**BARD Final Scientific Report
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BARD Project Number: US-3108-99

Project Title: Microbial Communities Active in Soil-Induced Systemic Plant Disease Resistance

Investigators

Institutions

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Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Biocontrol, Trichoderma hamatum 382, Chryseobacterium, Cucumber, Radish, Phytophthora, Pyrenomyces, Rhizosphere

Abbreviations commonly used in the report, in alphabetical order:

Denaturing Gradient Gel Electrophoresis (DGGE), Induced Systemic Resistance (ISR), Terminal Restriction Fragment Polymorphism (TRFLP), Terminal Restriction Fragment (TRF), Trichoderma hamatum 382 (T382)

Budget: IS: \$150,000

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Total: \$300,000

Signature
Principal Investigator

Signature
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)	1	4	2	7
Submitted, in review, in preparation	2	1	1	4
Invited review papers	0	0	0	0
Book chapters	0	0	0	0
Books	0	0	0	0
Master theses	0	0	0	0
Ph.D. theses	0	0	1	1
Abstracts	5	5	15	20
Not refereed (proceedings, reports, etc.)	1	0	0	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.

Sonia Tiquia 484-27-6649

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	2	3	7
Longer Visits (Sabbaticals)	0	1	0	1

Description of Cooperation:

D. Minz, Y. Hadar, F. Michel and H. Hoitink met at International Symposium on the Microbiology of Composting in Innsbruck, October 17-20, 2000. From Sept 1-Dec 31, 2001, S. Green, a graduate student at the Hebrew University, came to Ohio to learn T-RFLP techniques, perform analyses and return with DNA samples from ISR-active substrates. D. Minz, S. Green, F. Michel and H. Hoitink met at the International Composting and Compost Utilization Symposium in Columbus, Ohio from May 6-9, 2002 to discuss progress in research and plan second and third year activities. D. Minz, S. Green, and F. Michel met at the International Symposium on Microbial Ecology meeting in Mexico in August 2004 to present BARD funded research findings, discuss research results, prepare manuscripts and plan future research collaborations and activities.

Patent Summary (numbers)

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

BARD FINAL SCIENTIFIC REPORT

Microbial Communities Active in Soil-Induced Systemic Plant Disease Resistance

BARD Project number: **US-3108-99**

Evaluation of the research achievements:

In this project microbial consortia in the rhizosphere of plants grown in Induced Systemic Resistance (ISR)-positive and negative compost-amended substrates were characterized. The plants used included primarily cucumber but also tomato and radish. Rhizosphere microbial consortia were characterized in both field plots and potting media using multiple molecular tools including DGGE (Israel) and T-RFLP (Ohio). General bacterial, fungal as well as population-specific primers were utilized. A procedure was developed for inoculation of a compost-amended substrate with *Trichoderma hamatum* 382 (T382) which consistently induces ISR in cucumber against Phytophthora blight caused by *Phytophthora capsici* and in radish against bacterial spot (Ohio). Molecular tools were developed to detect and quantify T382 in compost-amended substrates (Ohio). In addition, a set of primers specific for *Pyrenomyces* was developed to monitor populations of *Trichoderma* and *Fusarium* in these substrates (Israel). These two groups of fungi harbor isolates which are active inducers of systemic resistance in plants. Microbial consortia associated with greenhouse cucumber plants grown in compost-amended potting mixtures were shown to be dominated by the phylogenetic taxon *Bacteroidetes*, including members of the genus *Chryseobacterium*, which in some cases have been shown to be involved in biocontrol (Israel). TRFLP analyses using universal bacterial primers showed few significant overall differences in microbial communities in ISR active and inactive substrates. In addition the community members which were significantly different varied when different ISR active composts were used (Ohio). Population specific molecular tools were employed to better characterize the shifts in microbial community structure during the development of ISR (Israel, Ohio).

Progress related to the specific research objectives is presented below.

