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United States Department of Agriculture–Agricultural Research Service research programs on microbes for management of plant-parasitic nematodes†‡

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Abstract: Restrictions on the use of conventional nematicides have increased the need for new methods of managing plant-parasitic nematodes. Consequently, nematode-antagonistic microbes, and active compounds produced by such organisms, are being explored as potential additions to management practices. Programs in this area at the USDA Agricultural Research Service investigate applied biocontrol agents, naturally occurring beneficial soil microbes and natural compounds. Specific research topics include use of plant growth-promoting rhizobacteria and cultural practices for management of root-knot and ring nematodes, determination of management strategies that enhance activity of naturally occurring *Pasteuria* species (bacterial obligate parasites of nematodes), studies on interactions between biocontrol bacteria and bacterial-feeding nematodes, and screening of microbes for compounds active against plant-parasitic nematodes. Some studies involve biocontrol agents that are active against nematodes and soil-borne plant-pathogenic fungi, or combinations of beneficial bacteria and fungi, to manage a spectrum of plant diseases or to increase efficacy over a broader range of environmental conditions. Effective methods or agents identified in the research programs are investigated as additions to existing management systems for plant-parasitic nematodes.
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1 INTRODUCTION

Concerns about public health and environmental safety have led to restrictions on chemical nematicide applications for the control of plant-parasitic nematodes. Cultural practices are also used for nematode management, but extensive annual losses in crop yields and quality[1] demonstrate a crucial need for new, environmentally friendly methods to enhance current management systems.

Application or manipulation of nematode-antagonistic microbes is one area being investigated to fill this need. Many microbes are active against plant-parasitic nematodes, and a few organisms, such as *Burkholderia*, *Paecilomyces* and *Myrothecium*, have been applied as biocontrol agents or cultured for production of active compounds.[2–9] A number of difficulties exist with development of biocontrol agents, including problems with culture and formulation, variable field performance, potential negative effects on non-target organisms, and expectations of broad-spectrum activity and rapid performance based on experience with chemical nematicides. Consequently, research programs at the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) are being pursued to contribute to development of efficacious biocontrol for plant-parasitic nematodes. These programs encompass a variety of approaches, including studies on application of microbial pest control agents, enhancement of naturally occurring beneficial soil microbes, interactions of biocontrol agents with other soil micro-organisms, use of microbially produced active compounds, and integration of biocontrol with other management techniques. Examples of research in these areas are provided in this paper.

2 BENEFICIAL BACTERIA

Bacteria exhibiting activity against plant-parasitic nematodes have potential for use as microbial

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pest management agents. In one programme, ARS researcher Nancy Kokalis-Burelle (US Horticultural Research Laboratory, Fort Pierce, FL) is working with university and private industry colleagues to study the combined effects of plant growth-promoting rhizobacteria (PGPR), soil-less transplant media, chitin and soil solarization on plant-parasitic nematodes and other pathogens. The focus of the work is transplanted crops, including tomato, pepper, strawberry, watermelon, muskmelon and cucumber.[10–13] For example, in Florida field trials with tomato and pepper, seedlings were grown in a potting mix with one of five formulations of PGPR, each mix containing *Bacillus subtilis* (Ehrenberg) Kohn, another *Bacillus* species or strain, and chitin.[11] The seedlings were transplanted into soil containing various soil-borne pathogens; *Meloidogyne incognita* (Kof & White) Chitwood (root-knot nematode) was the predominant nematode. The soil was untreated, or pretreated with either soil solarization or methyl bromide. The PGPR generally enhanced plant growth and improved transplant survival. PGPR treatments did not decrease *M incognita* galling of tomato, but one formulation significantly decreased the number of root-knot nematode galls on pepper.[11] Pepper yields with one PGPR formulation plus solarization were equivalent to yields with methyl bromide. The PGPR research program led to the development of a commercial transplant mix (Bio Yield™ Gustafson LLC) containing *Paenobacillus macerans* and *Bacillus amylolique-faciens* for use against plant-parasitic nematodes and other soil-borne pathogens on tomato, bell pepper and strawberry.[14,15]

