42534 5

973

Final Scientific Report

Cover Page

BARD Project Number: IS-3512-04

Date of Submission of the report:

July 2008

**Project Title: Ecology of seed-colonizing microbial communities:
Influence of soil and plant factors and implications for rhizosphere
microbiology**

Investigators**Principal Investigator (PI):**

Dror Minz

Co-Principal Investigator (Co-PI):

Eric Nelson

Collaborating Investigators:

Yitzhak Hadar

Institutions

ARO

Cornell

HUJI

Keywords *bacteria, Pythium.***Abbreviations commonly** used in the report, in alphabetical order:

DGGE denaturing gradient gel electrophoresis

MDSA multidimensional scaling analysis

T-RFLP terminal restriction fragment length polymorphism

Budget: IS: \$ 201,000

US: \$129,000

Total: \$330,000

Signature
Principal Investigator

State of Israel Ministry of Agriculture
Agricultural Research Organization
The Volcani Center

Signature
Authorizing Official, Principal Institution

630.72

BAR - MEN

Overall Summary and Statistics

Publication Summary (numbers)

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

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant. **None**

Cooperation Summary (numbers)

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

Description Cooperation:

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				
Licensed				

Abstract

Original objectives: Our initial project objectives were to 1) Determine and compare the composition of seed-colonizing microbial communities on seeds, 2) Determine the dynamics of development of microbial communities on seeds, and 3) Determine and compare the composition of seed-colonizing microbial communities with the composition of those in the soil and rhizosphere of the plants.

Revisions to objectives: Our initial work on this project was hampered by the presence of native *Pythium* species in the soils we were using (in the US), preventing us from getting accurate assessments of spermosphere microbial communities. In our initial work, we tried to get around this problem by focusing on water potentials that might reduce damage from native *Pythium* species. This also prompted some initial investigation of the oomycete communities associated seedlings in this soil. However, for this work to proceed in a way that would allow us to examine seed-colonizing communities on healthy plants, we needed to either physically treat soils or amend soils with composts to suppress damage from *Pythium*. In the end, we followed the compost amendment line of investigation, which took us away from our initial objectives, but led to interesting work focusing on seed-associated microbial communities and their functional significance to seed-infecting pathogens. Work done in Israel was using suppressive compost amended potting mix throughout the study and did not have such problems.

Our work focused on the following objectives: 1) to determine whether different plant species support a microbial induced suppression of *Pythium* damping-off, 2) to determine whether compost microbes that colonize seeds during early stages of seed germination can adequately explain levels of damping-off suppression observed, 3) to characterize cucumber seed-colonizing microbial communities that give rise to the disease suppressive properties, 4) assess carbon competition between seed-colonizing microbes and *Pythium* sporangia as a means of explaining *Pythium* damping-off suppression.

Background: Earlier work demonstrated that seed-colonizing microbes might explain *Pythium* suppression. Yet these seed-colonizing microbial communities have never been characterized and their functional significance to *Pythium* damping-off suppression is not known. Our work set out to confirm the disease suppressive properties of seed-colonizing microbes, to characterize communities, and begin to determine the mechanisms by which *Pythium* suppression occurs.

Major Conclusions:

- Compost-induced suppression of *Pythium* damping-off of cucumber and wheat can be explained by the bacterial consortia colonizing seeds within 8 h of sowing. Suppression on pea was highly variable.
- Fungi and archaea play no role in disease suppression.
- Potentially significant bacterial taxa are those with affinities to Firmicutes, Actinobacteria, and Bacteroidetes. Current sequencing efforts are trying to resolve these taxa.
- Seed colonizing bacteria suppress *Pythium* by carbon competition, allowing sporangium germination by preventing the development of germ tubes.
- Presence of *Pythium* had a strong effect on microbial community on the seed.

Achievements

Significance of the main scientific achievements.

Establishing that seed-colonizing bacterial communities are responsible for *Pythium* damping-off suppression is a major advance in our understanding of disease suppressive soils because it establishes the important role of the plant in regulating disease suppression but also the significance of understanding pathogen dynamics and the infection court as a site to examine microbial interactions associated with plants. Our work further illustrated the importance of the host plant in regulating these interactions by the highly variable results obtained with pea (*Pisum sativum*)

With the above observations as a framework, we were able to develop a unique strategy for identifying the seed-associated microbes involved in *Pythium* suppression. Identifying microbes involved in disease suppression is somewhat challenging. First, soils harbor massive populations of prokaryotic and eukaryotic microbes, many of which have little to do with disease suppression. Second, a large percentage of the microbes present in soil are not yet culturable, making detailed studies of microbial function and population development difficult. Third, individual microbes with disease suppressive properties can be isolated from both suppressive or non-suppressive soils or composts, making it impossible to understand the nature of suppressive microbial communities simply based on the suppressive activities of isolated individuals. Our work was significant in that our approach to identifying *Pythium* suppressive microbes took advantage of our knowledge of the temporal framework for *Pythium* pathogenesis and of selective inhibitors to reduce or eliminate suppressiveness and corresponding microbial populations.

