**GENOTYPIC AND ENVIRONMENTAL INFLUENCES ON THE HOST-PARASITE INTERACTION BETWEEN *MELOIDOGYNE ARENARIA* AND *PASTEURIA PENETRANS***

by

CHANG LIU

(Under the Direction of Patricia Timper)

ABSTRACT

Root-knot nematodes (*Meloidogyne* spp., RKN) are economically important plant-parasitic nematodes that have a wide distribution. *Pasteuria penetrans* is gram-positive bacterium that is an obligate parasite of RKN. Numerous field studies have shown the suppression of RKN by *P. penetrans*, demonstrating the potential to be developed into a biological control product. The endospores of *P. penetrans* attach to the surface coat of RKN and subsequently sterilize the female. Environmental factors influenced *P. penetrans* spore attachment. Exposure to root exudates of second-stage juveniles (J2) reduced spore attachment, suggesting the nematode surface coat was influenced by root exudates. Application of *P. penetrans* spores in furrow reduced egg production more than as a seed treatment. When applied in furrow, spores may have a wider distribution in the soil compared to spores applied to the seed, and J2s migrating through the soil would encounter *P. penetrans* spores before they were exposed to root exudates and become more resistant to spore attachment. The susceptibility of RKN progeny was shown to be influenced by the maternal environment. Mothers raised under stressed conditions produced

more resistant offspring than did mothers raised under favorable conditions. In a 4 year field study, rapid changes in the dominant spore phenotype were observed in each plot in each year, and the changes occurred on the local level. Such findings indicate the occurrence of negative frequency-dependence selection in this field. Single spore lines of a Florida *P. penetrans* population were used to test the phenotypic and immunological differences among spore lines. No difference in spore attachment phenotype was observed mainly because these single spore lines were obtained from single egg mass line (clonal) of *M. arenaria*; thus they may be the same spore attachment phenotype. This project provided much needed information on understanding the host-parasite interactions between *P. penetrans* and *Meloidogyne* spp, and provides suggestions for better developing *P. penetrans* into a biological control product.

INDEX WORDS: *Pasteuria penetrans*, *Meloidogyne* spp., biological control, root exudates, spore attachment, maternal effect, negative frequency-dependent selection, host-parasite interaction specificity

# GENOTYPIC AND ENVIRONMENTAL INFLUENCES ON THE HOST-PARASITE INTERACTION BETWEEN *MELOIDOGYNE ARENARIA* AND *PASTEURIA PENETRANS*

by

CHANG LIU

B.S. Shandong Agricultural University, China, 2012

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

**GENOTYPIC AND ENVIRONMENTAL INFLUENCES ON THE HOST-PARASITE INTERACTION BETWEEN *MELOIDOGYNE ARENARIA* AND *PASTEURIA PENETRANS***

by

CHANG LIU

Major Professor: Patricia Timper

Committee: Pingsheng Ji

Jim. P. Noe

Ronald Walcott

Electronic Version Approved:

Suzanne Barbour

Dean of the Graduate School

The University of Georgia

December 2017

# DEDICATION

Dear Dad and Mom, thank you for all your love and support. This is for you.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major advisor Dr. Patricia Timper for the strong support throughout my graduate study at the University of Georgia. Her wisdom in research and advising has significant impact on my future career. I would also like to thank my advisory committee members, Dr. Pingsheng Ji, Dr. Jim. P. Noe and Dr. Walcott for offering their suggestions and advises in this project. Thanks to Syngenta® for funding part of the research. I would like to thank Dr. Soumi Joseph, Dr. Tesfamariam Mekete from University of Florida, Dr. Amanda Gibson and Dr. Levi Morran from Emory University for their help. Special thanks are sent to Mr. David Clements and Ms Susan Drawdy in the Nematology lab in USDA ARS for their technique support through these years. My gratitude also extends to Ms. Xuelin Luo for her help in data analysis. I also acknowledge Dr. Harald Scherm and fellow graduate students in the Department of Plant Pathology for their help. In the end, I would also like to thank my family and my dear friends for their endless support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Introduction

Root-knot nematodes (*Meloidogyne* spp.) (RKN) are one of the three most economically damaging genera of plant-parasitic nematodes on horticultural and field crops. They cause more damage worldwide than any other genus of plant-parasitic nematode, and account for about 5% of global crop loss (Sasser, 1985). Root-knot nematodes have a world-wide distribution. They are found in all agricultural regions worldwide, but primarily in tropical to sub-tropical environments, and are devastating in subsistence agriculture. Approximately 100 species of *Meloidogyne* have been described, with some having several different races. The most widespread and economically important species are *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*.

RKN damage plants by acting as metabolic sinks, transferring energy from the plant to nematode, and by impairing root function. Infection of roots results in the formation of giant cells leading to the development of root-knot galls. For young plants, infection by root-knot nematodes may be lethal, and for mature plants, they can result in poor crop growth and decreased yield. Most root-knot nematodes also have extremely wide host ranges, with some species parasitizing a host range as wide as hundreds of different plant species. This wide host range can make it extremely difficult to control root-knot nematodes, particularly if the nematode can reproduce on weeds. In addition, the wound caused by nematodes can provide an entrance

for other pathogens, and lead to high yield losses. For example, the *Fusarium* wilt/root-knot nematode complex is one of the most widely recognized and economically important disease complexes in the world (Vernon, 2003).

The life cycle of RKN takes 3 to 6 weeks to complete, depending on the species and environmental conditions. Root-knot nematodes start their lives as eggs deposited into a gelatinous matrix on the surface of the root gall. All nematodes pass through an embryonic stage, four juvenile stages (J1–J4) and an adult stage. The first-stage juvenile (J1) resides entirely in the egg where it molts into the J2. The J2 is the mobile stage, and is also the only stage that can initiate infection of roots. They may reinvade the host plant of their parent or migrate through the soil to find a new host plant. The J2 penetrate the area behind root tip, and from there, they migrate intercellularly toward the vascular system, establish a permanent feeding site, then become sedentary (Eisenback, 1981). The juveniles eventually develop into adults after three more molts. The mature females are saccate, the males become veriform and leave the root without feeding. The length of a root-knot nematode life cycle varies depending on temperature, with temperature optima varying among species. The life cycle can be as short as 2 weeks, but is typically 3 weeks (Madulu & Trudgill, 1994). Nematodes in cooler regions have longer life cycles. Eggs hatch is generally not affected by the presence of host, which means they do not require exposure to root exudates. Eggs can survive in the soil for over one year under favorable conditions. The life span of an adult female may be up to 3 months, and many hundreds of eggs can be produced.

A major characteristic of root-knot nematodes is the variation in modes of reproduction, including amphimixis, facultative meiotic parthenogenesis and obligatory mitotic parthenogenesis. Amphimixis is sexual reproduction, and 90% root-knot nematode species have

this type of reproduction mode. However, the major root-knot nematode species are all parthenogenetic. Facultative meiotic parthenogenesis depends on the presence or absence of viable males. A female may undergo parthenogenesis if a male is absent from the habitat. When viable males are present, females can reproduce by amphimixis. *M. hapla* has this type of reproduction. Obligatory mitotic parthenogenesis is the process in which organisms exclusively reproduce through asexual means (Castagnone-Sereno, 2013). Fertilization does not occur even when males are present. A female will produce an ovum with a full set of genes provided solely by the mother. Thus, a male is not needed to provide sperm to fertilize the egg. However, males can be sporadically observed even in obligate parthenogenesis. In such cases, males develop as a result of sex reversal induced by unfavorable environmental factors (Triantaphyllou, 1985), such as crowding, poor nutrition from the host, or high temperature. Since male formation would reduce the reproductive rate, it would prevent populations from becoming too large for the host to support. Among the four major root-knot nematode species, *M. javanica, M. arenaria* and *M.incognita* reproduce exclusively by obligatory mitotic parthenogenesis.

The basic objective in the management of root-knot nematode is to increase crop quality and yield. The most effective practices include crop rotation with non-hosts, host resistance and nematicide application. Biological control has been utilized in agriculture for managing root-knot nematodes. Fumigants, such as Telone (1, 3-dichloropropene), are widely used as pretreatments. They are very effective when applied pre-plant, and can kill a broad spectrum of nematodes. But they are expensive to use, and can only provide a short-term control. Nematicides have a side effect of harming both human health and environment (Perry, 2009). For example, methyl bromide was a common fumigant used in controlling root-knot nematodes, but it has been totally banned due to the ozone depletion (Collange et al., 2011). Chemical nematicides are being

discouraged specifically as a sole management method, and alternative control strategies are in urgent need. Crop rotation with a poor or non-host for the nematode can decrease nematode populations, and is widely used in current cropping systems (Stirling et al., 2011). The advantage of rotation is that it can provide long-term nematode suppression. However, due to the wide host range of root-knot nematodes, it is difficult to find non-host crops with high economic value, and the crop rotation strategy is usually nematode specific. Host resistance is another effective control method. Resistance against *Meloidogyne* spp. has been reported in many agricultural crops, but is not widely used (Cook & Starr, 2006). Tomato is one of the few crops in which resistance is widely used. In tomato, genetic resistance to root-knot nematodes is conferred by the *Mi* gene (Gilbert, 1956), and commercial resistant cultivars and rootstocks are available. In peanut, several resistant cultivars are available, for example, the cultivars Tifguard and Georgia-14N with resistance to *M. arenaria* and *M. javanica.* However, the yield potential of these resistant cultivars are not as high as the susceptible cultivars, and the resistance is only effective against two species of nematodes. None of the above management strategies alone are adequate in providing nematode control. Biological control is very promising in reducing nematode populations and can readily be incorporated into integrated management. More studies are needed to improve biological control agents and develop effective products for managing root-knot nematodes.

*Pasteuria* species are gram-positive bacteria. Four species of *Pasteuria* have been described, and they have different host preferences. Three of them are parasites of nematodes: *P. penetrans* on *Meloidogyne* spp., *P. thornei* on *Pratylenchus* spp., and *P. nishizawae* on cyst nematodes. The other is *P. ramosa* that parasitizes water fleas of the genus *Daphnia*. *Pasteuria penetrans* is a widely distributed endospore-forming bacteria, which is a parasite of *Meloidogyne* spp. The

endospores of *P. penetrans* attach to the second stage juveniles (J2) as they migrate through soil. After adhesion, the endospores usually germinate within 4-10 days after J2 enters a plant root and begins to feed (Sayre, 1977). A germ tube forms to penetrate the nematode body wall, and further develops into a microcolony. The colony fragments go through sporogenesis, and sporangia proliferate within the nematode body. Egg production by infected females is inhibited, and the number of females in roots is reduced (Bird, 1988; Davies, 1991). The degeneration in the female's reproduction system leads to few, if any, eggs. When the plant root containing females decomposes, as many as $2x10^6$ mature spores are released into the soil. J2 attached by large numbers of spores (>15) becomes less mobile, leading to the decrease in ability to locate and penetrate host roots (Davies, 1991).The encumbered nematodes with a decrease in mobility caused a significant reduction on the number of females developing in the roots compared to unencumbered controls.

Endospores of *P. penetrans* have potential for long term survival. In the laboratory, the endospores remained viable for more than a year at 10 to 36 ℃ (Mani, 1988). The endospores can endure a wide temperature range as well as desiccation. The optimal develop temperature for *P. penetrans* is between 28 and 35℃, and the attachment to J2 increased with increasing temperature up to 30℃. Chemical resistance is another characteristic of *P. penetrans* endospores, and it is related to the metabolic dormancy and waxy coat layer of the endospore (Setlow, 1994; G. R. Stirling, 1979). This is a very important characteristic of the bacterium, which indicates the possibility of using *P. penetrans* together with nematicides to manage root-knot nematodes in the field. Soil texture and flows of water are important environment factors that influence the endospores in the soil. Leaching accounts for the absence of *P. penetrans* from the cultivated

layer in many cases. Heavy rain fall contributes to movement the spores down (Mateille, 1996; Trudgill, 2000). These factors can further influence the endospore abundance in soil.

An in-vitro spore production system has been developed by *Pasteuria* Bioscience Inc (acquired by Syngenta®), and they have successfully produced spores for several strains for *Pasteuria*, including *P. penetrans*. Syngenta has released a product named CLARIVA™, which is a proprietary *P. nishizawae* seed treatment for control of the soybean cyst nematode.

Suppression of RKN by *P. penetrans* has been documented in many field studies, demonstrating the potential of this bacterium to be developed as an ideal biological control product. *P. penetrans* does not affect other organism or the environment due to their specificity to RKN. According to previous studies, tomato planted in soil treated with *P. penetrans* showed reduced crop damage and nematode populations compared to non-treated soil, and the level of reduction was similar to the treatments with nematicides (Stirling, 1984). When soil was inoculated with $10^5$ spores/g of *P. penetrans*, peanut root galling was reduced by 81% in a microplot study (Chen, 1996). In field studies, *P. penetrans* decreased the number of both J2 and gall indices,suppressed damage by RKN, and increased yields by up to 30% (Trudgill, 2000).The decrease in root gall number indicated that the endospores have prevented the J2 from entering plant roots, and the decrease in nematode population indicates the endospores could affect the reproduction of the female nematodes. Crop rotation with non-hosts of root-knot nematodes can decrease the density of endospores, since the bacterium only completes its lifecycle after the nematode infects a host plant (Ciancio, 2000; Madulu & Trudgill, 1994; Timper, 2001). In a rotation study carried out from 1991 to 1999, highest spore attachment was observed in continuous peanut, while rotations with non-hosts showed lower spore densities (Timper, 2001). However, even with presence of continuous susceptible hosts, *P. penetrans* does not always lead

to obvious suppression of RKN. In a multi-national project, increasing the frequency of RKN host plants did not lead to an increase in *P. penetrans* density was not observed (Trudgill, 2000). Plots that were naturally infested with *P. penetrans* did not show suppression; however, increased suppression was documented where an exotic isolate of *P. penetrans* had been added, suggesting that the nematode population was resistant to the native *P. penetrans*.