Studies are also being conducted by Andrew Nyczepir (ARS Southeastern Fruit & Tree Nut Research Laboratory, Byron, GA) and university researchers on bacteria as biocontrol agents for the ring nematode, *Mesocriconema xenoplax*, on peach tree roots. In the southeastern USA, ring nematode infection predisposes peach to cold injury, bacterial canker or both, resulting in the disease complex peach tree short life (PTSL).[16] The root-colonizing bacterium *Pseudomonas* sp BG33R, which was isolated from a peach orchard suppressive to ring nematode, was found to inhibit ring nematode egg hatch *in vitro*, and to suppress reproduction in greenhouse tests.[17,18] When peach seedlings were planted in solarized field plots and inoculated with BG33R, the solarization-BG33R combination appeared to have a synergistic effect on management of *M xenoplax*.[19] In addition, population levels of *M xenoplax* remained at or below the South Carolina nematicide treatment threshold level for about eight months after planting trees in solarized soil-BG33R combination treatments, indicating that the tested treatments may be beneficial for nematode management (Reference 20 and Nyczepir AP, 2002, pers comm). An orchard experiment is currently investigating delivery of the beneficial bacterium BG33R through an irrigation system to trees established in solarized soils.

Biocontrol agents are often variable in field efficacy, and many biotic and abiotic factors can contribute to this phenomenon. Studies on microbial interactions and on soil ecology contribute to understanding the reasons for success or failure of biocontrol agents, and can lead to development of methods for enhancing the usefulness of beneficial microbes. In one such study, Lynn Carta (ARS Nematology Laboratory, Beltsville, MD), examined the interaction between biocontrol bacteria and bacterial-feeding nematodes. These beneficial nematodes, which recycle nutrients, and may thereby enhance plant productivity,[21,22] may consume beneficial microbes or be the target of microbes applied against plant-parasitic nematodes. In Petri dish tests, bacterial-feeding nematodes from various genera were placed on isolates of the bacterium *Burkholderia cepacia* Yabuchi *et al* ex Burkholder (all isolates with known biocontrol activity).[21] Responses to the *B cepacia* isolates ranged from normal to poor population growth; some nematode-bacterial isolate combinations resulted in distortion of eggs, and sluggish movement, loss of intestinal reserves, vulval eversion and premature death in adults (Fig 1).[21] When nematodes were presented with both *B cepacia* and *Escherichia coli* as food sources, preference for feeding on *B cepacia* varied with the tested nematode and the *B cepacia* isolate (independent of ability of an isolate to support nematode growth); variance with bacterial isolate is demonstrated with data from the nematode *Caenorhabditis elegans* (Maupas) Dougherty (Table 1).[21] Two of the tested nematodes, *Zeldia punctata* and *Pristionchus pacificus*, grew well when consuming the *B cepacia* isolates, and had good potential to reduce populations of the biocontrol isolates.[21] As knowledge increases in this area, effective selection of management agents may eventually take into account the taxonomy of predominant types of beneficial nematodes found in a field.

Examples of ARS research on the ecology of natural management agents include studies on the obligate nematode parasites in the bacterial genus *Pasteuria*. Enhancement of this naturally occurring antagonist could help to reduce yield losses without substantially increasing pest-control costs. Soybean cyst nematode (*Heterodera glycines* Ichinohe), the most economically important pathogen of soybean,[23] has been the subject of ARS *Pasteuria* studies. Field research conducted by Gregory Noel (ARS Soybean/Maize Germplasm, Pathology and Genetics Research Unit, Urbana, IL) and colleagues demonstrated that a species of *Pasteuria* kept *H glycines* populations below damage threshold levels.[24] A joint university-ARS project in Urbana that included Noel and Leslie Domier also characterized this *Pasteuria* species phylogenetically with 16S rDNA.[25] Another research programme involving *Pasteuria* focuses on the root-knot nematode *Meloidogyne arenaria* (Neal) Chitwood, which can reduce peanut yields 1–15% annually in the southern USA.[26] Patricia Timper (ARS Crop Protection and Management Research