Rapid transformations in seed development during the early stages of seed germination elicit rapid developmental changes in *Pythium* species but also in successions of microbial communities on seed surfaces. Therefore, to identify those microbial communities that interact directly with *Pythium* during seed infection, sampling of these communities must be synchronized with the initial stages of pathogenic development of *P. ultimum*. Furthermore, spermosphere microbial communities are less diverse than the bulk soil microbial communities, with individual taxa reaching higher populations over time in the spermosphere than they otherwise would in bulk soil. This suggests that *Pythium*-suppressive microbial communities that develop in the spermosphere should be much less complex than surrounding soil microbial

communities and also enriched in populations of microbes essential for disease suppression. This would facilitate the search for microbial communities involved in disease suppression.

Using such a strategy in combination with a T-RFLP analysis, we were able to identify 20 putative TRFs as being potentially involved in *Pythium* damping-off suppression. Among the more important were those taxa that have affinities with Firmicutes, Actinobacteria, and Bacteroidetes, with lower frequencies of Proteobacteria. Whereas the actual taxa can only be confirmed by excising, reamplifying, and sequencing individual TRFs, our results appear to be consistent with other reports of cucumber seed-colonizing bacteria from composts.

Our work was also significant because it provided information on the possible mechanisms of disease suppression that target the early stages of disease development, especially by providing carbon limiting conditions that induce germ tube abortions. While our results establish strong correlations between carbon availability, germ tube abortion, and *Pythium* damping-off suppression, our results are not entirely definitive.

Agricultural or Economic Impacts of Findings

Managing microbes in agriculture has been difficult to implement largely because little is known about the ecology of plant-associated and soil microbes. Our understanding is also incomplete in how they function to induce useful plant responses. Our work provides an important piece to that puzzle in providing important information on how seed-associated microbes influence disease development in an important plant pathogen. Our work has been important in generating a better understanding of seed-associated microbes so that useful microbial technologies may be developed for agriculture in the future.

The survival of cucumber plants amended with and without *Pythium* was assessed in various potting mixes (Fig.1). In general, the percent survival of plants infected by *Pythium* was lower than plants growing without the *Pythium*. The lowest survival rates were measured when cucumber plants were grown in peat, potting mix amended with straw compost or separate compost manure. The highest survival rate was measured in the potting mix amended with manure and chicken compost. These results suggest that some properties of the compost contribute to the survival of plants under *Pythium* amendments. These properties are thought to be of a microbial source and therefore the diversity of the microbial communities in the treatments amended with and without *Pythium* was studied.

Details of Cooperation

There was an exchange of procedures and experimental designs between the groups. All experiments were carried out in identical methodologies and using identical experimental system in both countries to allow comparison of the results. Hadar and Minz co-supervised two PhD students working in this project. Hadar visited Nelson for discussion, comparison of results and planning of the work.

List of Publications

- Chen, M.-H., and E. B. Nelson.** 2008. Seed-colonizing microbes from municipal biosolids compost suppress *Pythium ultimum* damping-off on different plant species. *Phytopathology, in press.*
- Chen, M.-H., McGuire, I.C., and E. B. Nelson.** 2008. Characterization of seed-colonizing bacterial communities associated with the suppression of *Pythium* damping-off in a municipal biosolids compost. *In preparation.*
- Chen, M.-H., and E. B. Nelson.** 2008. Carbon competition as a mechanism of *Pythium* damping-off suppression in a municipal biosolids compost. *In preparation.*
- Ofek M., Bucki P., Hadar Y. and D. Minz.** Microbial events related to cucumber seed infection by *Pythium aphenidermatum* as affected by suppressive compost amendment. *In preparation.*
- Bucki, P. Hadar, Y. and Minz. D.** The effects of plant pathogen the seed microbial community. *In preparation.*

