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131-0924-97	הגברת הפעילות האינסקטיצידית של נגיפי הבקולו על ידי

תקציר הדו"ח:
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1. הצגת הבעיה:
   1. הכנת תוארית חדשה לריסוס נגיפי בקולו המבוססת על איבוק וכוללת פתיון אכילה.
   2. הערכה של יעילות התוארית הנ"ל וכנגד זחלי הליוטיס.

2. מהלך ושיטות עבודה:
   1. הכנת התוארית החדישה.
   2. פיזור התוארית על צמחי כותנה.
   3. הערכת מניעת נזק של זחלי הליוטיס על הלקטי צמחי כותנה מרוססים בתוארית.
   4. פיתוח מבחן ELISA לכימות חלקיקי הנגיף בתוארית.

3. תוצאות עיקריות:
   התוארית החדישה שומרת על פעילות האינסקטיצידית של נגיף הבקולו ללא פגיעה ביעילות: הנגיף פעיל ב-100% שבוע לאחר חשיפה. נגיף שרוסס בתוארית מימית מאבד באותה תקופה 80% מפעילותו.
   מבחן ה ELISA מאפשר לבדוק את ההומוגניות של פיזור הנגיף בתוארית.

4. מסקנות והמלצות:

   יש צורך ללמוד את פעילות התוארית בתנאי חשיפה קיצוניים יותר בריסוסי שדה. בנוסף כדאי לבדוק האם יעילות התוארית נובעת מיכולתה להגן על שלימות החלקיק הנגיפי בתוארית.

FINAL REPORT

BACULOVIRUSES ENHANCED INSECTICIDAL ACTIVITY THROUGH GENETIC IMPROVEMENT AND NOVEL FORMULATION

Nor Chejanovsky and Amos Navon

Abstract

Extensive use of non-specific chemical insecticides against the lepidopterous polyphagous pests from the species *Spodoptera* and *Heliothis* (=Helicoverpa) , poses serious risks to the environment and stimulates the appearance of resistant insect species. Thus ,the search for alternative means of pest control brought back the attention to the insect-specific Baculoviruses. A major millstone in the implementation of these viruses in the field is their inactivation by environmental factors mainly, solar UV radiation. Our data suggested that photolysis of the viral particle ,occluded in the polyhedral body, occurs primarily through the , cleavage of UV-sensitive amino acids of its major component , the polyhedrin protein. In this project we treat this problem by a combination of basic and practical approaches: 1. The basic approach deals with the genetic modification of the polyhedrin protein (the major component of the polyhedral body which occludes the viral particles) to diminish its sensitivity to UV light and 2. The practical approach includes the preparation of baculovirus stocks with the novel dust formulation developed by A. Navon successfully tested with *Bacillus thuringiensis* (1).

Due to the level of support obtained for the project we directed our efforts to the practical approach. During the first phase of the it we prepared novel granular bait formulations of our baculovirus HaNPV-Is. These formulations were effective against *Helicoverpa (Heliothis) armigera* in our cotton leaf assays and in the field, conferring lasting activity to the viral polyhedra and showing improvement in their performance compared to the aqueous formulation. Also, an ELISA method developed for the analysis of the distribution of the viral particles in the formulations, showed that the viral component of the granular bait formulations is homogeneously distributed (at least to the 1 mg level).

These data allow us to test in the forthcoming years the degree of protection conferred by these formulations to viral particles against UV irradiation , allowing a more effective (persistent) insecticidal activity towards *Heliothis armigera* larvae.

We expect that this project will result in baculovirus stocks and formulations more resistant to inactivation by solar UV radiation with long-lasting anti-insect activity in the field, contributing to obtain pesticide-free agricultural products for internal consume and export markets, and improving the overall pest management (also, by facilitating the introduction of natural enemies against non-target insects in a insecticide-free environment).