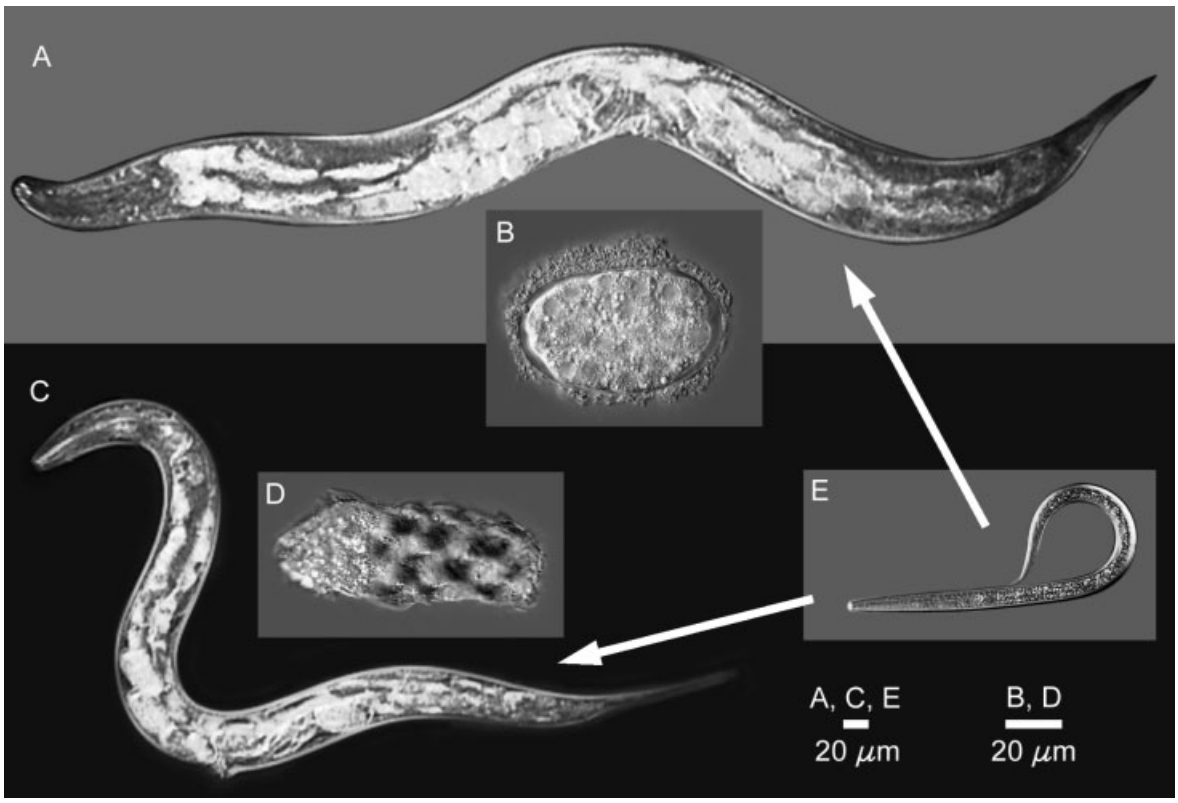

Figure 1. *Caenorhabditis elegans* (A, C) adult hermaphrodites and (B, D) eggs at early gastrula stage 48 h after (E) dauer juveniles fed on (A, B) *Escherichia coli* OP50 or (C, D) *Burkholderia cepacia* J82. Adults are reduced in size and eggs are distorted after feeding on *B cepacia*.

Table 1. *Caenorhabditis elegans* population growth on the bacteria *Escherichia coli* and *Burkholderia cepacia*, and attraction to the bacteria for feeding

Bacteria	Median number of nematodes per Petri plate on each bacterial isolate[a]	Nematode accumulation relative to *E coli* (attracting activity index)[b]
E coli OP50	44 358 A	0 AB
B cepacia Bc2	40 609 A	+3.5 A
B cepacia M36	38 725 A	−5.1 AB
B cepacia J82	2872 AB	−6.1 B
B cepacia BcF	1487 B	−3.4 AB
B cepacia PHQM 100	954 B	−5.2 AB

[a] For every *B cepacia* isolate, five young adult nematodes were placed on each of four bacterial plates. All living and dead progeny were counted after seven days at 24 °C, and each treatment was repeated at a later date ($n = 8$). Median nematode population numbers per Petri plate were then calculated. Medians followed by the same letter are not different ($P \leq 0.05$) with Tukey's pairwise multiple comparison procedure.

[b] Preferential accumulation. One hundred nematodes were dropped at the centers of 100-mm diameter Petri plates of 2% water agar, each plate containing opposing 30-μl spots of *B cepacia* and *E coli* OP506 (spots were located 6 cm apart from each other). Ten Petri plates were used per nematode–bacterial isolate combination. Nematode counts in the bacterial spots were made after 4 h. The attracting activity index was calculated as $(N_t - N_c)/N_c \times 10$, where N_t = nematodes accumulating in *B cepacia* spot and N_c = nematodes accumulating in *E coli* control spot. 0 = no preference, + = preference for *B cepacia*, and − = preference for *E coli*. Medians followed by the same letters are not different ($P \leq 0.05$) with Dunn's pairwise multiple comparison procedure.

Unit, Tifton, GA) and colleagues determined that the species *Pasteuria penetrans* (Thorne) Sayre & Starr is present in most Georgia peanut fields (Timper P, 2001, pers comm), and can build to levels at which the soil becomes nematode-suppressive.[27–29] In a field study, aldicarb did not affect numbers of *Pasteuria* endospores per nematode, but *Pasteuria* density was affected by crop rotation sequence (Fig 2A).[30] Rotations including poor hosts for root-knot nematode reduced endospore densities. In addition, research demonstrated that increasing *Pasteuria* populations suppressed root-knot nematode reproduction (Fig 2B).[31] Crop rotations that enhance levels of the naturally occurring biocontrol agent are being investigated.