The host range of *P. penetrans* isolates is limited to *Meloidogyne* spp. The initial phase of parasitism by *P. penetrans* is determined by the attachment of endospores to the J2, which is the first step in the infection process. Endospores show heterogeneous adhesion to root-knot nematodes, and a carbohydrate-protein as well as carbohydrate-carbohydrate interaction is believed to be responsible for the adhesion specificity (Davies, 2008).

Researchers found that polyclonal antibodies could recognize 100% of the tested spores, while different monoclonal antibodies (Mabs) recognized different portions of the spores, indicating that different subpopulations of the spores were specific to different nematode populations, and there was heterogeneity among the *P. penetrans* population (Davies, 1994). Stirling (1985) tested the ability of 4 *P. penetrans* isolates to attach 15 RKN populations. Some populations of *P. penetrans* could attach to different species of RKN, but not to every population within a species. A population of *P. penetrans* could attach to different species of RKN, but not to every population within a species (Tridill eg al., 2000). So the attachment occurs on a population and perhaps even at the individual level.

The difference in susceptibility of individuals within a *Meloidogyne* population provides opportunities for developing resistance. When repeatedly challenged with a *P. penetrans* population, a population of RKN showed decreased spore attachment, indicating the occurrence

of a shift to genotypes resistant to attachment (Channer & Gowen, 1992; Tzortzakakis, 1996). Evidence for this scenario was also observed in a long-term field study carried out from 2000 to 2008 (Timper, 2009). Five single-egg mass (SEM) lines from the field population plus a greenhouse population (originally from Gibbs farm) of *M. arenaria* were used to assay soil. Four SEM lines acquired numerous spores, while one SEM and the greenhouse population acquired less than 5 spores. Such observation indicates that the population of *M. arenaria* is heterogeneous for spore attachment.

Both molecular and immunological methods have been used to test the heterogeneity of *P. penetrans* populations. When compare the 16S rRNA sequences of two populations of *P. penetrans*, and no variation was found (Bekal et al., 2001). However, nucleotide polymorphisms (SNPs) in the *spoII*AB gene between two isolates of *P. penetrans* were detected, and a single individual could be infected by multiple *P. penetrans* genotypes (Nong et al., 2007).

Negative frequency- dependent selection (NFDS) is a key process in driving host-parasite coevolution in many natural communities. The fundamental mechanism is that an increase in fitness in a parasite may change the selection pressure on a population of the hosts, leading to an antagonistic coevolution (Ayala & Campbell, 1974; Clarke, 1976; Hamilton, 1982). For NFDS to occur, there must be genetic variation in both the host and parasite population (Auld et al., 2012; Castagnone-Sereno & Danchin, 2014; Ebert, 2008). The host population should have both resistant and susceptible genotypes. Correspondingly, the parasite population should have both virulent and avirulent genotypes for common host genotypes. Rare host genotypes have an advantage over common genotypes with more chances of escaping parasite infection (Jayakar, 1970; Tellier & Brown, 2007). The common host genotypes tend to decrease in frequency because of parasitism, and will be gradually replaced by rare genotypes that are resistant to the

parasite. In turn, the parasite genotypes will change after a time lag in response to the reciprocal selection imposed by the hosts (Hutson & Law, 1981; Bell, 1982; Seger & Antonovics, 1988). Fluctuation in *P. penetrans* endospore attachment to an *M. arenaria* greenhouse population was observed in a field study (Timper, 2009). In 1997 and 1998, the greenhouse population acquired numerous spores, however, endospore acquisition declined from, 2000 to 2007, and since 2008 endospore acquisition increased again. Since greenhouse population was not exposed to *P. penetrans* selection pressure, it is unlikely that the greenhouse population has changed. The changes in spore acquisition over time likely reflect the changes in the frequency of *P. penetrans* attachment phenotypes. Due to selection pressure, co-evolution may happen between *P. penetrans* and *M. arenaria*.

*Daphnia magna* is a fresh water planktonic crustacean that reproduces by cyclical parthenogenesis; *Pasteuria ramosa* is a gram-positive, endospore-forming bacterium that is an obligate parasite of *Daphnia* (Ebert, 2005). The bacterium sterilizes the host soon after infection, in a way similar to *P. penetrans*. Since *Daphnia* reproduces by facultative parthenogenesis, it has the advantage of reproducing clonaly as well as sexually. Additionally, *P. ramosa* can be kept with its host in culture. The *Daphnia- P. ramosa* system has been widely used as a model system in evolutionary and epidemiological studies (Ebert, 2008). The coevolution of *D. magna* and *P. ramosa* has been well studied, and is consistent with negative frequency-dependent selection. *D. magna* populations have been shown to be heterogeneous for *P. ramosa* infection . When clones of *D. magna* were treated with single isolates of *P. ramasa,* the isolates attached to some clones, and not at all to other clones (Luijckx et al., 2011). The host and the pathogen show a high specific genotype-genotype interaction (Luijckx, 2011). In the *D. magna-P. ramosa* interaction,

rapid co-evolution has been observed (Little, 2006), and this may be due to the multiple genotypes within population and the short life cycle of *D. magna* (Ben-Ami, 2008).

The surface coat of nematodes is an amorphous layer that overlays the cuticle regions, and is predominantly made up of glycosylated proteins (Davies & Curtis, 2011). There is a dynamic replacement of the nematode surface coat reflected by continuous shedding and replacement of surface-associated antigens (Blaxter et al., 1992). Nematodes can alter their surface composition in response to environmental signals, and evidence for this switching has been observed in changes in surface antigens, as well as changes in surface lipophilicity (Proudfoot et al., 1993; Olsen et al., 2007). The surface coat is the outmost layer as well as the first to interface with host plants and parasitic organisms. There is strong evidence that *P. penetrans* spores adhere to the nematode surface coat. When J2 were pretreated with detergents that removed the surface coat, fewer spores attached compared to untreated J2 (Spiegel et al., 1996). When spores were pretreated with an extract of the surface coat, spore attachment was also reduced. Furthermore, when J2 were pretreated with lectin or carbohydrates that can bind to the nematode surface coat, fewer spores attached to the J2 (Spiegel et al., 1996), suggesting a carbohydrate-protein mechanism for spore attachment. Many factors have been shown to affect attachment of *P. penetrans* to *M. arenaria*, including temperature, pH, ion concentration, and electrostatic and hydrophobic interactions (Ahmed and Gowen, 1991; Hatz and Dickson, 1992; Afolabi et al., 1995; Mateille et al., 1995). Root exudates have been shown to alter the surface coat of plant-parasitic nematodes (Curtis, 2008); however, no studies have evaluated the effect of exudates on attachment of *P. penetrans* spores to the surface coat of root-knot nematodes. Root exudates contain substances including ions, free oxygen, enzymes, mucilage and a diverse array of primary and secondary metabolites (Bais et al., 2006). They are also important sources of

organic carbon utilized by soil microbes. Through the exudation of a wide variety of compounds, roots impact the soil microbial community, alter the chemical and physical properties of the soil, and influence the microbe-nematode interactions (Bertin et al., 2003; Bais et al., 2006).

Maternal effects on disease susceptibility have been reported in both vertebrates and invertebrates (Grindstaff, 2003). The mechanism can be responses to various factors, such as nutrition, presence of biological enemies and mate quality (Gliwicz, 1992; Prasad, 2003). Maternal effects were observed in the *Daphnia-P. ramosa* system. The offspring of mothers that were raised under poor condition (low food supply) show more resistance to parasites than offspring of mothers that were raised under good conditions (Mitchell, 2005). This phenomenon may be part of a general phenomenon by which mother will optimize their reproductive allocation strategy. Mothers experience stressed environment will give more resistant and better quality progeny that show greater survivorship, and this may be related to the effectiveness of the innate immune system (Gliwicz, 1992; Moller, 1998).

It is estimated that $10^4$ endospores per gram of soil are required for suppressive of RKN (Stirling, 1991). But if the nematode population is heterogeneous, only a subset of these $10^4$ endospores will truly attach to RKN genotypes within the population. From the perspective of biological control, it is more important to focus on the proportion of compatible and incompatible RKN-*Pasteuria* interactions instead of spore number (Davies, 2009). Moreover, due to selection pressure, if the RKN population is diverse enough, the nematodes will develop resistance to a commercial formulation of *P. penetrans*. When we use *P. penetrans* as a biological control agent, if the spore population is not diverse enough, it will fail to adapt to the development of RKN resistance, which would lead to the failure of biological control. In addition to understanding the genetic variation in the nematode-*Pasteuria* interaction, it is also

important to understand the environmental factors associated with the nematode susceptibility. Soil is a complex environment with interactions between root, soil, soil microbial community, the nematode and *P. penetrans*. A good understanding of the environment influence can help integrate the biological control with other disease management strategies more efficiently.

**References**


Afolabi, P., O'Shea, P. S., & Davies, K. G. 1995. The electrostatic nature of the spore of *Pasteuria penetrans*, the bacterial parasite of root-knot nematodes. *Journal of Applied Bacteriology 79*, 244-249.

Ahmed, R. & Gowen, S. R. 1991. Studies on the infection of *Meloidogyne* spp. with isolates of Pasteuria penetrans. Nematologia Mediterranea 19, 229-233.

Atibalentja, N., Noel, G. R., & Domier, L. L. (2000). Phylogenetic position of the North American isolate of *Pasteuria* that parasitizes the soybean cyst nematode, *Heterodera glycines*, as inferred from 16S rDNA sequence analysis. *International Journal of Systematic and Evolutionary Microbiology, 50,* 605-613.

Auld, S. K. J. R., Hall, Spencer R., Duffy, & Meghan A. (2012). Epidemiology of a *Daphnia*-multiparasite system and its implications for the red queen. *PLoS One, 7,* 1-6.

Ayala, F. J., & Campbell, Cathryn A. (1974). Frequency-dependent selection. *Annual Review of Ecology and Systematics, 5,* 115-138.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology 57,* 233-266.

Bekal, S., Giblin-Davis, R.M., Becker, J.O., Borneman, J., & Springer, M.S. (2001). Phenotypic and molecular analysis of a *Pasteuria* strain parasitic to the sting nematode. *Journal of Nematology*m *33*, 110-115.

Bell, G. (1982). The masterpiece of nature : the evolution and genetics of sexuality. Berkeley : University of California Press.

Ben-Ami, F., Regoes, R. R., & Ebert, D. (2008). A quantitative test of the relationship between parasite dose and infection probability across different host-parasite combinations. *Proceedings of the Royal Society B-Biological Sciences, 275*, 853-859.

Bertin, C., Yang, X., and Weston, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil, 256*, 67-83.

Bird, A. F., & Brisbane, P. G. (1988). The influence of *Pasteuria penetrans* in field soils on the reproduction of root-knot nematodes. *Revue de Nématologie*, *11*, 75-81.

Blaxter, M. L., Page, A. P., Rudin, W., and Maizels, R. M. 1992. Nematode surface coats - actively evading immunity. *Parasitology Today*, 8, 243-247

Castagnone-Sereno, P., Danchin, E. G. J., Perfus-Barbeoch, L., & Abad, P. (2013). Diversity and evolution of root-knot nematodes, genus *Meloidogyne*: new insights from the genomic era. *Annual Review of Phytopathology*, *51*, 203-220.

Channer, A. G. D. R., & Gowen, S. R. (1992). Selection for increased host resistance and increased pathogen specificity in the *Meloidogyne* spp *Pasteuria penetrans* interaction. *Fundamentals of Applied Nematology*, *15*, 331-339.

Charles, L., Carbone, I, Davies, K.G., Bird, D., Burke, M., Kerry, B. R., & Opperman, C. H. (2005). Phylogenetic analysis of *Pasteuria penetrans* by use of multiple genetic loci. *Journal Of Bacteriology*, *187*, 5700-5708.

Chen. (1996). Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. *Journal of Nematology, 28,* 159-168.

Chen, S. Y., Charnecki, J., Preston, J. F., & Dickson, D. W. (2000). Extraction and purification of *Pasteuria* spp. endospores. *Journal of Nematology, 32,* 78-84.

Ciancio, A., & Queneherve, P. (2000). Population dynamics of *Meloidogyne incognita* and infestation levels by *Pasteuria penetrans* in a naturally infested field in Martinique. *Nematropica, 30,* 77-86.

Clarke, B. (1976). The ecological genetics of host-parasite relationships. Genetic aspects of host-parasite relationships. Oxford, London, Edinburgh, Melbourne: Blackwell Scientific Publications.

Curtis, R. H. C. 2008. Plant-nematode interactions: environmental signals detected by the nematode's chemosensory organs control changes in the surface cuticle and behaviour. *Parasite 15,* 310-316.

Davies, K. G. (2009). Understanding the interaction between an obligate hyperparasitic bacterium, *Pasteuria penetrans* and its obligate plant-parasitic nematode host, *Meloidogyne* spp *Advances in Parasitology* , *68,* 211-245.

