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BARD

FINAL REPORT

PROJECT NO. US-2858-97

**A Molecular Approach for Improving Disiccation
Tolerance of Insecticidal Nematodes**

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BARD Final Scientific Report**Date of Submission of the report: November, 27, 2002****BARD Project Number:** US-2858-97R ✓**Project Title: A Molecular Approach for Improving Desiccation Tolerance of Insecticidal Nematodes****Investigators****Institutions****Principal Investigator (PI):**

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נתקבל

Abbreviations commonly used in the report, in alphabetical order:**Budget:** IS: \$ 127,800

US: \$ 192,200

Total: \$ 320,000

Signature

Principal Investigator

Signature

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)			4	
Submitted, in review, in preparation	2		1	
Invited review papers				
Book chapters		1	1	
Books				
Master theses			2	
Ph.D. theses			2	
Abstracts		1		
Not refereed (proceedings, reports, etc.)		1	3	

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		
Longer Visits (Sabbaticals)				

Description of Cooperation:

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	-		-	
Issued (allowed)	-		-	
Licensed	-		-	

Abstract

Our overall goal is to improve insecticidal nematodes by genetically engineering strains capable of entering an enhanced state of dormancy that provides improved stability.

Objectives:

1. **Clone and sequence tps-1 homologue from *Steinernema carpocapsae*.**
(Revised: A failure to isolate the *tps* gene group from *Steinernema* precipitated a redirection to identifying other genes involved in Insecticidal nematode desiccation process.)
2. **Incorporate cloned tps-1 gene into *S. carpocapsae* to obtain overexpression, thereby, enhancing desiccation tolerance.** (Revised: Other stress genes in addition to tps-1 genes were cloned and efforts at expression in *S. carpocapsae* were conducted)
3. **Characterize the transgenic strains.**

No other biological control agent offers more impressive attributes than insecticidal nematodes. However, their potential is limited by the bane of nearly all biological control agents: poor stability. This leads to inadequate shelf-life and ultimately reduced field efficacy. Nematode storage is based on desiccation, yet insecticidal species are only capable of partial desiccation termed quiescent anhydrobiosis. Overwhelming evidence has shown that when the disaccharide compound trehalose is elevated in anhydrobiotic organisms such as yeast, plants, and nematodes it enables these organisms the ability to survive environmental stresses i.e., desiccation. Armed with this information our goal was to improve insecticidal nematodes stability by engineering trehalose overexpression.

Major conclusions, solutions, achievements:

1. Solomon *et al.* (2000b) identified in *S. feltiae* IS-6 a heat-stable, water stress-related protein with a molecular mass of 47 kDa (designated *desc47*). The LEA proteins are a diverse group of water stress related proteins which are expressed in maturing seeds.
It was the first time that such plant protein was recorded in animals.
2. Zitmann-Gal *et al.*, (2000) identified a novel gene, glycogen synthase (*Sf-gsy-1*), in *S. feltiae* IS-6 that exhibit changes in transcript levels upon dehydration. This is the first report of a gene related to the desiccation process in entomopathogenic nematodes
3. Over 90 genes appear to be differentially expressed in *S. feltiae* IS-6 during exposure to 97% RH when compared to nematodes obtained from water. Of the genes analyzed so far all of them appear to be upregulated during *S. feltiae* IS-6 dehydration, whereas transcription levels of their homologs were only slightly increased during *C. elegans* dehydration. These results demonstrate fundamental differences in the two nematodes responses to desiccation stress and unveiled some of the components of the genetic networks that are activated during dehydration.
4. Four stress genes from four different biological sources have been successfully isolated and cloned into *gfp* expression vectors.
5. We were unsuccessful in transforming (stable) *Steinernema carpocapsae* with stress genes. Therefore, a major finding from this study is the need for an alternative gene delivery system for *Steinernema*. We are in the process of collaborating with Dr. Fodor on a project to develop such a nematode transformation system.

In conclusion even though we were unable to complete objectives 2 and 3, we did isolate and demonstrate novel genes, for the first time, involved in nematode desiccation process. Also, we have shown that *C. elegans* can be employed as model for studying stress gene expression at the molecular level. As for applied aspects until a reliable gene delivery system is developed for *Steinernema* species their ability to be genetically improved through recombinant DNA technology will be impeded.

Achievements (three pages maximum, 1.5 spaced, font not smaller than 12), include:
Significance of main scientific achievements or innovations.

