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I am submitting herewith a thesis written by Katrina C. Burns entitled "Compatibility of the entomopathogenic nematode, *Steinernema carpocapsae*, with *Bacillus* spp. used in the biological control of plant pathogens." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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Compatibility of the Entomopathogenic Nematode, *Steinernema carpocapsae*, with *Bacillus* spp. Used in the Biological Control of Plant Pathogens

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Katrina C. Burns

December 1999

AG-VET-MED.

Thesis
99
.B87

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ACKNOWLEDGMENTS

I thank Dr. Roberto Pereira, who served as my major professor, for his assistance during this study, and for his guidance during the preparation of this thesis. I also am grateful to my graduate committee members, Dr. Ernest Bernard and Dr. Bonnie Ownley, for their advice and instruction.

Special thanks to Tina Richey, Mary Dee, and Pennie Jennings for their assistance with various areas. Also, thanks to Stephanie French, Melinda Gibbs, and Nongpanga Doungkeaw for their encouraging words. My most sincere thanks to Dr. Roberto Pereira and Dr. John Schneider for their invaluable assistance in the analysis and preparation of the data in this thesis.

Finally, I express my most sincere gratitude to my mother and family for their love, support, encouragement and patience during the course of this project.

ABSTRACT

Entomopathogenic nematodes in the order Rhabditida are bacteria-carrying nematodes that possess the ability to infect a variety of insects. They have generated interest due to their potential use as biological control agents of insects. Biological control of plant pathogens has also received increasing attention in recent years. Several *Bacillus* spp. can improve plant growth by suppressing major and minor pathogens and possibly by direct stimulation of plant growth.

The compatibility of entomopathogenic nematodes and Bacilli with biological control activity against plant pathogens, are examined in this research project. The objective of the first experiment was to determine whether bacterial isolates with biological control activity against plant pathogens would attract or repel an entomopathogenic nematode, *Steinernema carpocapsae.* A choice test was used with culture medium plugs cut from plates inoculated with *Bacillus* spp., or bacteria-free nutrient agar plates. Approximately 200 to 400 infective juveniles were released at a point distant from these plugs. Nematodes in the vicinity of the control or treatment plugs were counted at 4 and 8 hours after release. The average percentage of infective juveniles that were attracted to the treatments was 31%, with a range of 20 to 43%. *Bacillus* isolate BA77 was least attractive to the nematodes and E69 was the most attractive. The nine *Bacillus* isolates with biological control activity against plant pathogens attracted *S. carpocapsae*, but only minimally.

The objective of the second experiment was to monitor the development and reproduction of *S. carpocapsae* on four of the *Bacillus* isolates tested in the previous

experiment (BA77, E69, E727, and E61). *Steinernema carpocapsae* population growth was adversely affected in the presence of BA77, and the other isolates supported minimal nematode development when feeding on the tested bacteria. The results indicate that *S. carpocapsae* may not be able to develop and reproduce on the tested bacteria.

Entomopathogenic nematodes should not be introduced into soils containing the *Bacillus* isolate BA77, and to some degree E727. To integrate isolates E69 or E61 with entomopathogenic nematodes, the nematodes should probably be introduced first. Further investigations are necessary before the type of relationship (competition, antagonism, synergism) between entomopathogenic nematodes and the Bacilli examined is determined.

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CHAPTER 1

Literature Review

INTRODUCTION

Current societal pressures to decrease chemical pesticide usage has resulted in an increased amount of interest on alternative means of pest control. Some of the pressure stems from the adverse effects that some chemical pesticides have on the environment. Reports on the harmful effects of chemical pesticides on nontarget organisms, and the increasing number of insects developing resistance to the chemicals that once kept them under control, are common in the literature.

Since chemical pesticides became available, their use has had both positive and negative results. The positive aspects of chemical pesticide use include the production of fruits and vegetables that are free of insect damage and pleasing to the eye. Some problems with chemical pesticides are: 1) their adverse effect on nontarget organisms and the environment including humans, and 2) their promotion of pest resistance and resurgence of pest populations. The societal cost associated with the use of chemical pesticides is still an unanswered question.

Biological control is a promising alternative control option because it is an environmentally sound approach to pest control. It is defined as a method of pest control that employs parasitoids, predators and pathogens to reduce pest populations (Metcalf and Luckmann, 1994). A notable success story with biological control occurred in 1888 with the control of the cottony-cushion scale, *Icerya purchasi* Maskell. California citrus orchards were being heavily attacked by this introduced pest. To find a natural enemy,

two researchers visited Australia and brought back to the United States several potential options to control the cottony-cushion scale. The vedalia beetle, *Rodolia cardinalis* Mulsant, and a parasitic fly, *Cryptochaetum iceryae* (Williston), reduced the scale population two years after their introductions and are attributed with the project success. The increased use of biological control in agricultural systems has the potential to reduce chemical pesticide residues in groundwater, on fruits and vegetables, and at reduced production costs (Metcalf and Luckmann, 1994).

Entomopathogenic nematodes in the order Rhabditida are bacterium-carrying nematodes that possess the ability to infect a variety of insects (Gaugler and Kaya, 1990). They have generated interest due to their potential use as biological control agents. They usually have a broad host range under laboratory conditions, a high reproductive potential, and are exempt from registration as a pesticide in the United States (Gaugler and Kaya, 1990). There are about 40 nematode families that are associated with insects, of which two, Steinernematidae and Heterorhabditidae, generate the most interest (Gaugler and Kaya, 1990; Metcalf and Luckmann, 1994). Steinernematidae was described by Steiner in 1923 and Poinar described Heterorhabditidae in 1976.

Biological control of plant pathogens also has received increasing attention in recent years. Numerous organisms have been studied as potential biological control agents, including several isolates of bacteria in the genus *Bacillus* (Brannen, 1998; Fernando and Lindermann, 1995; Hebbar et al., 1992). Their potential for use as biological control agents is high because these bacteria are able to produce endospores that are heat and dessication-tolerant. Several *Bacillus* spp. are thought to improve plant growth by

suppressing major and minor pathogens and possibly by direct stimulation of plant growth (Ownley and Smith, 1998). They also have exhibited a high tolerance to many chemical and physical control tactics. *Bacillus* spp. belong the kingdom Procaryote, division Firmicutes and family Bacillaceae (Lacey, 1997).

GENERAL NEMATODE CHARACTERISTICS AND BRIEF HISTORY

Nematodes are one of the most abundant groups of multicellular organisms on earth (Poinar, 1983). In general, nematodes are characterized as minute, wormlike invertebrates with bilateral symmetry. They lack appendages, are unsegmented, and have a body cavity, a complete digestive system, a well-developed nervous system, an excretory system, and a set of longitudinal muscles that aid in locomotion (Poinar, 1983). They possess a noncellular, flexible and multilayered cuticle that protects them from predators and the environment. This cuticle is composed of various layers which are shed four times during their lifetime (Poinar, 1983). They occur in various habitats ranging from hot springs to polar areas. Their beneficial attributes include the ability to serve as biological control agents and their use in a wide array of experiments investigating genetics (Gaugler and Kaya, 1990). Harmful nematodes parasitize crops, humans, and livestock.

The date when the first nematode was described is uncertain because earlier they were earlier referred to as roundworms (Poinar, 1983). It is thought that nematodes arose in the Precambrian or Cambrian periods (Poinar, 1983). The first nematodes to receive attention were human parasites and were observed around 1550 B. C. (Poinar, 1983). In 1835, Owen discovered that nematodes were the causal agents of trichinosis (Poinar,

1983). The first free-living nematode to be recognized, the vine eelworm, was found in homemade vinegar in 1656 (Poinar, 1983) and the first plant parasitic nematode was described by Needham inside a diseased wheat kernel (Poinar, 1983). Early on, nematodes were placed in the Kingdom Vermes along with the other worms, but currently they are classified in the Kingdom Animalia and Phylum Nematoda or Nemata.

ENTOMOPATHOGENIC NEMATODES

Two genera of entomopathogenic nematodes are characterized by their mutualistic association with bacteria in the genera of *Xenorhabdus*, in steinernematid nematodes, and *Photorhabdus*, in heterorhabditid nematodes. These nematodes go through several developmental stages including an egg, four juvenile stages, and an adult (Poinar, 1983; Gaugler and Kaya, 1990). Only the third-stage infective juvenile is free-living and is capable of surviving outside an insect host (Gaugler and Kaya, 1990). This stage is ensheathed in the cuticle of the second-stage juvenile, which protects the nematode from adverse environmental conditions (Gaugler and Kaya, 1990). The infective juvenile enters the host via natural openings such as the mouth, spiracles and anus. The third-stage infective juvenile carries mutualistic bacteria in its intestine and releases them into the hemolymph of the host upon entrance (Gaugler and Kaya, 1990). Once inoculated into the insect hemolymph, the bacterial cells multiply and kill the host within 72 hours by septicemia. The environment inside the dead insect provides a suitable habitat for nematode development and reproduction for at least three generations (Gaugler and Kaya, 1990). Inside the host the nematodes develop into adults, mate, and lay eggs that hatch

and develop to the third-stage infective juvenile, then exit the insect cadaver in search of new hosts. This life cycle is completed within 6 to 18 days, depending on the nematode species and environmental conditions (Georgis and Kelley, 1997; Gaugler and Kaya, 1990). Steinernematids reproduce amphimicticly, which requires both sexes, whereas amphimictic and hermaphroditic reproduction (only females are required) occurs in heterorhabditids (Gaugler and Kaya, 1990).

Gotz et al. (1981) discovered that entomopathogenic nematodes possess immune inhibitors to prevent the host from exhibiting any defense mechanisms against the nematode and its bacterial symbionts. Immunized (with injection of *Enterobacter cloaca* cells) and normal diapausing pupae of the cercropia moth were examined. Various strains of *Steinernema carpocapsae* symbionts, *Escherichia coli,* and *E. cloaca* were tested. When axenic nematodes and *Xenorhabdus nematophilus* were introduced into immunized pupae, the bacterium became established. These results suggest that *S. carpocapsae* inhibited the antibacterial proteins of the Cercopia moth allowing *X. nematophilus* to become established.

THE MUTUALISTIC BACTERIA

The bacteria associated with entomopathogenic nematodes are Gram-negative, facultative anaerobes that belong to Enterobacteriaceae (Gaugler and Kaya, 1990). They do not form spores and have not been found in nature without being associated with a nematode (Georgis and Kelly, 1997). The bacteria are dimorphic under *in vitro* conditions; the forms are designated as Phase I and Phase II. Though both phases are pathogenic to

insects, Phase I is more suitable for nematode development (Gaugler and Kaya, 1990; Jackson et al., 1995). The differences in the two phases include colony morphology, absorption of dyes, and antibiotic production (Gerritsen and Smits, 1997). Normally, only Phase I is transmitted to the host, though both variants are equally pathogenic. Akhurst (1983) conducted a study to determine the significance of the relationship between the two forms of *X. nematophilus* in six strains of *S. feltiae*, nine strains of *S. bibionis*, one strain of *S. glaseri*, three strains of *Heterorhabditis bacteriophora*, and six strains of *Heterorhabditis* spp. Each bacterium examined exhibited dimorphism. Phase I was preferred by the infective juveniles and supported greater reproduction than Phase II. The role of Phase II was not identified.