3 BENEFICIAL FUNGI AND FUNGUS/BACTERIUM COMBINATIONS

Morphological studies and research on rhizosphere colonization have also been conducted to investigate the ecology of natural management agents. Susan Meyer and William Wergin (Nematology Laboratory, Beltsville, MD) used conventional

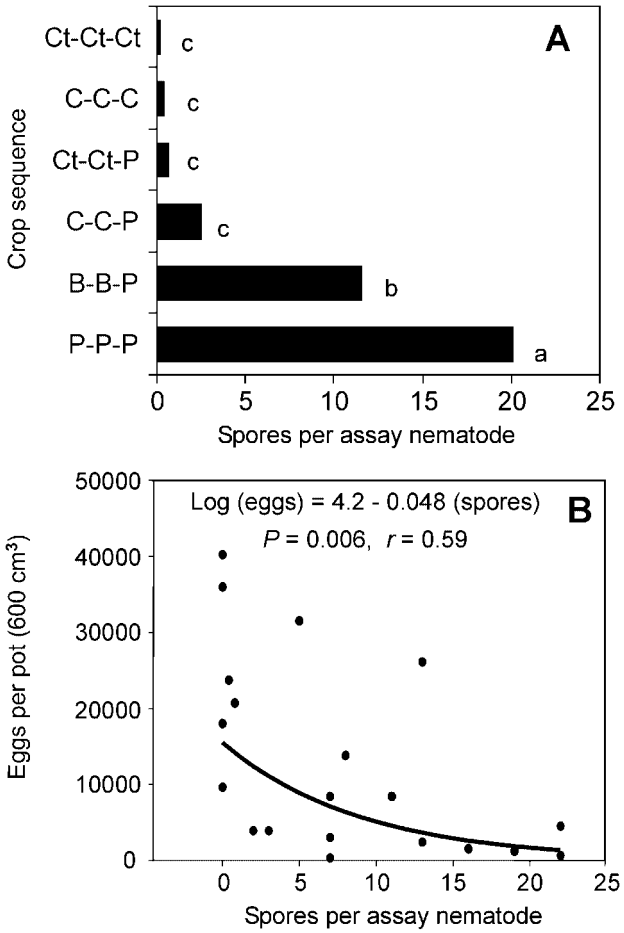

Figure 2. (A) Abundance of endospores of the bacterium *Pasteuria penetrans* following different crop sequences in field plots. Crops were (P) peanut, (B) bahiagrass, (C) corn, and (Ct) cotton. Bars with the same letter are not different ($P > 0.05$). Assay nematodes were second-stage juveniles of *Meloidogyne arenaria*; spore abundance was estimated by adding greenhouse-cultured *M arenaria* to soil–water suspensions of soil collected from the field plots. The number of spores per nematode was counted 24 h after addition of the nematodes to the suspensions. (B) Effect of spore abundance on reproduction of *M arenaria* on peanut in the greenhouse. Peanut seedlings were planted in the greenhouse into soil collected from the different field plots used in the rotation experiment. Each seedling was inoculated with 6000 eggs of *M arenaria*. The number of *M arenaria* eggs per pot was determined by extracting eggs from roots 60 days after planting; spore counts per assay nematode were made from second-stage juveniles of *M arenaria*.

and low-temperature scanning electron microscopy methods, combined with light microscopy, to demonstrate that a fungus active against plant-parasitic nematodes, *Verticillium lecanii* (Zimmerman) Viegas, colonizes *H glycines* females and cysts, and that parasitism of unhatched eggs is not a primary mode of action.[32] Daniel Roberts (ARS Sustainable Agriculture Systems Laboratory, Beltsville, MD) and Meyer also applied computer imaging techniques to study spatial distribution of *V lecanii* propagules in the soil and rhizosphere, demonstrating that the fungus is a poor colonizer of the soybean rhizosphere; this may in part explain the variable activity of *V lecanii* when applied against nematodes in soil.[33] These approaches can also be used to study the activity of other microbes and can therefore aid in identifying methods for enhancing biocontrol efficacy.