Specific objectives

1. Preparation of novel formulations of the wild type *Heliothis armigera* Nuclear Polyhedrosis Virus-Is strain (HaNPV-Is) utilizing the new granular formulation with feeding bate stimulation (see below).
2. Genetic modification of the HaNPV-Is polyhedrin amino acids sensitive to UV-light and, engineering the modified polyhedrin genes back into the HaNPV viral genome.
3. Evaluation of the produced viral stocks for infectivity and residual infectivity to *H. armigera* larvae.

Due to the level of support obtained (50 % of the approved suggested budget) we decided to concentrate on objectives 1 and 3.

A baculovirus strain highly virulent to *Helicoverpa armigera*: the HaNPV-Is was isolated and characterized and, a tissue culture system which allows the efficient replication of the virus for the isolation of appropriate recombinants was established (2). This isolate constitutes the basis for the development of our microbial insecticide.
Our approach adapts the above mentioned dry granular formulation to protect and enhance the insecticidal activity of our HaNPV-Is isolate. The formulation, method of application and initial results are presented in the following pages:

Section I: Insecticidal activity of the baculovirus dry granular formulation

Baculovirus in granular larval-bait preparation: 0.5 ml of HaNPV-IS polyhedra (11,150 PIB/ ul) and 0.5 ml water were mixed with 5 mg yeast and 1 g flour meal (< 100 mesh) in a pestle until a viscous homogeneous mixture was obtained. The mixture was dispersed on a glass plate and left overnight in a dry hood under a moderate sub-pressure. The dry powder was grinded by means of a mortar to a fine powder (20 mg wheat germ oil were added and mixed). Control powder was prepared similarly but without viral polyhedra.

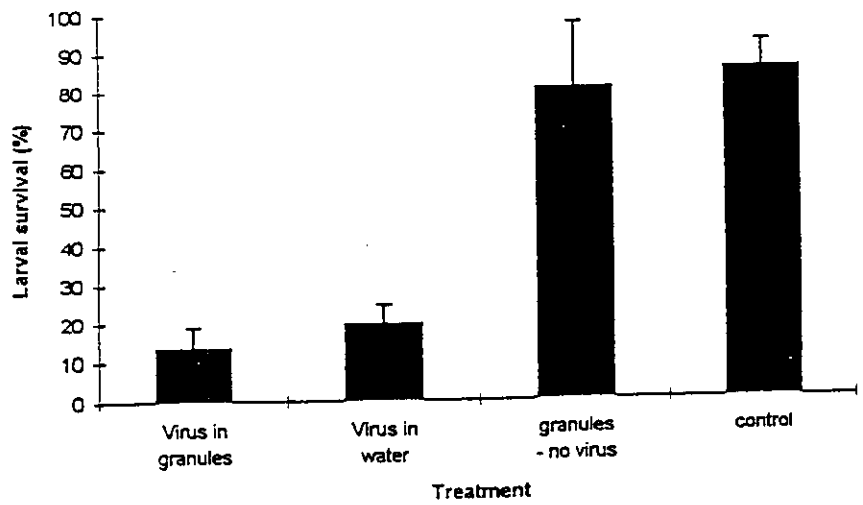
Leaf bioassay: A layer of 1 cm thick of 1 % aqueous agar-agar gel was set in glass scintillation vials. A single leaf of either cotton, *Gossypium hirsutum* (Acala SJ2) seedlings from plant plots outside the laboratory were used. The plants were hand-washed with 70% ethanol using a cotton swab and air dried. % or 10 mg of powder formulations were dispersed on both sides of the leaves. Five H. armigera neonates were used per leaf. S4 hours after leaf feeding the larvae were placed on single virus-free diet flakes in cells of a grid placed in sterile petri dishes. Larval mortality was observed after 5-6 days of feeding. Five replicates per treatments per date were used. The experiments were replicated 6-9 times at various dates.

The baculovirus assay treatments were:

1. Baculovirus in granular larval-bait formulation.
2. Baculovirus in aqueous mixture (same concentration of PIB per leaf as in 1).
3. Granular larval-bait virus-free formulation.
4. Untreated control.

