Effect of Storage Conditions on Physicochemical Properties of Spintor Formulation and their Biochemical Effects Against The Cotton Leafworm, *Spodoptera littoralis* (Boisd.)

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Abstract

The present study was conducted to investigate the effect of storage of bio-insecticide (Spintor 24% SC) under different temperatures (zeroºC, room temperature and 45ºC) and exposure to sunlight on its persistence and their physical properties. The biochemical effects of the stored Spintor formulations on the 2nd instar larvae of *Spodoptera littoralis* were also determined. Results showed differences in the active ingredients contents of Spintor after exposure to temperature and sunlight. The highest loss percentage of spinosyn A and D (38.96 and 56.18 %, respectively) was recorded after stored at 45ºC. No effect for foaming test in all samples and the pH values appeared lower than the normal range according to FAO specifications. Likewise the biochemical analysis showed a significant increase in acetylcholinesterase (AChE) and amylase activities in larvae homogenate treated with Spintor stored under the investigated conditions. Whereas, protease activity was reduced in all treatments as compared to control. The highest percentage of decrease (-56.41 % of control) was recorded after 7 days of treatment with Spintor stored at 45ºC. Also, treatment with Spintor exposed to sunlight caused significant reduction in protease activities in all periods of treatments.

Key words: Spintor, Storage conditions, Physicochemical Properties, Acetylcholinesterase, Amylase, Protease, *Spodoptera littoralis*

1. Introduction

Chemical control by using conventional insecticides became undesirable, due to the developing of resistance in insects to these insecticides after many successive applications, which can affect the implementation of effective pest control programs **(Knight and Norton, 1989)**, residual toxicity and environmental pollution **(Frank *et al.*, 1990)** and negative effect on non- target organisms **(Franz, 1974)**.

Therefore, the application of bio-pesticides with high selectivity to the pest and low toxicity to humans and environment is highly appreciated **(Defago *et al.*, 2006)**. In the past few decades, efficacy of many bio-pesticidal products, including microbial-based and plant-based botanicals against insect pests have been studied. Spinosad is one of the microbial insecticides which is widely used in Egypt for controlling a wide range of pests belonging to different orders such as Lepidoptera, Diptera, Thysanoptera, Coleoptera, Orthoptera, and Hymenoptera **(Sparks *et al.*, 1995)**. Spinosad has low effective against many beneficial insects **(Thompson *et al.*, 2000; Sparks *et al.*, 2001 and Jackson *et al.*, 2014),** and it has been certified for use in organic agriculture **(Racke, 2007).** Spinosad is produced naturally from soil Actinobacteria "Saccharopolyspora spinosa" **(Mertz and Yao 1990)**, and consists of a mixture of two components "spinosyn A and spinosyn D" (**Thompson *et al.*, 2000 and Anonymous 2013)**. It has rapid contact and ingestion activity in insects, causing excitation of the nervous system, leading to cessation of feeding and paralysis **(Thompson and Hutchins, 1999)**. It achieves this effect via its action on the nicotinic acetylcholine receptor (nAChRs) and gamma amino butyric acid receptor GABA in the insect's nervous system **(Salgado, 1997 and Sparks *et al.*, 2001).**

Regrettably bio-pesticides had short persistence when used under different environmental and field conditions such as temperature, moisture, pH extremes, sunlight, rain, etc. **(Satinder *et al.*, 2006).**

The low environmental risks posed by Spinosad were assessed by **Saunders and Bret (1997).** Research also showed that complete breakdown of the active spinosyns yielded the two sugars, rhamnose and forosamine, which commonly occur throughout nature and the macrolide ring was found to further degrade to form carbon dioxide and water.

In this light, the present study aimed to investigate the effect of storage conditions (temperature and sunlight) on persistence and physical properties of Spintor formulation as well as their effects on some vital enzymes (acetylcholinesterase, amylase and protease) in the 2nd instar larvae of *S. littoralis*.

2. Material and Methods

2.1. Bio-insecticide used:

Spintor analytical standard, mixture of (Spinosyn A & Spinosyn D) (purity>98%) and the formulation (SC 24%) were supplied by Central Agricultural Pesticide Laboratory, Dokki, Giza.

