Final Scientific Report

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BARD Project Number: IS-4106-08 R

Submission date: January 7, 2013

Project Title: Genomic and Organismal Studies to Elucidate the Mechanisms of Infectivity of Entomopathogenic Fungi to Ticks

Investigators

Institutions

Principal Investigator (PI):

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Co-Principal Investigator (Co-PI):

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Collaborating Investigators:

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Dr. Galina Gindin	ARO, The Volcani Center, Israel

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Keywords (*not* appearing in the title and in order of importance).

Metarhizium brunneum, *Metarhizium pingshaense*, *Metarhizium majus*, *Rhipicephalus (Boophilus) annulatus*, *Rhipicephalus sanguineus*, *Hyalomma excavatum*, *Galleria mellonella*, green fluorescent protein, host-pathogen interactions, invertebrate fungal pathogens

Abbreviations commonly used in the report (in alphabetical order):

cDNA = complementary DNA synthesized from RNA

EST = expressed sequence tag

GFP = green fluorescent protein

RNAseq = high throughput sequencing of cDNA to get information about a samples' RNA content

Budget: IS: \$ 172,000

US: \$ 172,823

Total: \$ 344,823

Signature of Prof. Itamar Glazer

PI

Signature ARO, The Volcani Center, Israel
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	2	1	0	3
Submitted, in review, in preparation	0	0	1	1
Ph.D. theses	0	0	1	1
Abstracts & not refereed (proceedings, reports, etc.)	4	0	5	5

Postdoctoral Training: none

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	0	1	2
Longer Visits (Sabbaticals)	0	0	0	0

Description of Cooperation: Collaboration of synergistic type.

Patent Summary (numbers): No patents resulted from this project. ??

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ABSTRACT

The overall goal of this research was to elucidate the factors affecting early development of *Metarhizium* spp. (previously named *M. anisopliae*) on ticks or tick cuticle extracts and the molecular basis of these early infection processes. The original objectives were: **1.** Characterize the pre-penetration events (adhesion, germination and appressorium formation) of spores of *M. anisopliae* strains with high or low virulence during tick infection. **2.** Create GFP-expressing strains of *M. anisopliae* tick pathogens having high and low virulence to compare their progress of infection by microscopy. **3.** Use microarray analyses, primarily with existing *M. anisopliae* EST sequences in GenBank, to identify and characterize fungal genes whose expression is regulated in response to host cuticle extracts. Objective 3 was later modified (as approved by BARD) to use RNAseq to characterize the early stages of fungal gene expression during infection of intact host cuticles. This new method provides a massively larger and more informative dataset and allows us to take advantage of a) recently published genomes of *Metarhizium robertsii* and *M. acridum* for RNAseq data analysis, and b) newly developed and highly efficient cDNA sequencing technologies that are relatively low cost and, therefore, allow deep sequencing of multiple transcriptome samples.

We examined pre-penetration and penetration events that differentiate high and low virulence strains of *Metarhizium* spp., focusing on spore adhesion, germination, appressorium formation, and penetration of tick integuments. Initiation of fungal infection was compared on susceptible and resistant tick species at different tick developmental stages. *In vitro* studies comparing the effects of protein and fatty acid profiles from tick cuticle extracts demonstrated that resistant tick cuticles contain higher concentrations of specific lipids that inhibit fungal development than do susceptible tick cuticles, suggesting one mechanism of Ixodidae resistance to fungal entomopathogens (Objective 1). We used molecular markers to determine that the three *M. anisopliae* strains from Israel that we studied actually were three distinct species. *M. brunneum* is highly virulent against the tick *Rhipicephalus annulatus*, *M. pingshaense* and *M. robertsii* are intermediate in virulence, and *M. majus* is of low virulence. We transformed all four *Metarhizium* species to express GFP and used them in pathogenicity assays against diverse tick species. Key findings were that a) resistant ticks inhibit *Metarhizium* infection prior to hemocoel invasion by reducing fungal viability on the cuticle surface (Objective 2), as was supported by the *in vitro* studies of Objective 1, and b) *Metarhizium* kills susceptible ticks after cuticle penetration but prior to hemocoel colonization. Transcriptome studies of the most virulent species, *M. brunneum*, are in progress and include analyses of ungerminated conidia and conidia germination and development on a low nutrient medium or on susceptible *R. annulatus* exoskeleton (Objective 3).