The nematode benefits from the relationship with the bacteria because the bacterium kills the host creating: 1) a suitable environment for the nematode, 2) the breakdown of host tissues into usable nutrients, and 3) a food source. According to Akhurst and Boemare (1988), *Xenorhabdus* species support entomopathogenic nematode development and reproduction by producing nutrients and antimicrobial agents that inhibit the growth of a wide range of organisms. The bacterium benefits in the relationship with the nematode because: 1) it is protected from the environment, and 2) it is transported to suitable hosts to ensure its proliferation.

Akhurst and Boemare (1988) studied the numerical taxonomy of 21 strains of *Xenorhabdus*. The objectives of their study were the identification of the significance of phase variants of *Xenorhabdus* spp. and the evaluation of taxonomic relationships within the genus. A close correlation existed between taxonomic groupings of *Xenorhabdus* spp.

and those of their entomopathogenic nematode associates. They also demonstrated that the phase variants of *Xenorhabdus* are important when characterizing the bacteria.

Jackson et al. (1995) identified a second bacterium present in some *Heterorhabditis* spp. This bacterium, *Providencia rettgeri*, infected *Galleria mellonella* more quickly than did the nematodes symbiont (*Photorhabdus* spp.) at 9°C. Although *P. rettgeri* was not used as a primary food source, it persisted in *Heterorhabditis*-infected cadavers.

Grewal et al. (1997b) examined Phase I variants of *Xenorhabdus* spp. to compare development of infective juveniles vs. non-infective juveniles and consequent reproduction of adults. The authors used a *Xenorhabdus* species from *S. scapterisci, X. nematophilus* from *S. carpocapsae*, and a *Xenorhabdus* species from *S. riobrave*. *Steinernema scapterisci* developed and reproduced on all the bacteria tested and also carried the bacteria in their intestine (Grewal et al., 1997b). In previous reports, *Steinernema* sp. and *Heterorhabditis* sp. were capable of reproducing on different symbiotic bacteria and other bacteria (Aguillera and Smart, 1993; Han et al., 1991). These findings suggest that entomopathogenic nematodes can be combined with species of *Xenorhabdus* other than their typical symbiont to augment their infectivity. A closely related study examined development and reproduction of five nematode species (*S. bibionis, S. feltiae, S. glaseri*, and two *Steinernema*. spp.) on monoxenic isolates of various *Xenorhabdus* spp. and *Photorhabdus luminescens* (Akhurst, 1983). None of the *Steinernema* spp. survived on *P. luminescens*, although all other bacteria supported development and reproduction. These findings also suggest that entomopathogenic nematodes can be recombined with bacteria

other than their ususal mutualistic symbiont.

Ehlers et al.(1990) fed axenic *S. carpocapsae* and *H. bacteriophora* both phases of *Xenorhabdus* spp. and *E. coli*. No significant difference occurred in reproductive ability when either phase of *Xenorhabdus* spp. or *E. coli* was introduced. However, in the *E. coli* trials, nematode development was delayed up to six days.

Dunphy et al. (1985) reared *S. glaseri* on Phase I forms of three subspecies of *X. nematophilus* including *X. nematophilus poinari*, *X. nematophilus nematophilus* var. *mexicanis*, and *X. nematophilus bovienii*. All isolates supported the development and reproduction of the nematode, but *S. glaseri* performed better on *X. nematophilus bovienii* than on its own mutualistic symbiont.

Gerritsen and Smits (1997) examined *Heterorhabditis* strains for their retention and selection of the two form variants of *P. luminescens*. The authors were not able to culture *H. bacteriophora* in the absence of its symbiont, although two *H. megidis* strains reproduced on the Phase I symbiont from *H. bacteriophora*. No significant difference was observed in selection of Phase I or Phase II by the nematode, but Phase I bacteria were the most abundant in the nematode intestine.

Ehlers et al. (1997) used crane fly and greater wax moth larvae infected with *S. feltiae* and its symbiont *X. bovienii* to determine how the nematode-bacterium complex was pathogenic. *Xenorhabdus bovienii* was highly pathogenic to *G. mellonella* but almost nonpathogenic to the crane fly. When axenic (intestine free of bacterial symbiont) and monoxenic (bacterial symbiont present in the intestine) nematodes were compared, monoxenic nematodes performed better, but axenic nematodes also could kill hosts.

Xenorhabdus bovienii was nonpathogenic to crane fly larvae, possibly because of the development of a defense mechanism in the insect hemolymph that prevented bacterial establishment.

Han and Ehlers (1998) examined the specificity of food signals from different *Photorhabdus* and *Xenorhabdus* isolates on development and reproduction of *H. indica* and *H. bacteriophora*. Neither nematode species developed or reproduced on *Xenorhabdus* isolates or on each others *Photorhabdus* symbiont. This finding suggested that there may be different strains of *P. luminescens*.

Non-mutualistic bacteria associated with entomopathogenic nematodes

There have been numerous reports on entomopathogenic nematode association with non-symbiotic bacteria (Aguillera and Smart, 1993; Grewal and Hand, 1992; Lysenko and Weiser, 1974). This relationship sometimes was more effective in killing insects than the normal mutualistic symbiont associations (Jackson et al., 1995). Poinar and Thomas (1966) tested *Pseudomonas aeruginosa*, *Bacillus cereus*, *Serratia marcescens*, and *X. nematophilus* to determine the importance of the nematode-bacterium relationship, using both axenic nematodes and host insects. *Galleria mellonella* larvae were first inoculated with 15 to 50 axenic *Steinernema* sp., then the bacteria were injected into the body cavity. Only *X. nematophilus* and *P. aeruginosa* supported nematode growth and reproduction. The authors concluded that the nematode-bacterium relationship is mutualistic, benefitting the nematode and the bacterium (Akhurst and Boemare, 1988; Poinar and Thomas, 1966).

Lysenko and Weiser (1974) showed that various quantities of *Alcaligenes*

odorans, P. flourescens, Xanthomaonas maltophilia, and *Acinetobacter* spp. are present in *Steinernema* spp. Most of the bacteria were isolated from the surface of the nematodes, indicating that bacteria can be transmitted in this manner into insect hosts. When compared to the nematode symbionts, *Xenorhabdus* spp., the other bacteria were not as pathogenic to insects. However, the authors hypothesized that these bacteria could be possible candidates for use in new nematode-bacterium combinations.

Xenorhabdus nematophilus, Pseudomonas sp., *Proteus* sp., *Alcaligenes* sp., and *Aerobacter* sp. were isolated from the intestinal tract of *Steinernema* sp. the DD-136 strain (Poinar,1966). As the nematodes fed on bacteria in the host cadaver, they also ingested other bacteria, but only the symbiont could persist in the intestine. However, Dunphy et al. (1985) and Grewal et al. (1997b) suggested that all bacteria could aid nematode development and reproduction.

Aguillera and Smart (1993) evaluated development, reproduction, and pathogenicity of *S. scapterisci* when associated with several bacteria. The bacterial species included *Ochrobactrum anthropi, Paracoccus dentrificans, E. coli, P. aureofaciens, P. flourescens* Biovar B, *Xanthomonas maltophilia, Xenorhabdus nematophilus,* and three *Xenorhabdus* spp. (two living isolates and one dead). All living bacterial isolates supported the development and reproduction of *S. scapterisci*. Therefore, this nematode would be a good candidate for mass production without its normal mutualistic symbiont.

Grewal and Hand (1992) examined the microbivorous rhabditid nematode *Caenorhabditis elegans* which is similar to *Steinernema* spp. and *Heterorhabditis* spp. in that it feeds on bacteria. The difference between *C. elegans* and the two

entomopathogenic nematodes is that *C. elegans* does not carry and is not associated with a bacterial symbiont. The authors isolated 10 different bacteria from *C. elegans: Acinetobacter calcoaceticus* var. *anitratus, B. cereus, Bacillus* sp., *E. amnigenus, E. cloacae, P. aeruginosa, P. agarici, P. fluorescens* biovar *reactans, Serratia liquefaciens,* and *X. maltophilia.* The *Bacillus* spp. inhibited nematode reproduction, while all other isolates supported development and reproduction.

BACILLUS SPP.

The use of *Bacillus* spp. with biological control activity against plant pathogens as biological control agents is receiving increasing attention (Ownley and McMahan, 1997; Brannen, 1998). Interest in *Bacillus* spp. is due to their unsurpassed spore resistance to chemical and physical agents, their developmental cycle, and biocontrol activity. *Bacillus* spp. are Gram-positive aerobic bacteria. Bacilli are found mostly in soil, but they have also been isolated from milk, plants (Skerman, 1967), and other materials. These bacteria contain round, oval, or cylindrical endospores and highly refractile structures within the bacterial cells. *Bacillus popilliae* was the first microbial agent registered in the United States for use as a biopesticide (Lacey, 1997). *Bacillus thuringiensis,* also an insect pathogen employed as a biopesticide, can be recognized by its formation of parasporal crystalline bodies.

Currently, scientists are evaluating other *Bacillus* strains that have activity against plant pathogens. Ownley et al. (1996) examined the effect of four *Bacillus* spp. on the management of target spot in tobacco. The authors found that disease severity was

decreased when the isolate BA55 was applied. Kim et al. (1997) obtained similar results when using *Bacillus* sp. L324-92. This *Bacillus* strain inhibited the development of three root diseases in wheat and increased yield. These results indicate that *Bacillus* species are effective control agents for plant pathogens.

Handelsman et al. (1990) isolated 700 bacterial strains from the roots of field-grown alfalfa plants to evaluate the biological control activity on seedling damping-off caused by *Phytophora megaserma* f. sp. *medicaginis*. The only isolate found to be effective was *B. cereus* UW85. This isolate reduced seedling mortality to zero and significantly increased the emergence of alfalfa. Thompson et al. (1996) tested 10 bacterial strains against summer patch in Kentucky bluegrass and found five effective strains. These included *P. fluorescens* 2-79 and 13-79, *B. subtilus* D-39Sr, and *E. cloacae* EcH-1 and EcCT-501. Over the course of four years, results varied from no reduction to approximately 50% reduction in disease severity. The authors concluded that more research was necessary to determine the persistence of introduced bacteria in the field.

ENTOMOPATHOGENIC NEMATODE ATTRACTION AND COMPATIBILITY WITH VARIOUS ORGANISMS

Entomopathogenic nematode attraction to various materials has been investigated in several studies (Agra Gothama et al., 1995; Grewal et al., 1997a; Kaya and Burlando, 1989; Khlibsuwan et al., 1992b; Koppenhofer and Kaya, 1997; Schmidt and All, 1979). Schmidt and All (1979) tested *S. carpocapsae*'s preference among insect feces and other components including uric acid, xanthine, allantoin, ammonia, arginine, and allantoic acid.

The nematodes were attracted to all tested components except allantoic acid. In a separate study, Schmidt and All (1978) showed that host detection by entomopathogenic nematodes involved insect-produced chemostimulants. Lepidopteran, dipteran and coleopteran larvae were examined and *S. carpocapsae* was attracted to all larvae tested. The authors also observed that a chemical gradient existed around the host which aided the nematode in locating the hosts.

