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Effect of sodium carbonate and mint oil as additives on the potency of the entomopathogenic bacteria, *Bacillus thuringiensis* against the cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract:
This study aimed to investigate the effect of adding the inorganic salt, sodium carbonate (Na_2CO_3) or mint oil to the bio-insecticide, *Bacillus thuringiensis* (Bay-8, 8% SC) on some of its physico-chemical properties as well as its toxicity in relation to its effect on some biochemical aspects in *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). The results clearly showed that adding 0.1% Na_2CO_3 or 0.3% mint oil to *B. thuringensis* solution increased its pH value, viscosity and its toxicity against *S. littoralis* 2nd instar larvae, where its LC_{50} value was reduced from 7.079ml/l to 3.972 and 3.549 ml/l after adding Na_2CO_3 or mint oil, respectively. The biochemical studies in the treated larvae with *B. thuringensis* combined with 0.1% Na_2CO_3 or 0.3% mint oil showed a significant reduction in the total protein content, α-esterase and phenoloxidase activities compared to treatment with *B. thuringensis* only. The obtained results confirm the effect of Na_2CO_3 and mint oil on the activity of these enzymes towards *B. thuringensis*, which may explain the increase in the toxicity of *B. thuringensis* when mixed with either Na_2CO_3 or mint oil against *S. littoralis.*

Introduction

The cotton leaf worm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is considered one of the major destructive pests in Egypt. The larval stage was known as a leaf eater accepting almost all herbaceous plants (Abdel-Wahab, 2002). The wide use of different chemical insecticides for controlling *S. littoralis* caused the development of pesticide resistance (Ishaaya and Klein, 1990). Therefore, there is always a need for an alternative method for controlling the pest to minimize chemical insecticide's application and avoid the problem of evolved resistance in insect's field populations. *Bacillus thuringensis* (*B.t.*) is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. These inclusions consist of protein which exhibited a highly specific insecticidal activity (Höfte and Whiteley, 1989). The main mode of action of this crystalliferous bacterium in different orders of insects was disrupting of the epithelial lining of the mid-

gut (Ignoffo *et al.,* 1981). *B.t.* crystal (d-endotoxin) protein must be ingested, solubilized and activated by larval gut enzymes to form its entomocidal effect (Salama *et al.,* 1984). Additives such as chemical or natural enhancers could be used to potentiate the bio-insecticide, *B.t.* The incorporation of these additives with entomopathogenic bacteria to achieve high efficacy was practiced either to extend the spectrum of activity or to overcome the short persistence of these insecticides in the field (Abdel-Hafez *et al.,* 2013). In addition, in order to increase *B.t.* potency, the conditions prevailing insect mid-gut must be modified. This might be achieved by using alkaline compounds, such as inorganic salts (Salama *et al.,* 1989). Moreover, the addition of vegetable oils could increase either the uptake of the toxicant by the insect or reduce its evaporation dissipation or both. Park and Lee (2007) cleared that low insecticide toxicity in pests may be due to biochemical mechanisms including target site insensitivity to pesticides and increased detoxification rate by enhancing the production of metabolic enzymes. Alteration in the detoxification enzymes might help in overcoming the insecticidal low toxicity that regulated by enzymes, where susceptibility variations were underlined mainly through three important tolerance mechanisms; decreased penetration, enhanced detoxification, and target-site insensitivity (Gunning *et al.,* 1996). Therefore, the current study aimed to investigate the role of each Na_2CO_3 or mint oil in the effect of *B.t.* on some biochemical parameters in *S. littoralis* larvae.

Materials and methods

1.Tested insect:

The 2nd instar larvae of the cotton leaf worm, *S. littoralis* was obtained from a laboratory strain maintained in the Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza. The culture was reared using the technique suggested by El–Defrawi *et al.* (1964).

2.Bioinsecticide:

Common name: *B. thuringiensis* (Berliner) subsp. *kurstaki*. Trade name: Bay-8 (8 % SC). It is a suspension concentrate (8% SC) formulation, that contains 8% lepidopteran active toxin and 92% an inert material. It was produced by global industrial chemical Co., Gintra- Egypt.

Additives:

2.1 Sodium carbonate: Molecular formula: Na_2CO_3. It was used at concentration 0.1% and it produced by (Adwic) El-Nasr pharmaceutical chemicals Co.

2.2. Vegetable mint oil. It contains menthol, menthone, carvenon, lemonin, and kadene. It was used at concentration 0.3% and it produced by El-captain Co. (CAP Pharm).

