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Title of Project

"Biocontrol of Armyworms with the Entomopathogenic Fungi *Nomuraea rileyi* and *Beauveria bassiana*"

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Section I: Technical Progress

I. A) Research Objectives:

This second annual performance report includes the full research report which was to have been submitted in April, 2004 and elements of a fifth semi-annual managerial report.

Of the four full-program research objectives listed immediately below, progress reported herein relates mainly to objectives (a) and (d):

(a) To select strains of *N. rileyi* adapted to the environment in the Middle East and effective against local populations of *H. armigera* and *S. littoralis*.

(b) To devise a procedure for mass-propagation of *N. rileyi* in a cost-effective manner, and to formulate a preparation that is relatively stable to environmental factors.

(c) To perform pilot field-tests with *N. rileyi* and a commercial preparation of *B. bassiana*, in order to establish the efficacy of the preparations and formulations under local field conditions in the coastal plain, mountains and inner valleys in this region.

(d) Host-pathogen interactions: This was initially formulated to examine whether the presence of the fungus in early stages of germination and penetration, alters the composition of cuticular lipids, thereby affecting larval activity and feeding behavior of the larvae. The differential activation of larval phenoloxidase activity in response to hyphal penetration now appears to be a more significant interaction affecting resistance or susceptibility of *H. armigera* larvae to *N. rileyi*.

I. B) Research Accomplishments:

These relate to the items cited in the **time-chart** of the 1st and 2nd years (i.e., the effects of physical and nutritional factors on fungal development, and host-pathogen interactions).

Time Chart

Activity	1st Year	2nd Year	3rd Year
Fungal development: Effect of physical factors			
Temperature			
Temperature-humidity-light			
Fungal development: Effect of nutritional factors			
Mass production and persistence			
Host- pathogen interactions			
Influence on larval behavior			
Field collections & experiments			
Training			

Experimental and results

1. Isolation of the fungus from soil (in Hebron)
1.1 Selective media

Several experiments were conducted to modify the Sneh selective medium (dodine selective medium) which is originally used for the isolation of *B. bassiana* from soil. This was done to adapt the medium for the isolation of *Nomuraea rileyi* fungus from soil. In this respect, dodine was added to *N. rileyi* standard medium (SMAY) with some slight modification in preparation of the medium.

Results revealed that:

1. The two isolates of *Nomuraea rileyi* failed to grow on selective SMAY when dodine was added to the medium in the concentration of 100-300 mg/l. However spores started to germinate at 90 mg dodine /l media.
2. In the 2nd experiments, SMAY was supplemented with dodine at various concentrations (100, 90, 80, 70, 60, 50, 40, 30, 20, 0 mg /l media). Spores of both isolates germinated in media supplemented with 0-90 mg/l dodine and germination was completely inhibited at 100 mg/l dodine. Germination percentages were correlated with dodine concentration in the media but there was no significant differences between the concentrations 100, 90, and 80 mg/l.

3. To evaluate the germination of *Nomuraea rileyi* in the presence of other fungi on the same plate, two experiments were conducted, using natural field soil inoculated with certain concentrations of spores of the two isolates. The result showed that SMAY amended with 90 mg/l dodine can be used efficiently used in the isolation of *Nomuraea rileyi* from soil.

1.2 Soil sampling (in Hebron)

Soil samples were collected from various agricultural sites in North of the West Bank. Samplings were done using dilute plate techniques on modified selective SMAY medium (with 90 mg/l dodine). Preliminary examination of cultures show that we have two new isolates of *Nomuraea* spp. from two separate soil samples.

1.3. Sampling of dead larvae of *H. armigera* (in Hebron)

Dead larvae of *H. armigera* were collected from agricultural fields in the West Bank, but so far we have not been successful in identifying *N. rileyi*, the target entomopathogenic fungus. However several other, as yet unidentified fungi were isolated in Hebron from the interior of some of the dead insects, which may be of some potential value. Meanwhile, we are trying to inoculate larval instars of *H. armigera* with these isolated fungi and any that seem important enough will be identified and exposed to further testing and trials.