Khlibsuwan et al. (1992b) tested the attractiveness of plasma from the cabbage worm, cutworm, and turnip moth to entomopathogenic nematodes. *Steinernema carpocapsae* was attracted to all plasma tested, but preferred the plasma of the cabbage worms.

Nematode behavioral response to hosts and environmental cues was tested by Wang and Gaugler (1998). *Steinernema glaseri* and *H. bacteriophora* were tested for their attraction to whole grass roots, wounded grass roots (cut into small pieces), grub gut fluid, and hemolymph. Both nematode species were attracted to grass roots. When wounded roots and whole roots were compared, the nematodes were attracted to wounded roots more strongly than to whole roots. This finding was important because Japanese beetles and possibly other soil-dwelling pests may ingest nematodes while feeding and thus increase entomopathogenic nematode infectivity.

Khlibsuwan et al. (1992a) evaluated the effect of several enzymes, chemicals and temperature treatments on attraction or repellence of *S. carpocapsae* to the plasma of the common cutworm. The materials examined included lectins, enzymes, spermidine, cetyl trimethlammonium bromide, sodium metaperiodate, and sodium hypochlorite. No effect

on nematode migration to host plasma existed when any lectin, enzyme, or the spermidine was introduced. Sodium hypochlorite and metaperiodate ions repelled nematodes. These findings suggest that other saccharides may be involved in mediating nematode attraction to host plasma. The authors concluded that carbohydrate moieties on the nematode cuticle and amphids were involved in mediating chemical signals from potential hosts.

The compatibility of two or more entomopathogenic nematodes

Since no one entomopathogenic nematode has been proven to be universally effective in field attempts, Kaya et al. (1993) tested the combination of two entomopathogenic nematodes with different search strategies against pests present at different levels of the soil. The nematodes examined were *S. carpocapsae* and *H. bacteriophora*, and the hosts tested were the black cutworm and the black vine weevil. The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) normally feeds at or near the soil surface, while the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) prefers plant roots. *Steinernema carpocapsae,* with the 'sit-and-wait' search strategy, performed well against the surface-feeding black cutworm, while *H. bacteriophora,* which actively cruises the soil profile in search of hosts, was effective against the black vine weevil. These results demonstrated how two entomopathogenic nematodes could be applied together to combat certain pest problems.

In experiments with *H. bacteriophora, S. feltiae, S. bibionis, S. glaseri* and lepidopteran hosts, Alatorre-Rosas and Kaya (1990) demonstrated that when infection by two nematode species occurred, both nematode species could not reproduce. However,

other results (Koppenhofer et al.,1995; Choo et al.,1996) contradict that finding and suggest that coexistence is possible and may result in an additive effect.

Grewal et al. (1997a) published a report on the interaction of several species of entomopathogenic nematodes in *G. mellonella*. *Steinernema anomaii, S. feltiae, S. glaseri, S. riobrave,* and *H. bacteriophora* were evaluated in parasitized and non-parasitized hosts. The *Steinernema* spp. did not show any recognition to hosts infected by conspecific and heterospecific nematodes. *Steinernema carpocapsae, S. glaseri,* and *S. anomaii* chose hosts containing conspecific nematodes over uninfected hosts. This type of recognition is thought to be controlled by odor-mediated responses.

The compatibility of fungi with entomopathogenic nematodes

Barbercheck and Kaya (1991) combined the fungus *Beauveria bassiana* with *H. bacteriophora* and *S. carpocapsae* to study what role the fungus may play in host detection. They observed that *S. carpocapsae* and *H. bacteriophora* preferred healthy hosts when given the choice between dead, fungus-infected, control, and healthy hosts. Neither *S. carpocapsae* nor *H. bacteriophora* chose *B. bassiana*-infected hosts. In another study, entomopathogenic nematodes and the fungus did not coexist within *G. mellonella* larvae that were treated with *B. bassiana* and exposed to *S. feltiae, H. bacteriophora,* or a combination of both one to five days later (Barbercheck and Kaya 1990). It is not known whether the nematodes or their symbiotic bacteria caused this behavior. The authors concluded that the relationship between *B. bassiana* and entomopathogenic nematodes is antagonistic.

The compatibility of chemical pesticides with entomopathogenic nematodes

Kaya et al. (1995) combined *H. bacteriophora* or *S. carpocapsae* with a pesticidal soap to determine their effect against the spotted cucumber beetle in the soil and all feeding stages of the cabbage aphid on plant foliage. Combining *H. bacteriophora* or *S. carpocapsae* with the pesticidal soap was effective in controlling the pests, so this combination could potentially be used in field control of certain pest populations.

Nishimatsu and Jackson (1998) combined entomopathogenic nematodes and chemical pesticides (tefluthrin, turbufos, or fonofos) to control the western corn rootworm. The highest mortality occurred when *S. carpocapsae* or *H. bacteriophora* was applied with tefluthrin. In a project testing the chemical pesticide oxamyl, Gaugler and Campbell (1991) determined that in the presence of oxamyl, host detection and infectivity by nematodes was negatively affected. Therefore, entmopathogenic nematodes should not be applied to an area where this chemical is present, and the equipment used to apply oxamyl must be thoroughly sanitized before applying nematodes.

The compatibility of viruses with entomopathogenic nematodes

Kaya and Brayton (1978) investigated *S. carpocapsae* development and reproduction in armyworms infected with a granulosis virus. *Steinernema carpocapsae* was capable of development and reproduction in healthy and granulosis virus-infected armyworms, and granulosis virus particles were found in the nematodes' lumen. Because the nematodes killed the armyworm prior to the granulosis virus becoming established, this pathogen combination could only be used in situations in which more than one pest is

creating problems.

Agra Gothama et al. (1995) reported on the use of *Spodoptera exigua* multinucleocapsid nucleur polyhedrous virus (SeMNPV) with *S. carpocapsae* to control the beet armyworm. Additive results were obtained with the combination when compared to mortality caused by each organism alone. The SeMNPV virus did not interfere with the reproductive ability of *S. carpocapsae*, thus it could possibly be used in conjunction with entomopathogenic nematodes.

The compatibility of Bacilli with entomopathogenic nematodes

Koppenhofer and Kaya (1997) combined *B. thuringiensis* ssp. *japonensis* with *S. glaseri*, *S. kushidai*, or *H. bacteriophora* and investigated their effect against *Cyclocephala hirta* and C. *pasadenae*. The combination of *B. thuringiensis* ssp. *japonensis* with the nematodes performed better than each nematode alone. The highest mortality rate was observed when the host was exposed to *B. thuringiensis* ssp. *japonensis* for at least one week before application of the nematode. This observation demonstrated that *B. thuringiensis* ssp. *japonensis* acts as a stressor to the host and allows the nematodes infection rate to increase. The authors suggested that the best management practice would be to apply *B. thuringiensis* ssp. *japonensis* initially and then apply entomopathogenic nematodes to reduce the pest population.

Bacillus popilliae also has been examined as a possible agent to be used in combination with entomopathogenic nematodes. Hurpin and Robert (1968) studied the effect of two or more disease organisms on an insect pest. The disease organisms *B.*

popilliae, *Rickettsiella melolonthae* and *Vagiovirus melolonthae* were applied in combinations or alone and insects were monitored biweekly. Their results showed that two microorganisms were capable of simultaneously developing within a single host. Thurston et al. (1993) used *B. popilliae* to enhance the infection of *C. hirta* by *H. bacteriophora* and to determine whether or not *H. bacteriophora* could develop and reproduce on *B. popilliae*. Grubs that were infected with *B. popilliae* were significantly more susceptible to *H. bacteriophora* than were healthy grubs. Nematode emergence from host cadavers that did not receive *B. popilliae* required two hours more than emergence from *B. popilliae*-infected grubs. Nematode development and reproduction were not supported by *B. popilliae*. *Bacillus popilliae* apparently acted as stressor to the host, which allowed easier infection by the entomopathogenic nematodes. In an additional study with greenhouse trials (Thurston et al.,1994), *S. glaseri* killed more *B. popilliae*-infected grubs than healthy grubs, suggesting that entomopathogenic nematodes may be attracted to *B. popilliae*-infected hosts.

Kaya and Burlando (1989) reported on the development of *S. feltiae* in hosts infected with *B. thuringiensis* subsp. *kurstaki* or nucleur polyhedrous virus. Nematodes performed well in nuclear polyhedrosis virus-infected hosts, but not in *B. thuringiensis* ssp. *kurstaki*-infected hosts. *Steinernema feltiae* did not respond well to *B. thuringiensis* ssp. *kurstaki* infected hosts, therefore infection was rarely initiated.

OBJECTIVES

There are no published reports on the compatibility of entomopathogenic nematodes with *Bacillus* spp. which have biological control activity against plant pathogens. In this study, the compatibility of entomopathogenic nematodes and bacterial isolates with biological control activity against plant pathogens was examined. The specific objectives of this study were to:

1) determine whether nine bacterial isolates with biological control activity against plant pathogens attract or repel an entomopathogenic nematode, *S. carpocapsae*;

2) monitor the development and reproduction of the nematodes on these nine bacterial isolates with biological control activity against plant pathogens.

CHAPTER 2

Attraction of *Steinernema carpocapsae* to Bacterial Isolates with Biological Control Activity Against Plant Pathogens

INTRODUCTION

Infective juveniles of entomopathogenic nematodes parasitize many types of insects (Gaugler and Kaya, 1990). They are not only stimulated by or drawn to various insect hosts, but also by other microorganinsms and signals emitted in the soil environment. For example, entomopathogenic nematodes are reportedly attracted to the fungus *Beauveria bassiana* (Barbercheck and Kaya, 1990), insect plasma (Khlibsuwan et al., 1992b), carbon dioxide (Gaugler and Kaya, 1990), and kale juice (Ishibashi and Kondo, 1989).

The sensory organs that aid entomopathogenic nematodes in locating stimuli and various hosts are the amphids and head papillae. The amphids are paired lateral organs that open on or near the lips of the nematode. Their size and shape vary depending on nematode species. The head papillae are cuticular elevations that are also located near the mouth. They are minute organs that assist the nematode in exploring its surroundings.

The mechanisms by which entomopathogenic nematodes locate stimuli and hosts vary among nematode species. Various factors affect host detection such as soil texture, moisture, and temperature. These factors may be more important for entomopathogenic nematodes known as "cruisers", which are nematodes that actively search for their hosts.

Steinernema carpocapsae is considered an "ambusher" because its search strategy consists of sitting and waiting, often rearing straight up on their tails, for a host to come to them. They attack the host as it passes by.

Entomopathogenic nematode behavior, development, and reproduction are affected in various ways when the nematodes are placed in favorable or unfavorable environments. According to some authors, many interactions between entomopathogenic nematodes and various soil constituents are antagonistic (Ishibashi and Choi, 1991; Kaya and Burlando, 1989). Ehlers et al. (1990) reported that in the presence of *Escherichia coli*, *S. carpocapsae*'s development is delayed up to six days. When the chemical pesticide oxamyl is present, entomopathogenic nematodes activity and movement increases. However, this chemical pesticide is not compatible with the nematodes because it adversely affects host detection and infectivity.

