Original Article

Toxic and sub toxic effects of *Bacillus Thuringiensis* svar. *kurstaki* Toward *Ectomyelois Ceratoniae* (Lepidoptera: Pyralidae).

Lebbouz Ismahane[1], Hadjeb Ayoub[2*], Mehaoua Mohamed Seghir[1], Merabti Ibrahim[2], Ouakid Mohamed Laid[3]

1. Genetics, Biotechnology and Bioresources valorization Laboratory Department of Nature and Life Sciences, Faculty of Exact Sciences and Sciences of Nature and Life, Mohamed Kheider University, Biskra, Algeria.
2. Ecosystem Diversity and Dynamics of Agricultural Production Systems in Arid Zones Laboratory Department of agriculture Sciences, Faculty of Exact Sciences and Sciences of Nature and Life, Mohamed Kheider University, Biskra, Algeria.
3. Applied Neuro-endocrinology Laboratory, Department of Nature and Life Sciences, Faculty of Exact Sciences and Sciences of Nature and Life, Badji Mokhtar University, Annaba, Algeria.

ABSTRACT

The objective of this study is twofold: first, to determine the toxicity of Bacillus thuringiensis var. kurstaki (Bt) on the first larval instar of Ectomyelois ceratoniae, and second, to study its deferred effect on other biological parameters, such as the development and reproduction of this pest. The treated larvae were then paired, and the following concentrations were utilized: The experiment was conducted using a series of concentrations, ranging from 0.25 grams per liter (250 parts per million [ppm]) to 2 grams per liter (2000 ppm). Six pairs of Petri dishes were utilized for each concentration, with the number of eggs laid being recorded. Subsequent to this, the number of eggs that hatched following their incubation period was tallied. In the context of Bt svar. kurstaki, the variable of interest is the rate of larval mortality. The results demonstrated a robust and positive correlation between the administered doses and the adjusted mortality of the larvae across a range of bioinsecticide exposure times. The five Bt svar. kurstaki concentrations utilized resulted in a corrected mortality of E. ceratoniae first instar larvae, exhibiting variation between a minimum of 50.78% and a maximum of 97.92%. It has been demonstrated that Bacillus thuringiensis var. kurstaki becomes increasingly toxic to larvae following exposure to the biopesticide. Therefore, the median lethal concentration (LC50) of Bt svar. kurstaki for E. ceratoniae larvae, calculated at concentrations of 250, 500, 1,000, 1,500, and 2,000 parts per million (ppm), exhibited an inverse proportionality to the different lethal times. Conversely, the Bt treatment exhibited a marked decrease in female insects' reproductive rate and egg viability. Consequently, the BT exerted a deleterious effect on the growth and reproductive parameters of E. ceratoniae.

Keywords: *Bacillus Thuringiensis, Ectomyelois Ceratoniae,* Mortality, Fecundity, Fertility.

1. Introduction

The date palm (*Phoenix dactylifera L.*) is a significant fruit crop that holds a strategic importance in many regions (1). Arid and semiarid regions have long relied on it as a crucial component of their economic and social fabric (2). The Ectomyelois ceratoniae continues to be a significant pest that poses a threat to Algerian palm trees. The substantial economic losses incurred as a result of this pest cannot be adequately mitigated through the implementation of chemical management strategies. The biology and feeding behavior of the pest render chemical treatments ineffective, thereby jeopardizing the viability of date production. The larvae are nourished and mature within the date fruit, where they are securely shielded (3), causing a significant decline in both their quality and their worth (4). The indiscriminate and irrational application of pesticides, in conjunction with farmers' lack of awareness regarding their hazards, serves to exacerbate their detrimental impact on human health, animals, and the environment. Moreover, this practice has been demonstrated to contribute to the depletion and destruction of beneficial fauna (5-7). The utilization of synthetic pesticides has been demonstrated to result in the accumulation of residues within the food chain, the contamination of the environment, and the development of pest resistance over successive generations (8-10). A salient drawback of these novel synthetic compounds is their lack of biodegradability. The molecules in question reproduce the substances from which they are composed, subsequently concentrating in organisms and being transmitted throughout food chains. This underscores the necessity for expeditious referral to alternative control methodologies that utilize natural compounds derived from the living world (i.e., from plants or micro-organisms). These alternative approaches have the potential to serve as the foundation for both preventive and curative treatments. The cultivation of these biological potentials facilitates the effective management of insect populations and mitigates. As posited by Mossini and Kemmelmeier in 2005, there is considerable potential for environmental pollution resulting from the usage of synthetic and non-biodegradable substances. The following text is intended to provide a comprehensive overview of the subject matter. In this context, the focus of biologists has been on the development of a new generation of biopesticides derived from natural oils, pathogenic bacteria, insect growth regulators (IGR), pheromones, nematodes, and marine toxins (9). In this regard, the objective of our research is to examine the toxicology of Bacillus thuringiensis var. kurstaki. Our primary goal is to ascertain the toxicity of this molecule to E. ceratoniae. Additionally, we seek to investigate its delayed impact on the growth and reproduction of this pest under laboratory conditions.