Fig. 1 shows that virus activity in the granular larval-bait formulation was somewhat higher than in the aqueous suspension and 6,3 times more active than in control, suggesting that the viral polyhedra were the cause of mortality and the other ingredients of the formulation were not detrimental to the larvae. Previous studies showed that the yeast and oil stimulated feeding in choice tests with the Styropor method. Thus feeding effect was responsible for larval acquiring of the granules resulting in virus-mediated mortality. Mortality was observed after 4 days latency and was accompanied by typical disease symptoms (body rupture and exudation of fluids full of polyhedra).

Figure 1. Survival of *H. armigera* neonate larvae in cotton leaf bioassays with baculovirus in granular feeding bait preparations (55,750 PIB/leaf)



Section II: detection of baculoviral polyhedra in granular bait formulations

Since our approach introduces a novel dry formulation, it is crucial to develop methods to monitor its homogeneity at the macro and micro levels.

For that purpose, we developed an Enzyme linked immunosorbent assay (ELISA) to detect the presence of the baculovirus in our preparations and to evaluate their homogeneity. This assay is based in the ability of an anti-polyhedrin antibody (produced by us by immunizing rabbits with the viral protein polyhedrin) to recognize the viral protein polyhedrin present in the polyhedral inclusion bodies (PIBs).

For that purpose , purified polyhedrin was coated on ELISA plates and further incubated with the anti-polyhedrin antibody. The extent of binding of this antibody was measured using a second anti-rabbit IgG antibody conjugated to the detector enzyme alkaline phosphatase.

Alkaline extractions of the polyhedrin present in the different formulations of HaNPV-Is were performed. The content of the polyhedrin in each formulation was determined by measuring the ability of the extracts to compete with the coated polyhedrin for the binding of the anti-polyhedrin antibody in the ELISA assay. A calibration curve was obtained by incubating extracts of known amounts of our control HaNPV-Is aqueous preparation in this assay. The results are presented in Figure 2. As can be seen this curve allows us to extrapolate the content of polyhedrin in various samples of polyhedral extracts derived from the granular bait formulation. This data is presented in table I.

This data show that the viral insecticide is homogeneously distributed in samples of 1 mg

Thus, to this step we can conclude that our initial formulations are quite homogeneous up to the milligram level (which is the maximal sensitivity of our test).

We are further developing an immunoblot method which will allow the detection of polyhedrin present in 100 g of sample.

Figure 2 Determination of polyhedrin by competitive ELISA

Extracts containing polyhedrin (derived from a known amount of polyhedral inclusion bodies) were added simultaneously with a fixed amount of anti-polyhedrin serum to the ELISA plate containing coated polyhedrin (derived from 11000 PIBs). The plate was incubated at 37 °C for an hour and washed . Anti-rabbit IgG-alkaline phosphatase conjugate was added and the plate was further incubated at 37 °C for 30 min. Alkaline phosphatase substrate was added to determine the degree of competition between free and bound polyhedrin.

