their bottom end was put inside the watered cylindrical containers. The larvae were fed *B. hyrcana* leaves. The pupae were transferred to rearing cages (length 60 cm × width 60 cm × height 60 cm), containing potted small boxwood plants and diluted honey (10%) for egg deposition and feeding of adults, respectively. The oviposited eggs were removed daily and transferred to new larval rearing containers.

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All developmental stages were reared under conditions of 27 ± 1 °C, 50 ± 5% relative humidity and a 16 : 8 h (L : D) photoperiod.

Laboratory bioassays. Up to 24 h-old 2nd instar larvae (mean size 7–9 mm) and 4th instar larvae (mean size 18–23 mm) were used in the bioassays. The average lifespan of 2nd and 4th instar larvae is 3.13 and 2.99 days, respectively (Farahani et al. 2021). Susceptibility of insects was assayed by feeding them treated leaves of boxwood. Each insecticide was diluted in distilled water to produce a sequence of five concentrations based on preliminary range-finding tests (Table 1). The effectiveness of all six insecticides was evaluated against 2nd instar, but, in accordance with these results, three insecticides (the microbial one Bt, one botanical Matrine® and one IGR insecticide, chromafenozide) were assayed on 4th instar larvae. Treatment with water served as control.

Leaves of the same size were dipped for 15 s into the particular concentrations and air-dried for 45 min. Treated Caspian boxwood leaves (8 leaves for 2nd and 12 leaves for 4th instar larvae) were placed in each Petri dish lined with wet cotton. Ten 2nd instar larvae or five 4th instar larvae (two Petri dishes per concentration per replication for 4th instars) were moved into each Petri dish and the experimental units were kept under the same conditions for rearing the insect. Six replicates were conducted for each treatment and the total number of 2 160 and 1 080 second and fourth instar larvae were bioassayed, respectively. Mortality of the larvae was recorded at 24 h, 48 h, 72 h and 96 h after treatment. Larvae were considered to be dead if they did not respond to prodding with forceps.

Statistical analysis. Mortality data were analyzed in a completely randomized design using one-way ANOVA (including homogeneity of variance test) followed by the comparison of the means, using the post hoc Duncan test at $P < 0.05$. The normal distribution of the data was evaluated and the results of the Shapiro-Wilk method, in the test of normality, were the basis. The non-normal data were subjected to square root transformation. For each insecticide, lethal concentrations (*LC*s), slopes, χ^2 and *P* values and 95% confidence limits were calculated using the Probit procedure. All the statistical analyses were performed using SPSS (Version 22, 2013).

RESULTS

Concentrations-response assessments

Bacillus thuringiensis. Survival of both instar larvae was significantly affected by increasing the Bt concentrations or exposure times ($P < 0.05$). While low mortality was recorded for both lar-

Table 1. Characteristics of the insecticides used in this work

Common name	Commercial name	Formulation	Chemical group	Manufacturer	Concentrations used (μL·L^{-1} or mg·L^{-1})
Bt	Dipel	WP	microbial (*Bacillus thuringiensis* sp. *Kustaki*)	Valent BioSciences Co. (USA)	200, 313, 490, 766, 1 200 (both 2nd and 4th instar larvae)
Bio1	Bio1	–	botanical (containing *Sophora flavescens* extract)	MR Innovation Co. Ltd. (Republic of Korea)	5, 7, 15.4, 27.5, 50 (2nd instar larvae)
Matrine	Matrine	0.6% SL		Beijing Kingbo Biotech Co. Ltd. (China)	0.4, 0.71, 1.26, 2.25, 4 (2nd) /0.3, 0.5, 0.86, 1.47, 2.5 (4th instar larvae)
Chlorfluazuron	Atabron	5% EC	insect growth regulator	Ishihara Sangyo Kaisha Ltd. (Japan)	20, 42, 89, 188, 400 (2nd instar larvae)
Chromafenozide	Matrik	5% SC		Nippon Kayaku Co. Ltd. (Japan)	100. 168, 238, 475, 800 (2nd) /10, 15, 22.3, 33.4, 50 (4th instar larvae)
Diflubenzuron	Dimilin	25% WP		Chemtura Co. (USA)	400, 891, 1 995, 4 466, 10 000 (2nd instar larvae)