2. Materials and Methods

2.1. Mass-Rearing of Carob Moth

The laboratory study of biological parameters in the date moth (Tephritidae) necessitates the mass rearing of the organism. The mass rearing of E. ceratoniae was conducted with the strain from infested dates of Biskra palm groves. The infested dates were placed in cages in a rearing room with controlled ambient conditions (temperature = 27 ± 2°C, relative humidity = 65 ± 10%) and a photoperiod (16: 8) (light: dark) (Al-izzi et al. 1987). The emergence of adults of E. ceratoniae was facilitated by utilizing a test tube, followed by their placement within a mating pot irrespective of their sex. Subsequent to mating, the females deposited their eggs within the pot coupling, and the eggs were subsequently transferred through the fine mesh tulle into large plastic boxes containing an artificial diet (11). Subsequent to the hatching of the eggs, the first instar larvae were collected for the bioassays.

2.2. Bio pesticide preparation

Dipel DF was utilized as a biological insecticide, comprising the active substances (spores and crystals) of Bacillus thuringiensis subspecies kurstaki strain ABTS-351. The formulation of the substance in question was of the WG type, characterized by water-dispersible granules, with a titration of 32,000 IU/mg. The kurstaki variety exhibits specificity in its targeting of lepidopteran larvae, engaging in an ingestion-based mechanism of action against these pests (12). Following a series of preliminary tests on the bio pesticide, a set of five concentrations was selected, with each concentration being twice the previous one. The selected concentrations were weighed and subsequently added to distilled water, in accordance with the established protocol for the utilization of this bio pesticide (0.1-1 kg/ha). The following concentrations have been approved: The concentrations of the aforementioned substances are 0.25 g/L (250 ppm), 0.5 g/L (500 ppm), 1 g/L (1000 ppm), 1.5 g/L (1500 ppm), and 2 g/L (2000 ppm).

2.3. Study of Bacillus Thuringiensis svar. Kurstaki Toxicity on the Date Moth Larvae

Twenty larvae of first instar were placed in Petri dishes containing the artificial diet treated by five concentrations of Bt (250, 500, 1000, 1500, and 2000 ppm), plus a control, all in three repetitions. The Petri dishes were hermetically sealed and transferred to the rearing room. Observations were conducted on a daily basis to enumerate deceased larvae, employing a binocular loupe for this purpose.

2.4. Effect of Bacillus Thuringiensis var. Kurstaki on Females and Eggs' Fertility

The larvae that demonstrated resilience to the toxic effects of Bacillus thuringiensis var. kurstaki were subsequently transitioned to an artificial diet, thereby facilitating the culmination of their development into the adult stage. Upon reaching adulthood, 30 pairs of treated larvae (six pairs for each dose) and six pairs of control larvae were individually placed in separate Petri dishes to quantify the number of eggs laid. The quantity of eggs that successfully hatched following the process of incubation was meticulously enumerated.

2.5. Statistical Analysis

In the context of Bt svar. kurstaki, the variable of interest is the rate of larval mortality. The mortality rate was adjusted using Abbott's formula (1925) (13), which provides an estimation of the actual toxicity of the bioinsecticide. The various rates undergo an angular metamorphosis in accordance with the Bliss tables (Fischer and Yates, 1975). The analysis of the normalized data was conducted using one-way analysis of variance (ANOVA). To characterize the insecticidal effect of the molecule under investigation, the median lethal concentration (LC50) was determined. The corrected mortality rates obtained were transformed into probabilities, and a linear regression line was established according to the decimal logarithms of the doses used. The determination of remarkable doses was accomplished through the implementation of a regression equation, employing the mathematical procedures outlined by Finney (1971) (14). The method developed by Swaroop et al. (1966) (15) was employed to calculate the LC50 confidence interval.