Our overall goal is to improve insecticidal nematodes by genetically engineering strains capable of entering an enhanced state of dormancy that provides improved stability. The following achievements were reached:

Activity at Rutgers University- US

Four stress genes from four different biological sources have been successfully isolated and cloned into *gfp* expression vectors. Stress genes include: glycogen synthase (*Sf gsy-1*) from *Steinernema feltiae* IS-6 (Zitman-Gal *et. al.*, 2000), trehalose phosphate-6-synthase (*Ce tsp-1*) from *Caenorhabditis elegans* (Brey *et. al.*, unpublished data), trehalose phosphate-6-synthase (*Sc tsp-1*) from *Saccharomyces cerevisiae* and hemolymph desiccation stress protein gene (*Tm dsp28*) from *Tenebrio molitor* (provided by V.K. Walker). Attempts to transform these genes in *S. carpocapsae* was not successful.

The USA team was unsuccessful in transforming (stable) *Steinernema carpocapsae* with stress genes (objective 2). András Fodor's laboratory, the only group reputed to have transformed *S. feltiae* with *tsp-1* yeast gene, has also failed in obtaining a stable *Steinernema* line (Fodor, personal communication). Therefore a major finding from this study is the need for an alternative gene delivery system for *Steinernema*. Dr. Fodor visited our lab recently to plan a joint project to develop such a transformation system (see appendix).

Within the last 6 months of the project a shift from *S. carpocapsae* to *C. elegans* was initiated to determine the effect of stress genes on *C. elegans* desiccation tolerance. Results have shown that *C. elegans* dauer larvae overexpressing the transgene *Ce-tsp-1* are similar to nontransgenic *S. carpocapsae* infective juveniles but 2X more desiccation tolerant than nontransgenic *C. elegans* dauer larvae at 85% RH.

Currently, we are in the process of microinjecting stress gene constructs [genes: ALDH (only recently received from Israel team), *gsy-1*, *tsp-1* and *dsp-28*] into *C. elegans* CB1372 (mutant strain that produces 100% dauers at 25°C). So far only *C. elegans* wild-type transformed with the *Ce tsp-1* gene has been assayed for desiccation tolerance. We plan on testing each stress gene construct within in the next year for its effect on *C. elegans* dauer survival under different RH regimes. Also, we will characterize gene expression by performing RNA interference on each of the *C. elegans* transgenic strains. We will also use

RNA interference technique to determine the role stress genes play in *S. carpocapsae* when they are silenced. (see appendix for future papers from this project)

Activity at ARO-Israel

- a. Solomon *et al.* (2000b) identified in *S. feltiae* IS-6 a heat-stable, water stress-related protein with a molecular mass of 47 kDa (designated *desc47*). This protein was accumulated 10-fold in infective juveniles (following exposure to 97% RH for 72 h). The *desc47* protein was found to be homologous to the *C. elegans* late embryogenesis abundant (LEA) homolog protein (g2353333) and to the LEA group 3-like protein from the fern *Onclea sensibilis* (g322390). The LEA proteins are a diverse group of water stress related proteins which are expressed in maturing seeds. It was the first time that such plant protein was recorded in animals.

(Items a-d were conducted as part of PhD work of Dr. Aaron Solomon).

- b. Following a failure to isolate the *tps* gene group from the nematode, as originally attempted (See appendix in ARO and Rutgers reports) Zitmann-Gal *et al.*, (2000) identified novel genes of *S. feltiae* IS-6 that exhibit changes in transcript levels upon dehydration. These included glycogen synthase (*Sf-gsy-1*). In this study, Zitmann-Gal *et al.* (2000) established changes in the steady-state level of *Sf-gsy-1* transcripts upon dehydration. We were the first to identify genes related to the desiccation process in entomopathogenic nematodes
- c. We continued to characterized the genes that are presumably involved in desiccation survival. We used "PCR-Selected cDNA subtraction kit" to compare between two cDNA libraries. The cDNA libraries were created from either infective juveniles of *S. feltiae* IS-6 obtained from water suspension or from infective juveniles which were exposed to 97% RH in a desiccator for 8, 24 or 48 h. This study yielded the identification of over 90 genes. We identified Expressed Sequence Tags (ESTs) that are differentially expressed during exposure to desiccation stress in infective juveniles of the semi-arid nematode *S. feltiae* IS6. All analyzed ESTs were upregulated during *S. feltiae* IS6 dehydration, whereas transcription levels of their homologs were only slightly increased during *C. elegans* dehydration. Our results demonstrated fundamental differences in the two nematodes responses to desiccation stress and unveiled some of the components of the genetic networks that are activated during dehydration. A manuscript describing this work was submitted to J. of Parasitology (see in Appendix)

(Items e-f were conducted as part of Master and PhD study of Mrs. Tali Zitmann-Gal).