SL – soluble concentrate; EC – emulsifiable concentrate; SC – suspension concentrate; WP – wettable powder

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vae at 24 h after treatment, it gradually increased so that the concentrations 490 mg·L^{-1}, 766 mg·L^{-1} and 1 200 mg·L^{-1} caused 75%, 80% and 90% mortality against 2nd instar larvae and 65%, 76.7% and 85% mortality against 4th instar larvae at 96 h after treatment (Figure 1). The results represented a slow release of Bt, as the mortality was significantly higher at 72 h and 96 h compared to 24 h after treatment for almost all concentrations against both instar larvae. As appropriate and cost-effective Bt concentrations are concerned, both 490 mg·L^{-1} and 766 mg·L^{-1} of the commercial formulation provided suitable results in laboratory experiments.

Botanical insecticides. In preliminary range-finding tests, 4th instar larvae were unexpectedly more susceptible to Matrine® than 2nd instar larvae. In main tests, mortality of 2nd instar of BTM was significantly correlated with ascending concentrations of Matrine® and Bio1® [except for Matrine® at 24 h after treatment ($F_{4,25}$ = 0.65, P = 0.66)] (Figure 2A, B), but in the case of 4th instar larvae (Figure 2C), it was only significant at 96 h after treatment with Matrine® ($F_{4,25}$ = 8.07, P < 0.05).