Choice tests

Choice tests are an efficient and effective screening procedure that can help determine compatibility, attraction, and various other aspects of the interaction between entomopathogenic nematodes and other elements in the environment. Choice tests are widely used in experiments evaluating entomopathogenic nematode behavior, food preference, and other variables. In a choice test performed by Schmidt and All (1979), the chemical components of feces from several insects were examined. The bioassay consisted of a test chemical on 1-cm^2 filter paper vs. a control (water only) or another chemical. The paper squares were placed 2 cm apart in a 100 x 20 cm petri dish containing 0.5% agar.

After 24 hours, infective juveniles were placed between the two squares. Following an additional 24-hour incubation, those juveniles within 0.5 cm of each square were recorded. The results indicated that infective juveniles were attracted to uric acid, xanthine, allantoin, ammonia, and arginine, but repelled by allantoic acid. The authors concluded that insect feces play an important role in entomopathogenic nematode host detection.

Barbercheck and Kaya (1991) slightly modified Schmidt and All's choice test to determine *S. carpocapsae's* response to dead, healthy, or fungus-infected hosts. The authors filled petri dishes with 0.5% water agar and inserted two pipette tips into two holes in the dish covers. The pipette tips held *Galleria mellonella* that were healthy, dead, or infected with *B. bassiana*. Empty pipette tips were used for controls. The dishes were held at 25°C for one day, and then 300 to 600 surface-sterile entomopathogenic nematodes were placed in the center of the dish. A square area was marked surrounding the pipette tips and nematodes inside each area were recorded after 24 hours. An average of 11% of the nematodes moved from the point of placement to their preferred host. *Steinernema carpocapsae* preferred healthy hosts. These results are significant because they show that entomopathogenic nematodes can distinguish diseased and stressed hosts from healthy ones.

The possible integration of insect control with entomopathogenic nematodes and plant pathogen control with bacteria raises the question of compatibility between these biological control agents. The relative attractiveness of bacterial isolates to entomopathogenic nematodes may affect how the nematodes perform against soil pests attacking plants treated with these biocontrol bacteria.

The objective of this experiment was to determine whether bacterial isolates with biological control activity against plant pathogens attract or repel the entomopathogenic nematode, *S. carpocapsae.*

MATERIALS AND METHODS

Nematode rearing

Steinernema carpocapsae All were obtained from Dr. Grover Smart, The University of Florida - Department of Entomology and Nematology. Nematodes were stored in sterile deionized water at 4°C throughout the duration of the experiment. Larvae of *Galleria mellonella*, the greater wax moth, were purchased from Grub Co. (Hamilton, OH) for *in vivo* production of *S. carpocapsae*.

The method for rearing *S. carpocapsae* was as follows. Between 10-25 living wax moth larvae were placed in an inverted petri dish with a 90-mm Whatman #1 filter paper in the lid. Approximately 200 infective juvenile nematodes were placed in the dish, and the dishes were held at room temperature or in a 24°C incubator for 5 days. After this incubation period, insect larvae which appeared yellowish brown and limp were moved to a White trap. This trap consisted of a 5.5 cm Fisherbrand filter paper that was draped over the lid of a 60x15 mm disposable petri dish inside a glass (100x15 mm) petri dish containing water. The White trap was kept in a 25°C incubator until infective juveniles emerged.

Infective juveniles emerged from the host, moved to the water, were extracted from the dish with a pipette and placed in a 12 x 75 mm test tube culture vial (Fisherbrand).

For use in the experiment, a dilution of the *S. carpocapsae* suspension from the White trap was prepared. An undetermined number of infective juveniles was pipetted out of the White trap. A single drop from the pipette was placed in a nematode counting dish containing 20 ml of distilled water, and the juveniles in the dish were counted. If the suspension contained more than 400 infective juveniles, a subsequent dilution was prepared by pipetting an undetermined number of juveniles from the nematode counting dish into a new container. The nematode suspension that was left in the nematode counting dish was placed in a 50-ml beaker and set aside. Subsequent dilutions were prepared in a similar manner until approximately 200 to 400 infective juveniles were obtained in a 20-ml sample. This dilution was set aside for future use.

***Bacillus* isolates**

Isolates of *Bacillus* spp. were obtained from Dr. B. H. Ownley, Department of Entomology and Plant Pathology - The University of Tennessee (Table 2.1). Cultures were maintained in the laboratory on nutrient agar media (Difco Laboratories, Detroit, MI) and stored at 4°C. When bacteria were needed for experiments, refrigerated cultures were removed from storage and streaked onto nutrient agar plates under a vertical laminar flow hood. Cultures were incubated for two days at 30°C to give a uniform layer of growth over the entire plate. Inoculated plates and an uninoculated nutrient agar plate were then randomly punctured with a sterile 0.5 cm cork borer. Cylindrical plugs of the

Table 2.1. *Bacillus* isolates used in choice test experiment and their identification using the Biolog System. **Source:** Smith (1997).

Bacterial isolates	Similiarity Index	Identified isolates
BA77	0.631	*Bacillus thuringiensis/cereus*
BA101	0.793	*Bacillus megaterium*
E726	0.505	*Bacillus subtilus* var. *globigii*
E727	0.388[y]	*Bacillus coagulans*[z]
E66	0.500	*Bacillus pasteurii*
E61	0.483[y]	*Bacillus megaterium*[z]
E69	0.662	*Bacillus coagulans*
E65	0.668	*Bacillus megaterium*
E21	0.706	*Bacillus brevis*

[y]Similarity index < 0.5 is not an accurate identification of species.
[z]Closest match, but not considered a valid identification.

culture medium, topped by a layer of bacterial growth were prepared for use in the choice tests. Control plugs obtained from the uninoculated plate had no bacterial growth.

Plate template

Choice test plates were prepared with a template also used when counting nematodes (Fig. 2.1). The template consisted of three equidistant 0.5 cm circles, 5.1 cm apart. These circles were used to mark the locations for release of the nematodes (N) , placement of the control plug (nutrient agar) (C1) and for the placement of the bacterial treatment (B1). Two concentric circles with diameter of 2 and 4 cm were made around the central points of C1 and B1, to mark the areas where the nematodes would be counted.

In order to position the treatments and the control on the plate, the template was marked on cut-out bottoms of 100 x 15 mm plastic petri dishes. Circles were marked on the cut out plastic plate with a compass fitted with two metal points. These templates were fitted under the water agar plates during preparation of choice test plates and when the nematodes were counted.

Choice test

Water agar plates (Difco Laboratories) were marked with a 0.5 cm cork borer at positions B1, C1, and N according to the plate template. The culture medium plugs cut out from plates with *Bacillus* spp., or from bacteria-free nutrient agar plates, were placed in the areas labeled B1 and C1, respectively. The agar plugs were removed from the

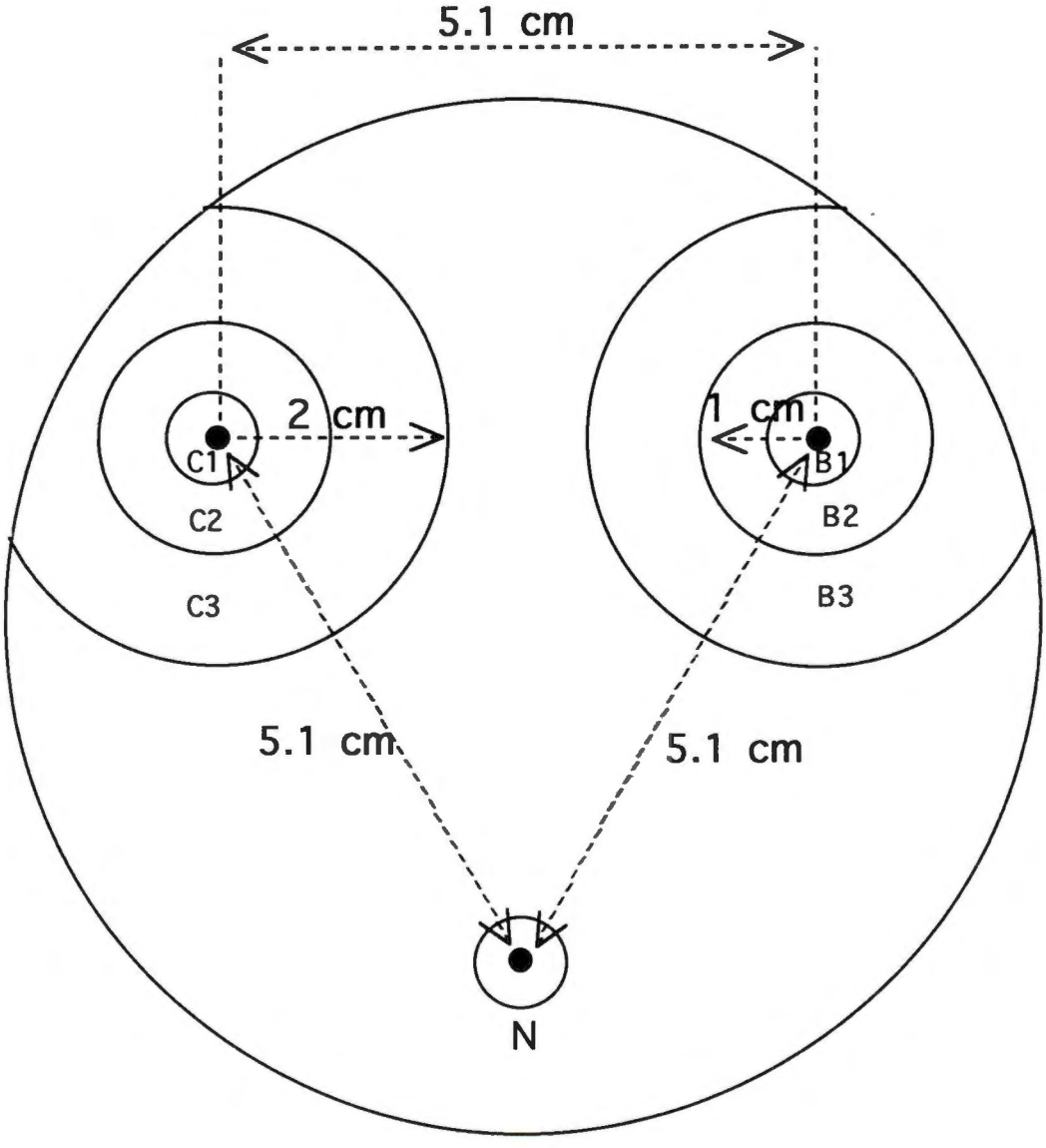


Figure 2.1. Schematic depiction of choice test template. N = Release site for infective juveniles, B1 = Placement of bacterial treatment plug, C1 = Placement of control plug, B1-3 and C1-3 = Areas where infective juvenile nematodes were counted for treatment and control, respectively.

numbers of infective juveniles in the treatment (B1+B2+B3) and dividing this total by the total number of juveniles observed (B1+B2+B3+C1+C2+C3). Data were analyzed with ANOVA and Repeated Measurements Analysis (SAS Institute, Cary, NC). The treatments served as the main effect and the evaluation times (4 or 8 hours) as the repeated measurement factor.