Abstracts

- Chen, M.-H., McGuire, C., and E. B. Nelson.** 2008. Characterization of seed-colonizing bacterial communities associated with the suppression of *Pythium* damping-off in a municipal biosolids compost. *Phytopathology* 98: S36.
- Chen, M.-H., and E. B. Nelson.** 2008. Carbon competition as a mechanism of *Pythium* damping-off suppression in a municipal biosolids compost. *Phytopathology* 98: S36.
- Bucki, P. Hadar, Y. and Minz. D.** 2006. How plant pathogen affects the seed microbial community. 11th International Symposium on Microbial Ecology, Vienna, Austria
- Karp, M.A., Arcate, J., and Nelson, E.B.** 2006. Dynamics and diversity of rhizosphere oomycete communities. *Phytopathology* 96: S59.
- Chen, M.-H., and E. B. Nelson.** 2006. Seed-colonizing microbial communities suppressive to *Pythium* damping-off. *Phytopathology* 96: S22.

Appendix

Diversity (unpublished results)

Microbial communities were analyzed by Degenerate Gel Gradient Electrophoresis (DGGE) in several experiments to assess whether the microbial community is affected by the presence of *Pythium*. This method, with the various analyses of the results obtained from the gels allows comparing large amount of samples without sequencing. In the first experiment, the bacterial community composition with and without *Pythium* was assessed in the potting mix and the rizosphere and on the seeds and roots. Bacterial community patterns of similar samples obtained from individual repeats (different pots) were highly similar (Fig. 2). The presence of *Pythium* in the potting mix caused a shift in the bacterial community composition in all samples examined (Figure 2). This could be observed with dominant bands in DGGE gel that represent Actinobacteriales population (2.6) in the samples from seed after one day of seeding and the root of 4 day-old seedling, which could not be seen in the same samples without *Pythium*. On the other hand, some dominant bands that represented *Flavobacteria sp.* (2.1); *Burkholderia sp.* (2.2); *Ralstonia sp.* (2.3); and uncultured soil bacteria (2.4) were present only in the samples without *Pythium*. This leads us to infer the existence of specific groups associated with the presence of *Pythium*. In addition, the experiment has shown that we can find an interesting relationship between bacteria and seeds and between bacteria and substrates in the initial hours following sowing.

Subsequently, a new experiment was designed with the same potting mix including and excluding *Pythium*. The aim was to compare the effect of *Pythium* on the bacterial community composition in seed and spermosphere. Seed and spermosphere samples were taken at seeding time and up to 12 hours after seeding. The community DNA was extracted and PCR amplifications were performed with general bacterial primer (341F/907R) and primers for the class actinobacteria (A235-F/1392-R). The fragments amplified with the actinobacteria primers were re-amplified with the general bacterial primer (341F/907R). The product of the PCR amplification from the DNA was used to perform the DGGE analysis.

The PCR-DGGE patterns of bacterial populations associated with seed or spermosphere were analyzed with a multidimensional scaling analysis (MSDA) and cluster analysis (Fingerprint II) (Fig. 3). The cluster analysis of the PCR-DGGE patterns performed with general bacterial primers revealed that there were two different clusters (Fig. 3a): one for spermosphere and

another one for seed. In the cluster corresponding to spermosphere, the bands were distributed from the middle to top of the gel. In the seed cluster there were populations in the middle area of the gel which were shared with the spermosphere, but there were also other seed distinctive populations at the bottom of the gel. Because of the complexity of analyzing populations through PCR-DGGE patterns, a multidimensional scaling analysis (MDSA) was performed (Fig. 3b). This analysis allows grouping individual patterns, avoiding the misrepresentation of distances inherent to hierarchical cluster analysis methodologies. MDSA was performed using pair-wise 1-Pearson correlations of densitometric curves obtained from the DGGE patterns (Fig. 3a). The MDSA analysis revealed a significant division between the seed samples and the spermosphere (Fig. 3b).

The analysis above was not sufficient to understand the dynamics of the population over time. For this reason, we analyzed the bacterial population associated with seeds or spermosphere separately, thus increasing the analysis resolution. There were bands representing bacterial populations observed only in samples with *Pythium*, and only at intermediate times (Fig. 4, band 3.1). Other populations only appeared at longer times of seed exposure to potting mix (band 3.2), while others only emerged at a particular time, such as 6 hours after introduction, and then disappeared. In contrast, there were populations not affected by the presence of *Pythium*, since they were present in samples with and without *Pythium* (band 3.7). Within these, there was a bacteria population dominant in both conditions but only after 12-hours contact of the seed and the potting mix (band 3.8). Finally, there were also populations which only appeared between 60 minutes and 4 hours of seed exposure to the potting mix (bands 3.5;3.6). The low stress value given by a MDSA analysis (0.031) indicated that the distances reproduced reliably the original pair wise distances. Therefore, MDSA analysis enabled us to infer that an axis could be created representing time and the points corresponding to the samples of both treatments, with and without *Pythium*, along the same axis, each one respecting its own trend.