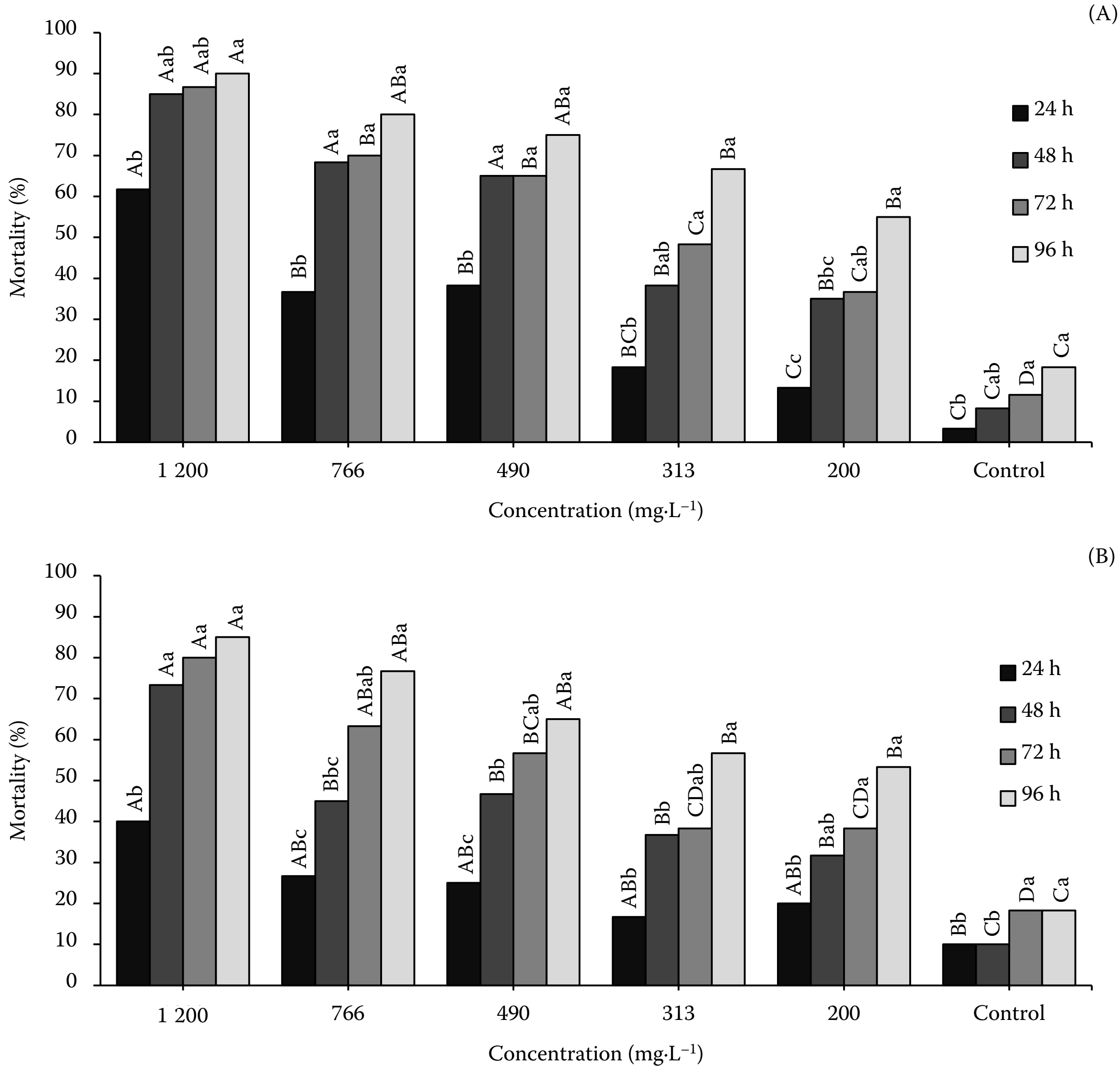

Figure 1. Percentage mortalities of 2nd (A) and 4th instar larvae (B) of *C. perspectalis* in response to the concentrations of formulated *Bacillus thuringiensis* (Bt) (Duncan test, P < 0.05) (different lower case in each concentration and uppercase in each time indicate the significant differences)

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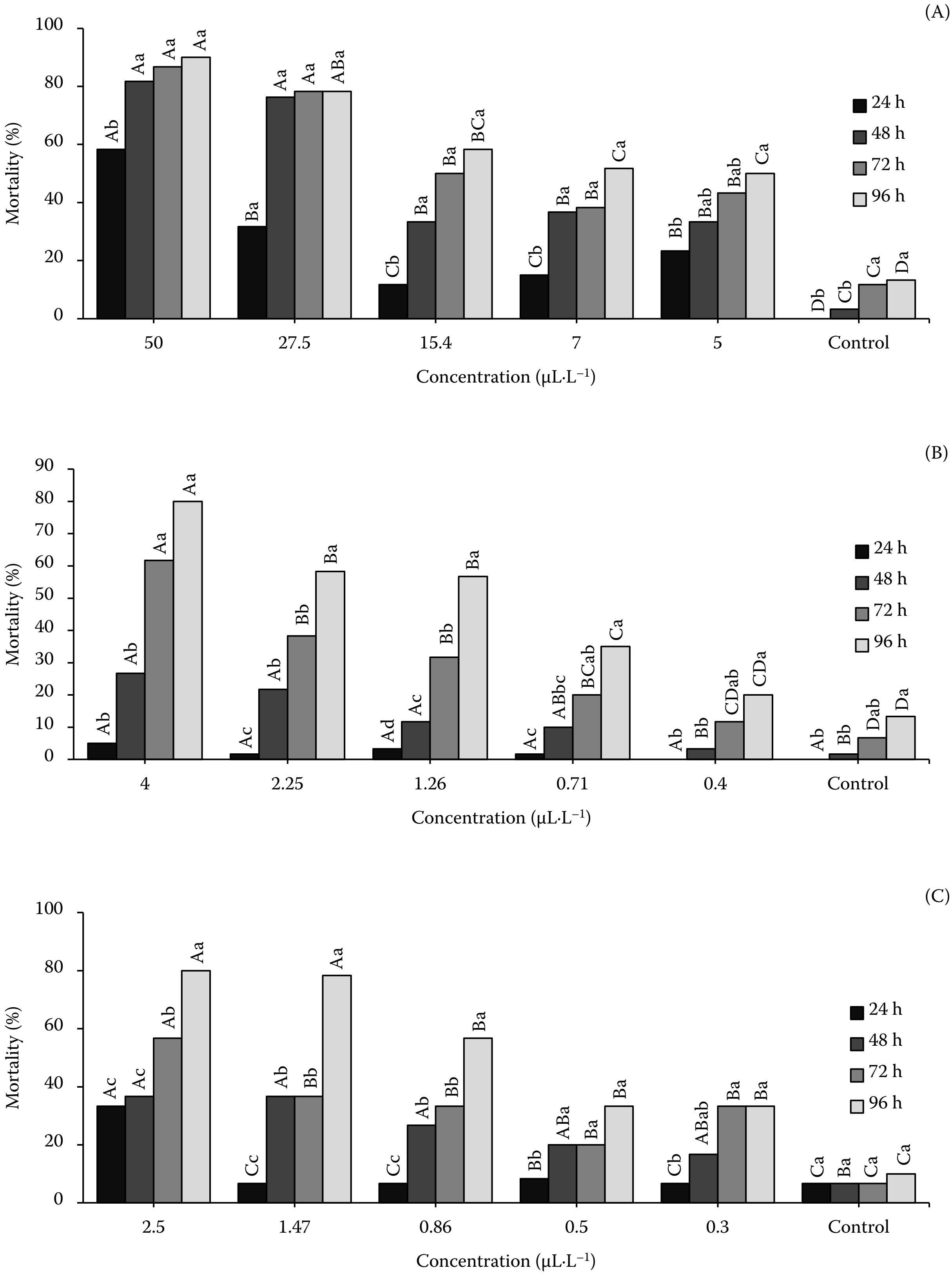