Significance of main scientific achievements or innovations.

Few composts naturally possess ISR activity. The frequency of natural ISR activity in composts is < 2% (Krause et al., 2002). During screening of several different batches of potting mixes prepared with composted sawdust-bedded cow manure, it was discovered that one batch naturally induced SR in Begonia to Botrytis blight and powdery mildew. The incidence and abundance of strains of *Bacillus* spp., *Pantoea agglomerans* and *Trichoderma* which earlier were shown to be the most ISR-active microorganisms in composts were monitored to develop a better understanding of why ISR activity in composts is such a rare phenomenon.

A process for inoculation of the ISR active strain *Trichoderma hamatum* T382 into composted cow manure-amended (15% v/v) sphagnum peat substrates was developed that consistently induced systemic resistance in plants to foliar diseases. The severity of Phytophthora blight and the percentage of diseased plants was reduced significantly ($P=0.05$) in the compost-amended mix fortified with T382 compared to in the uninoculated control (Khan et al. 2002). T382 was as effective as a drench with Mefanoxam (a systemic fungicide specific against *Phytophthora*) or BTH (Actigard; Benzothiodiazole). The inoculated substrate also consistently suppressed the severity of bacterial leaf spot on radish seedlings caused by *Xanthomonas campestris* cv *armoraceae*. T382 was as effective against disease as drenching of plants with BTH. In split root bioassays, inoculation of *Trichoderma hamatum* 382 into a compost-amended potting mix significantly reduced the severity of *Phytophthora* root and crown rot on paired roots in the peat mix. Based on area under disease progress curves, T-382 also significantly reduced the severity of *Phytophthora* leaf blight in transplants produced in the compost mix compared with controls not inoculated with T-382. Efficacy of T-382 did not differ significantly from that provided by a drench with BTH. T-382 remained spatially separated from the pathogen in plants in both the split root and leaf blight bioassays, suggesting that these effects were systemic in nature (Khan et al., 2004).

Ribosomal RNA genes from the ISR-active *Bacillus* and *Pantoea* strains were sequenced and used for phylogenetic placement of these isolates. They also were used to develop specific PCR primers and DNA probes to monitor populations of these organisms in different batches of composted cow manure. The dynamics of the populations were determined during the composting process and during curing and utilization of the compost to develop a better understanding of how ISR activity develops naturally in composts (Krause et al., 2003).

Further experiments were conducted in both ISR active field plots and in potting media amended with ISR active composts and/or inoculated with ISR active isolates to identify differences in community structure and bacterial strains/groups that correlate with ISR activity. A new computer program was developed that compares the individual TRF sizes obtained from a compost, soil or other environmental sample to the TRF sizes generated by a simulated amplification and restriction digestion of ribosomal RNA genes contained in a ribosomal database of over 80,000 isolates and environmental clones. This program enabled complex TRFLP data to be used to determine which microorganisms correlate significantly with experimental treatments.

In field plots expressing ISR, bacterial communities in the rhizosphere of cucumber (*Cucumis sativus* L. Straight Eight) seedlings grown in microcosms were analyzed using TRFLP of PCR amplified 16S rRNA genes and by most probable number (MPN) analysis of culturable heterotrophic fluorescent pseudomonads in King's B medium. Populations of heterotrophic bacteria and fluorescent pseudomonads were significantly greater in the composted yard waste-amended plots expressing ISR than in control fertilized plots that did not induce SR. Unique TRF peaks detected among treatments suggest that specific subcomponents of the microbial communities differed. A higher number of TRFs corresponding with biocontrol agents such as *Pseudomonas* and *Pantoea* spp. were observed in plots mulched with compost. However, the mulch treatments had more pronounced effects on soil chemical and microbial properties than on the T-RFLP-based bacterial community structure in the cucumber rhizosphere. Nonetheless, the data show clearly that mulching with ISR inducing compost strongly influenced the structure of the microbial rhizosphere community (Tiquia, et al. 2002). These findings, utilizing T-RFLP analysis, were in agreement with earlier work utilizing FAME analysis (Boehm, et al. 1997, AEM 63:162-168).