In addition the ARO team had conducted a background study on the physiological and behavioral aspects of desiccation tolerance of Entomopathogenic nematodes. This work was highly relevant and supportive of the overall objective of this project:

- a. Solomon *et al.* (2000a) studied the desiccation tolerance of the IS-6 strain of *S. feltiae*, isolated from the desert region of Israel. The desiccation tolerance of the IS-6 this strain strains was associated with a dispersal response of aggregated infective juveniles at the slow dehydration regime, which was followed by entrance into a state of anhydrobiosis as individually coiled infective juveniles. Survival of individual Infective juveniles at 85% RH was positively correlated to the clump size which ranged from 70 to 7700 infective juveniles per clump (Solomon *et al.*, 2000a).
- b. Increased Trehalose levels were determined in infective juveniles of the IS-6 strain after 3 days exposure to 97% RH (from 0.3 to 0.6 g Trehalose per g protein), while an opposite trend was observed in glycogen (from 0.09 to 0.02 g glycogen per g protein) (Solomon *et al.*, 2000a). Upon rehydration, glycogen returned to its original concentration after 24 h, while Trehalose reached only 50% from the original.
- c. Nematode virulence and invasion capability were not hampered by the dehydration and rehydration processes.
- d. Liu and Glazer (2000) determined the desiccation tolerance of heterorhabditid populations isolated from different climatic regions in Israel. Substantial differences in survival ability were observed among infective juveniles of 12 new heterorhabditid populations. We attempted to elucidate the causes for the variation among heterorhabditid strains in regard to desiccation tolerance by determination of water loss during desiccation and changes in Trehalose and glycogen levels. (this part of the study was *conducted as part of PhD work of Dr. Liu Qi-Zhi*).

Activity at Tel-Aviv University- Israel

The attempts to isolate heat-shock genes from *H. bacteriophora* (strain HP88) have yielded two such genes; HSP70C and HSP70A. Both were characterized. See annual reports in the appendix. Using the "PCR-Selected cDNA subtraction kit" approach an heat-shock gene was identified during the desiccation process of *S. feltiae* IS-6. The gene was cloned and sequenced its expression was characterized (*manuscript in preparation- this part of the study was conducted as part of Masters thesis of Mrs. Maria Goldsmith*)

Appendix (technical information supporting the research findings): Provide a table of contents and include the following:

Published papers.

ARO-Israel

- Liu Q-Z, and Glazer, I. 2000. Desiccation survival of the entomopathogenic nematodes, of the genus *Heterorhabditis*. *Pytoparasitica* 28: 331-340.
- Solomon, A. Solomon, R., Paperna, I. and Glazer, I. 2000. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable-protein. *Parasitology*. 121:409-416.
- Zitman-Gal, T., Solomon, A., Glazer I. and H. Koltai 2001. Alternations in the levels of glycogen and glycogen synthase transcripts in the insect-killing, nematode *Steinernema feltiae* IS-6 J. *Parasitology*. 87: 725-732
- Liu Q-Z, Piggott S. J, Solomon, A. and Glazer, I. 2002. Physiological and Biochemical Changes in Nematodes from the Genus *Heterorhabditis* Following Desiccation. *Phytoparasitica* 30: 253-261.
- Zitman-Gal, T., Glazer I. and H. Koltai 2002. Differential gene expression during desiccation stress in *Steinernema feltiae* IS6 and *Caenorhabditis elegans* nematodes. J. Exp. Parasitol. (Submitted- see appendix)

Book Chapters

ARO-Israel

- Solomon A., Paperna, I and Glazer, I. 2000. Desiccation survival of the entomopathogenic nematode *Steinernema feltiae* : Induction of anhydrobiosis. In 'Survival Strategies of Entomopathogenic Nematodes' Glazer, I., Richardson, P., Boemare, N. and Coudert, F. [Eds]. EUR EN Report
- Glazer, I. 2001. Survival biology. In 'Entomopathogenic nematodes' Gaugler R. [Ed]. CABI Publishing, UK pp. 169-187.

Rutgers University- USA:

- Brey, C.W. and Hashmi S. (2003). Genetic improvement of entomopathogenic nematodes for insect biocontrol. In: Upadhyay R. (ed.) *Advances in Microbial Control of Insect Pests*. Kluwer Academic / Plenum Publishers (in press).