Figure 2. Percentage mortalities of 2nd instar larvae of *C. perspectalis* in response to the concentrations of Bio1® (A) and Matrine® (B) and 4th instar larvae to Matrine® (C) (Duncan test, $P < 0.05$) (different lower case in each concentration and uppercase in each time indicate the significant differences)

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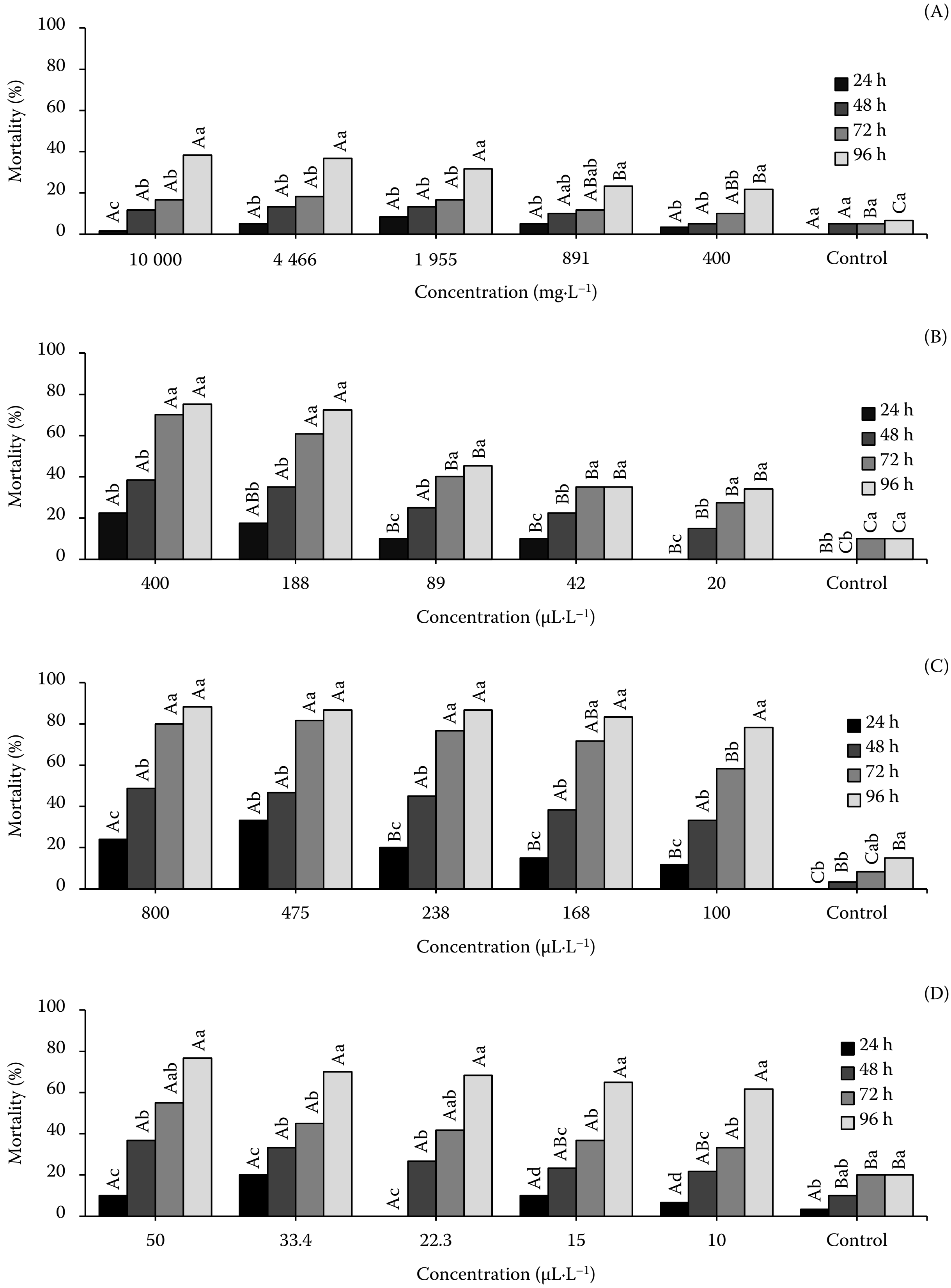