2.1.1.Experimental conditions:

Spintor formulation was kept at room temperature (25±2°C) in their original containers for 12 months during

the study period. Another batch of Spintor formulation were held in tightly container and stored at zero ºC and 45 ºC for 7 days. The third batch of Spintor formulation were exposed to direct sunlight in glass conical flask at dominating temperature that ranged from (30-35ºC) for 4 and 6 hours.

2.2.Chromatographic analysis:

Determination of Spintor active ingredient (Spinosyn A and D) was done by HPLC Agilent 1100 equipped with diode array detector. Zorbax SBC 8 (250mm×4.6mm i.d× 5 µm film thickness) was used as analytical column. The mobile phase was acetonitrile 2% ammonium acetate with flow rate was 1.3 ml/min. Detection wavelength for detection of pymetrozine was set at 250 nm. Retention time was 12.6 and 14.64 min for Spinosyn A and Spinosyn D, respectively.

2.3. Determination of Physical properties of Spintor formulation:

Physical properties of Spintor 24 % SC samples (under all storage conditions) with their spray solutions in soft and hard water were determined according to **CIPAC (1995)** handbook methods. Hard water was prepared by dissolving 0.304 g of anhydrous calcium chloride and 0.139 g of magnesium chloride hexahydrate in distilled water and made up to one liter. This provides total hardness equivalent to 342 ppm of calcium carbonate. Soft water was prepared by mixing one volume of hard water with five volumes of distilled water to provide water hardness of 57 ppm according to **CIPAC (2001).**

2.3.1. Persistent foam (MT 47.2): Foam volume was measured after 5 minutes after invert 30 times. The cylinder contained diluted pesticide.

2.3.2. pH values (MT 75): The spray solution was shaken well till homogeneity and pH value was measured by Schott Gerate pH meter.

2.3.3. Electric conductivity (MT32): Conductivity, salinity and total dissolved solids (T.D.S) were measured using conduct-meter YS1 model 35S-C-T (mMHOs) is the unit of electrical conductivity measurement.

2.4.Tested insect:

A laboratory strain of the cotton leafworm *S.littoralis* was provided by (CAPL), Dokki, Giza. The culture was reared using the technique described by **(El-Defrawi *et al.*, 1964).** Insects were reared on castor bean leaves in laboratory under constant conditions of 25±2ºC and 65±5% R.H.

2.5. Biochemical analysis:

2.5.1. Preparing of samples for enzyme analysis: To estimate the enzyme activities of (acetylcholinesterase, amylase and protease) induced by (LC_{50}) of Spintor stored at different tested conditions, the experiments were carried out using leaf-dipping technique **(Shepard, 1958).** Fresh castor bean leaves were dipped for 20 seconds in aqueous solution of Spintor at LC_{50} values (31.171, 25.775 and 43.938 ppm) after stored at zero ºC, room temperature and 45ºC, respectively and (33.883 and 34.312 ppm) after 4 and 6 hours of exposure to sunlight, respectively. Then left one hour to dry in room temperature, before being offered to the larvae for feeding on it. Ten healthy 2nd inster larvae with five replicates were subjected to each of the treated leaves. The larvae were exposed and fed on treated leaves for 48 h, the survival larvae were transferred to feed on untreated leaves for another 24 h. Control larvae were fed on water-tested leaves. The larval samples were collected after 3, 5 and 7 days from treatment, and starved for about 4 h before being homogenized in (1/5 w/v) homogenization buffer (pH 7.8). Homogenates were centrifuged at 10000 rpm for 15 minutes at 4ºC and the resultant supernatants were held in clear Eppendorf tube and stored at -20°C **(Mohamady, 2005).**

2.5.2. Enzymes activity: Acetylcholinesterase (AChE) activity was measured according to the method described by **Simpson *et al.* (1964).** Amylase activity was determined by the method described by **Ishaaya and Swirski (1976)**. Protease activity was determined by the casein digestion method described by **Ishaaya *et al.* (1971)**.

2.6. Statistical Analysis:

Data obtained from the enzymes analysis of different treatments are represented in tables as the mean ± standard error (mean ± SE). The significant differences between the mean values of the treatments were calculated using Student's t-test.