Abbot's formula: Corrected mortality Percentage (%) = X-Y / X ×100.
Where: X = Number of living in the control lot,
Y = Number of living in the treated lot.
Parametric tests were used to compare the means. The computations were performed using the XLSTAT software.

3. Results

3.1. Mortality study of E. ceratoniae larvae exposed to Bacillus thuringiensis svar. Kurstaki

After the exposure of *E. ceratoniae* first instars larvae to *Bt* svar. *kurstaki* during 24, 48, 72, 96, 120 and 144 hrs., the corrected mortality rates revealed significant differences among the five concentrations tested; $P = 0,0410$; $P = 0,0070$; $P = 0,0408$; $P = 0,0031$; $P < 0,0001$ and $P = 0,0271$, respectively (Table 1). After a 24- and 48-hour period, the lowest corrected mortality rates (38.86% and 43.95%, respectively) were recorded in the larvae treated with the lowest concentration (250 ppm). Conversely, mortality rates exceeded 50% for concentrations of 500, 1000, and 1500 ppm, reaching a maximum of 64.39% and 76.23% at the concentration of 2000 ppm (see Table 1 for complete mortality rates). For exposure times of 72, 96, and 120 hours, the five Bt svar. kurstaki concentrations used resulted in a corrected mortality rate of 50.78% to 97.92% among E. ceratoniae first instar larvae. Furthermore, the elevated concentrations (1500 and 2000 ppm) resulted in the maximum mortality rate (100%) over a prolonged lethal time period (144 hours) (Table 1). As demonstrated in Table 2, the maximum LC50 and LC90 concentrations (568.60 ppm and 64365.60 ppm, respectively) were documented at an exposure duration of 24 hours, exhibiting an R^2 of 0.942 and a regression equation of $y = 0.624x + 3.28$, with a slope of 39. The lowest LC50 and LC90 concentrations (76.86 and 2320.54 ppm, respectively) were

obtained for an exposure time of 144 hours, with an R^2 value of 0.822. The regression equation was $y = 3.323x - 2.41$, and the slope was 1.99. It has been demonstrated that Bacillus thuringiensis var. kurstaki becomes increasingly toxic to larvae following exposure to the biopesticides. Therefore, the median lethal concentration (LC50) of Bt svar. kurstaki for E. ceratoniae larvae, calculated at concentrations of 250, 500, 1,000, 1,500, and 2,000 parts per million (ppm), exhibited an inverse proportionality to the various lethal times (24, 48, 72, 96, 120, and 144 hours). As demonstrated in Table 2, the LC50 of Bt svar. kurstaki, calculated at the longest mortality duration (144 h), was lower (169.79 ppm) than that recorded at the lethal time of 24 h (568.60 ppm).

3.2. Study of E. ceratoniae female and eggs fertility

The highest number of eggs was laid by control females (145.00 ± 11.54), whereas those treated with different concentrations of Bacillus thuringiensis var. kurstaki exhibited a very low egg-laying number, oscillating between 24.17 ± 7.14 at the concentration of 1500 ppm and 46.00 ± 15.10 at the concentration of 250 ppm (Table 3). As illustrated in Table 3, the analysis revealed that the control females exhibited the highest mean hatching rate of eggs, with an average of 91.24 ± 1.93%. Conversely, the lowest recorded values were observed in the case of eggs from female larvae that had been treated with varying concentrations of Bt Svar. kurstaki (ranging from 37.82 ± 12.52% to 55.02 ± 6.21%). An analysis of the variance of the number of eggs laid per female was conducted, as well as an examination of the average rate of E. ceratoniae eggs hatched in a lot treated by four concentrations of Bt Svar. kurstaki (250, 500, 1000, 1500 ppm). The results obtained showed significant differences with $P < 0.0001$ and $P = 0.003$, respectively (see Table 3). The findings of the study demonstrated that Bt svar. kurstaki led to a significant reduction in female fertility, with an average of 83.34% decrease, and in egg hatching, with an average of 58.55% decrease, irrespective of the utilized concentration.