2a. Bioassay of pathogenicity (performed in Hebron)

The pathogenicity of the entompathogenic *N. rileyi* isolates (ARSEF 539 and ARSEF 1972) on the 4th stage larvae of *H. armigera* was evaluated by using the following treatments:

- Dipping the larvae in 10^9 spores/ml aqueous suspension for 30 seconds.
- Rearing the larvae on filter paper wetted with 500 µl of spore suspension.
- Rearing the larvae on 5 g of field soil wetted with 500 µl of spore suspension.
- Exposing the larvae to spores of 14 days old cultures of the fungus for 30 seconds (dry).
- Two control treatments.

The plates were incubated at 25°C and natural light. The experimental design was completely randomized with 12 replicates for each treatment. The mortality was evaluated after 11 days of incubation.

Table 1. The mortality (%) of the 4th stage larvae of *H. armigera* caused by the *N. rileyi* isolates (ARSEF 539 and ARSEF 1972) after 11 days incubation.

Treatment	ARSEF 539	ARSEF 1972
Dipping	60	40
Filter paper	80	100
Soil	40	20
Dry	83	60
Dipping (CK)	0	0
Filter paper (CK)	0	0

CK=control

2b. Bioassay of pathogenicity of single-spore cultures (performed in Rehovot)

The virulence of the two isolates 1972 and 539 were tested in Rehovot against 3rd and 4th larval instars of *H. armigera*.

Single-spore cultures from the two isolates ARSEF 1972 and ARSEF 539, and a single spore culture from the Israeli isolate (see first annual report), were prepared from the original cultures, after one passage through *H. armigera* larvae. The cultures were then sub-cultured for 3-4 months on the standard SMAY medium and stored at 4°C prior to use. Fungal pathogenicity was evaluated using 4th instar larvae of *H. armigera* and *Spodoptera littoralis*. Cultures were grown on SMAY (Sabouraud's Maltose Yeast extract Agar) for 2-3 weeks at 20-23°C in the dark and conidia were scraped from the surface, dispersed in soybean oil and filtered through gauze to remove hyphae. Serial dilutions of conidia were prepared and conidial viability determined.

Conidia were also prepared from laboratory-infected larvae. *H. armigera* and *S. littoralis* 4th instar larvae were topically treated with either soybean oil (control) or with conidial suspensions in soybean oil, then transferred individually to 50 mm diameter Petri dishes and fed with fresh cabbage leaves. The ambient temperature during these experiments was in the range of 24±2°C. Dead larvae were transferred to clean Petri

dishes with moist filter paper, kept in the dark at about 25°C and examined daily for fungal growth for 3-4 weeks after inoculation. Larvae with no visible sporulation were dissected to examine the presence of hyphae within the host.

Isolate ARSEF 1972 was the most effective of the three isolates and larvae of *S. littoralis* were affected more than were larvae of *H. armigera* by several criteria:
A concentration of 10^4 conidia of the isolate ARSEF 1972 was sufficient to obtain maximal infection of *S. littoralis* larvae (69%); Mycosed larvae were first observed at all conidial concentrations about 6 days after infection. Infected larvae died within the next 24 h when treated with a concentration of 10^5, within 48 h when treated with a concentration of 10^4 and within 9 days when treated with a concentration of 10^3 conidia.
ARSEF 1972 was less effective against *H. armigera* larvae: Conidial concentration to obtain maximal infection (36%) was about 10^4; Death of mycosed larvae was first observed at this conidial concentration 8 days after infection and occurred between 8-15 days. This could be expected, as this, and the other isolates tested, originated from field-infected *S. littoralis*

Isolate ARSEF 539 has been tested with *H. armigera* larvae:
Conidial concentration to obtain maximal infection (25%) was about 10^4 Mortality of *H. armigera* larvae was less than that obtained with ARSEF 1972; Death of mycosed larvae was first observed at conidial concentrations of 10^3-10^5 at 5 days after infection and occurred in the range of 5-16 days.
The Israeli isolate was also tested with *H. armigera* larvae at one concentration of $0.8*10^4$ conidia:
Maximal infection was about 33%, a percentage of mortality comparable to ARSEF 1972; Death of mycosed larvae was first observed by 7 days after infection and occurred in the range of 7-17 days.