2.3. Surfactant agent: Emulsifier (Sisi-6) was used at a concentration 0.3%. It provided by Central Agricultural Pesticides Laboratory (CAPL), Dokki, Giza.

3. Physico-chemical properties:

Some physico-chemical properties were estimated for the solutions of *B.t.* (only or associated with each tested additive). Suspensibility test was conducted to determine the compatibility of the resulting solutions according to WHO (1979) specifications. The pH value was measured through AD 11 pH meter and viscosity was determined by using Ostwald viscometer.

4. Bioassays:

To investigate the interaction between *B.t.* and each of the additives; Na_2CO_3 and mint oil, a series of six aqueous concentrations (1.25, 2.5, 5, 8, 10 and 15ml/L) of *B.t.* were prepared as *B.t.* only or in combination with each 0.1% Na_2CO_3 (Mohamed *et al.,* 2010) or (0.3% mint oil+0.3% emulsifier) according to the method of Abdel- Hafez *et al.* (2013). The leaf-dipping technique using fresh castor bean leaves was used according to the method of Shepard (1958). The percentages mortality

after 3 days of treatment of *S. littoralis* 2nd instar larvae were corrected using Abbott's formula, Abbott (1925). The LC_{50} values of *B.t.* (only and in combination with each additive) were determined after 3 days of treatment according to the method described by Finney (1971) through LDP line software computerized program. The synergistic ratio (SR) based on the LC_{50} values was calculated according to the method of Sun and Johnson (1960).

5. Biochemical studies:

5.1. Sample preparation for biochemical analysis:

Three groups of *S. littoralis* 2nd instar larvae were treated with the median lethal concentration (LC_{50}) value of *B.t.* (only or in combination with each tested additive). The 1st group was treated with *B.t.* only while the 2nd group was treated with *B.t.* in combination with 0.1% Na_2CO_3 and the 3rd group was treated with *B.t.* in combination with 0.3% mint oil. The survived healthy treated larvae in the three groups were separated after 5 days of treatment. These healthy treated larvae were starved for 4 hours, then kept frozen (-5°C) until larval homogenation. The larvae were weighted then homogenated in distilled water with the fixed ratio (0.5 gm.b.wt to 10 ml d. water). The samples were centrifuged at 8000 rpm for 15 min undercooling (4°C) to remove the remnant of tissues. The supernatant fluid was divided into small aliquots (0.5 ml) to be used in the enzymes assay. Percentage of change was calculated from the obtained data according to Elhadek *et al.* (2015).

5.2.Determination of total protein content:

Total protein content was determined by the method of Bradford (1976).

5.3.Determination of α-esterase activity:

α-esterase activity was determined according to the method described by Van Asperen (1962) using α-naphthyl acetate as substrate.

5.4.Determination of phenoloxidase activity:

Phenoloxidase activity was determined according to the method of Ishaaya (1971).

6. Statistical analysis:

The data were subjected to analysis of variance using (ANOVA) in SAS program, (SAS Institute, 1998). Mean separation was conducted using the least significant difference (LSD) in the same program at significant level $P \leq 0.05$.

Results and discussion

1. Physico-chemical properties:

Data presented in Table (1) summarized the physico-chemical properties of *B.t.* (Bay-8) only or in combination with each tested additive. Suspensability test showed that each of the two tested additives was compatible with *B.t.* solution, they gave good suspension without any separation or precipitation at the bottom of the cylinder. In addition, the obtained result indicated that the viscosity of *B.t.* solution increased from 11.1 to 12.0 mP after addition of mint oil additive. The current data clearly showed that the pH value of *B.t.* solution increased from 6.5 to 10 and 7.8 after addition of each Na_2CO_3 and mint oil additives, respectively to be more alkaline. The current results agree with those of Abdel-Hafez *et al.* (2013).

Table (1): Physico-chemical properties of *B.t.* solution (only or in combination with each tested additive).