Davies, K. G., & Curtis, R. H. C. 2011. Cuticle surface coat of plant-parasitic nematodes. *Annual Review of Phytopathology, 49,* 135-156.

Davies, K. G., Laird, V., & Kerry, B. R. (1991). The motility, development and infection of *Meloidogyne incognita* encumbered with spores of the obligate hyperparasite *Pasteuria penetrans*. *Revue de Nematologie, 14*, 611-618.

Davies, K. G., Redden, M., & Pearson, T. K. (1994). Endospore heterogeneity in *Pasteuria penetrans* related to adhesion to plant-parasitic nematodes. *Letters in Applied Microbiology, 19,* 370-373.

Davies, K. G., Rowe, J. A., & Williamson, V. M. (2008). Inter- and intra-specific cuticle variation between amphimictic and parthenogenetic species of root-knot nematode (*Meloidogyne* spp.) as revealed by a bacterial parasite (*Pasteuria penetrans*). *International Journal For Parasitology, 38,* 851-859.

Ebert, D. (2005). Ecology, epidemiology, and evolution of parasitism in *Daphnia*: National Library of Medicine National Library of Medicine.

Ebert, D. (2008). Host-parasite coevolution: insights from the Daphnia-parasite model system. *Current Opinion in Microbiology, 11,* 290-301.

Eisenback, J.D., & Triantaphyllou, H.H. (1981). Root-knot nematodes: *Meloidogyne*. In W. R. Nickle (Ed.), *Manual of Agricultural Nematology*.

Gilbert, J.C. (1956). The inheritance of resistance to severe root knot from *Meloidogyne incognita* in tomato. *Proceedings of Hawaii Academy of Sciences, 31,* 17.

Gliwicz, Z. M. & Guisande, C. (1992). Family Planning in *Daphnia*: Resistance to Starvation in Offspring Born to Mothers Grown at Different Food Levels. *Oecologia*, 463-467.

Grindstaff, J. L., Brodie, E. D., & Ketterson, E. D. (2003). Review paper. immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proceedings: Biological Sciences*, 270, 2309-2319.

Hamilton, W. D. (1982). Pathogens as causes of genetic diversity in their host populations. *Population biology of infectious diseases, 25,* 269-296.

Hatz, B., and Dickson, D. W. 1992. Effect of temperature on attachment, development, and

interactions of *Pasteuria penetrans* on *Meloidogyne arenaria*. *Journal of Nematology, 24*, 512-521

Hutson, V., & Law, R. (1981). Evolution of recombination in populations experiencing frequency-dependent selection with time delay. *Proceedings of the Royal Society of London, 213*, 345-359.

Jayakar, S. D. (1970). A mathematical model for interaction of gene frequencies in a parasite and its host. *Theoretical Population Biology, 1*, 140-164.

Little, T. J., Watt, K., & Ebert, D. (2006). Parasite-host specificity: experimental studies on the basis of parasite adaptation. *Evolution, 60*, 31-38.

Luijckx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., & Ebert, D. (2011). Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions. *Ecology Letters, 14*, 125-131.

Madulu, J. D., & Trudgill, D. L. (1994). Influence of temperature on the development and survival of *Meloidogyne javanica*. *Nematologica, 40*, 230-243.

Madulu, J. D., Trudgill, D. L., & Phillips, M. S. (1994). Rotational management of *Meloidogyne javanica* and effects on *Pasteuria penetrans* and tomato and tobacco Yields. *Nematologica, 40*, 438-455.

Mani, A. (1988). Studies on the bacterial parasite *Pasteuria penetrans*:I. Spore viability after storage. II. Culture on citrus nematode *Tylenchulus semipenetrans*. *International Nematology Network Newsletter, 5*, 24-25.

Mateille, T., Duponnois, R., Dabire, K., Ndiaye, S., & Diop, M. T. (1996). Influence of the soil on the transport of the spores of Pasteuria penetrans, parasite of nematodes of the genus *Meloidogyne*. *European Journal of Soil Biology, 32*, 81-88.

Mitchell, S. E., & Read, A. F. (2005). Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings: Biological Sciences, 272,* 2601-2607.

Moller, A. P., Christe, Ph, Erritzoe, J., & Mavarez, J. (1998). Condition, disease and immune defence. *Oikos, 83,* 301-306.

Nong, G., Chow, V., Schmidt, L. M., Dickson, D. W., & Preston, J. F. (2007). Multiple-strand displacement and identification of single nucleotide polymorphisms as markers of genotypic variation of *Pasteuria penetrans* biotypes infecting root-knot nematodes. *Fems Microbiology Ecology, 61,* 327-336.

Olsen, D. P., Phu, D., Libby, L. J. M., Cormier, J. A., Montez, K. M., Ryder, E. F., & Politz, S. M. (2007). Chemosensory control of surface antigen switching in the nematode *Caenorhabditis elegans. Genes, Brain and Behavior, 6,* 240-252.

Perry, R. N., Moens, Maurice, & Starr, James L. (2009). *Root-knot nematodes / edited by Roland N. Perry, Maurice Moens and James L. Starr*: Wallingford, UK ; Cambridge.

Prasad, N. G., Mallikarjun, Shakarad, Rajamani, M., & Amitabh, Joshi. (2003). Interaction between the effects of maternal and larval levels of nutrition on pre-adult survival in *Drosophila melanogaster*. *Evolutionary Ecology Research, 5,* 903-911.

Proudfoot, L., Kusel, J. R., Smith, H. V., Harnett, W., Worms, M. J., and Kennedy, M. W. (1993). Rapid changes in the surface of parasitic nematodes during transition from pre- to post-parasitic forms. *Parasitology, 107,* 107-117.

Sasser, J.N., & Carter, C.C. (1985). Overview of the International *Meloidogyne* Project 1975-1984. In J. N. Sasser & C. C. Carter (Eds.), *An advanced treatise on Meloidogyne. Vol. I. Biology and Control* (pp. 19-24). Raleigh, NC: North Carolina University Graphics.

Sayre, R. M., & Wergin, W. P. (1977). Bacterial parasite of a plant nematode: morphology and ultrastructure. *Journal of Bacteriology, 129,* 1091-1101.

Seger, J., & Antonovics, J. (1988). Dynamics of Some Simple Host-Parasite Models with More than Two Genotypes in Each Species. *Philosophical Transactions of the Royal Society of London, 319,* 541-555.

Setlow, P. (1994). Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. *Society For Applied Bacteriology Symposium Series, 23,* 49-60.

Spiegel, Y., Sharon, E., & Mor, M. (1996). Attachment of *Pasteuria penetrans* endospores to the surface of *Meloidogyne javanica* second-stage juveniles. *Journal of nematology, 28,* 328-334.

Stirling, G. R. (1979). Biological control of root-knot nematodes (*Meloidogyne* spp.) on peach. *Phytopathology, 69,* 806-809.

Stirling, G. R. (1984). Biological control of *Meloidogyne javanica* and *Bacillus penetrans*. *Phytopathology,* 74, 55-60.

Stirling, G. R. (1985). Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne. Nematologica, 31*, 203-209.

Stirling, G.R. (1991). Biological control of plant parasitic nematodes : progress, problems and prospects / by Graham R. Stirling: Wallingford, Oxon, UK : C.A.B International.

Stirling, G.R. & Nikulin, A. (1998). Crop rotation, organic amendments and nematicides for control of root-knot nematodes (*Meloidogyne incognita*) on ginger. *Australasian Plant Pathology, 27,* 234-243.

Tellier, A, & Brown, J. K. M. (2007). Stability of genetic polymorphism in host-parasite interactions. *Proceedings: Biological Sciences, 274,* 809-817.

Timper, P. (2009). Population Dynamics of *Meloidogyne arenaria* and *Pasteuria penetrans* in a long-term crop rotation study. *Journal of Nematology, 41,* 291-299.

Timper, P., Minton, N. A., Johnson, A. W., Brenneman, T. B., Culbreath, A. K., Burton, G. W. &Gascho, G. J. (2001). Influence of cropping systems on stem rot (*Sclerotium rolfsii*), *Meloidogyne arenaria*, and the nematode antagonist *Pasteuria penetrans* in peanut. *Plant Disease, 85,* 767-772.

Triantaphyllou, A. C. (1985). Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes *Advanced treatise on Meloidogyne / edited by K.R. Barker, C.C. Carter and J.N. Sasser*: Raleigh, North Carolina State University, 1985.

Trudgill, D. L., Bala, G., Blok, V. C., Daudi, A., Davies, K. G., Gowen, S. R., & Voyoukallou, E. (2000). The importance of tropical root-knot nematodes (*Meloidogyne* spp.) and factors affecting the utility of Pasteuria penetrans as a biocontrol agent. *Nematology, 2*, 823-845.

Tzortzakakis, E. A., & Gowen, S. R. (1994). Resistance of population of *Meloidogyne* spp. to parasitism by the obligate parasite *Pasteuria penetrans*. *Nematologica, 40*, 258-266.

Tzortzakakis, E. A., Gowen, S. R., & Goumas, D. E. (1996). Decreased ability of *Pasteuria penetrans* spores to attach to successive generations of *Meloidogyne javanica*. *Fundamental and Applied Nematology, 19*, 201-204.

Vernon, P.R & Colye, P. D. (2003). Managing fusarium wilt/root-knot nematode complex. *Louisiana Agriculture, 46,* 22-23.

# CHAPTER 2

# INFLUENCE OF ROOT EXUDATES AND SOIL ON ATTACHMENT OF *PASTEURIA PENETRANS* TO *MELOIDOGYNE ARENARIA*[1]

[1]Chang Liu, Patricia Timper, Pingsheng Ji,Tesfamariam Mekete and Soumi Joseph. 2017. *Journal of Nematology*. 49(3):304-310.

Reprinted here with permission of the publisher.

**Abstract**

The bacterium *Pasteuria penetrans* is a parasite of root-knot nematodes (*Meloidogyne* spp.). Endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) and subsequently sterilize infected females. When encumbered by large numbers of spores, juveniles are less mobile and their ability to infect roots is reduced. This study looked at different factors that influence spore attachment of *P. penetrans* to the root-knot nematode *M. arenaria*. Pretreatment of J2 with root exudates of eggplant (*Solanum melongena* cv. Black beauty) reduced spore attachment compared to pretreatment with phosphate-buffered saline (PBS), suggesting that the nematode surface coat was altered or the spore recognition domains on the nematode surface were blocked. Spore attachment was equally reduced following exposure to root exudates from both host and non-host plants for *M. arenaria*, indicating a common signal that affects spore attachment. Although phytohormones have been shown to influence the lipophilicity of the nematode surface coat, auxins and kinetins did not affect spore attachment compared to PBS. Root exudates reduced spore attachment more in sterilized soil than in natural soil. Sterilization may have eliminated microbes that consume root exudates, or altered the chemical components of the soil solution or root exudates. Root exudates caused a greater decrease in spore attachment in a loamy sand than in a sandy loam soil. The sandy loam had a higher clay content than the loamy sand, which may have resulted in more adsorption of compounds in the root exudates that affect spore attachment. The components of the root exudates could have also been modified by soil type. The results of this study demonstrate that root exudates can decrease attachment of *P. penetrans* endospores to root-knot nematodes, indicating that when these nematodes enter the root zone their susceptibility to spore attachment may decrease.

*Key words: Meloidogyne arenaria, Pasteuria penetrans,* root exudates, root-knot nematode, spore attachment.

## Introduction

*Pasteuria penetrans* is a widely distributed endospore-forming bacterium, which is a parasite of root-knot nematodes, *Meloidogyne* spp. (Sayre and Starr, 1985). Suppression of root-knot nematodes by *P. penetrans* has been evaluated on numerous crops, demonstrating the potential of this bacterium as a biological control organism (Stirling, 1984; Chen et al., 1996; Trudgill et al., 2000). The initial stage of infection occurs when endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) of *Meloidogyne* spp. as they migrate through soil. The spores usually germinate within 4-10 d after J2 enters a host plant root and begins to feed. The bacterium goes through sporogenesis and will finally dominate the nematode body. When a small number of *P. penetrans* spores adhere to the J2, the infected female produces few to no eggs; when encumbered by a large number of spores (5 to 15), juveniles are less mobile and their ability to enter roots is reduced (Stirling, 1984; Davies et al., 1988). Attachment of endospores to J2 is a fundamental step in the infection process, without which the infection will not occur (Chen and Dickson, 1998).

The surface coat of nematodes is an amorphous layer that overlays the cuticle regions, and is predominantly made up of glycosylated proteins (Davies and Curtis, 2011). The replacement of the nematode surface coat is a dynamic process reflected by continuous shedding and replacement of surface-associated antigens (Blaxter et al., 1992). The composition of the surface coat changes rapidly in response to environmental signals. When J2 of *M. javanica* were treated with detergents, their ability to bind red blood cells was reduced. However, the binding property was completely renewed after 24 hr, suggesting that the surface coat was replaced (Spiegel et al.,

1997). Nematodes can switch surface composition in response to environmental signals, which may be important in host-parasite interactions (Olsen et al., 2007). The surface coat is the outmost layer as well as the first to interface with host plants and parasitic organisms. Using the fluorescent lipid probe AF18 (5-N-(octodecanoyl) aminofluorescein), rapid changes in surface lipophilicity were observed after incubating *Globodera rostochinensis* with potato root exudates (Akhkha et al., 2002). Phytohomones such as auxin and cytokinins decreased AF18 uptake by *G. rostochiensis*, while they had the opposite effect on *M. incognita* (Akhkha et al., 2002).