If these preliminary results with single spore culture are corroborated in our ongoing experiments, this means that compared to the parent cultures, infectivity is reduced. If so, mass propagation and field tests, to be done later on, should be performed with preparations from heterogenous (multi-spore) populations.

3. Effect of *N. rileyi* culture age on the mortality of the 4th stage larvae of *H. armigera*

Virulence of old cultures of the isolates (ARSEF 539 and ARSEF 1972), and newly isolated ones from mycosed artificially inoculated larvae (ARSEF 539A and

ARSEF 1972A) was evaluated **in Hebron** by using conidial suspension at 10^8 conidia/ml of 0.05% Tween 20. Larvae were reared on filter paper wetted with 500 µl of the mentioned spore suspension, or dipped into the suspension directly. The experiment was completely randomized with 10 replicates in each treatment. Larvae mortality rate was evaluated after 6, 11, and 15 days of incubation. Data of the 15 days mortality are presented below (Table 2).

In a different experimental protocol, virulence of old cultures of the isolates (ARSEF 539 and ARSEF 1972), and newly isolated ones from mycosed artificially inoculated larvae (ARSEF 539A and ARSEF 1972A) was evaluated by using conidial suspension at 4.3* 10^7 conidia/ml of 0.05% Tween 80. Larvae were dipped in the spore suspension directly or fed dipped food (tomato leaves) for 72 hr before transferred again to regular artificial feed. The experiment was completely randomized with 10 replicates in each treatment. The larvae mortality rate was evaluated after 14 days of incubation (Table 3).

Table 2. Effect of *N. rileyi* isolates culture age on the mortality of 4th stage larvae of *H. armigera* after 15-days of incubation at 25° C.

Treatment	ARSEF 539	ARSEF 1972	ARSEF 539A	ARSEF 1972A
Dipping larvae	58	50	50	25
Filter paper	67	58	33	33
Control	0	0	0	0

Table 3. Effect of *N. rileyi* isolates culture age on the mortality of 4th stage larvae of *H. armigera* after 14-days of incubation at 25° C.

Inoculation	ARSEF 539	ARSEF 539A	ARSEF 1972	ARSEF 1972A	CK
Dipping larvae	50	50	50	33	0
Dipping Tomato Leaves	100	100	100	100	16

CK = control

These results apparently indicate that infectivity of *N. rileyi* isolates is appreciably maintained on standard SMAY medium and stored under refrigeration, and does not require serial passages through *H. armigera* larvae. This is an encouraging result, within limits, for future mass propagation and formulation of fungal conidia of *N. rileyi*: In experiments performed in Rehovot and Hebron we found that a culture kept for 9 weeks in the standard culture room had essentially lost most of its virulence. The fungus loses its viability and not only virulence and its growth cannot be maintained adequately on SMAY. In Hebron, cultures obtained from inoculated dead larvae were subcultured two times on SMAY (total time of 24 days) and then stored at 5 °C for 45 days and subcultured again on SMAY after refrigeration for 14days, after which they were then was used for inoculating larvae. This issue would benefit from further investigation, by examining several more storage periods.

4. Effect of *N. rileyi* introduced in sunflower oil on the mortality of the 3rd stage larvae of *H. armigera*.

The isolates ARSEF 539 & ARSEF 1972 were prepared **in Hebron** at a concentration of 10^8 conidia/ml in **sunflower oil**. Tomato leaves were then dipped for 5 sec. in the conidial suspension. Third stage larvae were used and the experiment was completely randomized with 10 replicates (larvae) in each treatment. Results revealed that all larvae died after 24 h **including the control**. Death occurred after feeding on treated leaves, and the assumed cause of death was oil either by direct intake or by surface contact with larvae.