RESULTS

Typically, the infective juveniles either remained near the point of inoculation or chose the control over the treatments. The juvenile nematodes seemed to move near the bacteria but did not remain there, instead they moved to the outside of the counted area or to the control. It was easier to count the plugs without any bacteria (control). The area where the plugs with bacteria touched the agar was cloudy, making it difficult to observe the juveniles.

A significant difference was observed among the percentage of nematodes attracted to the nine bacterial treatments (Table 2.2) (P=0.0025, df=8, 56). The average percentage of infective juveniles that were attracted to the treatments was 31%, and the range was 20-43% (Fig. 2.2). *Bacillus* isolate BA77 was significantly less attractive than isolates E21, BA101, E726, and E69. *Bacillus* isolate E69 was significantly more attractive than isolates E61, E66, E65 and BA77.

A significant difference was also observed in the replicate and the replicate x time interaction (Table 2.2) (P=0.0001; df=7, 56 and P=0.0004; df=7, 56, respectively). In addition, a great amount of variation was observed in the repetitions. For instance, the

Table 2.2. Analysis of variance for the percent attraction of *Steinernema carpocapsae* to the nine bacterial treatments.

Source	DF	Sums of Squares	Mean Square	F value	Pr>F
Replicate	7	1.351	0.193	10.283	0.0001
Treatment	8	0.522	0.065	3.477	0.0025
Error	56	1.051	0.019		
Time	1	0.079	0.079	13.988	0.0004
Time x Replicate	7	0.324	0.046	8.182	0.0001
Time x Treatment	8	0.060	0.008	1.336	0.2453
Error	56	0.316	0.006		

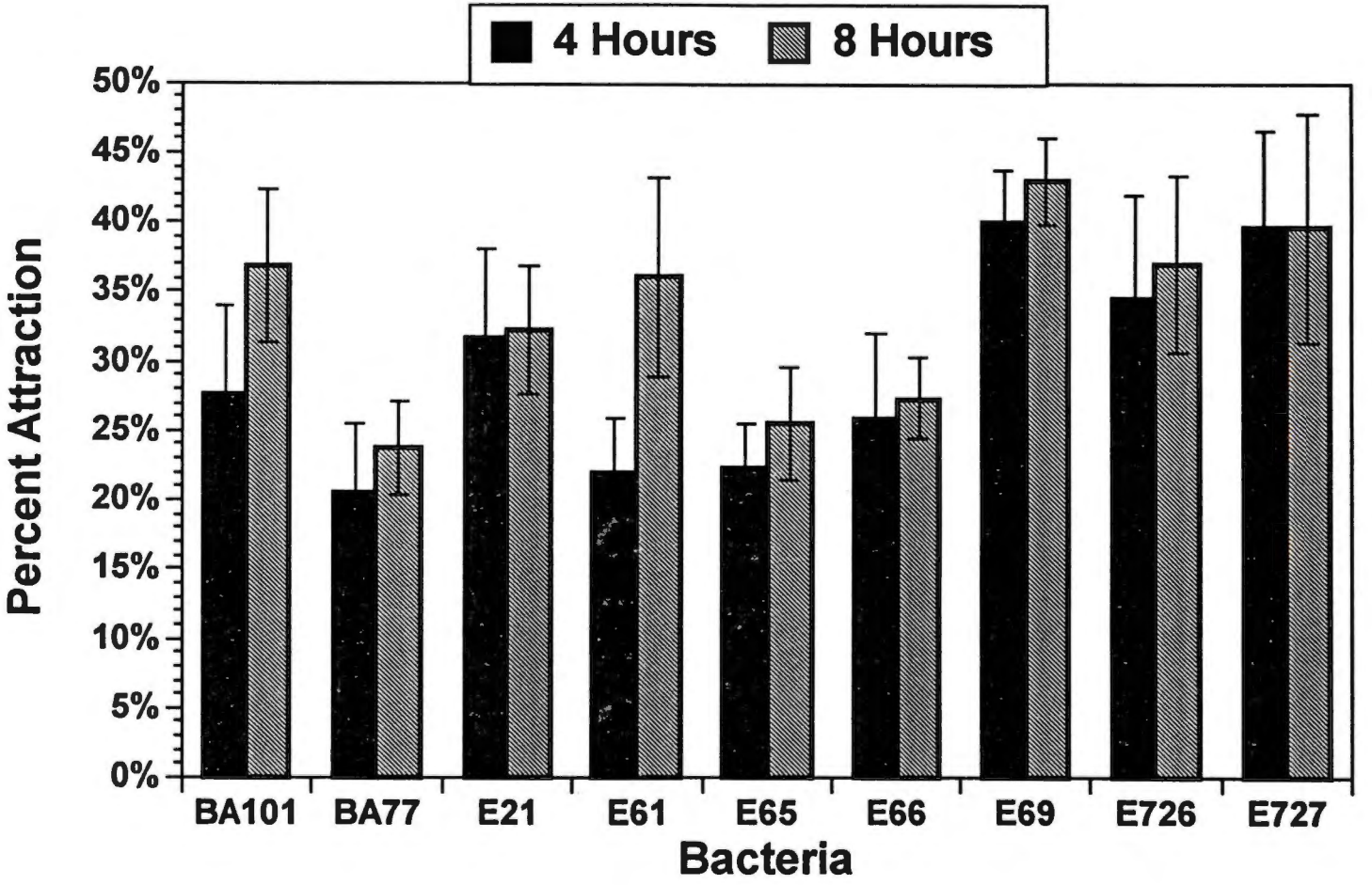


Figure 2.2. The mean percent *Steinernema carpocapsae* infective juveniles that were attracted to nine *Bacillus* isolates with biological control activity against plant pathogens when presented with the choice of a bacterial culture and uninoculated nutrient agar.

variation in the *Bacillus* isolate BA77 treatment was 54-97% attraction in the 8-hour evaluations, and 13-39% attraction at the 4-hour evaluations. The variation in the *Bacillus* isolate E69 was 27-57% attraction in the 8-hour evaluations, and 27-56% attraction at the 4-hour evaluations.

A significant difference existed between the eight- and four-hour time period evaluations (Table 2.2) (P=0.0004; df=1, 56). At the eight-hour evaluation more infective juveniles had been attracted to the bacterial cultures than at the four-hour evaluation (Fig. 2.3). Isolates E727, E726 and E69 had attracted a large percentage of juveniles by the 4-hour evaluation. Infective juveniles were only weakly attracted to isolates E61 and BA101 after four hours, but after eight hours the percent attraction levels were similar to those of the isolates E727, E726 and E69. In contrast, isolates BA77, E65, and E66 initially had low attraction percentages and increased only slightly after eight hours. The percent attraction of any isolate did not drop between the four- and eight-hour evaluations for any isolate. There were only four individual replicates where the *Bacillus* isolates were able to attract more juveniles than the control. These occurred in isolates BA101, E727 and E21.

There was no significant difference observed in the time x treatment interaction (Table 2.2) (P=0.2453, df=8, 56).

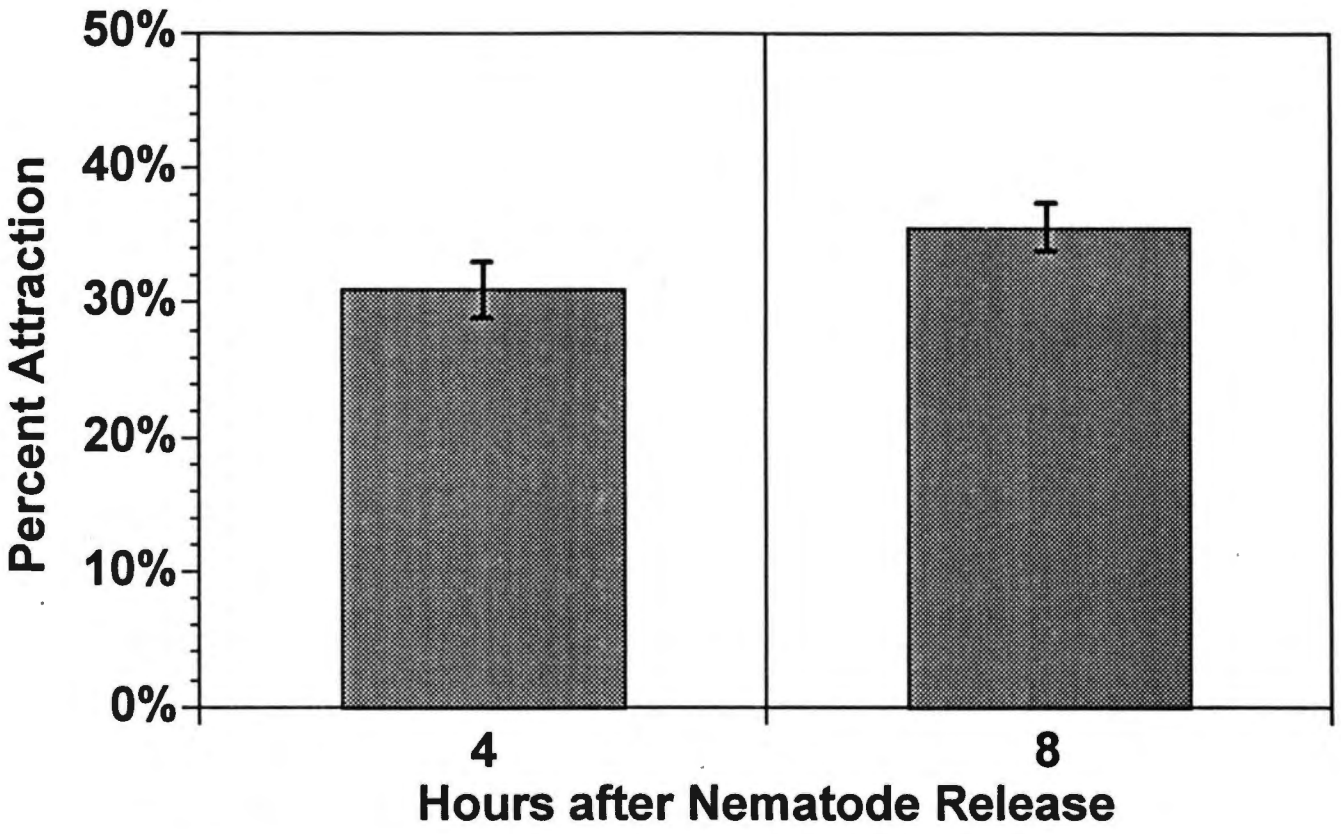


Figure 2.3. The mean percent *Steinernema carpocapsae* infective juveniles that were observed 4 and 8 hours after being released in choice plates with agar plugs of *Bacillus* isolates that have biological control activity against plant pathogens and uninoculated nutrient agar plugs. Small bars are standard errors.

DISCUSSION

This is apparently the first time that entomopathogenic nematodes have been tested with bacterial isolates with biological control activity against plant pathogens. Therefore, it is impossible to make direct correlations between the results obtained in this experiment and those in previous reports. However, similar studies have been conducted with other soil microorganisms used in biological control and their attraction ability in relation to entomopathogenic nematodes (Barbercheck and Kaya, 1991; Thurston et al., 1993). The results presented herein are in agreement with similar studies that show that entomopathogenic nematodes are attracted to the fungus *B. bassiana*, the insect pathogenic bacterium *B. popilliae*, and various soil bacteria (Aguillera and Smart, 1993).