3. Results and Discussion

3.1. Effect of storage conditions on the degradation rate of spinosyn (Spintor 24% SC) using HPLC:

To our knowledge this is the first study which has been determined the degradation rate and effect of different storage conditions on the efficacy of Spintor formulation (SC 24 %).

Data presented in Table (1) clearly showed that, the degradation rate of Spintor with all various conditions of storage was higher than the sample storage at room temperature (as control). Thus it appears that Spintor is strongly affected after exposed to 45 ˚C than zero ˚C and direct sunlight exposure. The percentage of loss at 45 ˚C was (38.96 % loss) and (56.18 % loss) for spinosyn A and D, respectively. While Spintor which was exposed to direct sunlight had a moderate degradation rate, with percentage (29.92 % and 31.21 % loss) after 4 hours of exposure and (31.68 and 29.58 % loss) after 6 hours of exposure for spinosyn A and D, respectively. The lowest degradation rate was recorded with Spintor which was exposed to zero˚C, with percentage (16.72 and 36.50 % loss) for spinosyn A and D, respectively.

$$\% \, Loss = \frac{Treated - Control}{Control} \times 100$$

The current results similar to the results obtained by **Pèrez *et al.* (2007)**, who study the persistence of Spinosad in sunny and shaded conditions and reported that Spinosad solution placed in sunny location (direct sunlight at experimental condition) was degraded and its toxicity was lost about ten folds faster than Spinosad solution placed in shaded condition. The obtained results were confirmed by the bioassay of Spintor on 2nd instar larvae of cotton leaf-

worm, *S. littoralis* in previous study (**Sleem, *et.al* 2012)**. Bioassay results reflected the loss of active ingredient in an increasing in LC_{50} values. The LC_{50} values were reached to 25.775, 31.171 43.938 ppm for Spintor storage at room temperature, zero ºC and 45ºC, respectively and (33.883 and 34.312 ppm) for Spintor exposed to direct sunlight after 4, 6 hours, respectively. **Subramanyam *et al.* (2006) and Daglish and Nayak (2006),** conducted the field trials in Kansas on stored wheat and in Indiana on stored corn using farm-size bins (60-125 metric ton capacity).Using Spinosad at the application rate of 1mg/kg. There was about 25-30% loss of insecticide during application resulting in 0.70-0.75 mg/kg Spinosad deposition

Table (1): Effect of storage conditions on the active ingredient content of Spintor 24% SC.

Treatments	Period of exposure	Spinosyn A Residue level (ng)	Loss %	Spinosyn D Residue level (ng)	Loss %
Standard	-	2000.00	-	2000.00	-
Room temperature (as control)	-	1961.59	-	1902.98	-
Storage at 0 ˚C	7 days	1633.45	16.72	1208.31	36.50
Storage at 45 ˚C	7 days	1197.24	38.96	833.86	56.18
Exposure to direct sunlight	4 hours	1374.51	29.92	1309.02	31.21
	6 hours	1419.08	31.68	1340.01	29.58

on grain. This percentage of loss of applied insecticide can be expected with any grain protectant due to the heterogeneous natural of the grain. Although Spinosad breaks down within a week when exposed to sunlight, in grain storage environments, Spinosad residues persisted for a period of 6 month to a year with minimal loss in insecticide activity. **Also Zubairi *et al.* (2015)** recorded a significant effect of thermal degradation or dissipation of rotenone content as bio-pesticides at higher operating temperature (greater than 40 ˚C) with a rapid rotenone reduction for the first 15 min of exposure.

3.2. Effect of tested conditions of storage on Physical properties of Spintor formulation:

Data summarized in Table (2) showed that, the spray solutions had no persistence foam was formed in soft and hard water**. (WHO, 1979 and CIPAC, 2001)** stated that in order to obtain successful of suspension concentrate foam test shouldn't exceed than 2 ml. The pH values were in range (5.86- 6.35) that were out of acceptance limit of (FAO) specification, because of Spinosad formulation pH rang 6.5 to 8.5 **(CIPAC 2001**). The tested samples having conductivity ranged from (123.7 to 668 mM), the highest values in soft and hard water were 139.2 and 668 mM that appeared in Spintor stored at 45ºC for 7 days and Spintor stored at room temperature; respectively.