In contrast to the seeds, changes in the composition of bacteria populations in spermosphere (Fig. 5) were less noticeable and overall sample repetitions for spermosphere were more homogeneous. Similar to the seeds, we also found bands representing populations present with and without *Pythium* (band 4.1). In the spermosphere samples without *Pythium*, we observed populations present at all sampled times but not in every repetition (bands 4.6 and 4.7), which confirms as well the variability of the system under study. Similarly to the seeds, there were

bacterial populations associated with longer exposures of the seed in the spermosphere (bands 4.30, 4.4). In the bacterial populations associated with *Pythium*, a population was identified to exist up to 120 minutes of exposure of spermosphere (band 4.5) while there was a population only present after 12 hours of exposure. The MDSA analysis (Fig. 5b) suggest that the samples were grouped according to sampling times, but not along an axis representing time, as it is the case of the seeds. Also, the points which represent the repetitions are grouped together, except for some cases which confirm the intrinsic variability of the system under study.

Since actinobacteria are suspected to be important in controlling *Pythium*, a detailed analysis of this group was done with specific primers set for the class actinobacteria (A235-F/1392-R) followed by a second PCR reaction with general bacterial primer (341F/907R). The analysis was done on the same samples described above for the general bacterial primers set. Similarly, the samples from seeds and spermosphere formed separate clusters. Two bacterial populations associated with the seeds without *Pythium* grouped into two clusters related with each other (Fig. 6a). A third external cluster grouped with the bacterial populations associated with the seeds in the treatment with *Pythium*, in which the seeds were exposed for 12 hours. At first sight, the bands representing actinobacteria populations are different from those representing seeds and spermosphere. A MSDA analysis also confirms the division of samples originating from seeds and spermosphere (Fig. 6b). In this space distribution, it is also very clear that the samples of seeds treated with *Pythium* and exposed for 12 hours are far from the samples corresponding to seeds exposed to potting mix with *Pythium*.

When increasing the resolution of the analysis to include only the samples associated with the seeds, the variability between repetitions is much larger (Fig. 7a) compare to the equivalent analysis with the general bacterial primer. As consequence, it is much more difficult to analyze the variations due to time in the aligned gel. When analyzing the banding patterns with MSDA, our conclusion is correct on the basis of the points corresponding to the treatment without *Pythium* which form a cloud but are not aligned –as in the case of the treatment with *Pythium*, in which a linear regression analysis gives significant statistically (R-square 0.77; p-value 3.21E-5). In contrast, the regression is not significant in the treatment without *Pythium* (p-value 0.3506). In the that treatment, it is worth mentioning the presence of two bacterial populations (band 5.1 and 5.3) which were only found 12 hr after sowing, and another population that appeared four hours after seed presence in the compost but remained present until the end of the experiment. In the

analysis of the results obtained from PCR-DGGE there were populations in the treatment with *Pythium* which were dominant from the beginning of the experiment to 1 hour after sowing and up to 4 hours after sowing in some samples and then disappear (bands 5.4 and 5.5). Two populations became important 6 hours after sowing and remain present until the end of the experiment (bands 5.6 and 5.7). Finally, similar to the treatment without *Pythium*, some populations only appear 12 hours after sowing (bands 5.8 and 5.9) (Fig. 7b).

In the analysis of the PCR-DGGE gel of samples from the spermosphere (Fig 8a), there was a similarity between the patterns of samples with and without *Pythium*. Populations represented by bands 6.1 to 6.5 were present in both treatments, although not in all the repetitions in the treatment with *Pythium*. In the treatment without *Pythium*, variability between repetitions of the different treatments was little as opposed to the treatment with *Pythium* in the potting mix, in which a dominant population (bands 6.12, 6.13 and 6.14) appeared after four hours only in some of the repetitions and the presence of the population represented in all of the samples with and without *Pythium* (bands 6.15 and 6.16) was maintained.