There is strong evidence that *P. penetrans* spores adhere to the nematode surface coat. When J2 were pretreated with detergents that removed the surface coat, fewer spores attached compared to untreated J2 (Spiegel et al., 1996). When spores were pretreated with an extract of the surface coat, spore attachment was also reduced. Furthermore, when J2 were pretreated with lectin or carbohydrates that can bind to the nematode surface coat, fewer spores attached to the J2 (Spiegel et al., 1996), suggesting a carbohydrate-protein mechanism for spore attachment. Davies and Danks (1993) demonstrated that the N-acetylglucosamine residues on the spore surface of *P. penetrans* can recognize the carbohydrate-recognition domains (CRD) on the nematode surface that are involved in spore attachment.

Root exudates have been shown to alter the surface coat of plant-parasitic nematodes (Curtis, 2008); however, no studies have evaluated the effect of exudates on attachment of *P. penetrans* spores to the surface coat of root-knot nematodes. Root exudates contain substances including ions, free oxygen, enzymes, mucilages and a diverse array of primary and secondary metabolites (Bais et al., 2006). They are also important sources of organic carbon utilized by soil microbes. Through the exudation of a wide variety of compounds, roots impact the soil microbial community, alter the chemical and physical properties of the soil, and influence the microbe-

nematode interactions (Bertin et al., 2003; Bais et al., 2006). Therefore, the objective of this study was to investigate the influence of root exudates on the attachment of *P. penetrans* to root-knot nematodes.

**Materials and methods**

*General methods:* Three single egg mass (SEM) lines of *M. arenaria* race 1 were obtained from a field population in Tifton, GA, and maintained on eggplant (*Solanum melongena*) in a greenhouse at 22-30°C. Eggs were separated from egg masses using 0.85% NaOCl (Hussey, 1973). Second-stage juveniles (J2) were hatched on a tissue paper supported by a screen placed on a hatching dish. Tap water was added to the hatching dish to just submerge the tissue paper. The hatched J2 migrated through the tissue paper into the water and they were collected every 2 days. After the final collection, J2 were left in tap water for 24 hr to develop their surface coat.

Two single spore lines of a population of *P. penetrans* originating from Florida were obtained from University of Florida. To propagate the spore lines, J2 of *M. arenaria* were incubated in phosphate-buffered saline, pH7.2 (PBS) with $10^5$ endospores from each single spore line for 4 hr, and 30 J2 were examined for spore attachment at 400 x magnification. Juveniles with an average of 2-5 spores/J2 were inoculated onto 4-wk-old eggplant seedlings (cv. Black Beauty) to produce spores. The plants were grown in a greenhouse for 4 mon before root harvest. Harvested roots were washed with running tap water and placed in a beaker containing 100 ml of 1:10 aqueous solution of Lallzyme EX-V (Lallemand, Montreal, Canada) to digest for 1 d on a shaker (100 rpm). Opaque infected females were freed from roots and hand-picked into glass dishes containing deionized water ($dH_2O$) with the aid of a dissecting microscope. Females were crushed with a dissecting needle to free endospores into $dH_2O$, and the eggs were vacuum

filtered (8-12 um) to remove the female cuticle. The spores that passed through the filter were enumerated on a hemocytometer at 1000x magnification. Spore concentration was adjusted to $10^6$ spores/ml for each single spore line and kept frozen.

For the endospore attachment bioassay, 200 infective J2 were incubated in 4 ml of 1x PBS in a small (150 mm × 25 mm) glass Petri dish with 0.1 ml of $10^6$ spores/ml of *P. penetrans*. Dishes were shaken horizontally with a shaker at 100 rpm for 6 hr at room temperature (24-26°C). The number of spores attached to 30 randomly selected J2 was determined using 400x magnification. Three single egg mass lines (SEM8, SEM14, and SEM40) of *M. arenaria* susceptible to the two single spore lines (SS16, SS17) of *P. penetrans* were used in the experiments.

Root exudates of eggplant, tomato (*Solanum lycopersicum*), corn (*Zea mays*), and cotton (*Gossypium hirsutum*) were obtained from plant seedlings grown in vermiculite. After approximately 4 weeks after planting, seedlings with root volumes equivalent to 5 ml (measured by water displacement) were submerged in 30 ml tap water in foil covered glass jars with the stem and leaves exposed to the air. Seedlings were incubated in the jars in a greenhouse for 24 hr and the root exudates were used immediately for experiments.

*Effect of root exudates on spore attachment:* To determine the effect of root exudates from eggplant cv. Black beauty and sodium dodecyl sulfate (SDS) detergent on the attachment of *P. penetrans* endospores to *M. arenaria*, the J2 were exposed to the following treatments: root exudates for 6 hr, root exudates for 6 hr followed by a 24 hr recovery, 1% SDS, and a PBS control. For the root-exudate treatments, J2 were incubated with 4 ml of eggplant root exudates for 6 hr, and rinsed with PBS three times before conducting the spore attachment bioassay. In

order to test if the surface coat would recover after incubating with root exudates, the J2 were rinsed and left in PBS for 24 hr before conducting the endospore attachment assay. For the SDS treatment, J2 were incubated for 30 min in PBS containing 1% SDS, and rinsed three times with PBS to remove the detergent. For the control, J2 were incubated in PBS for 6 hr. Incubation in exudates, SDS and PBS was done in 150 mm × 25 mm foil covered glass Petri dishes with 200 J2 on a horizontally shaker (100 rpm) at room temperature. Two SEM lines (SEM 8, 14) and two single spore lines (SS 16, 17) were used in this experiment, and it was conducted twice.

*Effect of phytohormones on spore attachment:* To determine the effect of phytohormones on attachment of *P. penetrans* to *M. arenaria*, J2 of SEM 40 were incubated in kinetin (6-furfurylaminopurine) with cytokinin activity and NAA (1-naphthaleneacetic acid) with auxin activity in foil covered glass dishes for 6 hr. A concentration of 10 μM of each phytohormones was prepared as described by Akhkha et al. (2002). As a control, J2 were incubated in PBS without phytohormones. After incubation, J2 were rinsed with PBS three times followed by an endospore attachment bioassay. Single egg mass lines 40 and SS 16 were used. There were three replicates for each treatment and the experiment was conducted twice.

*Effect of root exudates from non-host and host plants*: To determine whether root exudates from non-hosts of *M. arenaria* have the same effect on attachment of *P. penetrans* spores as host plants, exudates from eggplant cv. Black beauty, tomato cv. Rutgers, corn cv. Agra Tech, and cotton cv. DP 0935 were tested. Eggplant and tomato are hosts, corn is a poor host, and cotton is a non-host to *M. arenaria*. Root exudates were obtained as described earlier. Two hundred infective J2 of SEM 8 were incubated in 4 ml of each root exudate as treatments. As a control, the J2 were incubated in 4 ml of PBS. The J2 were incubated as described earlier before rinsing three times with PBS. The endospore attachment bioassay was conducted after exposure to root

exudates with SS16. There were four replicate dishes set up for each treatment and the experiment was conducted twice.

*Effect of soil microorganisms on root exudate activity:* To test if soil microorganisms can modify the effect of root exudates on attachment of *P. penetrans* to *M. arenaria*, soil types were used in this experiment. A Tifton loamy sand soil (fine-loamy, siliceous, thermic Plinthic Kandiudult; 85% sand, 11% silt, 4% clay, pH 6.1) was collected from the root zone of peanut at the Gibbs Farm, Tifton, GA, and a Greenville sandy loam soil (Fine, kaolinitic, thermic Rhodic Kandiudults; 69% sand, 15% silt, 16% clay; pH 5.6) was collected from the root zone of cotton from the Southwest Georgia Research and Education Center, Plains, GA. Soils were autoclaved for 30 min at 121°C, cooled for 24 hr, and autoclaved for another 30 min to kill microorganisms in soils. Natural and autoclaved soils were bagged and kept at 4°C for a month before use. The following treatments were used for both soils: natural soil with a plant, sterilized soil with a plant, and natural soil without a plant. Soils were added to small pots (120 $cm^3$). Both PBS and root exudates from eggplant seedlings incubated in foil covered bottles were used as controls. For treatments with plants, three 1-mon-old eggplant seedlings were planted in each pot. All treatments, except the PBS, were left in the greenhouse for 3 d. To extract the soil solution, soil was saturated with water and vacuum filtered (Whatman #1) using a Buchner Funnel at 72 kPa pressure. For treatments with the loamy sand, soil was vacuum filtered for 3 min and for treatments with sandy loam, soil was vacuum filtered for 4 min. The soil solutions were tested for pH and 5 ml was used to incubate J2 of SEM40 for 6 hr. Following incubation, the J2 were rinsed with PBS three times before conducting the endospore attachment bioassay with SS17. There were three replicates per treatment and the experiment was conducted twice.

*Statistical analysis:* Data were analyzed using the PROC GLIMMIX (generalized linear models) with a negative binomial distribution in SAS (v. 9.3). For the first experiment with root exudates, the spores per J2 for 30 individuals were used as data points. Single egg mass line, SS line, Treatment, and Trial were included in the model as well as the fully factorial interaction terms. Later we found that there was variation in spore acquisition among replicate dishes in the attachment bioassay. To account for this variation, the mean spore per J2 for the 30 individuals in a replicate was used as data points in all future experiments where only one SEM line and SS line was used. Treatment, Trial, Treatment * Trial and Reps were used to construct model effects. For all experiments, Tukey's HSD test was used to test pairwise comparison of model effects. For the experiment to test the effect of soil microorganisms on root exudate activity, contrasts were used to compare the effect of soil sterilization, root exudates, and different soil types on spore attachment.

**Results**

*Effect of root exudates on spore attachment:* The effect of root exudates on spore attachment was consistent among SEM lines, SS lines, and trials (no three- or four-way interactions). However, the effect of the root-exudate treatments on spore attachment differed between the trials (Treatment * Trial interaction, $P = 0.004$). In both trials, the J2 treated with root exudates and the detergent had lower ($P < 0.0001$) spore attachment than those treated with PBS (Fig. 2.1). In Trial 1, J2 pretreated with detergent had fewer attached spores than those pretreated with exudates, but this result was not confirmed in Trial 2. Incubating J2 in root exudates or detergent reduced spore attachment by 80 to 95% in Trial 1, and 76 to 84% in Trial 2 compared to J2 incubated in PBS. Even after 24 hr of recovery, the J2 incubated in root exudates

still had 80% lower spore attachment in Trial 1 and 84% lower spore attachment in Trial 2 than J2 incubated in PBS (Fig. 2.1).

*Effect of host plant and phytohormones on spore attachment*: The effect of root exudates from various plants on spore attachment differed between the two trials (Treatment * Trial interaction, $P < 0.0001$). In both trials, all root exudates reduced ($P < 0.0001$) spore attachment compared to the PBS control (Fig. 2.2). Among the root exudates, pretreatment of J2 with exudates from corn resulted in the lowest spore attachment, while pretreatment with exudates from cotton resulted in the highest spore attachment in Trial 1; the root exudates did not vary in their effect on spore attachment in Trial 2. . Pre-incubating J2 in phytohormones, including auxin and cytokinins, did not influence attachment of *P. penetrans* spores to *M. arenaria* J2 (data not shown).

*Effect of soil microorganisms on root exudate activity:* The effect of the soil treatments on spore attachment was not consistent between the two trials (Treatment * Trial interaction, $P = 0.001$). For both trials, pretreatment of J2 with either soil solutions (with and without plants) and root exudates reduced spore attachment compared to pretreatment with PBS (Fig. 2.3). The presence of plant roots in soil decreased ($P < 0.0001$) spore attachment compared to soil without plants (Table 2.1). However, in Trial 1 there was no difference between the sandy loam with and without plants (Fig. 2.3). The effect of root exudates on spore attachment tended to be greater in the loamy sand than in sandy loam. Root exudates reduced spore attachment more ($P = 0.013$) in sterilized soil than in natural soil (Table 2.1, Fig. 2.3). However, in Trial 1, there was no difference between natural and sterilized loamy sand with plants. Across all treatments, spore attachment was greater ($P < 0.0001$) in the sandy loam than in the loamy sand (Table 2.1). The pH of extracts from the natural soils without plants was 6.8-6.9, from natural soils with plants

was 7.0-7.1, and from autoclaved soil with plants was 7.2-7.3. The pH of the root exudates (without soil) and the PBS was 7.4 and 7.2, respectively.

**Discussion**

Our results demonstrate that exposure of J2 to root exudates reduced attachment of *P. penetrans* spores. The level of suppression of spore attachment was similar to when SDS was used to remove the nematode surface coat. It is noteworthy that even given a 24-hr recovery time, the spore attachment was still lower than the PBS control, indicating that this is a nonreversible process. Many factors have been shown to affect attachment of *P. penetrans* to *M. arenaria*, including temperature, pH, ion concentration, and electrostatic and hydrophobic interactions (Ahmed and Gowen, 1991; Hatz and Dickson, 1992; Afolabi et al., 1995; Mateille et al., 1995). In our study, we pre-exposed J2 to root exudates and soil solutions before conducting the attachment assay in PBS to exclude the influence of these factors. The compounds in root exudates that influence the surface coat are unknown. Root exudates from both host and non-host plants of *M. arenaria* reduced spore attachment to J2 suggesting that root exudates from diverse families share a common signal that affects spore attachment.