4. Discussion

In order to develop an integrated pest management program to combat the date moth, E. ceratoniae, the toxicological effects of Bacillus thuringiensis var. kurstaki were evaluated. However, the utilization of Bt svar. kurstaki is employed for the regulation of lepidopteran caterpillars, which are considered detrimental to both cultivated plants and forest species (16). Tests were conducted to ascertain the toxicity of Bt svarl. kurstaki towards E. ceratoniae larvae. The results of these tests appeared to provide a definitive conclusion. The initial mortality rates became apparent 24 hours after the larvae were exposed to the substance. The survival rate of Euprosterna elaeasa caterpillars (Lepidoptera: Limacodidae) was assessed 48 hours after exposure to Bt-strains at a concentration of 0.84

Table 1. Corrected mortality rate of first instars larvae of *Ectomyelois ceratoniae* treated with *Bacillus thuringiensis* svar. *Kurstaki.*

Exposure time (hours)	250 *ppm*	500 *ppm*	1000 *ppm*	1500 *ppm*	2000 ppm	*DOF*	*F*	*P*
24	38,86±6,78	50,88±5,23	54,04±11,48	57,54±6,56	64,39±10,06	4	3,748	0,0410
48	43,95±6,64	56,05±6,64	60,88±12,29	66,05±3,54	76,23±7,82	4	6,678	0,0070
72	50,78±6,76	58,38±9,93	63,74±7,52	70,86±3,70	83,33±16,67	4	3,754	0,0408
96	54,58±7,08	62,31±5,93	69,83±8,36	81,05±6,82	90,63±11,51	4	8,419	0,0031
120	62,89±5,20	78,38±3,71	88,21±0,69	96,06±3,43	97,92±3,61	4	33,074	<0,0001
144	82,90±10,49	89,67±9,20	95,82±3,64	100,00±0,0	100,00±0,00	4	4,346	0,0271

Table 2. Toxicological parameters of *Bacillus thuringiensis* svar. *kurstaki* after different intervals.

Exposure time (hours)	Regression equation	R^2	LC_{16}	LC_{50}	LC_{84}	LC_{90}	Slope
24	Y = 3,28 + 0,624 * X	0,942	14,49	568,60	22312,99	64365,60	39,24
48	Y = 2,872 + 0,833 * X	0,932	22,95	358,61	5604,02	12392,45	15,63
72	Y = 2,74 + 0,919 * X	0,845	23,83	287,87	3478,16	7140,92	12,08
96	Y = 2,243 + 1,152 * X	0,832	33,88	247,30	1805,14	3204,27	7,30
120	Y =0,703 + 1,893 * X	0,971	55,54	186,18	624,16	885,04	3,35
144	Y = -2,41 + 3,323 * X	0,822	85,24	169,79	338,21	412,66	1,99

Table 3. Average number of eggs lay per female and hatchability.

	Control	Concentrations (ppm)				*F*	*P*
		250	500	1000	1500		
Average number of eggs laid per female	145,00	46,00	34,50	32,17	24,17	27,497	< 0,0001
Standard deviation	11,54	15,10	2,98	6,05	7,14		
Average rate of eggs hatched (%)	91,24	55,02	50,80	39,30	37,82	5,392	0,003
Standard deviation	1,93	6,21	5,45	12,69	12,52		

milligrams per milliliter. The survival rate exhibited a decline from 99.9% in the control group to 52.79% with SA-12 var. kurstaki, 51.37% with GC-91 var. aizawai, 35.62% with HD-1 var. kurstaki, and 23.12% with ABTS-1857 var. aizawai (Plata-Rueda et al., 2020). According to Chaufaux (1995) (17), the death of the insect occurred within 24 to 48 hours after ingestion of the Bt crystals. The bacteria produce a toxin that, when ingested by the larvae, results in the destruction of its digestive system. Consequently, the larvae cease feeding and perish within a few days following the treatment (18). The kurstaki subspecies has been observed to exhibit toxicity against larvae; however, the extent of this toxicity varies depending on the specific species of larvae. The author further noted that the insects against which Bt was toxic ceased feeding in less than a few hours and perished after 2-5 days. The mortality rate exhibited a decline when the exposure duration was limited to 24 or 48 hours, irrespective of the utilized concentration. According to the information available and cited above, the mortality observed in these young larvae (L1) following exposure to Bt was significant. Therefore, the efficacy of treatments utilizing Bacillus