In experiments performed **in Rehovot**, with conidia dispersed in **soybean oil**, and topically applied (10 microliters per larva) no mortality was observed in controls. These are not comparable experiments: the oils differ in origin; ingestion delivers the oil to a different location from topical application; the sunflower oil may have oxidized. The results obtained with the two oils will be repeated under the exact same conditions, to resolve this issue. Perhaps a formulation need not be composed of oil only.

6. The effect of spore concentrations of both isolates (ARSEF 539 and ARSEF 1972) on the mortality rate of the 4th stage larvae of *H. armigera* (in Hebron).

The effect of spore concentrations of the isolates ARSEF 539 and ARSEF 1972 on mortality were tested by using conidial suspension at the concentrations of 0 (ck??), 10^5, 10^6, 10^7 and 10^8 conidia/ml of 0.02% tween 80. The conidia were harvested from 17 days old culture and the suspensions were stored at 5°C for 3 days. Tomato leaves were dipped in the prepared suspensions of conidia, and were placed on tissue paper for 10 minute. The 2nd stage larvae were fed on the treated tomato leaves for 72 h and then transferred to artificial diet and incubated at 25 °C. The experiment design was completely randomized with 10 replicates in each treatment. The larvae mortality rate was evaluated after 6-14 days of incubation. The LD50 and LT50 were also calculated as well.

Table 4. The effect of spore concentrations of both isolates ARSEF 539 and ARSEF 1972 on the mortality of the 4th stage larvae of *H. armigera*.

	ARSEF 1972								ARSEF 539							
Conc.	**6D**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**6D**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
10^5	0	0	0	0	0	0	0	0	0	25	37	37	37	37	50	75
10^6	0	0	0	25	25	37	50	50	25	25	25	37	37	37	50	63
10^7	0	13	25	75	75	75	88	100	38	75	88	88	88	100	100	100
10^8	0	50	63	63	63	88	100	100	38	38	75	100	100	100	100	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

7. Effect of *N. rileyi* isolates spray application to tomato plants on the mortality of the 4th stage larvae of *H. armigera* (in Hebron)

Forty-day old tomato plants (in pots) were spraying with 60 ml of conidial suspension (10^8 conidia/ ml 0.02% tween 80) of the isolates ARSEF 539 & ARSEF 1972. The inoculated plants were covered with plastic mesh after introducing four 4th stage larvae for each pots. The percent mortality of larvae was evaluated 10 day afterwards

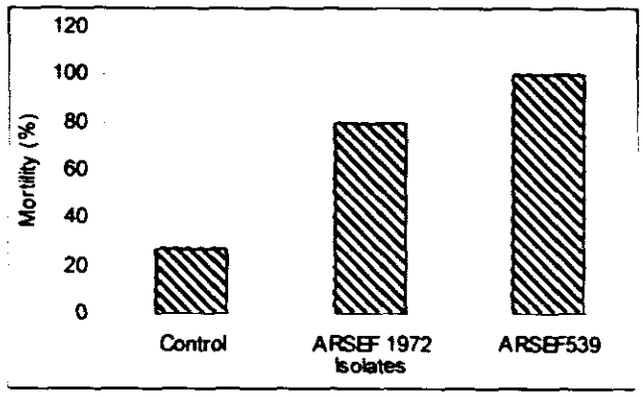

Fig.1 Effect of *N. rileyi* isolates spray application to tomato plants on the mortality of the 4th stage larvae of *H. armigera*.

7. Host-pathogen interactions (in Rehovot)

Scientific background

The invasion of a pathogen into a host insect leads to its recognition as "non-self" by a front-line defense system involving proteins that bind to bacterial peptidoglycan or lipopolysaccharide, or to fungal β-1,3-glucan. Following this foreign recognition event, an immune response takes place, consisting of cellular and humoral defenses against the invader.