Nematodes can alter their surface composition in response to environmental signals, and evidence for this switching has been observed in changes in surface antigens, as well as changes in surface lipophilicity (Proudfoot et al., 1993; Olsen et al., 2007). *Caenorhabditis elegans*, widely studied as a model for environmental induction of surface changes, was found to switch its surface antigens based on environmental cues, and the switch involved chemosensory neurons (Grenache et al., 1996; Olsen et al., 2007). Changes in the lipophilicity of *G. rostochiensis* and *M. incognita* were observed after exposure to phytohormones (auxin and kinetin) and root exudates

(Mendoza et al., 2000; Akhkha et al., 2002). Phytohormones decreased the uptake of the fluorescent surface probe AF18 in *G. rostochiensis*, while they increased the uptake of AF18 in *M. incognita*. Root exudates increased AF18 uptake by *G. rostochiensis*, but had no effect on *M. incognita*. Although phytohormones are constituents of root exudates (Martinez-Toledo et al., 1988; Muhammad, 1998), our results showed that they had no effect on attachment of spores to J2. It is likely that the changes in lipophilicity observed previously are not involved in attachment of *P. penetrans* spores to the surface coat of *M. arenaria*.

The nematode surface coat can be shed upon exposure to host signals (Maizels et al., 1993; Modha et al., 1995), and this may explain the reduced spore attachment following exposure to root exudates. The level of reduction in spore attachment following pretreatment with root exudates was similar to pretreatment with SDS, which has been shown to remove the surface coat. However, if the surface coat was shed, replacement would have been rapid. Studies with monoclonal antibodies raised against the surface coat of *Globodera pallida* and *M. javanica*, showed a rapid replacement of the nematode surface (Fioretti et al., 2002). For instance, antibodies recognizing the nematode surface impaired nematode movement, but movement fully recovered after 1-6 hr indicating the rapid replacement of antigens on the surface. Human blood cells also have been used in studying nematode surface properties because of their ability to bind to the surface of several plant-parasitic nematode species (Spiegel and McClure, 1995). Spiegel et al. (1997) showed that binding of human blood cells to *M. javanica* decreased after treating J2 with detergents, but binding was completely renewed after 24 hr. In studies with animal-parasitic nematodes, changes in the nematode surface coat may be an important process for evading the immune response of hosts. The surface coat of *Romanomermis culicivorax*, a parasite of mosquito larvae, is shed continuously to avoid the mosquito immune system (Shamseldean et al.,

2007). The shedding of the surface coat can effectively remove host immune products from the surface coat.

In our study, spore attachment did not increase after 24 hr suggesting that the J2 surface coat was either shed permanently or was replaced with a new surface coat resistant to spore attachment. Similarly, Modha et al. (1999) observed shedding of the surface coat in *Trichinella spiralis* after exposure to host cues. When juveniles are activated by exposure to host cues, the probe PKH26, which is bound to the surface coat, is lost and the nematode cannot be re-labeled. However, when an unactivated juveniles (not exposed to host cues) sheds its surface coat, it can be re-labled with PKH26, suggesting that the host cues are triggering a prolonged or irreversible change in the surface of the nematode. In our study, we hypothesize that the surface coat was shed upon exposure to root exudates, and replaced with an altered surface coat.

Carbohydrate, lipid, and protein constituents of the nematode surface have been characterized (Robertson et al., 1989). A carbohydrate recognition domain (CRD) on the nematode surface coat recognizing N-acetylglucosamine residues on the *P. penetrans* spore surface has been reported to be involved in the endospore attachment to root-knot nematodes (Davies and Danks, 1992). Lectins concanavalin A (Con A) and wheat germ agglutinin (WGA) containing N-acetylglucosamine residues were found to bind to the surface of *M. javanica* inhibiting attachment by *P. penetrans* spores (Bird, et al., 1989; Sharon and Spiegel, 1996). Carbohydrate residues such as mannose or glucose, which are also found on the spore surface, can also be candidates for binding to nematode surface CRD. Pretreatment of *M. javanica* with carbohydrates fucose or α-methyl mannoside decreased *P. penetrans* spore attachment (Spiegel et al., 1996). Lectins and carbohydrates are common components of root exudates (Badri and Vivanco, 2009; De Hoff et al., 2009), and they may have bound to CRDs on the nematode

surface, thus preempting attachment of *P. penetrans* spores. However, this blockage should be transient with the continual replacement of the surface coat.

Prior exposure of *M. arenaria* juveniles to soil extracts in the absence of plants reduced attachment of *P. penetrans* spores to the nematode surface, and attachment was lower in extracts from a loamy sand than from sandy loam soil. It is not clear whether this reduction in spore attachment is due to an altered surface coat or to blockage of CRD domains on the nematode surface. However, the presence of root exudates in the soil extract further reduced spore attachment compared to extracts without root exudates confirming the results obtained with root exudates collected in tap water.

Root exudates reduced spore attachment more in sterilized soil than in natural soil. With sterilization, microbes that consume root exudates were eliminated, presumably resulting in greater availability of root exudates than in natural soil. Sterilization can also alter the components of soil solution, including the physicochemical characteristics (Egli et al., 2006; Dao, 2014; Mahmood et al., 2014). Studies showed that autoclaving can increase soluble organic matter as well as a small increase in soil pH (Berns et al., 2008; Liegel, 1986). These physicochemical differences can have an impact on the plant rhizosphere, which could influence root exudates in sterilized and non-sterilized soil.

We observed an interaction between soil type and root exudates; spore attachment decreased more in the loamy sand than in sandy loam soil, indicating that root exudates had a greater effect on attachment in the sandier soil. The higher percentage of clay in the sandy loam compared to the loamy sand (16 vs 4%) may have reduced the bioavailability of some components of root exudates due to adsorption onto the clay particles (Jones and Edwards, 1998).

The active component of the root exudates which triggers changes in the surface coat may have been bound to the clay particles resulting in lower concentrations in the soil solution from the sandy loam compared to the loamy sand soil. Furthermore, the constituents of root exudates can differ among soils (Huang et al., 2014; Neumann et al., 2014). A study with lettuce cultivated in three different types of soil showed that there were large quantitative differences, particularly for sugars and amino acids, among the three soil types (Neumann et al., 2014). This difference in root exudate profile may, in part, explain the interaction between soil type and root exudates on spore attachment.

In conclusion, the attachment of *P. penetrans* endospore to root-knot nematodes is a complex interaction with many factors influencing the outcome, including exposure to root exudates. In the field, the first generation nematodes that migrate to the roots are likely to be more susceptible to *Pasteuria* spore attachment because of lower exposure to root exudates than future generations that will be hatching around the roots. Understanding the interaction among root exudates, the nematode surface coat, and spore attachment is important to help develop *P. penetrans* as a biological control agent of root-knot nematodes.

**Literature cited**

Afolabi, P., O'Shea, P. S., and Davies, K. G. 1995. The electrostatic nature of the spore of *Pasteuria penetrans*, the bacterial parasite of root-knot nematodes. Journal of Applied Bacteriology 79:244-249.

Ahmed, R., and Gowen, S. R. 1991. Studies on the infection of *Meloidogyne* spp. with isolates of *Pasteuria penetrans*. Nematologia Mediterranea 19: 229-233.

Akhkha, A., Kusel, J., Kennedy, M., and Curtis, R. 2002. Effects of phytohormones on the surfaces of plant-parasitic nematodes. Parasitology 125:165-175.

Badri, D. V., and Vivanco, J. M. 2009. Regulation and function of root exudates. Plant, Cell and Environment 32: 666-681.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology 57:233-266.

Berns, A. E., Philipp, H., Narres, H. D., Burauel, P., Vereecken, H., and Tappe, W. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. European Journal of Soil Science 59:540-550.

Bertin, C., Yang, X., and Weston, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil 256: 67-83.

Bird, A. F. , Bonig, I., and Bacic, A. 1989. Factors affecting the adhesion of micro-organisms to the surfaces of plant-parasitic nematodes. Parasitology 98:155-164.

Blaxter, M. L., Page, A. P., Rudin, W., and Maizels, R. M. 1992. Nematode surface coats - actively evading immunity. Parasitology Today 8:243-247

Chen, Z. X., Dickson, D. W., McSorley, R., Mitchell, D. J., and Hewlett, T. E. 1996. Suppression of M*eloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. Journal of Nematology 28:159-168.

Chen, Z.X., Dickson. 1998. Review of *Pasteuria penetrans*: Biology, ecology, and biological control potential. Journal of Nematology 30: 313-340.

Curtis, R. H. C. 2008. Plant-nematode interactions: environmental signals detected by the nematode's chemosensory organs control changes in the surface cuticle and behaviour. Parasite 15:310-316.

Dao, T. 2014. Influence of sterilization methods on selected soil microbiological, physical, and chemical properties. Journal of Environmental Quality 18:39-44.

Davies, K. G., and Curtis, R. H. C. 2011. Cuticle surface coat of plant-parasitic nematodes. Annual Review of Phytopathology 49:135-156.

Davies, K. G., and Danks, C. 1992. Interspecific differences in the nematode surface coat between *Meloidogyne incognita* and *M. arenaria* related to the adhesion of the bacterium *Pasteuria penetrans*. Parasitology 105:475-480

Davies, K. G., and Danks, C. 1993. Carbohydrate/protein interactions between the cuticle of infective juveniles of *Meloidogyne incognita* and spores of the obligate hyperparasite *Pasteuria penetrans.* Nematologica 39:53-64.

Davies, K. G., Flynn, C. A., and Kerry, B. R. 1988. The life cycle and pathology of the root-knot nematode parasite *Pasteuria penetrans.* Conference paper, Brighton Crop Protection Conference, London: British Crop Protection Council.

De Hoff, P. L., Brill, L. M., and Hirsch, A. M. 2009. Plant lectins: the ties that bind in root symbiosis and plant defense. Molecular Gnetics and Genomics 282:1-15.

Egli, M., Mirabella, A., Kaegi, B., Tomasone, R., and Colorio, G. 2006. Influence of steam sterilisation on soil chemical characteristics, trace metals and clay mineralogy. Geoderma: 131:123-142.

Fioretti, L., Porter, A., Haydock, P.J., and Curtis, R. 2002. Monoclonal antibodies reactive with secreted–excreted products from the amphids and the cuticle surface of *Globodera pallida* affect nematode movement and delay invasion of potato roots. International Journal for Parasitology 32: 1709-1718.

Grenache, D. G., Caldicott, I., Albert, P. S., Riddle, D. L., and Politz, S. M. 1996. Environmental induction and genetic control of surface antigen switching in the nematode *Caenorhabditis elegans.* Proceedings of the National Academy of Sciences of the United States of America 93:12388-12393.

Hatz, B., and Dickson, D. W. 1992. Effect of temperature on attachment, development, and interactions of *Pasteuria penetrans* on *Meloidogyne arenaria*. Journal of Nematology 24:512-521.

Huang, X. F., Chaparro, J. M., Reardon, K. F., Zhang, R. F., Shen, Q. ,R., and Vivanco, J. M. 2014. Rhizosphere interactions: root exudates, microbes, and microbial communities. Botany 92: 267-275.

Hussey, R.S., Barker, K.R. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57: 1025-1028.

Jones, D.L., and Edwards, A.C. 1998. Influence of sorption on the biological utilization of two simple carbon substrates. Soil Biology and Biochemistry 30:1895-1902.

Liegel, L. H. 1986. Effects of sterilization procedures on the biological, chemical, and physical properties of soils: a review. Turrialba 36: 11-19.

Mahmood, T., Mehnaz, S., Fleischmann, F., Ali, R., Hashmi, Z. H., and Iqbal, Z. 2014. Soil sterilization effects on root growth and formation of rhizosheaths in wheat seedlings. Pedobiologia 57:123-130.

Maizels, R. M., Blaxter, M. L., and Selkirk, M. E. 1993. Forms and functions of nematode surfaces. Experimental Parasitology 77: 380-384.

Martinez-Toledo, M. V., de la Rubia, T., Moreno, J., and Gonzalez-Lopez, J. 1988. Root exudates of *Zea mays* and production of auxins, gibberellins and cytokinins by *Azotobacter chroococcum*. Plant and soil 110: 149-152.

Mateille, T., Duponnois, R., and Diop, M. T. 1995. Influence of abiotic soil factors and the host plant on the infection of photoparasitic nematodes of the genus *Meloidogyne* by the actinomycete parasitoid *Pasteuria penetrans*. Agronomie 15:581-591.

Mendoza, M. E. L. de, Modha, J., Roberts, M. C., Curtis, R., and Kusel, J. R. 2000. Changes in the lipophilicity of the surfaces of *Meloidogyne incognita* and *Haemonchus contortus* during exposure to host signals. Parasitology 120:203-209.

Modha, J., Kennedy, M. W., and Kusel, J. R. 1995. A role for second messengers in the control of activation-associated modification of the surface of *Trichinella spiralis* infective larvae. Molecular and biochemical parasitology 72: 141-148.

Modha, J., Roberts, M. C., Robertson, W. M., Sweetman, G., Powell, K. A., Kennedy, M. W., and Kusel, J. R. 1999. The surface coat of infective larvae of *Trichinella spiralis*. Parasitology 118: 509-522.

Muhammad, A., Frankenberger, K.T., Jr. 1998. Plant growth-regulating substances in the rhizosphere: microbial production and functions. Advances in Agronomy 62: 145-151.