Representative results:

Figure 1: Survival of cucumber plants in different potting mix sources with and without *Pythium*. CMO: Manure and Orange Peel compost, CM: Manure and Chicken manure compost, CS: Straw compost, SCM: Separate cow Manure Compost, GM: Grape Marc Compost and SSC: Sewage Sludge compost

Figure 2: **Bacterial** community composition in **potting mix, seeds, roots, and rhizosphere** at seeding time (0d), one, four and 21 days after seeding, using DGGE analysis. DNA samples were loaded after enzymatic restriction (*Bsr*BI) and PCR amplification with general bacterial primers (341F- 907R). Dominant bands are indicated by circles (2.1: *Flavobacteria* sp.; 2.2: *Burkholderia* sp.; 2.3: *Ralstonia* sp; 2.4: uncultured soil bacteria; 2.5: *Streptomyces hygroscopicus*; 2.6: Actinobacteria, related by sequence analysis)

3.a)

3.b

Figure 3: Effect of *Pythium* on bacterial community composition of spermosphere and seeds in potting mix (50% V:V :straw compost:perlite) up to 12hrs after seeding. a) Bacterial community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were analyzed using the Fingerprint II software. a) A Ward tree was calculated from Pearson correlation distance matrix. b) Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves. MDSA was performed based on 1-pearson r distance matrices between the densitometric curves, using STATISTICA software. The 1st and the 2nd dimensions are presented (Stress = 0.03148).

4.a)

4.b)

Figure 4: Effect of *Pythium* on bacterial community composition on seeds in potting mix (50% V:V :straw compost:perlite) up to 12hrs after seeding. a) Bacterial community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were aligned using Fingerprint II software. DNA samples were loaded on acrylamide gel after PCR amplification with general primers (341F- 907R). Relevant bands are indicated by arrows. b) Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves. MDSA was performed based on 1-pearson r distance matrices between the densitometric curves, using STATISTICA software. The 1st and the 2nd dimensions are presented (Stress = 0.0311634).

5.a)

5.b)

Figure 5: Effect of *Pythium* on **bacterial** community composition of **spermosphere** after seed seeding on potting mix (50% V:V :straw compost:perlite) up to 12hrs after seeding. a) Bacterial community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were aligned using Fingerprint II software. DNA samples were loaded on acylamide gel after PCR amplification with general primers (341F- 907R). Relevant bands are indicated by arrows. b) Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves. MDSA was performed based on 1-pearson r distance matrices between the densitometric curves, using STATISTICA software. The 1st and the 2nd dimensions are presented (Stress = 0.036).

6.a

Figure 6: Effect of *Pythium* on *Actinobacteria* community composition of **spermosphere and seeds** seeding on potting mix (50% V:V :straw compost:perlite) up to 12 hrs after seeding. a) *Actinobacteria* community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were analyzed using the Fingerprint II software. a) A Ward tree was calculated from Pearson correlation distance matrix. b) Multidimensional scaling analysis (MDS) of DGGE patterns densitometric curves. MDS was performed based on 1-pearson r distance matrices between the densitometric curves, using STATISTICA software. The 1st and the 2nd dimensions are presented (Stress =0.0496).

7. a)

7. b)

Figure 7: Effect of *Pythium* on **Actinobacteria** community composition of **seeds** seeding on potting mix (50% V:V :straw compost:perlite) up to 12hrs after seeding. a) Actinobacteria community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were aligned using Fingerprint II software. DNA samples were loaded on acylamide gel after PCR amplification with specific primer for Actinobacteria (A235-F/1392-R) follows by general primers (341F- 907R). Relevant bands are indicated by arrows. b) Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves. MDSA was performed based on 1-pearson r distance matrices between the densitometric curves, using STATIATICA software. The 1st and the 2nd dimensions are presented (Stress =0.0264852).

8.a)

8. b)

Figure 8: Effect of *Pythium* on **Actinobacteria** community composition of **spermosphere** after seed seeding on potting mix (50% V:V :straw compost:perlite) up to 12hrs after seeding. a) Actinobacteria community compositions were compared based on PCR-DGGE patterns of 16S rRNA gene fragments. PCR-DGGE patterns were aligned using Fingerprint II software. DNA samples were loaded on acylamide gel after PCR amplification with specific primer for Actinobacteria (A235-F/1392-R) follows by general primers (341F- 907R). Relevant bands are indicated by arrows. b) Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves. MDSA was performed based on 1-pearson r distance matrices between the densitometric curves, using STATISTICA software. The 1st and the 2nd dimensions are presented (Stress = 0.0416108).