We anticipate these studies will contribute to identifying fungal genetic factors that increase virulence and speed of kill and may help reveal tick chemistries that could be included in biocontrol formulations to increase efficacy. Methodologies developed to screen tick cuticle extracts for ability to support conidia germination and development may help in the selection of wild fungi with increased virulence against resistant ticks. The overall knowledge gained should contribute not only to the improvement of tick control but also to the control of other blood-sucking arthropods and related plant pests. Use of bio-based agents for controlling arthropods

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will contribute to a healthier, more sustainable environment and serve a growing number of organic food farmers.

ACHIEVEMENTS

Research achievements

Objective 1. Characterize pre-penetration events of *M. anisopliae* strains having high or low virulence against ticks. Previously, we reported that *Metarhizium* spp. developing on resistant ticks ultimately die without penetrating tick cuticles, leading to the assumption that the cuticles of resistant ticks contain antifungal compounds (Ment et al. 2010, J. Invertebr. Pathol. 103:132-139). The Israeli team investigated the possible influence of protein or lipid extracts of tick cuticle on *Metarhizium brunneum* conidial germination and differentiation. Conidia exposed to lipids from the susceptible tick *R. annulatus* germinated and differentiated into appressoria *in vitro* at high rates. Conidia exposed to lipids from the resistant tick *Hyalomma excavatum* demonstrated significantly lower rates of germination (ca. 20%) with no further differentiation. A comparative analysis of the fatty acid profiles of lipid extracts from the epicuticles of susceptible and resistant ticks was performed. The two profiles exhibited similar qualitative compositions, but the abundances of C16:0, C18:0, C18:1 ω 9C and C20:0 were 5 to 13 times higher in extracts from resistant ticks. In *in-vitro* assays, each of these four fatty acids inhibited conidial germination at 10 and 100 ppm/4.84 cm² of agarose, but C20:0 stimulated appressorium differentiation at low concentrations (0.1 ppm/4.84 cm² agarose). This is the first report demonstrating a possible link between the concentration of specific cuticle compounds and Ixodidae tick resistance to fungal infection. Proteinaceous extracts from susceptible ticks stimulated conidial germination but did not support further development; extracts from resistant ticks had no affect on fungal development.

Objective 2. Use GFP-expressing *Metarhizium* strains to understand early fungus/tick interactions. The use of green fluorescent protein (GFP)-expressing fungi to study development on resistant and susceptible ticks proved to be highly informative. Live ticks of different developmental stages were inoculated with GFP-expressing *Metarhizium* strains and evaluated by confocal microscopy over time until death (Ment et al. 2012, Environ. Microb. 14:1570-1583). The main findings included: 1) The first report of DNA-mediated transformation of *M. brunneum*, *M. pingshaense*, and *M. majus*, 2) Comparisons of the development and relative levels of virulence of

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four *Metarhizium* spp. against two susceptible tick species, two resistant tick species and the lepidopteran *Galleria mellonella*. Conidia of all four species germinated on all five hosts examined, but on resistant hosts, sustained hyphal growth was inhibited and GFP emission steadily decreased over time, suggesting a loss of fungal viability. Cuticle penetration was observed only for the three most virulent fungal species infecting the susceptible hosts. This is the first report (i) of tick mortality occurring after cuticle penetration but prior to hemocoel colonization and (ii) that resistant ticks do not support development of *Metarhizium* germlings on the outer surface of the cuticle. The U.S. team created the fungal transformation vector (B.G.G. Donzelli), generated the GFP-expressing *Metarhizium* strains (A.C.L. Churchill and B.G.G. Donzelli), and conducted the molecular taxonomy studies (S.A. Rehner). The Israeli team conducted the pathogenicity and confocal microscopy assays on ticks using the U.S.-generated GFP-expressing *Metarhizium* strains.

Objective 3. Use RNAseq approaches to identify and characterize fungal genes whose expression is regulated in response to intact susceptible host cuticle.