The entomopathogenic nematode and the *Bacillus* isolates tested may be compatible since the infective juveniles were not repelled by the bacteria. Thurston et al. (1993) combined entomopathogenic nematodes with *B. popilliae* and noticed that entomopathogenic nematode infectivity was enhanced. The authors also demonstrated that the two organisms were compatible. In an additional study with greenhouse trials (Thurston et al.,1994), *S. glaseri* killed more *B. popilliae*-infected grubs than healthy grubs. This suggests that entomopathogenic nematodes may be attracted to *B. popilliae* infected hosts.

Since the average soil contains 70% sporulating *Bacilli* (Burges and Raw, 1967) and entomopathogenic nematodes are one of the most abundant microorganisms on earth (Poinar, 1983), an interaction between entomopathogenic nematodes and *Bacilli* in the soil is likely.

Potential adverse effects of the *Bacillus* spp. used for plant disease control on entomopathogenic nematode populations is one of the concerns when integration of these biological control agents is attempted. In the presence of some chemicals (Gaugler and Campbell, 1991 and Khlibsuwan et al.,1992a) entomopathogenic nematodes behavior is adversely effected. If the *Bacillus* spp. caused similar effects, their integration into agricultural systems could be a problem. However, the results suggest that the *Bacillus* isolates tested do not seem to interfere with nematode behavior. If *Bacillus* spp. are applied to control plant pathogens, it seems possible that entomopathogenic nematodes could also be introduced if an insect problem arose.

The results from this experiment indicate that entomopathogenic nematodes may feed on these and other Bacilli. These findings are in agreement with similar reports. Poinar (1966) suggested that entomopathogenic nematodes ingest all bacteria present in the host cadaver, and that they also carry cells of various bacteria on the surface of their cuticle. Aguillera and Smart (1993) reported that *S. scapterisci* was compatible with a number of soil bacteria including *P. aureofaciens, P. flourescens* , and *Xanthomonas maltophilia*. Lysenko and Wieser (1974) isolated *Alcaligenes odorans, Ps. flourescens, Ps. maltophilia, Ps. alcaligenes,* and *Acinobacter* sp. from the cuticle surface and gut of *S. carpocapsae.*

The observed significant differences between the replicates were expected because the replicates were performed at different times and on different dates over a course of a 45-day period.

The numbers of nematodes observed in the control and the bacterial treatment

zones in the choice test were low compared to the number of nematodes released onto the plates. The behavior of nematodes in agar plates may explain this observation. Barbercheck and Kaya (1991) reported that *S. carpocapsae* infective juveniles are not as mobile on agar plates as in soil bioassays. In agar plate choice tests only an average of 11% of the juveniles moved from the point of placement to the observation or counting site; in contrast, in the soil bioassays with organic, clay and fine sandy loam soil an average of 21.6, 20.2 and 18.1% juveniles, respectively, moved from the release site. This low mobility may possibly be explained by the host search behavior of *S. carpocapsae* to sit and wait on a host or environmental cue.

In conclusion, the nine *Bacillus* isolates with biological control activity against plant pathogens were able to at least moderately attract the entomopathogenic nematode, *S. carpocapsae.*

CHAPTER 3

Development and Reproduction of *Steinernema carpocapsae* on Four *Bacillus* Isolates with Biological Control Activity Against Plant Pathogens

INTRODUCTION

In the laboratory, entomopathogenic nematodes have a wide host range that encompasses almost all soil-dwelling pests (Gaugler and Kaya, 1990). Each entomopathogenic nematode species is also associated with its own mutualistic symbiont that provides the nematode with the food resources that are necessary for its development and reproduction. In addition, entomopathogenic nematodes reportedly ingest other species of bacteria if they are encountered (Poinar, 1966). Despite the fact that entomopathogenic nematodes ingest other species of bacteria along with their mutualistic symbiont, it is not clear whether or not they can use these bacteria for their development and reproduction.

The mutualistic bacteria associated with entomopathogenic nematodes are medium to long motile rods that are Gram-negative. They are facultative anaerobes that do not form spores and thus do not exist outside entomopathogenic nematodes or nematode-infected hosts. The relationship between the bacterium and the nematode is mutualistic because neither organism is self-sufficient. The bacteria benefit from the relationship because they are dispersed and their proliferation is ensured. The nematodes benefit from

the relationship because they are provided with a food source and a suitable environment for development and reproduction.

There are conflicting reports regarding the ability of entomopathogenic nematodes to use other species of bacteria to support their growth and infectivity. Poinar (1966) isolated *Pseudomonas* sp., *Proteus* sp., *Alcaligenes* sp., and *Aerobacter* sp. from *S. carpocapsae* and determined that only the symbiont, *Xenorhabdus nematophilus,* was pathogenic to wax moth larvae, and only the symbiont could support development and reproduction of the nematode. Lysenko and Weiser (1974) reported that entomopathogenic nematodes are capable of development, reproduction and infectivity on other bacteria besides their symbionts. The bacteria tested included: *Alcaligenes odorans, Xanthomonas maltophila, Pseudomonas alcaligenes, Enterobacter agglomerans, Serratia liquefaciens,* and *P. flourescens.*

Although the use of entomopathogenic nematodes as biological control agents against insect pests appears promising in laboratory studies, this potential has not been in some field trials. To improve performance some researchers have hypothesized that entomopathogenic nematodes could be combined with other biological control agents to augment their infectivity. The use of two or more biological control microorganisms to control a pest problem has been evaluated by several authors (Agra Gothama et al., 1995; Jaques and Morris, 1981; Kaya et al., 1993). To increase entomopathogenic nematode efficacy in the field, it may be advantageous to combine them with other biological control agents. The combination of entomopathogenic nematodes with bacterial isolates that have biological control activity against plant pathogens represents a novel idea. If these bacteria

can perform a dual function, i.e., combat plant diseases and serve as a nutrient source for nematodes used to control insects, some advantages may occur when these biological control agents are integrated. Mass production of bacteria that could be used for plant disease control and as a substrate for nematode cultures would have obvious effects in lowering production costs. Applications of mixed cultures in the field (bacteria and nematodes), could also lower pest control costs. Additionally, survival of both organisms may be enhanced.

With the increased use of Bacilli to control plant pathogens and the commercial availability of entomopathogenic nematodes, it is necessary to understand their interactions. The objective of these experiments was to monitor the development of *S. carpocapsae* on four *Bacillus* isolates with biological control activity against plant pathogens.

MATERIALS AND METHODS

Nematode rearing

Steinernema carpocapsae All were reared by the same procedure as outlined in Chapter 2, except that wax moth larvae were not transferred to White traps.

***Bacillus* isolates**

Bacillus spp. were produced by the same method outlined in Chapter 2, except that when bacteria were needed for this experiment, refrigerated cultures were removed from storage and a ±10 cm line was streaked across the surface of Yeast-Peptone-Cholesterol (YPC) medium plate (2 g yeast extract [Difco Laboratories], 10 g soy peptone [Sigma], 25 g agar [Difco Laboratories], 0.2 g cholesterol [Sigma] dissolved in 20 ml of heated 95% ETOH, and 1 l distilled water). Prior to streaking the plates with the bacterial isolates, templates were inoculated with axenic nematode eggs as described below. For the following experiments, only isolates BA77, E69, E61, and E727 (Table 2.1) were tested.

Axenic egg extraction

Axenic eggs were obtained by following the procedures outlined by Aguillera and Smart (1993). Nematode-infected wax moth larvae that appeared yellowish brown were placed in a dish that contained a solution of sterile saline solution (7.5 ml NaCl, 0.35 ml KCL, 0.21 ml CaCl, 1,000 ml distilled water). *Galleria mellonella* larvae were dissected under a dissecting microscope by taking two forceps and tearing away the cuticle to open the cadaver. More sterile saline was added to the dish to separate the females from the host tissues. A metal hook prepared with a minuten pin was used to lift approximately 300 female nematodes out of the solution. The females were placed in a separate sieve inside a 100 x 15 mm petri dish containing sterile saline solution. The sieve was prepared by cutting out the bottom of a 35 x 10 mm petri dish and sealing a 500-mesh screen to the

bottom. After obtaining 300 females, the procedure was moved to a vertical laminar flow hood to ensure the process was performed under sterile conditions.

The 500-mesh sieve containing approximately 300 females was picked up with a sterilized forceps and the solution of sterile saline was allowed to drain. The sieve then was placed in a digesting solution (1.0 ml 5.25% NaOCl, 2.5 ml 0.4 M NaOH, and 21.0 ml distilled water) for one minute. The digestion solution was then drained and the sieve was placed back into new sterile saline solution for one minute. The sterile saline was again drained and the sieve was placed in the digestion solution to release the eggs from the females. After ten minutes, eggs were rinsed three times in sterile saline solution and saved to be used in experiments.

Plate preparation

A 100 μl aliquot of a suspension containing approximately 500 eggs in sterile saline solution was extracted from the sieve and placed on YPC plates. Plates were maintained in a 25°C incubator for 24 hours and then examined for the presence of contaminants. Plates that were not contaminated were streaked with ±10 cm of the test bacterial isolate and placed back into the incubator from which they were removed for daily observations.

Data collection and analysis

The experiment consisted of four bacterial treatments: *Bacillus* spp. strains BA77, E69, E726, and E61. There were four replicates of each treatment and the experiment was repeated three times. The plates were examined daily over the course of eight days and experiments were conducted over a period of approximately two months.

The numbers of nematodes on the plates were counted by placing the plates under a dissecting microscope on top of a nematode counting dish. The counting dish was a square petri dish (90 mm x 15 mm depth) marked with a grid of lines determining 6 x 6 squares (1.27 cm side) . Each individual square was examined for the presence or absence of entomopathogenic nematodes. Control plates received no bacteria. An error in the setup of the second experiment caused the loss of the control plates.

An index was calculated to measure nematode population growth. This growth index was calculated by dividing the number of nematodes observed on Day 8 by the number of nematodes observed on Day 1. Thus, an index greater than 1 represented a growing population, whereas an index less than 1 represented a decreasing population. Data were analyzed with ANOVA and Repeated Measurements Analysis (SAS Institute, Cary, NC). The bacterial treatments served as the main effect and the number of days the plates were observed as the repeated measurement factor.

RESULTS

Due to the low number of eggs that hatched in Experiment 1, it was excluded from the statistical analysis (Fig. 3.1). Entomopathogenic nematodes did not appear to move around much on the YPC plate, instead they remained in the area where they hatched. However, visual observations of sinuous tracks in the agar indicated that some nematode movement occurred on the plates. The medium appeared to have water-like spots on the surface, as if some component of the medium was precipitating. Also, when female adults were observed, they were usually coiled and immobile. Additionally, nematodes may have disintegrated in the medium because dead nematodes were not observed on the plates.

Analysis of variance (Table 3.1) of the population growth index demonstrated that differences existed among the treatments ($P = 0.0219$, $df = 4, 27$). Treatments E69 and E61 were significantly different from the BA77 treatment and the control (Table 3.2). In addition, they were the only treatments that seemed to support nematode population growth in Experiment 2 (Fig. 3.2). In Experiment 3, the only treatment that supported minimal growth was E69 (Fig. 3.3). With all other treatments in Expepp eriment 3, a reduction in nematode populations occurred.