Neumann, G., Bott, S., Ohler, M. A., Mock, H. P., Lippmann, R., Grosch, R., and Smalla, K. 2014. Root exudation and root development of lettuce (*Lactuca sativa L.* cv. Tizian*)* as affected by different soils. Frontiers in Microbiology 5:2-2.

Olsen, D. P., Phu, D., Libby, L. J. M., Cormier, J. A., Montez, K. M., Ryder, E. F., and Politz, S. M. 2007. Chemosensory control of surface antigen switching in the nematode *Caenorhabditis elegans.* Genes, Brain and Behavior 6:240-252.

Proudfoot, L., Kusel, J. R., Smith, H. V., Harnett, W., Worms, M. J., and Kennedy, M. W. 1993. Rapid changes in the surface of parasitic nematodes during transition from pre- to post-parasitic forms. Parasitology 107:107-117.

Robertson, W. M., Spiegel, Y., Jansson, H. B., Marbanmendoza, N., and Zuckerman, B. M. 1989. Surface carbohydrates of plant parasitic nematodes. Nematologica 35:180-186.

Sayre, R. M., and Starr, M. P. 1985. *Pasteuria penetrans* (ex Thorne, 1940) nom. rev., comb.n.,sp.n. a mycelial and endospore-forming bacterium parasitic in plant-parasitic nematodes. Proceedings of the Helminthological Society of Washington 52:149-165.

Shamseldean, M. M., Platzer, E. G., and Gaugler, R. 2007. Role of the surface coat of *Romanomermis culicivorax* in immune evasion. Nematology 9: 17-19.

Sharon, E., and Spiegel, Y. 1996. Gold-conjugated reagents for the labelling of carbohydrate recognition domains and glycoconjugates on nematode surfaces. Journal of Nematology 28:124-127.

Spiegel, Y., Kahane, I., Cohen, L., and Sharon, E. 1997. *Meloidogyne javanica* surface proteins: characterization and lability. Parasitology 115:513-519.

Spiegel, Y., and McClure, M. A. 1995. The surface-coat of plant-parasitic nematodes - chemical-composition, origin, and biological Role - a review. Journal of Nematology 27:127-134.

Spiegel, Y., Sharon, E., and Mor, M. 1996. Attachment of *Pasteuria penetrans* endospores to the Surface of *Meloidogyne javanica* second-stage juveniles. Journal of nematology 28:328-334.

Stirling, G. R. 1984. Biological control of *Meloidogyne javanica* and *Bacillus penetrans.* Phytopathology 74:55-60.

Trudgill, D. L., Bala, G., Blok, V. C., Daudi, A., Davies, K. G., Gowen, S. R., and Voyoukallou, E. 2000. The importance of tropical root-knot nematodes (*Meloidogyne* spp.) and factors affecting the utility of *Pasteuria penetrans* as a biocontrol agent. Nematology 2: 823-845.

Table 2.1. Contrast comparisons between treatment groups on attachment of *Pasteuria penetrans* endospores to *Meloidogyne arenaria* second-stage juveniles.

| Treatment | Contrast $P > F$ | Mean comparison |
|---|---|---|
| Plant vs no plant[a] | < 0.0001 | 3.8 vs 5.8 |
| Sterilize vs natural soil (with plant)[b] | 0.0133 | 3.3 vs 4.4 |
| Loam vs sand[c] | < 0.0001 | 5.3 vs 3.7 |

[a] Comparison includes natural and sterilized soil (sandy loam and loamy sand) with plants and without plants (N = 12).

[b] Comparison includes treatments with plants in sterilized and natural soil (N = 6).

[c] Comparison includes treatments with and without plants, both sterilized and natural sandy loam (loam) and loamy sand (sand) (N = 18).

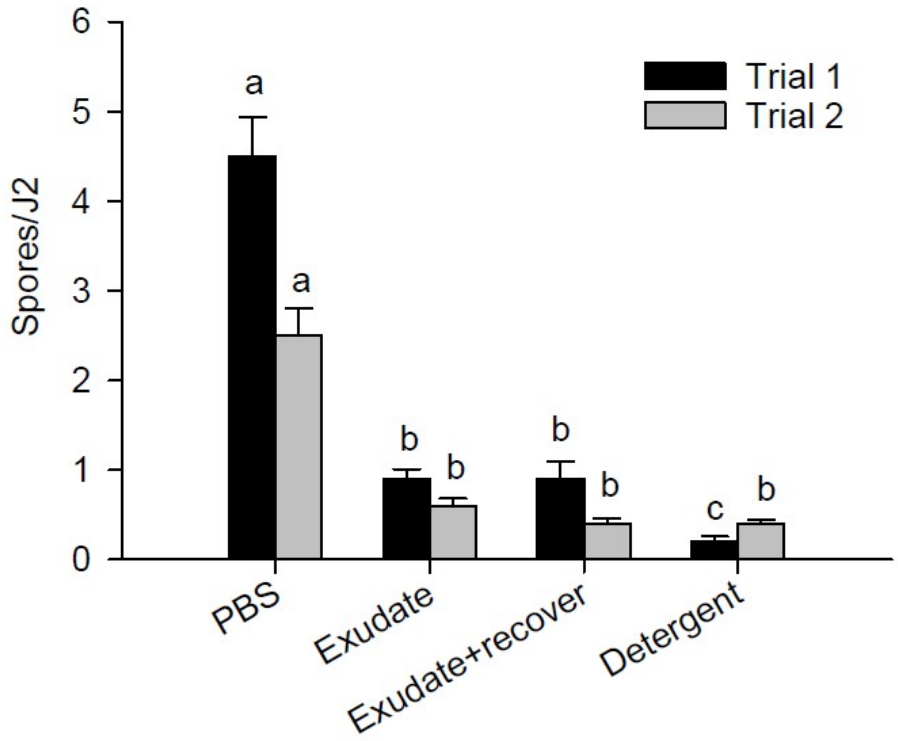


Fig. 2.1. Effect of root exudates on attachment of *Pasteuria penetrans* endospores to *Meloidogyne arenaria* second-stage juveniles (J2). PBS = phosphate-buffered saline. Within a trial, means followed by the same letter are not significantly different ($P > 0.05$). Each bar represents the mean spores per 30 J2 (± SE).

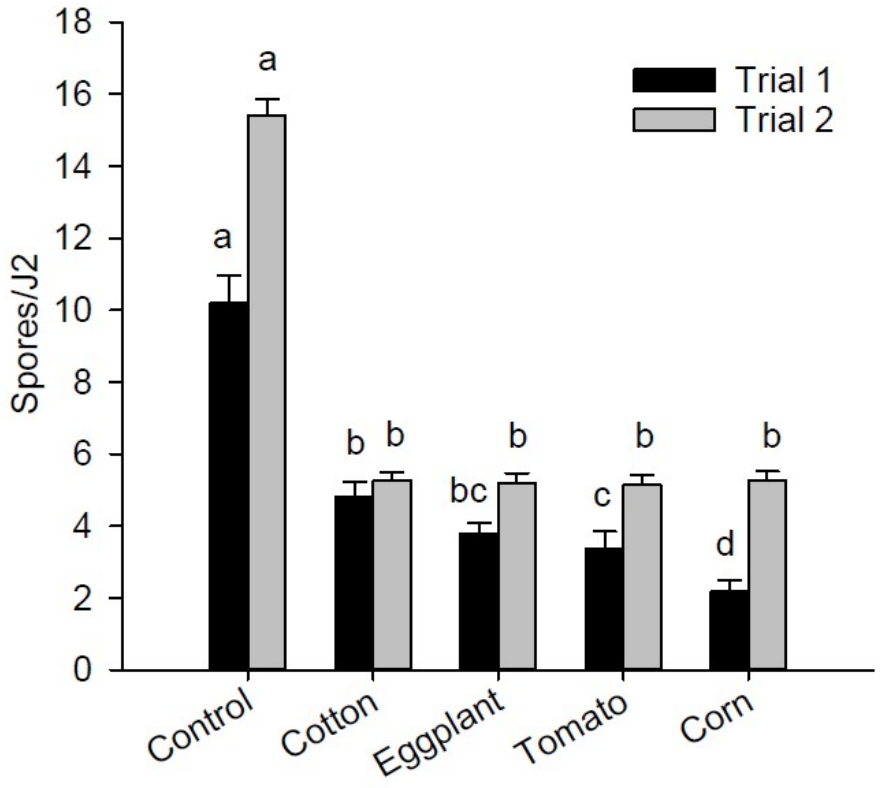


Fig. 2.2. Effect of root exudates from host (corn, eggplant, tomato) and non-host (cotton) plants on attachment of *Pasteuria penetrans* to second-stage juveniles (J2) of *Meloidogyne arenaria*. Within a trial, means followed by the same letter are not significantly different ($P > 0.05$). Each bar represents the mean of four replicates (± SE).

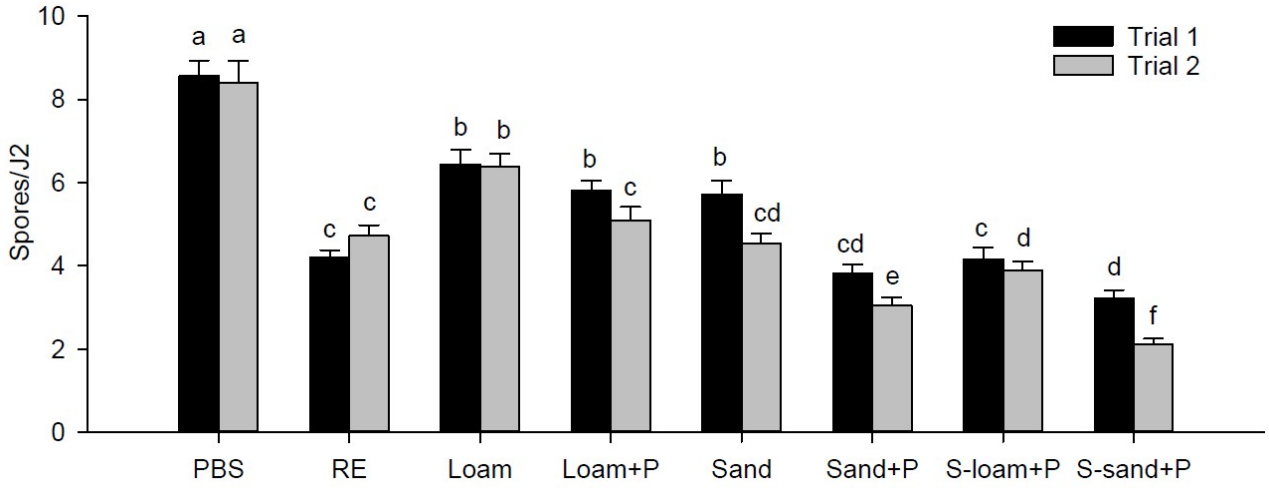


Fig. 2.3. Effect of root exudates and soil type on attachment of *Pasteuria penetrans* to *Meloidogyne arenaria*. PBS: phosphate-buffered saline. RE: root exudates. Loam: natural sandy loam. Loam+P: natural sandy loam with plant. Sand: natural loamy sand. Sand+P: natural loamy sand with plant. S-loam+P: sterilized sandy loam with plant. S-sand+P: sterilized loamy sand with plant. Within a trial, means followed by the same letter are not significantly different ($P > 0.05$). Each bar represents the mean of three replicates (± SE).

# CHAPTER 3

## EFFECTIVENESS OF *PASTEURIA PENETRANS* AS A SEED TREATMENT COMPARED TO AN APPLICATION IN FURROW[1]

[1] Chang Liu and Patricia Timper. To be submitted to *Journal of Nematology.*

**Abstract**

*Pasteuria penetrans* is a parasite of root-knot nematodes (*Meloidogyne* spp.). Endospores of *P. penetrans* attach to the cuticle of second stage juveniles (J2) and subsequently sterilize the infected female. When encumbered by large numbers of spores, juveniles are less mobile and their ability to infect roots is reduced. Exposure to root exudates can influence the nematode surface coat, and reduce *P. penetrans* spore attachment. This study compared the effect of applying *P. penetrans* spores in furrow versus as a seed treatment on the egg production of root-knot nematodes. Applying *P. penetrans* spores in furrow reduced egg production more than as a seed treatment. When applying *P. penetrans* as a seed treatment, the spores would distribute along the roots as the seed germinates. Second stage juveniles hatching from eggs in the soil will be exposed to root exudates as they enter the root zone and may, thereby, become more resistant to spore attachment. However, when *P. penetrans* were applied in furrow, the spores would have a wider distribution than just along the roots. The nematodes, therefore, may have more chances to encounter the spores before their surface coat is influenced by root exudates. The results of this study indicate that it is important to apply *P. penetrans* spores early in the season, and application in furrow is more effective than as a seed treatment in reducing nematode egg production.

**Introduction**

Root-knot nematodes (RKN; *Meloidogyne* spp.) are important pests in the southeastern US, causing significant yield losses in various crops. The basic objective in the management of root-knot nematode is to increase crop quality and yield. The most effective practices include crop rotation with non-hosts, host resistance, and nematicide application (Bridge, 1996; Collange et

al., 2011). Biological control has recently been utilized in agriculture for managing root-knot nematodes, and *Pasteuria penetrans* has shown to be an effective biological control agent. *Pasteuria penetrans* is widely distributed endospore-forming bacteria, and it is an obligate parasite of *Meloidogyne* spp. The endospores of *P. penetrans* attach to the surface coat of second stage juveniles (J2) of the nematode. When a small number of *P. penetrans* spores adhere to the J2, the infected female produces few to no eggs; when encumbered by a large number (>15) of spores, juveniles are less mobile and their ability to enter roots is reduced (Davies et al., 1988; Stirling, 1984).