Figure 3. Percentage mortalities of 2nd instar larvae of *C. perspectalis* in response to the concentrations of diflubenzuron (A), chlorfluazuron (B) and chromafenozide (C) and 4th instar larvae to chromafenozide (D) (Duncan test, $P < 0.05$) (different lower case in each concentration and uppercase in each time indicate the significant differences)

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As the time variable is concerned, second instar larval mortality changed significantly throughout time for all Matrine® concentrations, but in the case of Bio1® concentrations it was not statistically significant at 7 ($F_{3,20}$ = 2.69, P = 0.07) and 5 μL·L^{-1} ($F_{3,20}$ = .01, P = 0.41). It was not also significant for three concentrations of Matrine®; 0.086 ($F_{3,20}$ = 1.63, P = 0.25), 0.50 ($F_{3,20}$ = 2.58, P = 0.12) and 0.30 μL·L^{-1} ($F_{3,20}$ = 2.38, P = 0.14) against fourth instar larvae.

Given that both formulations contained the same plant extract, while Matrine® possessed a more lethal effect, Bio1® exerted more a time effect.

IGR insecticides. Due to the low lethal effect of diflubenzuron, some very high concentrations (up to 10 000 mg·L^{-1}) were examined against 2nd instar larvae, nevertheless, the highest mortality after 96 h was only 38.5% (Figure 3A).

The concentrations of chlorfluazuron significantly affected larval mortality (second instar) only at 96 h after treatment ($F_{4,25}$ = 3.11, P < 0.05). Their survival was slightly affected at 24 h and 48 h and the highest control measures were achieved at 96 h after treatment (Figure 3B); the highest mortality was 75.2% for 400 μL·L^{-1} of chlorfluazuron.

In the case of chromafenozide, 4th instar larvae were more susceptible than 2nd instar larvae. There was a significant difference between the concentrations in terms of mortality of 2nd instar BTM larvae at all four times, but only at 96 h after treatment for 4th instar larvae (P < 0.05). Furthermore, the mortality of both instar larvae was significantly changed throughout time for all concentrations. Within 48 h after treatment, the insecticide slightly exerted its effect; it was 48.7% and 36.7% at the highest concentration for second and fourth instar larvae, respectively. Mortality then increased, and it reached 88.3% and 76.7% at 96 h after treatment for these concentrations.