thuringiensis var. kurstaki was found to be higher when administered during the youngest larval stages (17). In their seminal study, Lereclus and Chaufaux (1986) (19) observed that when a larva in its early stage consumed crystals, they were rapidly metabolized, resulting in the production of a toxin that paralysed the digestive tract. Consequently, insects exposed to the toxin exhibited symptoms consistent with toxaemia or septicemia, ultimately leading to their demise within a few days. The majority of lepidopteran species exhibit sensitivity to the crystals produced by the kurstaki and aizawai strains (20). According to Lambert (2010) (18), the Bt subspecies kurstaki (Btk) exhibited efficacy solely against juvenile larvae of the gypsy moth (Lymantria dispar Linnaeus). In the study conducted by Mazollier, it was determined that the Bt subspecies kurstaki exhibited its activity exclusively through ingestion, manifesting its effects on young larvae. Consequently, the early larval instars exhibit heightened sensitivity to Bt, underscoring the necessity to target these stages in the larval phase. The mortality rate observed in the present experiment was likely associated with the quantity and duration of food intake. In his 1971 study, Ghy examined the effects of Bt on the growth and development

of the migratory locust (Locusta migratoria). The study indicated that when the toxin was ingested by the locust at the beginning of the larval stage, the locust's development was slowed at this instar. The greater the quantity of toxin ingested, the more pronounced the retardation. The author further noted that the majority of mortalities occurred between the second and sixth days following treatment, with 30% of mortalities resulting from high doses of Bt toxin occurring within the first three days after treatment. The mortality rate reached 50% after four days, 60% after five days, and 80% after six days. However, the mortality rates exhibited an upward trend with increasing bio pesticide concentrations and exposure durations, reaching a maximum after six days (100%) in treatments involving high concentrations (1500 and 2000 ppm). The larval mortality rate exhibited a significant correlation with the period of exposure to the biopesticide. The obtained results indicated a gradual decrease in the LC50 over time. Consequently, the Bt exhibited an escalating degree of toxicity in proportion to the prolongation of exposure of the larvae to the product. The toxicity of Bt may also be attributable to the age of E. ceratoniae larvae treated (L1). In the context of the study, Bt was found to be less toxic to E. ceratoniae larvae than to the last larval stages of Simulium vittatum. The median lethal concentration (LC_{50}) of Bt for S. vittatum larvae was determined to be between 1 and 1.1 parts per million (ppm) after a 24-hour exposure period. Abid et al. (2021) (21) demonstrated that biological control is the most ecologically friendly and effective option for combating E. ceratoniae. The lipopeptide biosurfactant produced by Bacillus subtilis SPB1 demonstrated notable efficacy against the insect species infesting preserved dates. Bacillus thuringiensis (Bt) toxins have demonstrated significant efficacy in managing dangerous insects that impact human health and agriculture. These toxins are utilized as the primary biological component in the production of bioinsecticides, owing to their ability to selectively target certain insect orders. Chapa et al. (2019) (22). In a controlled laboratory setting, an evaluation was conducted to ascertain the larvicidal efficacy of Bacillus thuringiensis var. kurstaki. The study focused on the initial larval stage of E. ceratoniae, and the results indicated a notable susceptibility to the Bt strain. The sensitivity, which exhibited a direct proportionality to the fatal period, demonstrated heightened values when the concentration level was elevated. The LC50 value exhibited a negative correlation with the duration of exposure of the larvae to the bio pesticide. The duration of lethality was found to be prolonged during periods of weakness and shortened during periods of strength. The collective outcomes of the study indicated that Bacillus thuringiensis var. kurstaki displays considerable larvicidal potential against E. ceratoniae.

Authors' Contribution
O. ML, BS. MK designed and coordinated the study. H. AM, MS. were performed the experiment, the statistical study was done by M. B, L. wrote the paper and send it to publication. All authors read and approved the final manuscript

Ethics
We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

Data Availability
All data generated or analyzed during this study are included in this published article.