Despite the fact that, Experiment 3 had the largest amount of eggs hatching, all isolates examined, including the control, experienced nematode die off. Isolate E727 had a severe decline in population on day 2. On day 1, a mean of 260 nematodes hatched, but the population decreased to 85 by day 2. This represents a loss of more than half of the nematodes. Overall, the other isolates and the control went through a steady decline in nematode population. In Experiment 1, the mean number of nematodes on day 1 with

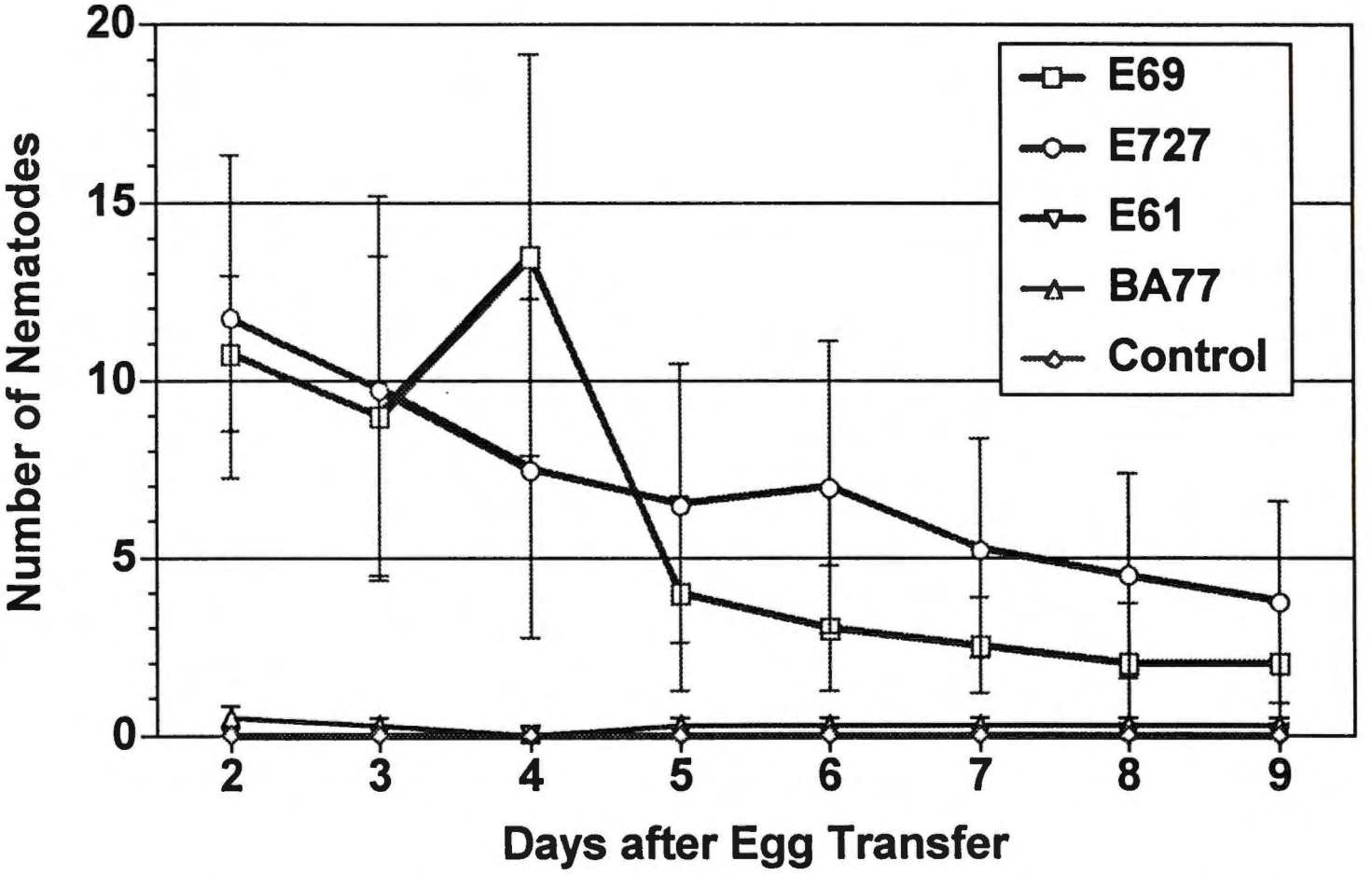


Figure 3.1. *Steinernema carpocapsae* populations in Experiment 1 after axenic eggs were added to YPC medium plates inoculated with four *Bacillus* isolates that have biological control activity against plant pathogens.

Table 3.1. Analysis of variance of the population growth in *S. carpocapsae* after axenic eggs were placed on the four bacterial treatments.

Source	DF	Sums of Squares	Mean Square	F value	Pr>F
Experiments	1	1.388	1.388	5.597	0.0266
Treatment	4	2.150	0.537	2.168	0.0219
Interaction	3	2.566	0.855	3.450	0.0128
Error	27	6.694	0.248		

Table 3.2. Mean population growth indices[a] for *Steinernema carpocapsae* grown on four bacterial treatments.

Treatment	Mean ± Standard error[b]
Control	0.33 ± 0.22 a
BA77	0.61 ± 0.09 a
E727	0.82 ± 0.36 ab
E61	1.12 ± 0.17 bc
E69	1.18 ± 0.10 c

[a] Population growth index = Population on Day 8/ Population on Day 1.
[b] Means followed by different letters are significantly different (P = 0.05) according to Fisher's Protected Least Significant Difference test.

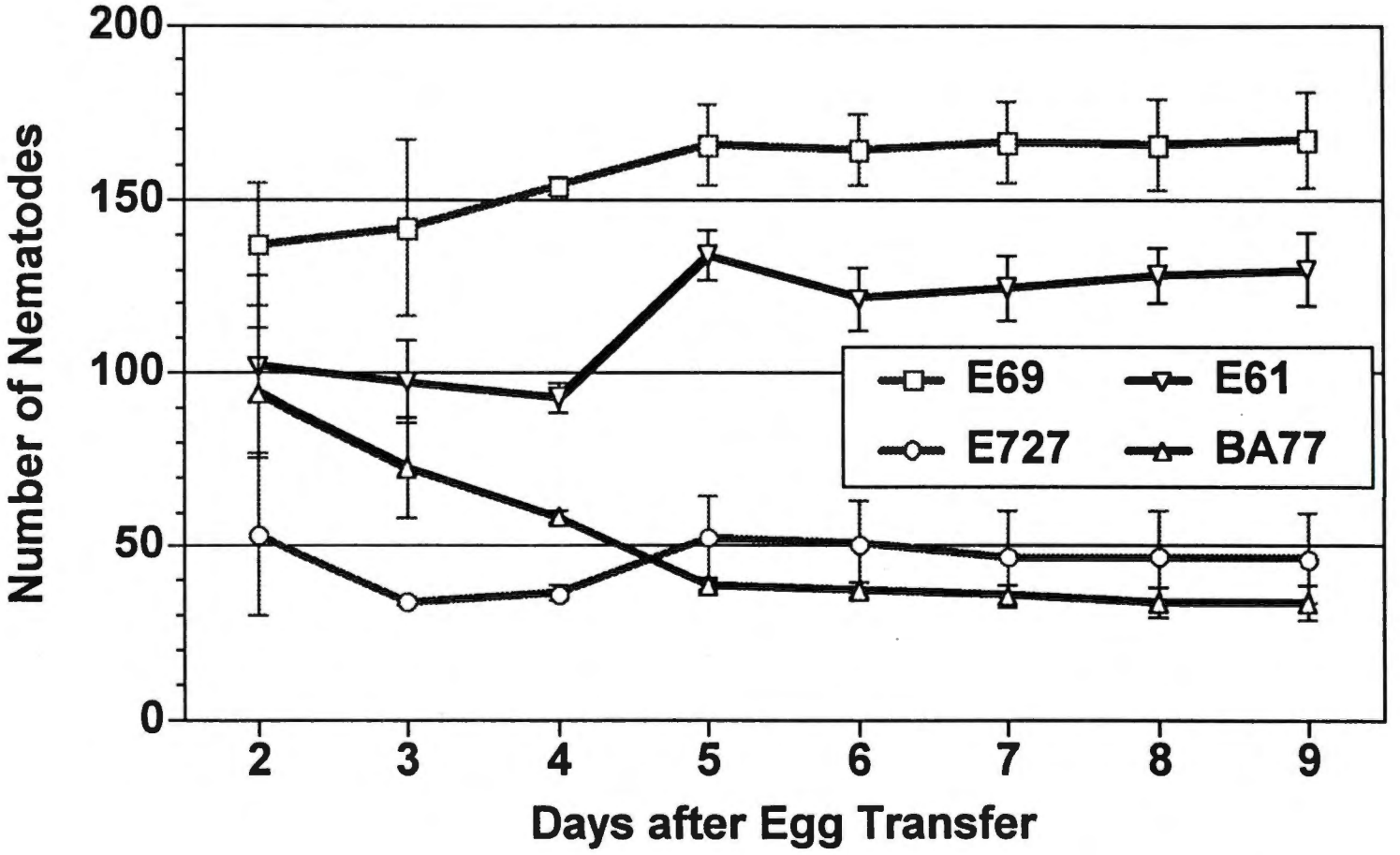


Figure 3.2. *Steinernema carpocapsae* populations in Experiment 2 after axenic eggs were added to YPC medium plates inoculated with four *Bacillus* isolates that have biological control activity against plant pathogens.