In another set of experiments, microbial communities in the rhizosphere and in bulk potting mix of compost-amended potting mixes that consistently induce systemic resistance were characterized using T-RFLP analysis. The potting mixes were amended with composts and/or inoculated with *Trichoderma hamatum* T-382 (Grewal et al., 2005). The diversity, similarity and structure of the rhizosphere communities in five replicates of the four treatments were estimated by analyzing amplified 16S rRNA gene 3' and 5' terminal restriction fragment length polymorphisms after *Hha*1, *Msp*1 and *Rsa*1 digestions.

The TRFLP based analysis showed that while the diversity of microbial communities in potting mixes amended with composts and inoculants known to be ISR did not vary significantly with these treatments, bacterial community composition did vary with treatment as indicated by clustering of T-RFLP profiles and specific TRFs that were significantly different in the different treatments. Although some TRFs varied significantly with treatments, no specific members of the bacterial communities could be identified that consistently correlated with the ISR active mixes.

The succession of bacterial populations in suppressive potting mixes and the rhizosphere was also examined using denaturing gradient gel electrophoresis (DGGE) of PCR-generated DNA fragments, bacterial and fungal populations from potting mixture and plant samples. Cucumber plants were grown under greenhouse conditions in peat-based potting mixes amended either with composted wheat straw or sawdust-bedded cow manure. Several trends were detected in these analyses. First, An overwhelming dominance of members of the taxonomic group *Cytophaga-Flavobacterium-Bacteriodes* (now designated *Bacteroidetes*) was observed in most rhizosphere samples. Among the *Bacteroidetes* detected in this study, *Chryseobacterium* spp. were most frequently recovered. Our initial studies revealed that the distribution of *Chryseobacterium* varied significantly with treatment; in the peat-only treatment *Chryseobacterium* were not detected in analyses of the roots, while in compost treatments these populations were among the most persistent. Since prior studies have reported contradictory results regarding the survival of *C. gleum* in potting mixes amended with compost (Krause et al., 2001; Ryckeboer, 2001) we cannot definitively conclude that the compost is critical for the maintenance of bacteria from the genus *Chryseobacterium*. However, survival in the potting mix during plant development may be critical for root colonization by non-motile bacteria such as *Chryseobacterium*. We further explored the provenance and persistence of *Chryseobacterium* spp. Via a series of nested-PCR-DGGE analyses coupled with long 16S rDNA sequencing, we determined that the *Chryseobacterium* spp. detected on seed and root in compost-amended treatments were in fact derived from the compost itself. A similar provenance for bacteria closely related to the genus *Telluria* could not be made due to the high similarity of populations detected in control (peat-only) and compost-amended treatments. These population specific analyses revealed that the effect of compost amendment could vary according to bacterial population. For example, the *Chryseobacterium* spp. detected on seeds and roots were largely defined by those present in high

levels in the potting mixes; these populations were not particularly sensitive to plant development. Alternatively, the provenance of bacteria closely related to *Telluria* could not be determined; however, these populations were highly sensitive to shifts in environment and proximity to root. These results indicate a highly complex interaction between compost amendment and plant-associated microbial populations. However, we were also able to show that given adequate amendment of compost, certain microbial populations could be introduced and maintained in root environments, at least during the early development of plants (i.e. 3 weeks from sowing).

A two-tiered effect was observed for the development of bacterial populations. Profiles of bacterial populations from samples (seed, potting mix and root-rhizosphere) taken during the first week of the experiment showed a high level of similarity to those of the original potting mixes. However, bacterial populations that colonized seed surfaces on day 1 were generally absent from root bacterial communities on roots of 3 week-old seedlings. Thus, root-associated bacterial communities shared relatively little commonality with either the initial potting mix or seed-colonizing bacterial communities.