A USDA-APHIS Veterinary Services permit was obtained by Churchill in August 2012 to receive a shipment of sterilized, excised *R. annulatus* tick exoskeletons from Israeli colleagues. These are being used as the substrate for *in vivo* transcriptome assays. Methods for the isolation of RNA from *M. brunneum* grown on tick exoskeleton are being developed so as to minimize RNA contamination from the tick. Transcripts are being isolated from ungerminated conidia, conidia germinated on a minimal medium, conidia that have germinated and developed on susceptible tick exoskeleton, and tick exoskeleton alone. Cornell's Core Laboratories Center has been contracted to construct the sequencing libraries in addition to conducting paired-end Hi-Seq Illumina sequencing of the transcriptomes. Bioinformatics analyses will be conducted at Cornell University.

Description of cooperation

Synergistic cooperation, based on very good personal relationships and the sincere intentions of each scientist to contribute to the highest quality research yielded success for this project (see list of publications). The cooperation was technically based on the many meetings and long detailed discussions (visits in labs, attending the same conferences) and mainly the very many detailed e-mails on each technical or theoretical question.

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- U.S. collaborator received three *Metarhizium* strains from Israeli colleagues for DNA-mediated transformation to GFP expression and glufosinate ammonium resistance and for RNAseq studies (Objectives 2 and 3).
- Israeli collaborators received five GFP-expressing transformants of each of three strains from U.S. collaborator (Objective 2).
- U.S. collaborator received sterilized, excised cuticles of engorged *R. annulatus* females prepared by the Israeli lab for RNAseq analyses at Cornell University (Objective 3).
- Mutual exchange of new publications and information from the literature, and discussion and planning of confocal microscopy and transcriptome analysis experiments together.
- Mutual participation and contributions to writing collaborative abstracts, manuscripts, and research reports.
- Participation of the Israeli team in a conference of the Israeli Society of Entomology in October, 2010: Ment, D., G. Gindin, A.C.L. Churchill, E. Belausov, I. Glazer, A. Rot, and M. Samish (2010). The development of *Metarhizium anisopliae* on susceptible and resistance hosts. The 29th Conference of the Entomological Society of Israel.
- Participation of the Israeli team in a conference of the Israeli Phytopathology Society in January, 2011: Ment, D., G. Gindin, A.C.L. Churchill, E. Belausov, I. Glazer, A. Rot, and M. Samish (2011). Interactions between the entomopathogenic fungus *Metarhizium anisopliae* and susceptible and resistant arthropod hosts. The 32nd Conference of the Israeli Phytopathology Society, *Phytoparasitica* 39:244.
- Participation of Israeli and U.S. team members in the international 26th Fungal Genetics Conference, Pacific Grove, CA, in March, 2011: Ment, D., G. Gindin, A.C.L. Churchill, A. Rot, B.G.G. Donzelli, E. Belausov, I. Glazer, and M. Samish (2011). GFP-expressing *Metarhizium* spp. of diverse virulence demonstrate differential development on various arthropod hosts. *Fungal Genetics Reports* 58(Suppl.): #523, p. 242.
- Participation of the Israeli team in an IOBC conference in June, 2011: Ment, D., G. Gindin, A.C.L. Churchill, A. Rot, E. Belausov, I. Glazer, and M. Samish (2011). The development of GFP-expressing *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) on susceptible and resistant ticks (Acari: Ixodidae). 13th Meeting of the Working Group of "Insect Pathogens and Insect Parasitic Nematodes," Innsbruck, Austria, IOBC/WPRS Bulletin 66:129.

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BARD PUBLICATIONS 2009 –2012

I -- Refereed (published, in press, accepted) BARD support acknowledged

Ment, D., Churchill, A.C.L., Gindin, G., Belausov, E., Glazer, I., Rehner, S.A., Rot, A., Donzelli, B.G.G., & M. Samish (2012). Resistant ticks inhibit *Metarhizium* spp. infection prior to hemocoel invasion by reducing fungal viability on the cuticle surface. *Environmental Microbiology* 14: 1570-1583 (Impact Factor of 5.84).

Ment, D., Gindin, G., Churchill, A.C.L., Rot, A., Belausov, E., Glazer, I. & M. Samish (2011). The development of GFP-expressing *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) on susceptible and resistant ticks (Acari: Ixodidae). *13th Meeting of the Working Group of "Insect Pathogens and Insect Parasitic Nematodes," Innsbruck, Austria, IOBC/WPRS Bulletin* 66:129 - 132.

Rohlf, M. & Churchill, A.C.L. (2011). Special Issue: Fungal Secondary Metabolism. Fungal secondary metabolites as modulators of interactions with insects and other arthropods. *Fungal Genetics and Biology* 48:23–34 (Impact Factor of 3.74).