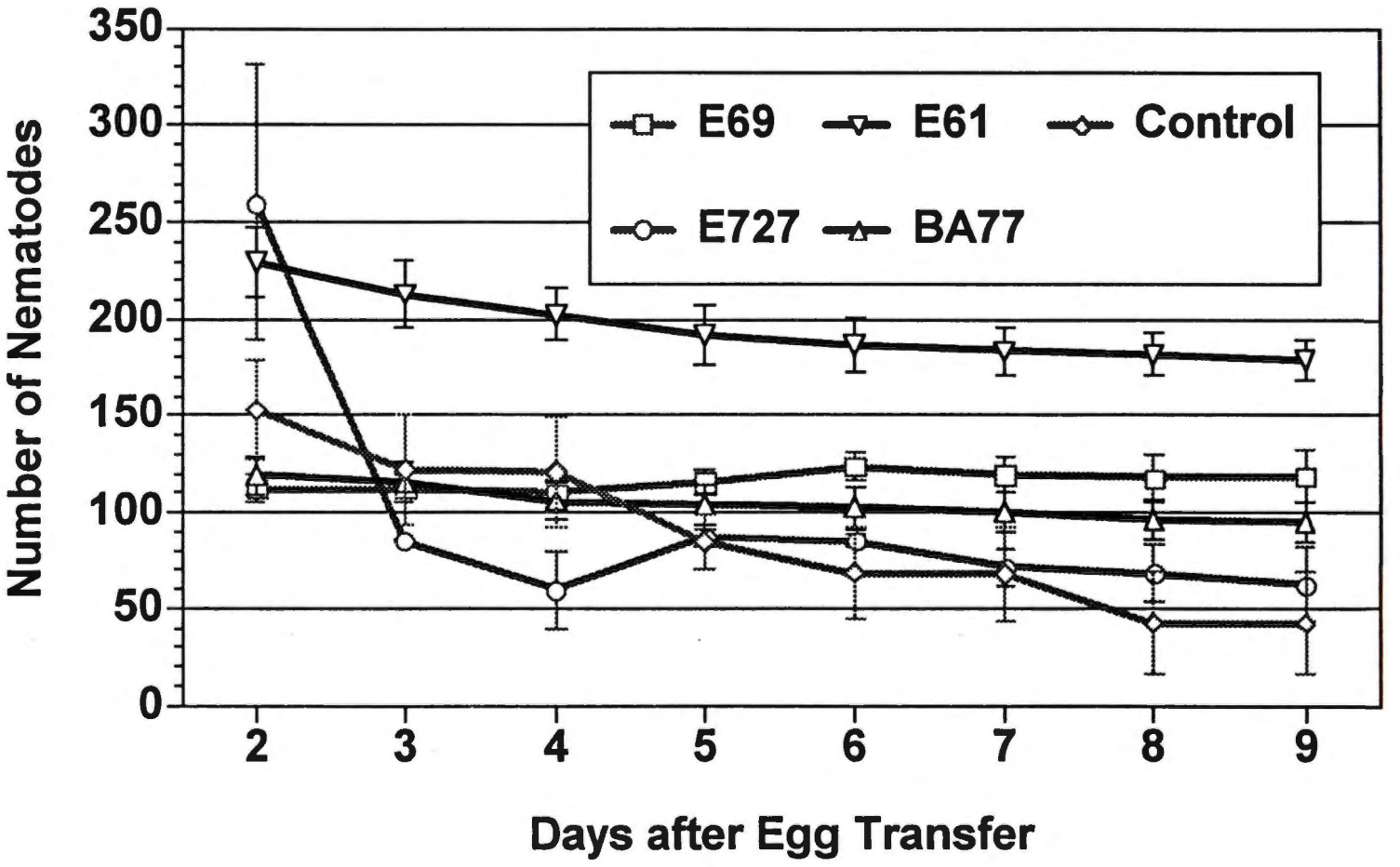


Figure 3.3. *Steinernema carpocapsae* populations in Experiment 3 after axenic eggs were added to YPC medium plates inoculated with four *Bacillus* isolates that have biological control activity against plant pathogens.

isolate E69 was 10, but by day 8 the mean number of nematodes present was only 2.

The number of eggs hatching in Experiments 2 and 3 with isolate E69 were similar (137 and 112, respectively). Similar results also were observed in the two experiments with the isolate BA77 (94 and 119, respectively).

Egg hatch was extremely low in Experiment 1. All repetitions of isolate E61 and the control, as well as one repetition of isolate BA77, had no hatch. Eggs hatched on the control plates in Experiment 3, but not Experiment 1.

There were significant differences between the two experiments (Experiment 2 and 3) (Table 3.1) (P=0.0266, df=1, 27) from which population growth indices were analyzed. In Experiment 2, isolate E69 had a steady increase in nematode population, while BA77 seemed to do the opposite. Isolate E61 had a surge in population growth on day 4. All the treatments experienced a growth in nematode population between days 3 and 4, except isolate BA77. In Experiments 2 and 3, more of the nematode populations decreased than experienced growth. However, more nematodes had population growth in Experiment 2 than Experiments 1 and 3.

The interaction between the experiments and the bacterial treatments was also significant (Table 3.1) (P=0.0128, df=3, 27).

DISCUSSION

The mutualistic symbionts of entomopathogenic nematodes go through a phase variation (Phases I and II) that interferes with their mass production. One reason for this phenomenon is the low nutritional value of the phase II variant compared to the Phase I variant (Jackson et al., 1995). The possibility of using different bacteria, including the *Bacillus* isolates tested, to mass-produce entomopathogenic nematodes would perhaps eliminate the complications experienced when using the mutual symbiont.

Entomopathogenic nematodes were expected to develop and reproduce on the *Bacillus* isolates examined since published reports indicated that these nematodes use a wide range of soil bacteria for their reproduction (Grewal et al., 1997b; Poinar, 1966). However, the results obtained herein indicate that *S. carpocapsae* may not be able to develop and reproduce on all soil bacteria as reported (Grewal et al., 1997b; Poinar,1966). It is apparent that entomopathogenic nematodes somehow differentiate among the bacteria they consume. They must avoid antagonistic bacteria in nature that do not provide enough nutrients for their growth and reproduction. Lysenko and Weiser (1974) proposed that other soil bacteria are present in the intestine of entomopathogenic nematodes, but in certain proportions that the entomopathogenic nematode must be able to regulate. This may explain how entomopathogenic nematodes protect themselves from the soil bacteria that were examined.

The *Bacillus* isolates BA77 and E727 had an adverse effect on entomopathogenic nematode populations in Experiment 2. These results are in agreement with previous reports that entomopathogenic nematodes cannot develop and reproduce in the presence

of *B. cereus* (Poinar and Thomas, 1966), the same species as the BA77 isolate. In our experiments, nematode populations declined, similar to a previous finding observed when *G. mellonella* was inoculated with cells of *B. cereus* (Poinar and Thomas, 1966). In another study, *Bacillus* spp. inhibited *C. elegans* reproduction, while all other isolates supported development and reproduction (Grewal and Hand, 1992). In contrast, in the current study isolates E69 and E61 were able to support development and possibly reproduction of *S. carpocapsae*, as was demonstrated by population growth indices above 1.

In a report by Han and Ehlers (1998), the authors observed that a signal from the nematodes mutualistic symbiont was required for the nematode egg to complete its development. However, in our experiments the eggs hatched in the presence of all the bacteria examined, as well as on the control plates where no bacteria were present. It is possible that some ingredient in the medium permitted development of the axenic eggs. The absence of growth factors in the YPC plates may explain why entomopathogenic nematodes did not reproduce.

Likewise, it may be possible that components in the media interfered with the development of the entomopathogenic nematodes, or that this medium was not a good choice for the bacteria to develop adequately. Gaugler and Georgis (1991) stated that culture conditions are important when examining entomopathogenic nematodes.

Soil applications of *Bacillus* spp. to control plant pathogens must be planned appropriately, if entomopathogenic nematode populations are to be protected. Soil examination prior to releasing entomopathogenic nematodes that detect large populations

of Bacilli could be used to avoid adding entomopathogenic nematodes to a harsh environment in the soil. The presence of detrimental Bacilli in soils may explain why entomopathogenic nematodes are not effective in some field trials, as others, or laboratory experiments.

The results of this study do not support the idea of using *Bacillus* isolates tested in the mass production of *S. carpocapsae*. Also, it does not appear to be advantageous to the entomopathogenic nematode to be mixed with the *Bacillus* spp. tested since the nematodes do not seem to develop on a diet with these bacteria.

CHAPTER 4

Conclusion

Currently, biological insecticides are not widely used by producers, even though they offer a environmentally safe way to combat pest populations. One factor limiting the increased use of biological insecticides is their variable efficacy in field trials. Georgis and Gaugler (1991) hypothesized that predictability is the key to improving the success of biological insecticides. Therefore, an increased knowledge is needed on the behavior, compatibility, host range, and interactions of biological control agents.

The employment of *Steinernema carpocapsae* as a biological control agent has not been as successful in the field as in laboratory trials. The reason why this phenomenon occurs should be examined. Although there are many published reports about entomopathogenic nematodes, the studies of their interactions with other microorganisms in the soil environment are somewhat limited.

Bacillus sp. with biological control activity against plant pathogens are receiving an increasing attention (Brannen, 1998; Fernando and Lindermann, 1995; Hebbar et al., 1992). It is necessary, though, that the compatibility of Bacilli with other potential biological control agents used in the soil be examined. Since sporulating Bacilli account for 70% of the total culturable microorganisms in soil (Burges and Raw, 1967), and bacteria are the most common microorganisms associated with insects, there is a good chance for an interaction between entomopathogenic nematodes and soil bacteria to occur.

The objectives of these experiments were to determine whether or not the *Bacillus* isolates examined would attract and also support development and reproduction of *S.*

carpocapsae. Reasons that lead to the speculation that these biological control agents would be compatible are previous reports showing potential beneficial interactions between entomopathogenic nematodes and other soil microorganisms. Various reports suggest entomopathogenic nematodes consume any soil bacteria they encounter (Poinar, 1966; Grewal et al., 1997b). Thus, it is possible that the entomopathogenic nematode, *S. carpocapsae,* and Bacilli with biological control activity against plant pathogens would come into contact with one another. Also, examinations of the behavior of entomopathogenic nematodes when, combined with Bacilli could lead to improved entomopathogenic nematodes or *Bacillus* spp. efficacy. The nature of the relationship between these biological control agents was examined since both receive considerable attention for their use as biological control agents.

Bacillus cereus (BA77), a bacterium widely distributed in the soil, moderately attracted *S. carpocapsae* but it adversely effected growth of the nematode population.

When examining all experiments conducted, it seems that although *S. carpocapsae* infective juveniles were somewhat attracted to the nine *Bacillus* isolates, they were not able to use every isolate as a food source to support their development and reproduction. For example, *Bacillus* isolate BA77 did attract infective juveniles (22%) (Fig. 2.2), but was not suitable for their development and reproduction (Figs. 3.1-3.3). In contrast, *Bacillus* isolate E727 had relatively high attraction percentages (Fig. 2.2), but like BA77 was not able to support development and reproduction.

The reason nine isolates were examined was so it could be determined which isolates would be better to integrate with entomopathogenic nematodes and which

combinations should be avoided. It appears that isolate E69 and to some extent E61 are compatible with *S. carpocapsae.* Isolate E69 had the highest attraction percentage (43%), and was the only isolate that consistently supported egg development (Figs. 3.1-3.3). Other isolates that were not examined in the second experiment may also be compatible with the nematode, but this will require further study. As previously mentioned, isolate BA77 is not compatible with entomopathogenic nematodes and isolate E727 needs to be further investigated.

Soil applications of *Bacillus* sp. to control plant pathogens must be planned appropriately, if entomopathogenic nematode populations are to be protected. Soil examination prior to releasing entomopathogenic nematodes that detect large populations of Bacilli could be used to avoid adding entomopathogenic nematodes to a harsh environment in the soil. The presence of detrimental Bacilli as seen in our experiments in soils may explain why entomopathogenic nematodes are not effective in some field trials.

In conclusion, entomopathogenic nematodes may not be very effective in soils to which *B. cereus* (BA77), and perhaps E727, are introduced. To integrate isolates E69 or E61 with entomopathogenic nematodes, it probably would be best to first introduce the nematodes since they require heavy irrigation. This heavy irrigation might wash the *Bacillus* inoculum from the *Bacillus*-treated seeds and decrease their efficacy against plant pathogens. Further investigations are necessary before the type of relationship (competition, antagonism, synergism) between entomopathogenic nematodes and the *Bacillus* spp. examined is determined.

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