Entomopathogenic fungi, unlike bacteria or viruses, have the ability to directly penetrate the cuticle to establish infection, and thus may induce a different set of defense responses to that induced by bacterial or viral infections.

Cellular defenses against invading microorganisms consist of phagocytosis and encapsulation of the foreign objects.

Humoral defense reactions include a challenge-induced synthesis by the fat body of antimicrobial, fungicidic or fungistatic peptides that are secreted into the haemolymph. The humoral reactions also involve several proteolytic cascades, of which the melanization cascade is among the more important responses. This cascade, taking place locally, leads to the production of melanin, which is deposited around invading microorganisms and wounded tissue. A final role in the process of melanization is reserved to the enzyme phenoloxidase. Phenoloxidase is synthesized as an inactive penultimate proenzyme termed prophenoloxidase (PPO). The latter is activated in response to foreign challenge by a serine protease cascade. This is a rapid

reaction that takes place in the haemolymph. The cleavage of PPO into phenoloxidase is performed by specific proteases, termed PO-Activating Enzyme (PAP) or ProPhenoloxidase Activating Factor (PPAF).

The activated phenoloxidase, in turn, catalyzes the hydroxylation of tyrosine and other phenols into reactive quinone compounds that polymerize into melanin. Additionally, cytotoxic molecules produced in this process, including quinones and reactive oxygen intermediates, may kill the invading microorganisms that are trapped by melanin.

The exact pathogenic mechanism of *N. rileyi* and its putative effect on melanization in the *H. armigera* larvae are unresolved. Based on previous findings of the mode of action of entomopathogenic fungi, our **working hypothesis** was that infection would increase hemolymph phenoloxidase activity.

The issue is compounded by the fact that phenoloxidase plays an important role not only in the insect immune responses, but is also crucial in the developmental process of molting in insects, where it regulates cuticular melanization and sclerotization. These processes occur at several developmental stages: the final stages of the formation of a new cuticle, during ecdysis (the shedding of the old cuticle), pupation and adult emergence are all developmental milestones.

As the relation between the insect's age and its immune activity is unclear, it is also not certain whether ecdysis, especially at pupation, is necessarily a means of evasion from entomopathogenic fungi.

We assume that changes occur in the haemolymph titers of the enzyme after challenge with a pathogen, including entomopathogenic fungi, due to developmental events.

It is known that the steroid hormone ecdysone, which regulates a variety of developmental processes in insects, is involved in regulation of PPO expression. This emphasizes the link between age, the molting process and phenoloxidase levels.
These considerations could have an effect on mortality elicited by fungal infection.

The allocation of haemolymph phenoloxidase between its roles in immune responses and its role in the molting process, if such exists, is yet to be studied. It is also as yet unknown whether the age of larvae – the instar as well as the developmental state within the instar, if indeed affecting the enzyme's level, would have an effect on the phenoloxidase immune activity, and if so, on the susceptibility of larvae of different ages - to fungi.

A thorough understanding of fungal-induced immune responses and their relation with insect development is essential for identifying determinants of fungal virulence that could be manipulated to accelerate host death in a biological pest control system.

Experimental, results and interim conclusions

It is reasonable to assume that physical barriers during the molting process in insects, or conversely, their absence, may affect the invasion of fungi into the insect and the establishment of infection, irrespective of the demands of the immune system. Additionally, apolysis (the detachment of the old cuticle from the underlying epidermis), followed later on by the shedding of the old cuticle, may present further obstacles to hyphal penetration through the cuticle into the haemocoel, and could facilitate the insect in evading the pathogen.

Based on this scenario, one **working hypothesis** of this study is that the *H. armigera* larvae would be most susceptible to *N. rileyi* infection at the beginning of each instar (e.g., the short period after ecdysis). We would also expect to see a decrease in susceptibility to fungi after apolysis, with barriers of molting fluid – and its complement of hydrolytic enzymes.