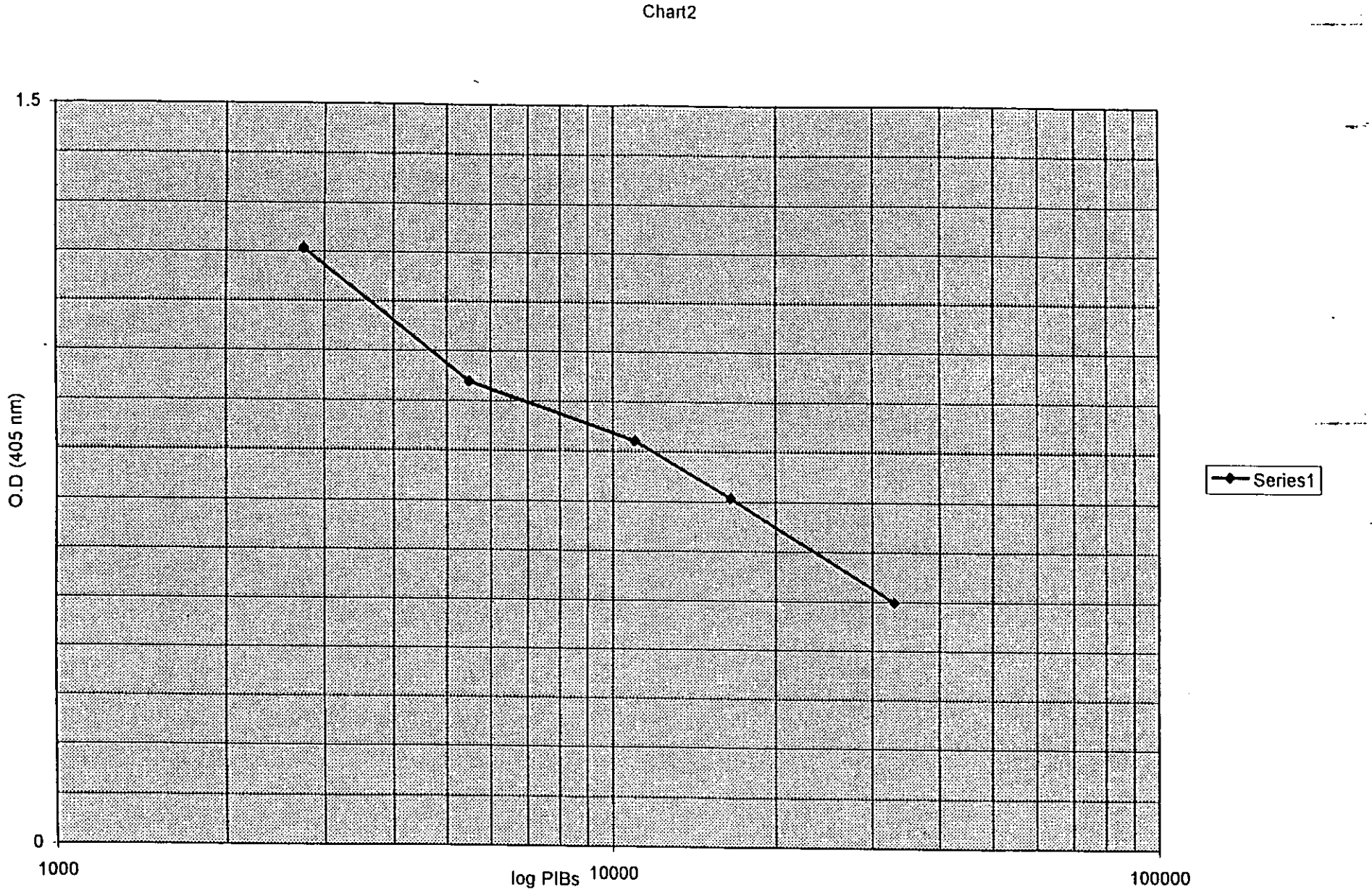


Chart2
1.5
O.D (405 nm)
0
1000
log PIBs
10000
100000
Series1

Table I Quantitative estimation of the polyhedrin content of baculovirus dry granular formulation samples

Formulation	Polyhedral Inclusion Bodies/ mg sample		
A (25\7\96)	3600	2400	3400
B (20\9\96)	2200	2200	2600
C (26\10\96)	3000	2600	3000

Extracted Polyhedrin from 1 mg samples was incubated as competing antigen in the competitive ELISA. A ±20% of error in the detection of polyhedrin is estimated.