Probit analysis

The standard concentration-response results were achieved at 72 h after treatment, therefore, the LC_{50}s for both instar larvae were calculated using linear regression only at this time (Table 2). With the exception of Bio1 against second BTM instar larvae (P = 0.002), the goodness-of-fit test revealed the normal distribution of expected response. Based on the LC_{50}s, the toxicity of the insecticide against 2nd instar larvae was as follows: Matrine® (2.87 μL·L^{-1}) > Bio1® (8.07 μL·L^{-1}) > chlorfluazuron (173.3 μL·L^{-1}) > Bt (326.3 mg·L^{-1}). The fourth instar larvae were more susceptible to Matrine® (1.75 μL·L^{-1}) than to Bt (335.8 mg·L^{-1}). By considering the 95% confidence intervals, all LC_{50}s at each larval instar were significantly different.

Table 2. Toxicity of *Bacillus thuringiensis* (Bt), Bio1®, Matrine® and chlorfluazuron on larvae of *Cydalima perspectalis* at 72 h after treatment

Instar of larvae	Insecticide	Number of insects	LC_{50} (95% CI) (μL·L^{-1} or mg·L^{-1})	LC_{90} (95% CI) (μL·L^{-1} or mg·L^{-1})	Slope (± SE)	χ^2 (df)	P-value
2nd	Bio1*	360	8.07 (5.6–11.7)	81.84 (49.4–208.8)	1.43 (0.21)	14.66 (4)	0.002
	Bt		326.3 (149.1–378.4)	1 756.6 (1 101–5 163)	1.33 (0.29)	2.4 (4)	0.486
	chlorfluazuron		173.3 (108.2–246.2)	4 355.1 (2 235–6 871)	0.53 (0.19)	644 (4)	0.921
	Matrine®		2.87 (2.16–4.45)	23.07 (11.3–88.7)	1.41 (0.233)	1.15 (4)	0.764
4th	Bt		335.8 (213.6–444.6)	3 924.2 (1 957.1–22 833)	1.2 (0.275)	5.75 (4)	0.124
	Matrine®		1.75 (0.98–8.8)	61.22 (9.3–181.7)	0.831 (0.322)	3.22 (4)	0.358

LC – concentration of the insecticides required for 50% and 90% mortality of second or fourth instar larvae of the insect; CI – upper and lower limits of the 95% confidence level

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DISCUSSION

Few kinds of research have been conducted on the susceptibility of BTM larvae to biorational insecticides. The results of this study showed that two botanical commercially available insecticides, Matrine® and Bio1®, IGRs including chromafenozide and chlorfluazuron along with Bt microbicide are suitable options for controlling this pest.

The use of insecticides to control insect pests of forest and urban trees is mainly carried out with the purpose of preventing complete infection of an area, eliminating pest populations, reducing pest damage and especially slowing the spread of the pest. Regarding the adverse environmental effects of insecticides, safe compounds should be used in these places. The introduction of effective biorational insecticides makes it possible to battle this pest within urban areas and small scale in the forests (Nair 2007).

B. thuringiensis subsp. *kurstaki* is the most effective microbial agent for controlling lepidopteran larvae of forest and urban pests. Application of commercial Bt formulations along with pheromone and biological control are three methods that have been proposed based on a temperature- and photoperiod-driven model (Suppo et al. 2020).

The susceptibility of BTM larvae to Bt formulation has not been investigated so far, however, in a field trial, the spraying of 1 000 $mg \cdot mL^{-1}$ of the formulation containing this subspecies caused more than 60% mortality of 4th instar larvae within four days after treatment (Gottig, Herz 2018). In the present study, LC_{50} s of the same subspecies for 2nd and 4th instar larvae after three days were 326.3 $mg \cdot L^{-1}$ and 335.8 $mg \cdot L^{-1}$, respectively. In addition, after four days, the concentration of 1 200 $mg \cdot L^{-1}$ caused 86.9% and 81.6% mortality, respectively. These results confirm the suitability of Bt as a safe and effective option for BTM control.