In experiments performed during this reporting period, the highest mortality rates in *H. armigera* larvae inoculated with *N. rileyi* were indeed achieved when inoculation was performed on the 1st day of the 4th instar, within a few hours post-ecdysis.

We have calculated the LT_{50} ratio (Figure 2) and find that 50% mortality was achieved only when larvae were inoculated on the first and second days of the 4th instar.

After inoculation on the first day after ecdysis, LT_{50} occurred at about 10 days, while if performed on the second day after ecdysis, LT_{50} was at about 14 days.. On the third day after ecdysis, and later on the first day of the 5th instar, with just a few hours separating between them, LT_{50} rates were after about 11 days. In contrast, for late 3rd instar larvae, inoculated a matter of hours prior to ecdysis to 4th instar, LT_{50} was about 19 days.

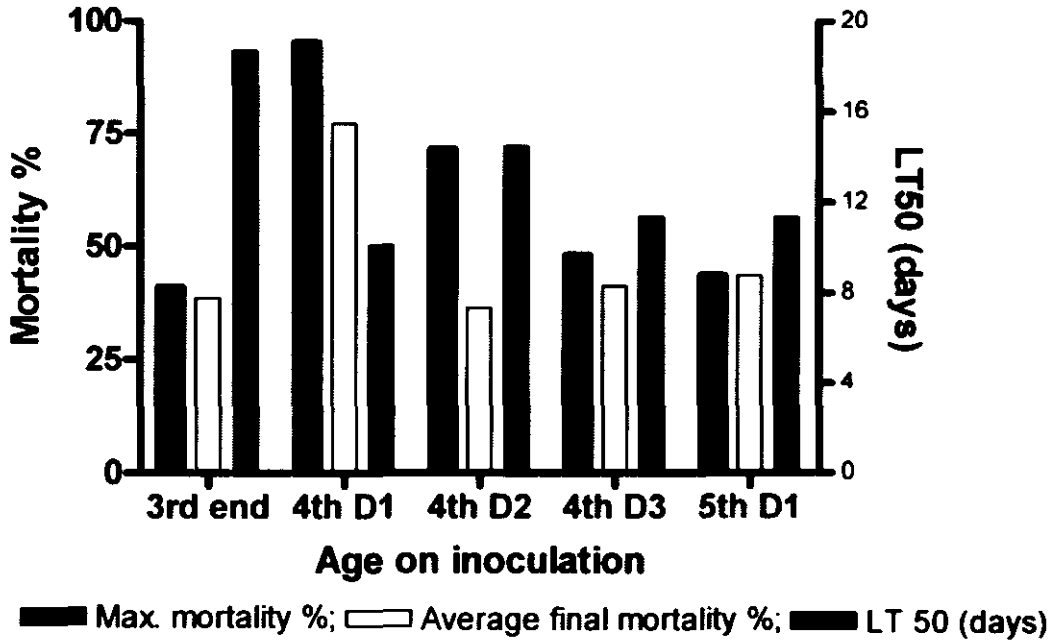

Figure 2: Percentage mortality and LT 50 of larvae inoculated at different ages

Another interesting observarion was that some of the larvae inoculated and infected during the 4th instar, ecdyse and mostly die as 5th instar larvae, which suggests that ecdysis does not necessarily evade fungal infection. Furthermore, some of these larvae manage to pupate but then died as pupae. We have conclusively determined that in these cases, mortality is attributable to the original fungal inoculation.

The effect of larval age on its susceptibility to *N. rileyi* should be examined further, at different stages within the instar, and at shorter time intervals.

Establishing the connection between molting and phenoloxidase levels in the haemolymph is done by testing **haemolymph phenoloxidase activity levels in non-inoculated larvae** at different developmental times. Our **working hypothesis** in this case is that the enzyme's levels will vary in accordance with timing of melanization and sclerotization during molting, peaking during the first hours post ecdysis.