Section III: Effects of *Helicoverpa* NPV (HaNPV)in granular-feeding bait formulation against *H. armigera* in cotton flower buds

In 1996, we conducted controlled field experiments in cotton Acala SJ-2 (Bet Dagan Farm) with the new NPV-granular formulation. The application of our viral preparation was directed towards cotton flower buds. Twenty flower buds were powdered each with 15 mg of the granular formulation. Other flower buds were smeared with aqueous mixture of the NPV. The amounts of polyhedra in the two treatments equaled $2.5.10^5$ PIBs. The control was not treated. Flower buds were picked up at zero time and seven days thereafter and bioassayed in the laboratory for 4-5 days in glass vials with gelled agar for water supply to the plant tissues. Two neonate larvae of *Helicoverpa armigera* were used per vial. The experiment was repeated two times at separate dates.

Date	NPV exposure	Mortality (%) of neonate larvae on:		
	in cotton (days)	NPV-Granular	NPV-Aqueous	Control
30/7-5/8	0	95	100	5
1/8-5/8	5	80	25	15
12/8-18/8	7	20	20	25

Table II. **Mortality of *H. armigera* larvae on cotton flower buds treated with granular and aqueous formulations of HaNPV**

Table II shows that after seven days from the time of application, the aqueous NPV formulation lost its activity whereas the granular formulation of NPV exhibited high activity. Damage to the NPV-treated flower buds was minimal but the control larvae infested the inner parts of the flower. In the granular NPV formulation the polyhedra were embedded in the carrier (Wheat

formulation.

Section IV: Effects of the HaNPV granular larval-bait formulation in cotton leaves.

1. Determination of the active viral concentrations in our formulations

Neonate larvae of *H. armigera* were offered series of virus concentrations in the granular formulation tested in leaf bioassays in glass vials with agar-agar. 15 mg of the granular formulation and 20ul of an aqueous mixture of the virus in equal virus concentration was applied to each leaf. Mortality was recorded after days. Experiments was replicated three times.

Virus concentration (PIB/ g)	Larval Mortality (%)	
	Granules	liquid
1.28. 10^6	100	100
1.28. 10^5	100	100
1.28. 10^4	70	90
1.28. 10^3	60	68
1.28. 10^2	44	24
1.28. 10^1	0	44
1.28. 10^{-1}	20	8
1.28. 10^{-2}	16	24
1.28. 10^{-3}	0	16

Table III shows that in the laboratory, the differences in mortality between the two formulations were minimal, and for both formulations the concentration of 1.28. 10^5 PIBs caused 100% mortality. Mortality in the control ranged 0-20%. Therefore, mortality at concentrations lower than 1.28. 10^3 PIBs can be ignored.

2. Field work

The work was done in a cotton plot outside the laboratory. The concentration of 1.28. 10^5 PIBs causing 100% mortality in the laboratory was tested in the field. The same virus concentration for both granular and aqueous preparations was applied to leaves. The granules were hand dispersed on the leaves and the aqueous formulation was smeared on the leaves. The granular formulation without the virus was one control treatment and a control without any treatment was used, as well. Leaves were picked up at different dates after the virus application

Larval feeding of larvae (days after application)	Larval mortality (%)			
	Granular virus formulation	Aqueous virus mixture	Control granular	Control no treatment
0	52	92	33	32
5	96	52	40	46
12	32	46	40	42

Table IV shows that at zero time the aqueous mixture was more effective than the granular formulation of the virus. But, after 5 days the granular formulation was superior to the aqueous mixture. After 12 days both preparation were not active. Probably, on the leaf, the virus in aqueous mixture was more available to the larvae than in the granules. On the other hand, the, granular virus formulation showed persistence that was higher than in the aqueous virus preparation.. The high mortality in the control resulted probably from low quality of leaves due to insect damage caused by whiteflies, aphids and more).