Application of microbes active against nematodes and other plant pathogens, or of biocontrol microbe combinations, could improve the efficacy of a biocontrol formulation, allowing for activity against more pathogens or over broader environmental conditions. Research in this area involved the fungus *Trichoderma virens* (Miller, Giddens & Foster) v Arx and the bacterium *B cepacia*. Both of these organisms are commercially formulated for management of fungi; *Burkholderia* is also sold for control of nematodes. Meyer *et al* tested strains of these organisms individually against the root-knot nematode *M incognita* on tomato, and also applied strains singly and in combinations against *M incognita* on bell pepper.[34,35] In these studies, individual microbes suppressed nematode populations on pepper compared to untreated controls, but did not suppress *M incognita* populations on tomato roots. The tested combinations were less effective against root-knot nematode on pepper than individually applied agents.[35] With careful crop selection, the individual test organisms might be applied for suppressing multiple plant pathogens, including plant-parasitic nematodes. To aid in finding methods for increasing the usefulness of introduced microbial agents, beneficial microbes will continue to be tested for activity against multiple soil-borne diseases or pests, and combinations of biocontrol agents will also be evaluated.

4 NATURAL COMPOUNDS

Active natural products for use against nematodes are being sought from microbes maintained in USDA collections. Fungi from two locations, the ARS Collection of Entomopathogenic Fungi (ARSEF) and the ARS Beltsville Nematology Laboratory collection of nematode-associated fungi, are being screened to identify environmentally friendly, biobased nematicides. Since many of the ARSEF fungi have not been evaluated for secondary metabolites, Donna Gibson (Plant Protection Research Unit, US Plant, Soil and Nutrition Laboratory, Ithaca, NY) and colleagues are using a molecular approach to identify those fungi that have the genes to produce two biologically active classes of compounds, polyketides[36] or peptides (Gibson DM, 2001, pers comm.) A study conducted by Meyer and colleagues at Beltsville focuses on 253 fungal isolates collected from *H glycines* eggs in China (centre of origin of soybean and soybean cyst nematode). When culture filtrates from these isolates were assayed in microwell plates for activity against *H glycines* and *M incognita*, results from active compounds in the filtrates included decreased mobility of hatched juveniles and effects on egg hatch ranging from stimulatory to inhibitory (Fig 3).[37–39] Active compounds (trichothecenes and flavipin) were identified from two of these isolates by Beltsville

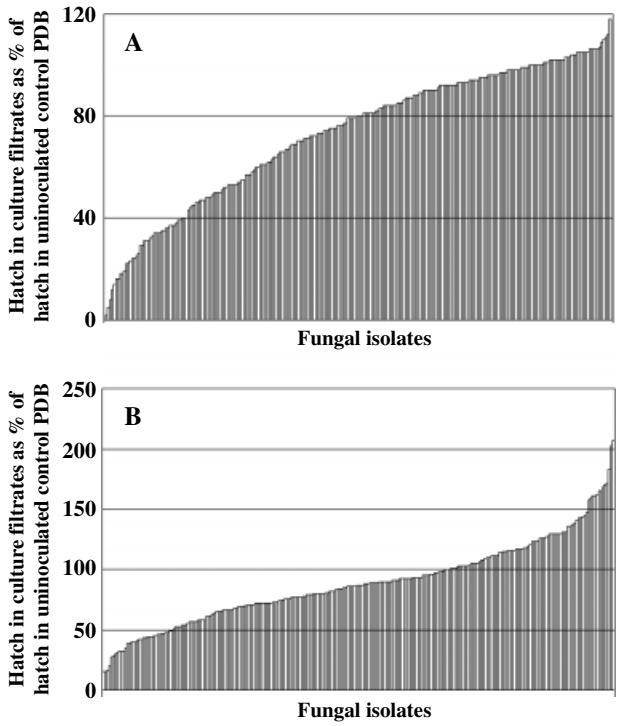

Figure 3. A total of 253 fungal isolates were collected from soybean cyst nematode (*Heterodera glycines*) in China, and filtrates from broth cultures of these fungi were assayed *in vitro* (in 24-well tissue culture plates) for presence of compounds active against (A) *Meloidogyne incognita* (root-knot nematode) and (B) *H glycines*. Culture broth filtrates were prepared by growing each fungus for 7 days in potato dextrose broth (PDB) on a rotary shaker, followed by removal of biomass with centrifugation and then filtration. Each vertical bar represents activity of filtrate from one fungal isolate, as percent egg hatch in the culture broth filtrate compared with egg hatch in uninoculated PDB. Affects of the filtrates on egg hatch of each nematode ranged from inhibitory to stimulatory.

researcher James Nitao and colleagues, demonstrating that the assay system assists in detecting chemicals affecting plant-parasitic nematodes.[40,41] Research is continuing on the isolates in this collection, to determine whether active compounds can be identified that have potential for enhancing current management practices for plant-parasitic nematodes.

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