Suppression of RKN by *P. penetrans* has been documented in many field studies, demonstrating the potential of this bacterium as a biological control product. According to previous studies, tomato planted in soil treated with *P. penetrans* had reduced crop damage and nematode populations compared to non-treated soil, and the level of control was similar to that achieved by nematicides (Stirling, 1984). In a microplot study, $10^5$ spores/g *P. penetrans* in soil reduced peanut root galling by 81% (Chen, 1996). When applied as a seed treatment in greenhouses, *P. penetrans* reduced egg production on tomato and cucumber (Kokalis-Burelle, 2015). Clariva™ is a seed treatment marketed by Sygenta contains *Pasteuria nishizawae*, Sedaxane, Thiamethoxam, Fludioxonil and Mefenoxam for use against soybean cyst nematode (Mohr, 2014). *Pasteuria nishizawae* is an obligate parasite of *Heterodera glycines*, the soybean cyst nematode, and has a life cycle similar to *P. penetrans* (Noel et al., 2010). It is likely that future products containing *P. penetrans* will also be applied as seed treatments.

The attachment of *P. penetrans* spores to J2 is a fundamental step in the infection process, without which the infection will not occur (Chen & Dickson, 1998). The nematode surface coat is an amorphous layer predominantly made up of glycosylated proteins (Davies & Curtis, 2011),

and there is strong evidence that *P. penetrans* spores adhere to the nematode surface coat (Spiegel et al., 1996). When J2s were pretreated with detergents that removed the surface coat, fewer spores attached compared to untreated J2s. Nematode can rapidly change their surface composition in response to environmental signals, and root exudates have been shown to alter the surface coat of plant-parasitic nematodes (Curtis, 2008). When exposed to root exudates, J2 showed more than 76% reduction in spore attachment compared to J2 incubated with phosphate-buffered saline (PBS) (Liu et al., 2017). Root exudates appear to have changed the nematode surface coat, increased resistance to *P. penetrans* spore attachment. Such observation led us to wonder whether using *P. penetrans* as a seed treatment is the most effective application method. If *P. penetrans* is applied as seed treatment, the spores would be distributed along the roots, and surface coat of the nematodes are likely to be changed as they approach the root. In this study, we compared the application of *P. penetrans* as a seed treatment versus in furrow. Our hypothesis is that applying *P. penetrans* in furrow will reduce egg production more than when used as a seed treatment.

**Material and Methods**

A *P. penetrans* single spore line SS 16 obtained from University of Florida was used in this experiment as spore inoculum. For the nematodes, a population of *M. arenaria* race 1 was maintained on eggplants (cv. Black Beauty) in a greenhouse at 22-30°C. Four months after nematode inoculation, roots of *M. arenaria* infected eggplants were cut off and washed with running water. Roots were cut to 5 cm pieces and mixed well. Mixed roots were weighed and evenly distributed to 22 parts. There were 260 g and 210 g roots put in each pot in trial 1 and trial 2 respectively. One part was set up on a mist chamber to estimate the number of hatched J2, and the rest were buried to a depth of 10 cm into each of 21 pots (dia=35 cm, hight=26 cm)

containing steam-heated soil. For the seed treatment, peanut seeds were planted into a 4 cm hole every 10 cm in the pot with $10^5$ spores inoculated right on the seed as seed treatment for a total of $3x10^5$ spores per pot. For the furrow treatment, a 3 cm wide, 4 cm deep furrow was made in each pot, and three seeds were evenly distributed in 10 cm intervals , and $3x10^5$ spores were evenly distributed along the furrow. For the Control group, three peanut seeds were planted in a 4 cm hole every 10 cm in the pot. Thirty days after seed germination, roots from all plants were cut off and washed well with running water. Roots were drained and left on paper towel to dry for 2 hr before weighed. Roots from the same pot were combined. Root weight per pot was recorded and eggs were separated from egg masses using 0.5% NaOCl (Hussey, 1973). Eggs were counted under 100X magnification, and egg per gram root and per pot were calculated. There were seven replicates set up for each treatment, and this experiment was conducted twice.

Statistical analysis

Data were analyzed using the Standard Least Squares Option (analysis of variance) in JMP Pro (v. 11). Egg per gram root and root weight per pot in each replicate were used as data points. Treatment, trial, as well as their interactions were used to construct the model effect. LS Means Student's t-test was used to test pairwise comparison of model effects.

**Results**

The estimated inoculum of *M. arenaria* J2 was 2000 and 1600 in trial 1 and trial 2, respectively. There was no treatment*trial interaction, so data from trial 1 and trial 2 were combined. Egg per gram root differed ($P<0.05$) among three treatments (Fig. 3.1). Control had highest egg production and the seed treatment had the lowest. *Pasteuria penetrans* reduced egg

production more in the furrow than in the seed treatment. There was no difference among treatments for root weight ($P$=0.91).

**Discussion**

*Pasteuria penetrans* applied both in furrow and as a seed treatment decreased egg production of *M. arenaria* in this study. Such observations agreed with previous studies which have shown that either applying *P. penetrans* as a seed treatment or used as a soil mix can reduce nematode population by decrease the number of eggs (Kumar et al., 2006; Chen, 1996; Kokalis-Burelle, 2015). This is the first study comparing in furrow and seed treatment of *P. penetrans*. We found that application of *P. penetrans* in furrow reduced egg production more than used as a seed treatment. Similar observations were made when comparing *P. penetrans* as a soil mix versus as a concentrated post-plant inoculation around the plant. Although no significant differences were found between these two treatments, egg production was consistently lower when used as a soil mix than as a post-plant inoculation (Kokalis-Burelle, 2015). Such results agreed with the observation that exposure to root exudates can reduce spore attachment of *P. penetrans* to RKN (Liu et al, 2017). In this study, when applying *P. penetrans* as a seed treatment, the spores would distribute along the roots as the seed germinates. When the eggs in the soil hatched into J2s and migrated through the soil, exposure to root exudates as they approach to root zone may increase their resistance to *P. penetrans* attachment. However, when *P. penetrans* were applied in furrow, the spores would have a wider distribution than just along the roots. The nematodes, therefore, may have more chances to encounter the spores before their surface coat was influenced by root exudates.

There are many factors to consider when commercializing a biological control agent. With a seed treatment, it is very convenient for the growers to use because they do not need to apply *Pasteuria* spp. before planting, and it can be formulated with other pesticides to make this product multi-functional. However, our results showed that applying *P. penetrans* spores in furrow is more effective than as a seed treatment. It is important to apply *P. penetrans* early in the season. In the field, the first generation nematodes are likely to be more susceptible to spore attachment because of the lower exposure to root exudates than future generations, which would be hatched around the root zone.

**References**


Bridge, J. (1996). Nematode management in sustainable and subsistence agriculture. *Annual Review of Phytopathology, 34*, 201-225.

Chen. Y.S. (1996). Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. *Journal of Nematology, 28*, 159-168.

Chen, Z. X., & Dickson, D. M. (1998). Review of *Pasteuria penetrans*: Biology, ecology, and biological control potential. *Journal of Nematology, 30*, 313-340.

Collange, B., Navarrete, M., Peyre, G., Mateille, T., & Tchamitchian, M. (2011). Review: Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. *Crop Protection, 30*, 1251-1262.

Curtis, R. H. C. (2008). Plant-nematode interactions: environmental signals detected by the nematode's chemosensory organs control changes in the surface cuticle and behaviour. *Parasite, 15*, 310-316.

Davies, K. G., & Curtis, R. H. C. (2011). Cuticle surface coat of plant-parasitic nematodes. *Annual Review of Phytopathology, 49*, 135-156.

Davies, K. G., Flynn, C. A., & Kerry, B. R. (1988). The life cycle and pathology of the root-knot nematode parasite *Pasteuria penetrans. Proceeding of British Crop Protection Conference.*

Kokalis-Burelle, N. (2015). *Pasteuria penetrans* for control of *Meloidogyne incognita* on tomato and cucumber, and *M. arenaria* on snapdragon. *Journal of Nematology, 47*, 207-213.

Kumar, A., Walia, R. K., & Ashima, K. (2006). Efficacy of *Pasteuria penetrans* (ex Thorne) Sayre & Starr as seed treatment in controlling *Meloidogyne javanica* (Treub) Chitwood on three succeeding crops. *Journal of Biological Control, 20*, 237-239.

Liu, C., Timper, P., Ji, P., I., Mekete, T., & Joseph, S. (2017). Influence of root exudates and soil on attachment of *Pasteuria penetrans* to *Meloidogyne arenaria*. *Journal of Nematology, 49*, 304-310.

Mohr, P. (2014). Grower tests new SCN seed treatment. *Farmer, 132*, 38.

Noel, G. R., Atibalentja, N., & Bauer, S. J. (2010). Suppression of *Heterodera Glycines* in a soybean field artificially infested with *Pasteuria nishizawae*. *Nematropica, 40*, 41-52.

Setlow, P. (1994). Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. *Society For Applied Bacteriology Symposium Series, 23,* 49S-60S.

Spiegel, Y., Sharon, E., & Mor, M. (1996). Attachment of *Pasteuria penetrans* endospores to the surface of *Meloidogyne javanica* second-stage juveniles. *Journal of nematology, 28*, 328-334.

Stirling, G. R. (1979). Biological control of root-knot nematodes (*Meloidogyne* spp.) on peach. *Phytopathology, 69,* 806-809.

Stirling, G. R. (1984). Biological control of *Meloidogyne javanica* and *Bacillus penetrans*. *Phytopathology*, 55.

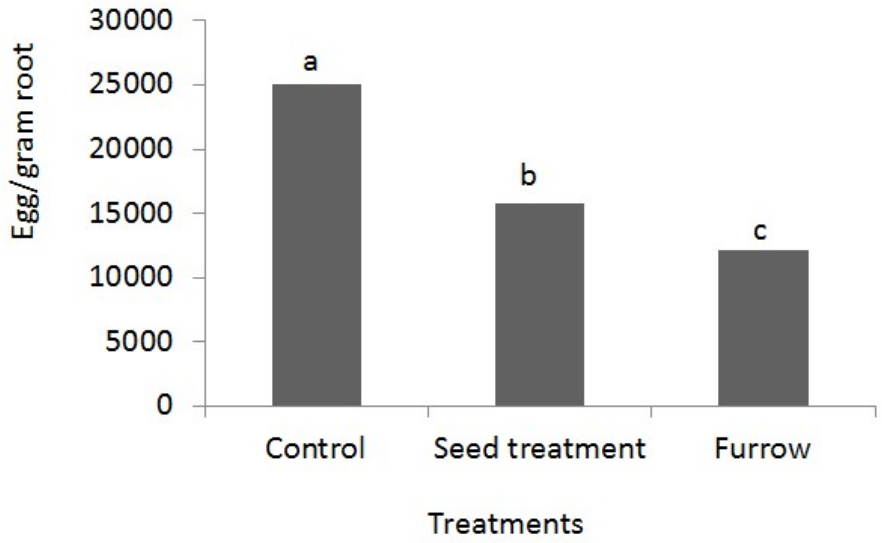


Fig. 3.1 Comparison of mean egg production by *Meloidogyne arenaria* in different treatments with *Pasteuria penetrans*. Bars with different letters indicate significant difference ($P$ <0.05). Each bar represents the mean of two trials.

# CHAPTER 4

# MATERNAL STRESS REDUCES THE SUSCEPTIBILITY OF ROOT-KNOT NEMATODE *MELOIDOGYNE ARENARIA* PROGENY TO *PASTEURIA PENETRANS* [1]

[1] Chang Liu and Patricia Timper. To be submitted to *Nematology*.

**Abstract**

*Pasteuria penetrans* is an obligate parasite of root-knot nematodes (*Meloidogyne* spp.). Endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) and complete their life cycle within the nematode female body. Infected females will be filled with spores and will be sterilized. Studies with *Daphnia magna* and its parasite *Pasteuria ramosa* showed that a poor maternal environment can lead to offspring resistant to *P. ramosa*. Therefore, we hypothesized that *Meloidogyne arenaria* females raised under a stressed environment would produce progeny that were more resistant to *P. penetrans*. Two treatments were set up in this study, a stressed environment created by crowding and low food supply, and a non-stressed environment. No difference in spore attachment was observed between the two treatments. However, infection rate of *P. penetrans* in the stressed treatment was significantly lower than that in the non-stressed treatment (8% vs. 18%). Mothers raised under stressed conditions produced more resistant offspring than did mothers raised under favorable conditions. When food supply is low, *M. arenaria* mothers may invest their progeny with enhanced survival traits. Our study indicates that *M. arenaria* can transfer immunity maternally to provide better protection to the progeny. In the field, nematodes often reach the carry capacity of their host plant by the end of the season, and their progeny may become more resistant to the infection by *P. penetrans*.