Abstracts and Proceedings

- Liu Q-Z, Solomon A. and Glazer. I. 2001. Desiccation Survival of *Heterorhabditis*: Physiological and Biochemical Changes Following Dehydration. IOBC Meeting, Athens, Greece.
- Zitman-Gal, T., Solomon, A., Glazer, I. and Koltai, H. 2001. Isolation and Characterization of Genes Involved in Desiccation Tolerance of *Steinernema feltiae* IS6. Soc. of Invertebrate Pathol., 34th Meeting, Noorwijkerhout, Holland.
- Zitman-Gal, T., Glazer, I. and Koltai, H. 2002. Isolation and characterization of genes induced by desiccation stress in the insect- killing nematode *Steinernema feltiae*. *Fourth Intr. Congress of Nematology, Tenerife, Spain*.

Rutgers-USA

- Brey, C.W. and Gaugler R. Enhancement of *Steinernema carpocapsae* desiccation tolerance by genetic engineering. International *C. elegans* meeting. UCLA, CA, 22-26 June 2001.

Brey, C.W. and Gaugler R. Enhancement of *Steinernema carpocapsae* desiccation tolerance by genetic engineering. International *C. elegans* meeting. UCLA, CA, 22-26 June 2001.

Brey, C.W. Gaugler R. and Glazer I. *Caenorhabditis elegans*: A model for genetic characterization of stress genes in entomopathogenic nematodes. National Entomological society of America Meeting. Fort Lauderdale, FL, 17-20 November 2002.

Papers in preparation (2):

Brey, C.W., Gaugler, R., Hashmi, S. and Glazer, I. Suppression of glycogen synthase gene expression in *Steinernema carpocapsae* and *Caenorhabditis elegans* by RNA interference. (For *Journal of Molecular and Biochemical Parasitology*)

Brey, C.W., Gaugler, R., Zitman-Gal, T. and Glazer, I. Molecular characterization of nematode stress genes in *Caenorhabditis elegans* (For *Journal of Biological Chemistry*)

Brey, C.W. Gaugler R. and Glazer I. *Caenorhabditis elegans*: A model for genetic characterization of stress genes in entomopathogenic nematodes. National Entomological society of America Meeting. Fort Lauderdale, FL, 17-20 November 2002.

Papers in preparation (Rutgers-USA)

Brey, C.W., Gaugler, R., Hashmi, S. and Glazer, I. Suppression of glycogen synthase gene expression in *Steinernema carpocapsae* and *Caenorhabditis elegans* by RNA interference. (For *Journal of Molecular and Biochemical Parasitology*)

Brey, C.W., Gaugler, R., Zitman-Gal, T. and Glazer, I. Molecular characterization of nematode stress genes in *Caenorhabditis elegans* (For *Journal of Biological Chemistry*)

Unpublished data, briefly summarized.

Rutgers University- Four stress genes from four different biological sources have been successfully isolated and cloned into *gfp* expression vectors. Stress genes include: glycogen synthase (*Sf gsy-1*) from *Steinernema feltiae* IS-6 (Zitman-Gal *et al.*, 2000), trehalose phosphate-6-synthase (*Ce tsp-1*) from *Caenorhabditis elegans* (Brey *et al.*, unpublished data), trehalose phosphate-6-synthase (*Sc tsp-1*) from *Saccharomyces cerevisiae* and hemolymph desiccation stress protein gene (*Tm dsp28*) from *Tenebrio molitor* (provided by V.K. Walker). See Second Annual report for vector construction details.

Objective 2: Incorporate cloned genes into *S. carpocapsae* to obtain overexpression, thereby enhancing desiccation tolerance.

From the beginning of the second to the middle of the third year an intensive effort was made to enhance the desiccation tolerance of *S. carpocapsae* by genetic transformation using the microinjection method. In *C. elegans*, transgene DNA (i.e., plasmids) injected into the syncytial cytoplasm of a hermaphrodite's gonad undergoes intermolecular ligation and recombination to form multicopy extrachromosomal arrays. Based on previous microinjection transformation successes (within our laboratory) with *H. bacteriophora*, we expected transformation of *S. carpocapsae* (although mating was an extra variable) utilizing the same transformation tools as *C. elegans* and *H. bacteriophora* would have been easily achievable. However, as our intensive transformation attempts show we were unable to successfully transform *S. carpocapsae* with *gfp*, a universal reporter gene, let alone the four stress genes using the microinjection method.

Over 2,500 *S. carpocapsae* young females were injected with four different desiccation gene constructs (Table 1). Only once were we able to observe *gfp* expression in *S. carpocapsae* using the trehalose-6-phosphate and glycogen synthase gene constructs. However, the progeny that were expressing *gfp* died within 2-3 days. Although extrachromosomal arrays created by germline injection have been successful in the past for *C. elegans*, they do have their limitations and barriers. For instance the same transgene that expresses in *C. elegans* may have a completely different expression pattern in another organism (i.e., *S. carpocapsae*) or tandemly repeated sequences in an array may trigger gene silencing.