Mixtures	Conc. ml/l + %	Suspensibility (ml of additive separation)	Viscosity *mP	pH value
B.t.	**LC_{50}**	**0.0**	**11.1**	**6.5**
B.t. **+ Na_2CO_3**	**LC_{50} + 0.1**	**0.0**	**-----**	**10.0**
B.t. **+ mint oil**	**LC_{50} + 0.3**	**0.0**	**12.0**	**7.8**

***mP = millipoise**

2. Synergistic action of adding each 0.1% Na_2CO_3 and 0.3% mint oil on *B.t.* toxicity against *Spodoptera littoralis* 2nd instar larvae:

The results illustrated in Table (2) showed the LC_{50} values after 3 days of treatment of *S. littoralis* 2nd instar larvae. Data revealed that the LC_{50} value (7.079ml/l) of *B.t.* was reduced sharply when combined with 0.1% Na_2CO_3 or 0.3% mint oil to reach 3.972 and 3.549 ml/l, respectively. This synergistic action of Na_2CO_3 may be due to its alkaline nature, which could facilitate the endotoxin breakdown and thereby, the crystals toxic fragments will be released (Nickerson, 1980). Salama *et al.* (1985) reported that augmentation of alkaline compounds concentration in the insect gut might directly affect the crystalline protein solubility. On the other hand, synergistic action of mint oil addition could be attributed to oil properties that might increase toxicity of the insecticide. Natural oil additives may have a significant role in increasing the persistence of the bio-insecticides on the treated leaves. Bode *et al.* (1976) stated that the synergistic effects of vegetable oils on the bio-insecticide activity might be due to changing its physical properties through increasing its viscosity by oil addition which would increase the insecticide deposit on plant leaves, minimize the drift and enhance the insecticide persistence. In addition, adding vegetable oils might increase the uptake of the toxicant by the insect or reduced its evaporation dissipation or both (Abdel-Hafez and Abdel-Aziz, 2010). Vegetable and mineral oils could increase the adhesion, wetting and spreading properties of pesticides on the surface of the targets, decreasing pesticide loss and improving pest control (Abhilash and Patil, 2006). The present result is agreed with Abdel-Hafez and Abdel-Aziz (2010) and Abdel-Hafez *et al.* (2013) they reported that emulsified oils of targets and sesame enhanced the toxicity and persistence of the *B.t.* bio-products; Protecto, Dipel 2x against *S. littoralis* 2nd instar larvae.

Table (2): Synergistic effect of adding each 0.1% Na_2CO_3 and 0.3% mint oil on *B.t.* toxicity against the 2nd instar larvae of *Spodoptera littoralis* after 3 days of treatment.

Treatments	Time after exposure (days)	LC_{50} (ml/l)	95%(FL)		Slope ± SE	*SR
			Lower	Upper		
B.t.	3	7.079	6.458	7. 767	3.048±0.235	----
***B.t.* + Na_2CO_3**	3	3.972	2.118	6.066	2.411±0.177	1.782
***B.t.* + mint oil**	3	3.549	2.335	4.805	2.321±0.174	1.995

***SR= Synergistic ratio**

3. Biochemical studies:

The effect of the LC_{50} value of *B.t.* (only or in combination with each tested additive) was evaluated on some biochemical aspects of *S. littoralis* 2nd instar larval body homogenate after 5 days of treatment.

3.1. Total protein content in the treated and untreated larvae of *Spodoptera littoralis*:

Data represented in Table (3) indicated that treatment with *B.t.* and its mixtures with Na_2CO_3 or mint oil showed a significant reduction in total protein content of the treated larvae compared with the untreated one. The percentages of change were - 11.655, - 20.835 and - 24.446, respectively. The present data also revealed that treatment with *B.t.*-additive mixtures induced significant reduction compared to treatment with *B.t.* only. This reduction of total protein in *B.t.* -additive mixtures may be related to the increased toxicity of its mixtures with both additives than that of treatment with *B.t.* only. According to Ahmed *et al.* (1985) the depletion in protein contents in the treated larvae might be due to binding with foreign compounds as the tested insecticides. El-Shershaby *et al.* (2008) observed that *B.t.* gradually suppressed protein synthesis in *S. littoralis* 4th instar

larvae as post treatment period increased and reached its maximum effect after120h (5 days) of treatment. The present data coincided with those of Kamel *et al.* (2010) who found that the commercial formulations of *B.t.*; Agerin, Dipel 2x and Dipel DF showed significant reduction in total protein contents in larvae of *S. littoralis* after 120h of exposure compared to those in the untreated larvae.

Table (3): Total protein content in *Spodoptera littoralis* 2nd instar larval body homogenate after 5 days of treatment with *B.t.* (only or combined with each tested additive).

Treatments	Total protein (mg/gm.b.wt)	
	Mean ± SE	Change %
Control	64.633 [a] ± 0.736	
B.t.	57.100 [b] ± 1.274	- 11.655
B.t. + Na_2CO_3	51.167 [c] ± 0.601	- 20.835
B.t. + mint oil	48.833 [c] ± 0.841	- 24.446
LSD	2.932	------

Means followed by different letters are significantly different, (P < 0.05).