Conclusions

At this stage of the project we were able to prepare granular bait formulations of our baculovirus HaNPV-Is. This formulations were effective against *Heliothis armigera* in our cotton leaf assays. Moreover we obtained improvement in their performance and lasting insecticidal activity in the field compared to the aqueous formulation . However, more work is needed to improve availability of the virus in the granular bait formulation to the larvae.
Our ELISA method of detection showed that the viral component of the granular bait formulations is homogeneously distributed (at least to the 1 mg level).
These data allow us to test in the forthcoming years the degree of protection conferred by these formulations to viral particles against UV irradiation , allowing a more effective (persistent) insecticidal activity towards *Heliothis armigera* larvae. Further experiments are required to determine the degree of protection against *H. armigera* conferred by our formulation.
Achieving a long-lasting insecticidal activity to the viral polyhedra will enable the incorporation of our HaNPV granular larval-bait formulations into Israeli pesticide-free sustainable agriculture practices.

References

1. Navon, A. (1994) A novel dry form with granular feeding bate which protects and enhances the insecticidal activity of *Bacillus thuringiensis*. (patent application, ARO).

2. Rivkin, H., Mor, M. and Chejanovsky, N.(1997). Isolation, replication and polyhedrin gene sequence of an Israeli *Helicoverpa armigera* Nuclear Polyhedrosis Virus (Virus Genes, accepted)

3. Navon, A., Klein, M. and Braun, S.(1990). *Bacillus thuringiensis* potency bioassays against *Heliothis armigera, Earias insulana* and *Spodoptera littoralis* based on standardized diets. J. Invertebr. Pathol. 55:287-393.

3. סיכום חדש לדו"חות מחקר 1997

נא לענות על כל השאלות, בקצרה ולעניין, ב- 3 עד 4 שורות מכסימום לכל שאלה (לא תובא בחשבון חריגה מגבולות המסגרת המודפסת).

שיתוף הפעולה שלך יסייע לתהליך ההערכה של תוצאות המחקר. תודה.

הערה: נא לציין הפניה לדו"ח אם נכללו בו נקודות נוספות לאלה שבסיכום.

1. מטרות המחקר לתקופת הדו"ח תוך התייחסות לתוכנית העבודה.
1. הכנת תוארית חדשה לריסוס נגיפי בקולו המבוססת על איבוק וכוללת פתיון אכילה. 2. הערכת יעילות התוארית הנ"ל כנגד זחלי הליותיס.
2. עיקרי הניסויים והתוצאות שהושגו בתקופה אליה מתייחס הדו"ח.
1. הכנת התוארית החדישה. 2. פיזור התוארית על צמחי כותנה. 3. הערכת מניעת נזק של זחלי הליותיס על הלקטי צמחי כותנה מרוססים בתוארית. 4. פיתוח מבחן ELISA לכימות חלקיקי הנגיף בתוארית.
3. המסקנות המדעיות וההשלכות לגבי יישום המחקר והמשכו.
התוארית החדישה שומרת על הפעילות האינסקטיצידית של נגיף הבקולו ללא פגיעה ביעילותו: הנגיף פעיל ב-100% שבוע לאחר חשיפתו. נגיף שרוסס בתוארית מימית מאבד באותה תקופה 80% מפעילותו. מבחן ה-ELISA מאפשר לבדוק את ההומוגניות של פיזור הנגיף בתוארית.
4. הבעיות שנותרו לפתרון ו/או השינויים שחלו במהלך העבודה (טכנולוגיים, שיווקיים ואחרים); התייחסות המשך המחקר לגביהן
יש צורך בלימוד פעילות התוארית בתנאי חשיפה קיצוניים יותר בריסוסי שדה. בנוסף כדאי לבדוק אם יעילות התוארית נובעת מיכולתה להגן על שלמות החלקיק הנגיפי בתוארית.
5. האם הוחל כבר בהפצת הידע שנוצר בתקופת הדו"ח - יש לפרט: פרסומים - כמקובל בביליוגרפיה, פטנטים - יש לציין מס' פטנט, הרצאות וימי עיון - יש לפרט מקום ותאריך.
בשלב זה הופץ המידע במסגרות מצומצמות של ימי עיון.