As a result, we tried numerous vector concentrations (1, 10, 50 100 to 200, 250 ng/μl), *C. elegans* promoters (express: *sqt-1* cuticle, *myo-1* muscle, *mec-4* nerve,

sqv-4 vulva, *mab-3* intestine, *unc-54* nucleolar), and co-injection combinations [stress construct with pRF4 (roller), genomic *C. elegans* and *S. carpocapsae* carrier DNA] against *S. carpocapsae*. In total we injected (*gfp* expression vectors with or without stress genes) over 10,000 *S. carpocapsae* without obtaining a single stable transformation line.

Why were we not successful in transforming *S. carpocapsae*? This maybe answered as follows: *Heterorhabditis bacteriophora* and *C. elegans* are in the same phylogenetic clade, whereas *Steinernema carpocapsae* is in a more distant clade than *C. elegans*. This may explain why genes, genetic process and pathways are conserved between *C. elegans* and *H. bacteriophora*. In addition, transcriptional factors may not be the same in *Steinernema carpocapsae*. Also, extrachromosomal expression is not uniform and many times expression is at a lethal high copy number (mosaic expression) in contrast to transposon (currently no viable entomopathogenic nematode transposons have been isolated) transformation which results in a relative save low copy number.

A physiological or behavioral barrier may contribute to the inability to transform *S. carpocapsae*. Whereas *S. carpocapsae* produce amphimictic male and females that have to mate in order to produce progeny, *H. bacteriophora* and *C. elegans* form hermaphrodites that self-fertilize and produce homozygous clones. We observed no physical or reproductive differences between injected *S. carpocapsae* females and non-injected females to explain transformation failure. We surmise that the barrier lies within the vector mechanism. That is the current limitation in *Steinernema* transformation is the lack of a suitable promoter to drive the identified genes of interest.

Table 1. *Steinernema carpocapsae* injected with four different desiccation gene constructs.

Vector Construct	Promoter Expression	Desiccation Gene	Number Injected	Percent Expressing <i>gfp</i>
pJGSY1.8	Cuticle	<i>Sf-gsy-1</i>	489	> 1%
pJTy.1	Cuticle	<i>Sc-tsp-1</i>	789	>0.01
pTreCe.5	Nucleolar	<i>Ce-tsp-1</i>	1230	> 1%
pJdsp-28	Cuticle	<i>Tm-dsp28</i>	223	0 %

Other laboratories have experienced similar difficulties in *Steinernema* gene transformation. The people in András Fodor's laboratory, the ones who supposedly transformed *S. feltiae* with *tsp-1* yeast gene, have also failed in obtaining a stable *Steinernema* line by recombinant DNA microinjection (Fodor, personal communication). Therefore an alternative gene delivery vector system is needed for *Steinernema* species. Theoretically a technique that would directly insert the transgene into the chromosome would be ideal. One method is the construction of a "mini-chromosome". Mini-chromosomes have the capacity to carry many beneficial genes, survive in a heterologous system, and remain stable for many generations. Another method would be to use microparticle bombardment to create low-copy integrative transformants in *Steinernema* species.

Within the last 6 months a shift from *S. carpocapsae* to *C. elegans* was initiated to determine the effect of stress genes on *C. elegans* desiccation tolerance.

Fig. 1. Percent survival of pre-conditioned (97% RH) dauer larvae of CeNT(non-transgenic), ScAll, and CeT (transformed with *tsp-1Ce*) at 85 and 93% RH (n=2; error bars = $\pm$ S.E.; rep. = 85 ± 35 dauer larvae).

When dauer larvae of transgenic and non-transgenic *C. elegans* and *S. carpocapsae* were exposed to 93% RH, no difference in percent survival was observed among the three strains (Fig. 1). However, at 85% RH the percent survival of CeT was similar to desiccation tolerant Sc, but 2X as many CeT dauer larvae survived compared to CeNT. These results suggest that the overproduction of *tsp-1Ce* may play a role in protecting *C. elegans* dauer larvae from desiccation.

Currently, we are in the process of microinjecting stress gene constructs [genes: ALDH (only recently received from Israel team), *gsy-1*, *tsp-1* and *dsp-28*] into *C. elegans*. So far only *C. elegans* transformed with the *tsp-1*Ce gene has been assayed for desiccation tolerance. We plan on testing each stress gene construct within in the next couple of months for its effect on *C. elegans* dauer survival under different RH regimes. Also, we will characterize gene expression by performing RNAi on each of the *C. elegans* transgenic strains.