References

1. Bedjaoui H, Benbouza H. Assessment of phenotypic diversity of local Algerian date palm (Phoenix dactylifera L.) cultivars. Journal of the Saudi Society of Agricultural Sciences. 2020;19(1):65-75.

2. CHAABAN SB, MNAFFED A, MAHJOUBI K, BEN JM. Efficacy of essential oils to control the carob moth, Ectomyelois Ceratoniae Zeller (Lepidoptera: Pyralidae). International Journal of Agriculture Innovations and Research. 2019;7(4):2319-1473.

3. Peyrovi M, Goldansaz S, Jahromi KT. Using Ferula assafoetida essential oil as adult carob moth repellent in Qom pomegranate orchards (Iran). African Journal of Biotechnology. 2011;10(3):380-5.

4. Jouve P, Loussert R, Mouradi H, editors. La lutte contre la dégradation des palmeraies dans les oasis de la région de Tata (Maroc). Colloque international; 2006.

5. Ben Saad A. Evolution des systèmes de production oasiens dans le contexte de désengagement de l'état. Cas des oasis du grand Gabes Manuel gouvernance foncière et usage des ressources naturelles FONCIMED INRA. 2010.

6. Bazzi L. Etude de la persistance de quelques pesticides dans la culture de l'haricot vert dans la région de Sous Massa. 2010.

7. Zohra BF, Mahmoud Y, Farid B, Bahia D-M. Activité biologique d'un biopesticide le Green muscle sur le tégument du criquet pélerin Schistocerca gregaria (Forskål, 1775)(Orthoptera, Acrididae). Nature & Technology. 2012(6):51.

8. Bélanger A, Musabyimana T. Le Neem contre les insectes et les maladies. Journées Horticoles au Canada en. 2005.

9. Abdullah MA. Toxicological and histopathological studies of Boxus chinensis oil and precocene II on larvae of the red palm weevil Rynchophorus ferrugineus (Oliver)(Coleoptera: Curculionidae). Egyptian Academic Journal of Biological Sciences A, Entomology. 2009;2(2):45-54.

10. Richard I. Les pesticides et la perte de biodiversité. Comment l'usage intensif des pesticides affecte la faune et la flore sauvage et la diversité des espèces, Pesticide Action Network Europe, Belgium. 2010:p3.

11. Mediouni J, Dhouibi M. Mass-rearing and field performance of irradiated carob moth Ectomyelois ceratoniae in Tunisia. Area-wide control of insect pests: from research to field implementation: Springer; 2007. p. 265-73.

12. Dhouibi M. Bacillus thuringiensis Kurstaki contre la pyrale des dattes Ectomyelois ceratoniae Zeller (Lepidoptera Pyralidae). Tunis, Tunisie, INAT, 54 p, document technique. 1993.

13. Culver J, Vienna V, BURGESS PA. A method of computing the effectiveness of an insecticide. Journal of economic entomology. 1925;18:265.

14. Finney D. Probit analysis, Cambridge University Press. Cambridge, UK. 1971.

15. Swaroop S, Satya S. Statistical methods in malaria eradication. 1966.

16. HABBACHI W. Etude des Blattellidae (Dictyoptera): essais toxicologiques, synergie et résistance aux insecticides et aux biopesticides: Université de Annaba-Badji Mokhtar; 2013.

17. Chaufaux J. Utilisation de biopesticides contre les ravageurs des cultures: le point sur Bacillus thuringiensis. 1995.

18. LAMBRET P. Dynamique de populations d'adultes de Lestes macrostigma (Eversmann, 1836) et implications pour son suivi: l'exemple de la Camargue.

19. Lereclus D, Chaufaux J. Etat actuel de la lutte biologique a l'aide de Bacillus thuringiensis: ce bioinsecticide permettra t-il demain d'atteindre le doryphore? 1986.

20. Drummond J, Pinnock DE. Host spectrum of Bacillus thuringiensis. Agriculture, ecosystems & environment. 1994;49(1):15-9.

21. Abid I, Laghfiri M, Bouamri R, Aleya L, Bourioug M. Integrated pest management (IPM) for Ectomyelois ceratoniae on date palm. Current Opinion in Environmental Science & Health. 2021;19:100219.

22. Fernández-Chapa D, Ramírez-Villalobos J, Galán-Wong L. thuringiensis: An Overview. Protecting rice grains in the post-genomic era. 2019;2:183.