Overall, the effects of compost amendment to soils or potting mixes on plant surfaces appear to be less dramatic than those found in the initial soils or potting mixes. However, compost effects were not negligible. For example, seed colonizing populations in compost treatments were detected to varying extents on one week roots. By three weeks, only *Chryseobacterium* spp. detected in the original compost potting mixes were still detected in the root samples, while the *Chryseobacterium* sp. from the peat-only treatment was absent from the root. Conversely, in the peat-only treatment, profiles of seed and root bacterial communities shared only a single band, representing a population from the family *Oxalobacteraceae*. In addition to those populations detected in potting mixes, compost amendments modified the developing root-colonizing bacterial populations. For example, methylotrophic β -proteobacteria, detected in several other studies were detected exclusively in root samples from compost treatments. Conversely, a *Bacteriovorax* sp. was detected in the 1- and 3-wk root samples from the peat-only treatment; this population was not detected in root samples of cucumber grown in compost treatments. These and other populations detected only in compost-treatment seed or root samples, indicate that compost amendments can play a profound role in influencing plant-associated bacterial populations. The substantial differences in specific microbial populations

detected in root samples via sequence analysis were somewhat offset by broad similarities in bacterial populations between the treatments, when observed from low phylogenetic resolution.

In addition to general bacterial and fungal TRFLP and PCR-DGGE analyses, two additional molecular techniques were developed to aid in the analysis of samples. First, a general bacterial primer set was designed to mitigate the strong PCR amplification of plastid DNA in root samples. Secondly, a nested-PCR approach with general and specific PCR primers was employed to concomitantly analyze DNA samples. Via this nested-PCR technique (the basis of an in-submission manuscript), phylum level (*Bacteroidetes*) and genus level (*Chryseobacterium* and *Telluria*) phylogenetic information was generated for DGGE bands without sequencing. The specificity and validity of the methodology was confirmed via sequence analyses, but can be employed in future studies without such sequencing. The abundance of *Bacteroidetes* populations present on cucumber plant surfaces (seeds and roots) led to the search for specific primers for this large phylogenetic taxon. Primers already developed for this group were employed for population-specific studies.

As described previously, we have also developed a series of other primers (also applicable as probes) for identification of *Pseudomonas*, *Xanthomonas* and *Streptomyces* populations. These primer sets are currently being employed in identification of bacterial populations isolated from seed surfaces of cucumber seeds planted in compost-amended potting mixes. In addition, the results of primer specific analyses of *Pseudomonas* and *Streptomyces* populations using PCR and DGGE were presented at the Compost Utilization Symposium in Ohio this past year. We note, however, that *Pseudomonas* spp. were not dominant in our peat-based potting mixes even when amended with compost. Instead, beta-proteobacteria belonging to the genera *Telluria* and *Massilia* were detected to a much greater frequency. *Telluria* spp. were previously misidentified as *Pseudomonas* spp., and their presence in this environment would appear to fill the niche of fast-growing aerobic, heterotrophic rhizobacteria. We have also developed specific primers to detect these organisms.

Agricultural and/or economic impacts of the research findings.

The degree of ISR induced by uninoculated composts is highly variable and the basis for this effect is still not well understood. Results showed that only 1 of 79 potting mixes prepared with different batches of mature composts produced from several different types of solid wastes

suppressed the severity of bacterial leaf spot of radish caused by *Xanthomonas campestris* pv. *armoraciae* compared with disease on plants produced in a nonamended sphagnum peat mix. An additional batch of compost-amended mix that had been inoculated with *Trichoderma hamatum* 382 (T-382) also suppressed the disease. A total of only 11 out of 538 rhizobacterial strains isolated from roots of radish seedlings grown in these two compost-amended mixes that suppressed bacterial leaf spot were able to significantly suppress the severity of this disease when used as inoculum in the compost-amended mixes. The most effective strains were identified as *Bacillus* sp. based on partial sequencing of 16S rDNA. These strains were significantly less effective in reducing the severity of this disease than T-382.