3.2. α-esterase activity in the treated and untreated larvae of *Spodoptera littoralis*:

Data represented in Table (4) recorded a significant increase in α-esterase activity in the treated 2nd instar larvae of *S. littoralis* with *B.t.* only by percentage of change 10.812 compared with the untreated larvae. The two mixtures, *B.t.*–Na_2CO_3 and *B.t.*– mint oil induced significant reduction in α-esterase activity with percentages of change - 19.831 and - 6.918, respectively compared with the untreated larvae. General esterases are a large and diverse group of hydrolases that hydrolyse numerous substrates including esters and certain non-ester compounds. Numerous studies have demonstrated that esterases play an important role in conferring or contributing to insecticide detoxifications in insect and other arthropod species (Mouches *et al.*, 1986). Thus, the increase in this enzyme activity in the present study might be referred to *B.t.* resistance in the treated pest. The current results are in agreement with those of Hamama *et al*. (2015) who reported that treatment with the bio-insecticide, *B.t.* (Profect) resulted in a significant increase in the α-esterase activity in all treated larvae of *S. littoralis* after 48h of exposure compared to the control. In contrast, Rizk (2014) reported that α–esterase activity was significantly decreased after treating the 4th instar larvae with the bio-insecticide; Protecto.

Table (4): α- esterase activity in *Spodoptera littoralis* 2nd instar larval body homogenate after 5 days of treatment with *B.t.* (only or combined with each tested additive).

Treatments	α-esterase (µg α-naphthol/min./gm.b.wt)	
	Mean ± SE	Change %
Control	2491.000 [b] ± 5.859	
B.t.	2760.333 [a] ± 5.783	10.812
B.t. + Na_2CO_3	1997.000 [d] ± 6.245	- 19.831
B.t. + mint oil	2318.667 [c] ± 2.028	- 6.918
LSD	17.171	-----

Means followed by different letters are significantly different, (P < 0.05).

3.3. Phenoloxidase activity in the treated and untreated larvae of *Spodoptera littoralis*:

Results represented in Table (5) showed that treatments with (*B.t.* only, *B.t.*+Na_2CO_3 and *B.t.*+mint oil mixtures) indicated significant increase in the activity of phenoloxidase with percentages of change 42.405, 16.566 and 33.972, respectively compared to the untreated larvae. Therefore, these data could explain the enhanced toxicity of the tested *B.t.* +additive mixtures than that of *B.t.* only. For that may be using Na_2CO_3 or mint oil interrupted the defense mechanism of the pest

to the bio-agent. The present results are in accordance with those of Valadez-Lira *et al.* (2012) they reported that PO activity in the treated *P. interpunctella* 2nd instar larvae with *B.t.* (Biobit) was 10 times higher than that of the untreated ones. They reported that Insects defend themselves against pathogens through innate mechanisms; as increased phenoloxidase activity. In contrast to the obtained data, Kamel *et al.* (2010) indicated that after 120h of treating *S. littoralis* larvae with the bio-product; Agerin, PO activity was significantly reduced in the treated larvae compared to the untreated ones. Whereas after 48h of treatment, PO had a greater activity levels in treated larvae. This might be due to PO had been activated once entry of bacteria into the insect hemolymph.

Phenoloxidase is an important component of insect immune systems. Its activity had been shown to be correlated with resistance to some parasites or pathogens across species (Nigam *et al.*, 1997). Using phenoloxidase activity as a physiological parameter might also help in the determination of immune response activation against entomopathogenic microbial infections (Narayanan, 2004).

Table (5): Phenoloxidase activity in *Spodoptera littoralis* 2nd instar larval body homogenate after 5 days of treatment with *B.t.* (only or combined with each tested additive).

Treatments	Phenoloxidase (O.D.units/min./gm.b.wt)	
	Mean ± SE	**Change %**
Control	6.320 [d] ± 0.117	
B.t.	9.000 [a] ± 0.208	42.405
B.t. + Na_2CO_3	7.367 [c] ± 0.120	16.566
B.t. + mint oil	8.467 [b] ± 0.088	33.972
LSD	0.4591	-----

Means followed by different letters are significantly different, (P < 0.05).

It is concluded that the present laboratory investigation suggests that adding Na_2CO_3 or mint oil could enhance *B.t.* performance and it is considered a useful addition for controlling *S. littoralis* larvae affecting its enzymes activity, enhancing its susceptibility to the tested bio-agent and reducing the cost of its control via reducing the insecticidal rates used, but further laboratory, semi-field and open field experiments are still needed to confirm the results.

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