II – Submitted to reviewed journals

Ment, D., Gindin, G., Rot, A., Eshel, D. P., Teper-Bamnolker, I., Ben-Ze'ev, I., Glazer, I., & M. Samish, (Submitted). Susceptibility of ticks to fungal infection: Possible role of cuticular lipids and proteins. Submitted to "Bicontrol Science & Technology" Nov. 2012. [see PDF file added].

III – Ph.D. theses

Dana Ment (2013). The role of biotic and a-biotic factors on the interaction between arthropods (ticks [Acari: Ixodidae]) and fungi (*Metarhizium anisopliae* [Hypocreales: Clavicipitaceae]). Ph.D. theses, The Agricultural Faculty, The Hebrew University of Jerusalem.

IV -- Abstracts published in proceedings (No acknowledgement to BARD)

Ment, D., Gindin, A., Churchill, A.C.L., Glazer, I., Rot, A. & M. Samish (2009). Evaluation of host-pathogen interactions of a fungal arthropod pathogen – ultrastructural observations. *The Israeli Society for Parasitology, Protozoology and Tropical Diseases, Annual Meeting 2009 (Tel Aviv, Israel)*. p. 38.

Ment, D., Gindin, G., Rot, A., Samish, M. & I. Glazer (2009). The potential use of entomopathogenic fungi for bio-control of ticks – application methods, problems and solutions.

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37th Conf. of The Israeli Society of Ecology & Environmental Sciences, The Weizmann Institute of Science (Rehovot, Israel). 2 pp.

Ment, D., Gindin, G., Soroker, V., Barel, S., Rot, A., Samish, M. & I. Glazer (2009). A method for quantification of the amount of *Metarhizium anisopliae* conidia adhering to the cuticle of ticks. *The 28th Annual Meeting of the Israeli Society of Entomology* (Tel Aviv, Israel) p. 51.

Rot, A., Samish, M., Gindin, A., Ment, D. & I. Glazer (2009). The effect of entomopathogenic fungi on tick behavior. *The Israeli Society for Parasitology, Protozoology and Tropical Diseases, Annual Meeting 2009* (Tel Aviv, Israel). p. 39.

Ment, D., Gindin, G., Rot, A., Churchill, A.C.L., Belausov, E., Glazer, I., & M. Samish (2010). *Metarhizium anisopliae* development on susceptible and resistant hosts. *The 29th Annual Meeting of the Israeli Society of Entomology* (Bet Dagan, Israel). p. 51.

V -- Proceedings (No acknowledgement to BARD)

Ment, D., Gindin, G., Glazer, I., Perl, S., Elad, D. & M. Samish (2009). *Metarhizium anisopliae* chlamydospores in tick eggs. *International Org. Biol. Integrated Cont. (IOBC) Bull.* 45:303-306.

Glazer, I., Gindin, G., Ment, D. & M. Samish (2010). Host-pathogen interaction between ticks and entomopathogenic fungi. *The 43rd Annual Meeting of the Society for Invertebrate Pathology*, Trabzon, Turkey, p. 104.

Ment, D., Gindin, G., Churchill, A.C.L., Rot, A., Belausov, E., Glazer, I., & M. Samish (2011). Interactions between the entomopathogenic fungus *Metarhizium anisopliae* and susceptible and resistant arthropod hosts. *The 32nd Conference of the Israeli Phytopathology Society* (Bet Dagan, Israel). *Phytoparasitica* 39:244.

Ment, D., Gindin, G., Churchill, A.C.L., Rot, A., Donzelli, B.G.G., Belausov, E., Glazer, I. & M. Samish (2011). GFP-expressing *Metarhizium* spp. varying in virulence demonstrate differential development on diverse arthropod hosts. *26th Annual Fungal Genetics Meeting, Pacific Grove, CA, USA, March 2011. Fungal Genetics Reports* 58(Suppl.): #523, p. 242.

APPENDIX

Copy of 'submitted' paper

Ment, D., Gindin, G., Rot, A., Eshel, D., P., Teper-Bamnolker, I., Ben-Ze'ev, I., Glazer, & M. Samish, (Submitted). Susceptibility of ticks to fungal infection: Possible role of cuticular lipids and proteins. *Submitted to "Bicontrol Science & Technology" Nov. 2012. [see PDF file added]*.