The lowest values in soft and hard water were 123.7 mM and 650 mM that appeared in Spintor exposed to sunlight for 4 hours and 6 hours, respectively. In salinity %, all samples detected values 0.1 % in soft water and 0.3% in hard water. As for T.D.S the values ranged from (59 to 66 mg/L) in soft water and (317- 322 mg/L) in hard water.

Table (2). Physical parameters in (soft and hard water) of Spintor 24% SC at different storage conditions.

Storage conditions		Physical parameters									
		Foam layer (ml)		pH		Conductivity (mM)		Salinity (%)		T.D.S. (mg /L)	
		Hard water	Soft water	Hard water	Soft water	Hard water	Soft water	Hard water	Soft water	Hard water	Soft water
Room temperature (23-25 ºC)		0.00	0.00	6.25	6.14	668	138.9	0.3	0.1	322	66
ZeroºC	7 days	0.00	0.00	5.94	6.23	657	126.7	0.3	0.1	317	60
45ºC	7 days	0.00	0.00	5.99	6.31	664	139.2	0.3	0.1	319	66
Exposure to sunlight	4 hrs	0.00	0.00	5.86	6.13	658	123.7	0.3	0.1	317	59
	6 hrs	0.00	0.00	6.01	6.35	650	137.3	0.3	0.1	317	65

3.3. Enzymes activity:

Influence of environmental conditions (storage temperature and sunlight) on the efficiency of Spintor against some vital enzymes (AChE, amylase,

3.3.1.AChE activity: As demonstrated in Table (3), AChE activity was increased significantly by 24.64 and 34.06% after 3 and 5 days from treatment of larvae with Spintor stored at room temperature as compared to control, and showed non-significant decrease (-3.1) after 7 days from treatment. No significant changes in AChE activity was observed in larvae homogenate treated with Spintor stored at zero ºC for 7 days at all time intervals, the percent of change were (-5.35, -2.18 and -6.25 % of control), after 3,5 and 7 days of treatment, respectively. On the other hand, the AChE activity was increased significantly as compared to control when larvae treated with Spintor stored at 45ºC for 7 days. The highest percentage of increasing (35.7 % of control) was occurred

after 3 days of treatment. Also exposure of Spintor to sunlight for 4 and 6 hours increased significantly the AChE activity in larvae homogenate at all periods of treatment. protease) in the 2nd instar larvae of *S. littoralis* was also investigated (Tables 3-5). The percentage of increasing reached its maximum level (53.48 %) after 7 days of treatment with Spintor exposed to sunlight for 4 hours and reached its minimum level (17.05 %) after 7 days of treatment with Spintor exposed to sunlight for 6 hours. Results in the present study agree with that obtained by **Elbarky, *et al.* (2008) and Farag and Mead (2009). Farag and Mead (2009)** recorded significant increase in AChE activity in the larvae treated with Spintoram with percent (248.05 %). The hyperactivity of AChE which is found in this study may be explained by **Salgado *et al.* (1998)**, who has demonstrated that Spinosad could attack the nicotinic acetylcholine receptor (nAChR) with acetylcholine Ach simultaneously, as well as acting on a new site differing from the site on which Ach acts. They gave a hypothesis that there were two special sites on nAChR for Spintoram and AChE individually. When both Spintoram and AChE are absent, the receptor channel will keep closed. When either of them is present or both of them are present, the channel will open up and subsequently the receptor will be activated. This assumption may be able to explain the overproduction of both AChE and ACh. However, there is no evidence demonstrate that Spinosad directly links to a site on nAChR, and it probably means that Spintoram indirectly regulates the nAChR. Furthermore, **Watson (2001)** indicated that Spintoram could also act on γ-amino butyric acid (GABA) receptor, and increase neural activity of pest in excess and subsequently make the pest fall into a decline and be dead eventually. On the other hand, our results disagree with that obtained with **Abd El-Mageed and El-Gohary (2006)** who found that Spinosad gave decrease in the acetylcholinesterase activity (-39.29%) lower than control in the laboratory strain of *S. littoralis* (Boisd) and **Abd El-Mageed (2007)** reported that treatment of 4th instar larvae with LC_{50} of Spinosad caused significant reduction in the activity of AChE compared with untreated insects by 49.58 %. Also **El-Sheikh *et al.* (2009)** recorded a decrease in activity of (AChE) by about 27.46 % for Spinosad compared to control and **Rashwan (2013)** revealed that Spinetoram exhibited a remarkable reduction in AChE activity reached -37.47%.