Results so far are in accord with this: A large increase was seen in phenoloxidase activity a few hours post-ecdysis into the 5th instar, when sclerotization of the new cuticle takes place. A lower peak was seen in the middle of 4th instar, and then a rise, probably following apolysis with formation of the new cuticle, followed

by a drop in phenoloxidase activity a few hours before ecdysis – possibly tp prevent sclerotization and allow flexibility of the newly exposed cuticle during the completion of the molting process.

Comparable **haemolymph phenoloxidase activity levels in inoculated larvae** at different developmental times was examined in larvae inoculated shortly after ecdysis into the 4th instar (Figure 3). Thus "24 hours post inoculation" is roughly comparable to 24 hours of age within the 4th instar. Ecdysis in these experiments usually took place within the time interval of 48-60 hours post inoculation, roughly in the middle of that interval, as previously mentioned. Apolysis is expected to take place about in the middle of the instar, which, in the case of the 4th instar, is between 24 and 36 hours into the instar (in inoculated larvae, post inoculation). Thus, "60 hours post inoculation" is comparable to about 6 hours into the 5th instar.

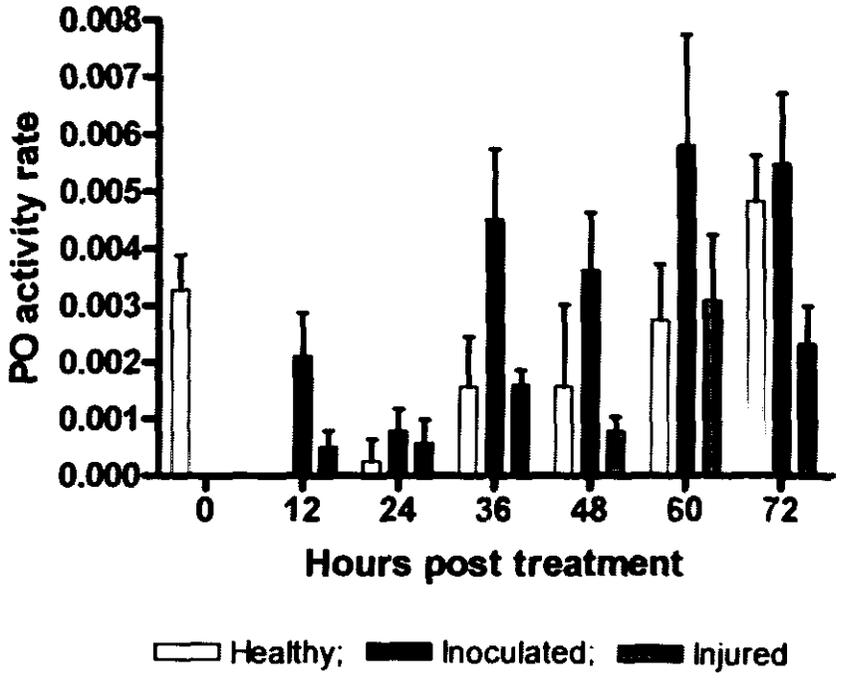

Figure 3: Phenoloxidase-DOPA reaction rates for haemolymph of "healthy"' inoculated and injured larvae.

The allocation of phenoloxidase for cuticle melanization, immune response and wound healing is unresolved at this point. It can be seen that at the same period of time when larvae are **most susceptible** to fungi – within hours after ecdysis, a **high**

peak of phenoloxidase activity was observed, indicating that an increase in the enzyme's activity at this time does not protect the larva from infection by *N. rileyi*.

We posit that during this vulnerable state of a soft, new cuticle, a significantly larger number of fungal conidia do manage to penetrate the cuticle and invade the insect, but that phenoloxidase levels are primarily allocated to cuticular sclerotization, and may be less available for immune responses. As the immune system presumably consists of more than one mode of defense against invading pathogens, neglecting sclerotization in favor of combating invading fungi might at this crucial stage pose an even greater danger to the insect.