A combined inoculum consisting of T-382 and the most effective rhizobacterial *Bacillus* strain was less effective than T-382 alone. A drench applied to the potting mix with the systemic acquired resistance-inducing chemical acibenzolar-S-methyl was significantly more effective than T-382 in several, but not all tests. We concluded that systemic suppression of foliar diseases induced by compost amendments is a rare phenomenon and that inoculation of compost-amended potting mixes with biocontrol agents such as T-382 that induce systemic resistance in plants can significantly increase the frequency of systemic disease control obtained with natural compost amendments (Krause et al., 2003). However bacterial ISR active isolates identified to date were not able to confer a response equivalent to that of T-382. The agricultural and economic impact of this research are that it has increased the ability of agricultural producers to control systemic plant diseases with composts that consistently induce systemic resistance.

APPENDIX: List of publications

Table of Contents

Published Peer-Reviewed Publications

1. Green, S.J., Freeman, S., Hadar, Y. and D. Minz. 2004. Molecular tools for isolate and community studies of *Pyrenomyces* fungi. *Mycologia* 96(3):439-451.
2. Green, S.J., Michel, F.C. Jr., Hadar, Y. and D. Minz. 2004. Similarity of bacterial communities in sawdust- and straw-amended cow manure composts. *FEMS Microbiol. Lett.* 233:115-123.
3. Khan, J., Ooka, J.J., Miller, S.A., Madden, L.V., Hoitink, H.A.J. 2004. Systemic resistance induced by *Trichoderma hamatum* 382 in cucumber against phytophthora crown rot and leaf blight. *Plant Disease* 88:280-286.
4. Krause, M.S., De Ceuster T.J.J., Tiquia S.M., Michel Jr., F.C., Madden L.V., Hoitink, H.A.J. 2003. Isolation and characterization of rhizobacteria from composts that suppress the severity of bacterial leaf spot of radish. *Phytopathology* 93:1292-1300.
5. Krause, M.S., Madden, L.V., Hoitink, H.A.J. 2001. Effect of potting mix microbial carrying capacity on biological control of *Rhizoctonia* damping-off of radish and *Rhizoctonia* crown and root rot of poinsettia. *Phytopathology* 91:1116-1123.
6. Tiquia, S.M., J. Lloyd, D.A. Herms, H.A.J. Hoitink, and F.C. Michel, Jr. 2002. Effects of mulching and fertilization on soil nutrients, microbial activity and rhizosphere bacterial community structure determined by analysis of T-RFLPs of PCR-amplified 16S rRNA genes. *Applied Soil Ecology* 21:31-48.

Submitted and In Press manuscripts (will be submitted to BARD after publication)

1. Green, S.J., Inbar, E., Michel, F.C. Jr., Hadar, Y. and D. Minz. 2005. Succession of bacterial communities during early plant development: transition from seed to root and effect of compost amendment. *Environmental Microbiology*. *submitted*.
2. Green, S.J., Michel, F.C. Jr., Hadar, Y. and D. Minz. 2005. Seed and root colonization by bacteria from the genus *Chryseobacterium* and the family *Oxalobacteraceae*. *To be submitted*.
3. Grewal, S., McSpadden B. Gardener, H. A. Hoitink and F.C. Michel Jr. 2005. Effects of compost and *Trichoderma hamatum* (T382) amendments that induce systemic resistance on rhizobacterial community structure in cucumber. *Biology and Fertility of Soils*. *submitted*.
4. Inbar, E., Green, S.J., Hadar, Y. and D. Minz. 2005. Competing factors of compost concentration and proximity to root affect the distribution of *Streptomyces*. *Microbial Ecology*. *In press*.