3.3.2.Protease activity: Data in Table (4) revealed that, protease activity was reduced in all treatments as compared to control**.** The highest percentage of decrease in protease activity was (-56.41 % of control) occurred after 7 days of treatment with Spintor storage at 45ºC followed by Spintor stored at room temperature (-50.33% of control). As well as the highest percentage of reduction in protease activity (-51.23 and - 49.60%) were occurred after 3 and 5 days of treatment with Spintor exposed to sunlight for 6 and 4 hours, respectively. While a non-significant decrease (-9.76 and -7.53%) were observed after 7 days of treatment with Spintor exposed to sunlight for 4 and 6 hours, respectively. Some proteases play roles in insect digestion and development, the exposure to Spintor in the present study resulted in decrease of the protease activity which agreed with suggestion of **Rodigue- Ortegua *et al.*, 2003** and **Moselhy, *et al.* (2015)** who recorded the same effect on *C. pipiens* larvae after Spinosad treatment. Whereas **El-Sheikh *et al.* (2009)** found a significant increase in protease activity in larvae treated with Spinosad and the highest level of the enzyme activity relative to control, was (66.54%).

3.3.3.Amylase activity: As shown in Table (5**)** it is clear that, the storage of Spintor at 45ºC for 7 days caused great increase in amylase activity by about (523 and 273.1 % of control) after 3 and 5 days of treatment, respectively. In contrast a significant reduction was observed after 7 days of treatment with Spintor storage at room temperature by about (-52.94 % of control) followed by Spintor stored at zeroºC by about (- 49.57% of control).

It can notice that, exposed Spintor to sunlight for 4 and 6 hours increased the amylase activity at all periods of treatment except after 7 days of treatment with Spintor exposed to sunlight for 6 hours recorded non-significant decrease in amylase activity by (-24.08 % of control) .

In the present study, the activity of amylase increased after treatment with Spintor which agreed with results of **Abd El- Mageed and El-Gohary (2006)** who recorded the same effect on *S. littoralis* larvae after Spinosad treatment by (46.115%) as compared to control; also **Abd El- Mageed (2007)** noticed significant increase in amylase activity in homogenate of 4th instar larvae of *S. littoralis* after 6 days of treatment with the LC_{50} of Spinosad reached to 109.54%. In contrary **Farag and Mead (2009)** recorded a significant reduction in amylase activity in 4th instar larvae of *S.littoralis* treated with LC_{50} of Spintoram.

From the above results, it can notice that stored of Spintor at different conditions cause fluctuated effects between increased and decreased in the enzymes activity as compared to that stored at room temperature. No information has been available regarding to this point; so it should be done more researches to understand and explain this effect.

Conclusion

The present study showed differences in declared active ingredients contents of Spintor after exposure to temperature and sunlight and this reflected on the activity of tested enzymes. No persistence foam was formed in all samples of Spintor diluted with soft and hard water and all

of them exhibited acidic pH value. Treatment of 2nd instar larvae of *S. littoralis* with Spintor under all tested stored conditions induced remarkable changes in the activity of tested enzymes.

Table (3): Effect of Spintor* storage at different storage conditions on the activity of AChE enzyme in the larval homogenate of *S.littoralis*.

Storage conditions		AChE activity (µg AChBr /min/mg protein)								
		Treatment by days								
		3days			5 days			7 days		
		M±SE		Change %	M±SE		Change %	M±SE		Change %
		Control	Treated		Control	Treated		Control	Treated	
Zero ºC	7 days	2.8 ±0.052	2.65 ±0.28	-5.35 ns	2.29 ±0.11	2.24 ±0.04	-2.18 ns	1.29 ±0.06	1.2 ±0.05	-6.25 ns
Room temperature (23-25)ºC		2.8 ±0.052	3.49 ±0.08	24.64**	2.29 ±0.11	3.07 ±0.14	34.06*	1.29 ±0.06	1.25 ±0.1	-3.1 ns
45ºC	7 days	2.8 ±0.052	3.8 ±0.08	35.7***	2.29 ±0.11	3.03 ±0.03	32.3**	1.29 ±0.06	1.71 ±0.06	32.5**
Expo-sure to sunlight	4 hrs	2.8 ±0.052	3.95 ±0.11	41.07***	2.29 ±0.10	2.9 ±0.12	26.63*	1.29 ±0.06	1.98 ±0.08	53.48**
	6 hrs	2.8 ±0.052	3.7 ±0.11	32. 14**	2.29 ±0.10	2.93 ±0.04	27.94**	1.29 ±0.06	1.51 ±0.11	17.05 ns