Results show a pattern of hemolymph phenoloxidase activity level highly similar in both inoculated and non-inoculated larvae. However, the absolute level in inoculated larvae was higher than in non-inoculated healthy larvae, suggesting that indeed phenoloxidase is recruited in response to fungal challenge.

In the experimental protocol used, it is not certain that each inoculated larva, used for the phenoloxidase assays, was actually infected with the fungus. However, in all times following inoculation, we can assume that the inoculated larvae are involved in combating the fungus, either to fail and become infected or to prevail and remain healthy. Importantly, when larvae were infected within hours into the 4th instar, this was found to be the most sensitive time, reaching, in one case, a mortality rate as high as 95%.

Another factor examined was a presumptive wound effect (Figure 3). Fungal infection begins with penetration of the insect's cuticle by the appressorium, which secretes a blend of substances facilitating the penetration. This contains an element of mechanical injury, but we assumed that mechanical injury.alone is not expected to mimic all aspects of hyphal penetration . We mechanically injured larvae using fine scissors to pierce the cuticle. Hemolymph was then sampled in time intervals of 12 hours after injury, and phenoloxidase activity levels were measured.

Results of these experiments (shown in Figure 3) indicate that hemolymph phenoloxidase activity is suppressed in a 72 hours period post injury, relative to both healthy and inoculated larvae of similar age. This stands in contrast to our **working hypothesis** in this case, that injury should trigger an immune response (in order to combat pathogens which might invade through the wound) as well as melanization (to block the wound), both procedures involving phenoloxidase activity. Perhaps phenoloxidase activity in these mechanically wounded larvae is recruited and

relocated to the specific wound site, thus depleting the apparent whole hemolymph pool.

During the coming year we intend to examine the role of serine proteases presumably involved in the release of active phenoloxidase from its precursor PPO and possible serpins (serine protease inhibitor) involvement in the regulation of phenoloxidase release for PPO, as affected by fungal challenge.

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I. C) Scientific Impact of Cooperation:

The interaction of the two research groups from Rehovot and Hebron continues to be restricted to direct meetings in Rehovot and Jerusalem between the two cooperating investigators, and to telephone and e-mail communication, due to circumstances beyond our control.

Dr. Aviva Uziel was employed in Rehovot on a temporary basis during part of this reporting period. She periodically contacted the Hebron laboratory by e-mail, in order to exchange details of experiments and results.

The technicians in Hebron and a Palestinian M.Sc. student, **Mr. Izaldien Qtait**, who is now registered at Hebron University, have participated in this project during this reporting period.

An Israeli M.Sc. student, **Ms. Dana Yehieli**, is studying the physiology of the host-pathogen interaction in respect to phenoloxidase activity. The research topics of Ms. Yehieli and Mr. Qtait are intended to complement each other.

I. D) Description of Project Impact:

Impact can be evaluated only after this project is field-tested and students complete their training in these aspects of insect pathology.

I. E) Strengthening of Middle Eastern Institutions:

As noted in our previous progress report, full mobility of students and technical staff between the two locations is necessary for optimal training to take place. This cannot be realized presently, but we intend to request this when feasible.

Section II: Project Management and Cooperation

II. A) Managerial Issues:

No major issues at the moment.

II. C) Cooperation, Travel, Training and Publications:

Cooperation has been detailed above.

We have agreed to publish a joint paper on part of the research performed hitherto but critical data is still absent.

II. D) Request for USAID Actions:

As mentioned previously, the lack of direct contact is hindering the personal interaction, coordination and ultimately, the optimal progress of this project. It is clear that in order to succeed more fully, we shall need an extension of the tenure of this grant, for at least one year (**no** additional budget allocation). We presently request such **extension for one year** (total of four years). We still hope that (earlier than later) issues of security will be resolved and that restrictions on mobility in both directions eased, so that we are able to realize fully our initial plans and intentions. In the event that this is not so during the year of extension, we may have to request an additional fifth year in order to perform some of the field experiments jointly.