Botanical insecticides belong among the integrable components into organic systems (Reddy, Chowdary 2021). Although the efficacy of a number of plant-derived compounds has been tested on BTM (Gottig et al. 2017; Szelényi et al. 2020; Gokturk et al. 2021), commercially available formulations are limited. In one related research, NeemAzal® (containing the active ingredient Azadirachtin A) was able to control 3rd instar larvae of BTM after 14 days in laboratory conditions. The concentrations, 0.3% and 0.5%, both caused about 60% mortality. However, the application of these concentrations did not have any promising results in field conditions (Gottig, Herz 2018). In the present study, BTM larvae were susceptible to Matrine® and Bio1®, two plant-derived formulated insecticides. While Bio1® caused faster mortality than Matrine® against 2nd instar larvae, Matrine® was more lethal; their LC_{50} s after 72 h were 2.87 $\mu L \cdot L^{-1}$ and 8.07 $\mu L \cdot L^{-1}$, and after 96 h they were 1.26 $\mu L \cdot L^{-1}$ and 7.47 $\mu L \cdot L^{-1}$, respectively. The highest mortalities caused by these two insecticides were 88.4% (50 $\mu L \cdot L^{-1}$) and 76.9% (4 $\mu L \cdot L^{-1}$), respectively. The effectiveness of Matrine® against some agricultural pests has been documented (Zanardi et al. 2015; Saleem et al. 2019) and our results showed, for the first time, its suitability for BTM control. Final approval of this option requires proving its effectiveness in field conditions.

Diflubenzuron is one of the oldest IGRs used in integrated forest pest management programs (Berry et al. 1993) and is one of the recommended insecticides for BTM control. In a field trial, diflubenzuron controlled about 56% of larvae after 72 h (Somsai et al. 2019). Our laboratory bioassays indicated the possibility of resistance to diflubenzuron in Iranian populations of BTM. The study of the genetic diversity of BTM populations in different countries of Asia and Europe showed that Iranian populations belong to the two most abundant haplotypes in other parts of the world (HTA1 and HTB1 haplotypes) (Bras et al. 2019). Confirmation of the resistance requires further research using its technical material on different BTM populations. Chlorfluazuron, another tested IGR, has a long history in integrated pest management (IPM) programs for lepidopteran pests (Ishaaya et al. 1986). Furthermore, this insecticide is an effective option for the control of fall webworm, *Hyphantria cunea* (Dury), a main forest pest (Edosa et al. 2019). In our study, chlorfluazuron revealed appropriate toxicity on 2nd instar larvae of BTM (75.2% by using 400 $\mu L \cdot L^{-1}$ at 96 h after treatment). There is a report of resistance to chlorfluazuron in BTM larvae in China (Wan et al. 2014), but no detailed data has been provided. However, the documented high resistance to chlorfluazuron in populations of other lepidopteran pests (Su, Sun 2014; Zhang et al. 2016) is a warning against the irregular and excessive use of chlorfluazuron for BTM management. Chromafenozide, the third tested IGR insecticide in this research, also targets lepidopteran pests (Yanagi 2000). This insecticide is used

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as an eco-friendly option for agricultural pest control (El-Zahi et al. 2021). Despite being slow-acting, chromafenozide showed high toxicity against both BTM larval instars, so mortality increased rapidly at 72 h and 96 h after treatment, even at the lowest concentration. Such a rapid increase in mortality has also been reported in chromafenozide-treated *H. cunea* 3rd instar larvae; the mortality rate caused by six concentrations (6.25–200 mg·L^{-1}) was 90–100% at 96 h after treatment (Gong et al. 2020).

CONCLUSION

We investigated the efficacy of three IGR insecticides (chlorfluazuron, chromafenozide and diflubenzuron), two botanical insecticides (Bio1® and Matrine®) and the commercial formulation of Bt on 2nd and 4th instar larvae of BTM. We found, except for diflubenzuron, the other insecticides caused significant mortality against both instar larvae. Chromafenozide and Matrine® revealed the highest potential as suitable biorational insecticides. These results provide information that can improve practical usage of chlorfluazuron, chromafenozide, Bio1®, Matrine® and Bt in BTM management programs.

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