*Treatment concentration at LC_{50} Data are presented as the mean ± stander error (M±SE) : M= Means : SE= Stander Error
*** highly significant ($p < 0.001$) : ** moderately significant ($p < 0.01$) : * significant ($p < 0.05$) ns : non-significant : % Change as compared to control

Table (4): Effect of Spintor* storage at different storage conditions on the activity of protease enzyme in the larval homogenate of *S.littoralis* .

Storage conditions		Protease activity (O.D. units x10^3/min/mg protein)								
		Treatment by days								
		3 days			5 days			7 days		
		M±SE		Change %	M±SE		Change %	M±SE		Change %
		Control	treated		Control	treated		Control	treated	
Zero ºC	7 days	24.75 ±1.98	23.8 ±0.77	-3.83 ns	27.2 ±1.00	22.4 ±0.71	-17.64*	24.16 ±1.51	23.15 ±1.45	-4.18 ns
Room temperature (23-25)ºC		24.75 ±1.98	13.75 ±0.79	-44.4**	27.2 ±1.00	15.7 ±1.17	-42.27**	24.16 ±1.51	12 ±1.01	- 50.33**
45 ºC	7 days	24.75 ±1.98	14.6 ±0.84	-41.01**	27.2 ±1.00	18.71 ±0.87	-31.21**	24.16 ±1.51	10.53 ±0.50	-56.41**
Exposure to sunlight	4 hrs	24.75 ±1.98	12.49 ±0.67	-44.65**	27.2 ±1.0	13.7 ±1.31	-49.60 ns	24.16 ±1.51	21.8 ±1.75	-9.76 ns
	6 hrs	24.75 ±1.98	12.07 ±1.02	-51.23**	27.2 ±1.0	16.8 ±0.88	-38.30**	24.16 ±1.51	22.34 ±0.45	-7.53 ns

*Treatment concentration at LC_{50} , Data are presented as the mean ± stander error (M±SE) : M= Means : SE= Stander Error
** moderately significant ($p < 0.01$). : * significant ($p < 0.05$) ns : non-significant : % Change as compared to control

Table (5): Effect of Spintor* storage at different storage conditions on the activity of amylase enzyme in the larval homogenate of *S. littoralis*.

Storage conditions		Amylase activity (µg glucose x10^3/min/mg protein)								
		Treatment by days								
		3 days			5 days			7 days		
		M±SE		Change %	M±SE		Change %	M±SE		Change %
		Control	Treated		Control	Treated		Control	Treated	
Zero ºC	7 days	221 ±11.57	593 ±17.79	168.3***	369 ±6.36	374 ±15.0	1.35 ns	714 ±28.68	360 ±9.95	-49.57***
Room temperature (23-25)ºC		221 ±11.57	472 ±14.81	113.5***	369 ±6.36	407 ±13.44	10.20 ns	714 ±28.68	336 ±12.07	-52.94***
45 ºC	7 days	221 ±11.57	1377 ±61.90	523***	369 ±6.36	1359 ±69.0	273.1***	714 ±28.68	1049 ±53.7	46.9**
Expo-sure to sunlight	4 hrs	221 ±11.57	1173 ±33.8	430.76***	369 ±6.36	1079 ±60.6	192.4***	714 ±28.68	825 ±28.6	15. 54 ns
	6 hrs	221 ±11.57	455 ±14.8	105.88***	369 ±6.36	545 ±26.97	47.69**	714 ±28.68	542 ±15.30	-24.08 ns

*Treatment concentration at LC_{50} Data are presented as the mean± stander error (M±SE) : M= Means : SE= Stander Error
*** highly significant ($p < 0.001$). ** moderately significant ($p < 0.01$). ns : non-significant : % Change as compared to control.

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