**Introduction**

Maternal effects on disease susceptibility have been reported in both vertebrates and invertebrates (Grindstaff et al, 2003). It arises where the mother makes a contribution to the phenotype of her progeny over and above that which results from the genes she contributes to the progeny (Mather & Jinks, 1982). Phenotype is a complex interaction between genotype and

environment (Bernardo, 1996). Maternal effects are important in influencing progeny phenotypes, and are believed to play a role in increasing population variability (Benton et al., 2001). Many studies have looked at the mechanism of maternal effects on disease susceptibility in vertebrates. Vertebrates mothers can pass on immune factors, such as antibodies, to their progeny through colostrum, milk, yolk eggs, etc. (Brambell, 1970; Grindstaff et al., 2003; Kowalczyk et al., 1985). Few studies have investigated maternal effects on invertebrates, mainly because invertebrates were thought to lack adaptive immunity in the form of T-cell receptors and major histocompatibility complex on all surfaces that mediate vertebrate immune responses (Anderem & Ulevitch). Maternal effects on invertebrate immunity have been observed in several organisms in response to various factors, such as nutrition, presence of biological enemies and mate quality (Gliwicz & Guisande, 1992; Rossiter, 1996). Huang and Song (1999) were first to report maternal transmition of resistance to viral infection in an invertebrate. Adult shrimp treated with glucan were slightly more resistant than non-treated adults to a virus that causes white spot syndrome; however, their offspring showed a 23% increase in survival compared to offspring from non-treated adults when challenged with the virus. Little et al. (2003) reported that in water fleas (*Daphnia magna*), when mothers were challenged with *Pasteuria ramosa*, their offspring showed lower infection levels when exposed to the same bacterial strain.

There is evidence in invertebrates that maternal environmental conditions may affect the disease susceptibility of progeny. With *D. magna*, the offspring of mothers that were raised under poor conditions (low food supply) showed more resistance to parasites than offspring of mothers that were raised under good conditions (Mitchell & Read, 2005). Similarly, Boots and Roberts (2012) reported that with Indian meal moth (*Plodia interpunctella*), offspring from mothers in poor environments were more resistant to an insect virus PiGV . These observations

may be part of a general phenomenon by which mothers optimize their reproductive allocation strategy. Mothers experiencing harsh environmental conditions produced more resistant and better quality progeny that show greater survivorship, and this may be related to the effectiveness of the innate immune system (Gliwicz & Guisande, 1992; Moller et al., 1998). Maternal effect can vary between different host genotypes, indicating there are genotype by maternal environment interactions (Stjernman & Little, 2011). When different *D. magna* genotypes were raised under low-food conditions, their offspring showed varying levels of resistance to parasites. Such differences maintain variation within populations even when food quantity fluctuates in nature (Hall & Ebert, 2012).

*Meloidogyne arenaria*, the peanut root-knot nematode, is an economically important plant parasite. They reproduce exclusively by mitotic parthenogenesis, in which fertilization does not occur even when males are present (Sasser & Carter, 1985). Thus, a male is not needed to provide sperm to fertilize the egg. However, males can develop as a result of sex reversal induced by unfavorable environmental conditions (Triantaphyllou, 1985), such as crowding or poor nutrition from the host or high temperature. Such a reproductive strategy is believed to prevent populations from becoming too large for the host to support. No previous study has looked at the maternal effects in root-knot nematodes.

*Pasteuria penetrans* is a widely distributed endospore-forming, gram positive bacterium. It is an obligate parasite of root-knot nematodes. The infection process of P. *penetrans* has multiple steps: (a) attachment of endospores to the cuticle of the second-stage juvenile (J2); (b) penetration of nematode cuticle by infection tube; (c) sporulation and proliferation of spores within the female body. It is noteworthy that the spores do not kill the nematode female, but

sterilize them. The objective of this study was to investigate the influence of maternal stress in *M. arenaria* on the susceptibility of progeny to parasitism by *P. penetrans*.

**Materials and Methods**

Two treatments were set up for this experiment: Stressed, in which the nematodes were raised under crowded conditions and non-stressed, in which the nematodes were raised under uncrowded conditions. Two single egg mass (SEM) lines (SEM23, 40) of *M. arenaria* race 1 were obtained from a field population in Tifton, GA, and maintained on eggplants (cv. Black Beauty) in a greenhouse at 22-30°C. The experimental design was a two by two factorial with two treatments and two SEM lines. To initiate the experiment, 4-week-old eggplant seedlings were transferred to 10 cm x 10 cm pots with sterilized soil 1 week before nematode inoculation. Nematode inoculum was obtained from 4-mon-old eggplants by cutting the roots from the plant, washing them in tap water, and placing the roots in a mist chamber for eggs to hatch. Fresh second stage juveniles (J2) were collected every two days.

A single spore line (SS 17) of *P. penetrans* was obtained from University of Florida (Soumi et al., 2015). Spores of *P. penetrans* were produced by inoculating 200 J2s attached with an average of 2-5 spores/J2 onto 4-week-old eggplant seedlings. The plants were grown in a greenhouse for 4 mon before root harvest. Harvested roots were washed with running water and placed in a beaker containing 100 ml of 10 g Lallzyme EX-V (Lallemand, Montreal, Canada) to digest root tissue for 1 d on a shaker (100 rpm). Fully opaque infected females were freed from roots and hand-picked into a glass petri dish containing deionized water. Females were crushed with a dissecting needle to free endospores into $dH_2O$. The spore solution was vacuum filtered

(8-12 um) to remove the female cuticle. Spore concentration was determined under 1000x magnification on a hemocytometer.

For the stressed treatment, 5000 J2 were inoculated into each pot and the lower two leaves were pruned to reduce root growth. For the non-stressed treatment, 1000 J2 were inoculated into each pot and the leaves were not pruned. Six replicates were set up for each treatment. Two months later, five soil cores were removed from each pot with a small sampling probe (130 mm dia.). The nematodes were extracted from 100 $cm^3$ of soil by the centrifugal sugar floatation method (Jenkins, 1964) and observed under x100 magnification to determine the number of *M. arenaria* males. The presence of males was used to indicate that the nematodes were under stress. To obtain progeny of *M. arenaria*, roots with egg masses from each pot were cut off, washed well and placed separately in a mist chamber for eggs to hatch. Hatched J2 were collected 3-4 d later. A spore attachment bioassay was carried out with J2 from each pot. About 1 ml of $10^5$ spores were added to a small (150 mm x 25 mm) glass Petri dish with 500 J2 and 4 ml of 1x phosphate buffered saline (PBS, pH=7.4). Dishes were placed on a horizonal shaker at 100 rpm for 6 hr at room temperature (24-26 °C). The number of spores attached to 25 randomly-selected J2 were determined under 400x magnification. These spore-incubated J2 were inoculated into new pots (planted with 4-week-old eggplant seedlings) immediately after examination to determine the rate of infection by *P. penetrans*. One month after inoculation, eggplant roots were cut off and washed well with tap water. Roots from each pot were treated with Lallzyme EX-V as described above. After the root tissue was softened, 30 females were randomly selected and hand-picked into glass dishes containing water with the aid of a dissecting microscope. Spore-infected females were fully opaque, while non-infected females were semi-transparent. The number of infected vs. non-infected females were recorded. The experiment was conducted twice.

Statistical analysis

For the spore attachment assay, data were analyzed using the Standard Least Squares Option (analysis of variance) in JMP Pro (v.13). The mean spores per J2 for 25 individuals was used as data points. Single egg mass lines, Treatment and Trial were included in the model as well as all two-and three-way interactions. LS Means Student's t-test was used to test pairwise comparision of model effects. For female infection rate, binary variables were used, where infected females were represented by 1 and non-infected femalew were represented by 0. Data were analyzed using the Binomial Distribution Option in JMP. Single egg mass lines, Treatment, Trial and their interactions were included in the model.

**Results**

Mean male number per 100 $cm^3$ soil was 3.4 in Trial 1 and 3.3 in Trial 2 in the stressed treatment. No males were detected in the non-stressed treatment. Males were observed in each pot in the stressed treatment. The effect of stress on spore attachment was consistent among SEM lines (no SEM*Treatment interaction) and among trials, so the results of two SEM line and two trials were combined. There was no difference ($P$=0.053) in attachment of spores to progeny from stressed and non-stressed environments, both treatments averaged 5.8 spores / J2. However, the two treatments showed a large difference in infection rate of the female progeny, and this difference was consistent between the SEM lines and trials. Progeny produced by mothers in crowded conditions were less ($P$=0.0016) susceptible to parasite infection compared to those produced under good conditions (Fig.4.1). In the stressed treatment, 8% of females were infected with *P. penetrans*, while it was 18% were infected in the non-stressed treatment. None of the infected females produced eggs in this experiment.

## Discussion

In this study, we showed a reduced susceptibility of *M. arenaria* progeny from mothers in a stressed environment. When mothers were raised under low food levels and crowding, the progeny were less susceptible to *P. penetrans* spore infection compared to progeny produced from mothers raised under adequate food levels. Few studies have focused on maternal effects on disease susceptibility in invertebrates, and our observation was consistent with the findings in other studies looking at the maternal effects in invertebrates (Mitchell & Read, 2005; Boots & Roberts, 2012).

It seems intuitive that a good maternal environment would lead to better quality provisioning of progeny, and an overall higher resistance to harsh environments and disease. The activation and deployment of the immune system may be costly, and when the food level is low, there is a conflict between defense and other fitness attributes for limited resources (Boots & Begon, 1994; Moret & Schmid-Hempel, 2000). However, several studies have shown that when the maternal environment is poor, the mother may invest more in each offspring (Beckerman et al, 2006; Boots &Roberts, 2012). Smaller clutch size but bigger eggs were produced by *D. magna* females that were raised under stressed conditions (Gliwicz & Guisande, 1992). Similarly, when *Drosophila melanogaster* mothers were reared on poor food, they laid heavier eggs (Prasad et al, 2003). Such trade-offs in offspring size and number may be a strategy for mothers to provision their offspring for greater survival (Gliwicz & Guisande, 1992; Rossiter, 1996; Cleuvers et al, 1997). We did not determine the number or size of *M. arenaria* eggs in this study; however, we predict that fewer but larger eggs will be produced by females under crowded conditions.

It is well known that vertebrates transfer immunity to progeny via antibodies (Dennis & Jan-Åke, 2009). The transfer of acquired immunity across generations was thought to be lacking in invertebrates because they lack immunoglobulins. However, studies with *D. magna* and *Bombus terrestris* in recent years showed that the transmission of immunity also exists in invertebrates, but the mechanism remains unclear (Grindstaff et al, 2003; Little et al, 2003; Sadd & Schmid-Hempel, 2006). Our study further enhances this view with the evidence that *M. arenaria* can transfer immunity maternally to provide better protection to progeny. The generation time of *M. arenaria* is short (about 30 days). Since females lay eggs next to its body, progeny of *M. arenaria* were likely to encounter the same environment as their mother. Mothers under stressed environment may invest their progeny with enhanced survival traits.

In this study, the maternal stress in *M. arenaria* was caused by low food supply imposed by crowding. The density effect on disease susceptibility has been observed in insects. Reeson et al. (1998) found that noctuid larvae reared at a high density were more resistant to virus than those reared in isolation. Crowding chemicals were shown to impact *D. magna* female size, brood size and juvenile densities (Mitchell & Carvalho). We do not know if crowding under adequate food supply makes a difference as we could not distinguish between low food supply and high density. Further research is needed to see if crowding pheromones in mothers plays a role in progeny susceptibility to *P. penetrans*.

There was no difference between the two SEM lines with respect to the different treatments. Hall and Ebert (2012) reported that host genotype can interact with maternal environment to influence the infection of *P. ramosa* to *M. magna*. They also showed that the maternal environment can influence different stages of the host-parasite interaction, including parasite penetration of the host, parasite clearance, and the proliferation of the parasite. In our study,

maternal stress had no influence on the spore attachment of *P. penetrans* to *M. arenaria*; differences were only observed in infection rate. It could be that the mechanism of enhanced resistance of *M. arenaria* to *P. penetrans* in stressed condition occurs after the spore attaches to the host cuticle. More *M. arenaria* and *P. penetrans* genotype combinations are worth testing to investigate the genotype-environment interaction.

Rao et al. (1997) reported that the infection rate of *P. penetrans* to *M. incognita* was about 20-28% when J2 were attached by one or two spores. The infection rate in this experiment was lower than this number, even in non-stressed conditions. The reason could be that the spore attachment assay in this study was done in petri dishes, and the spores may not attach as firmly as in natural soil. Or, since we were using single spore lines of *P. penetrans* instead of using a population of spores, the low genetic variability may be the reason for the low infection rate.

In this study, we emphasized the role of maternal environment in root-knot nematode resistance to *P. penetrans* spores. When the nematodes reach the carry capacity of their host plant, which usually happens at the end of the season, the progeny may become more resistant to infection by *P. penetrans*. These resistant progeny will be the initial inoculum for next spring, which may reduce the efficacy of *P. penetrans*. More studies are needed to investigate this maternal stress effect, including whether maternal stress would influence progeny egg size and egg number, or influence the spore reproduction in the female progeny. Moreover, it would be of great interest to identify the mechanism of the resistance caused by maternal effect.

**References**

Anderem, A. & Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature, 406,* 782.

Beckerman, A.P., Benton, T, G; Lapsley, C.T. & Koesters, N. (2006). How effective are maternal effects at having effects? *Proceedings: Biological Sciences, 273,* 485-493.

Benton, T.G., Ranta, E., Kaitala, V., & Beckerman, A. P. (2001). Maternal effects and the stability of population dynamics in noisy environments. *Journal of Animal Ecology, 70,* 590-599.

Bernardo, J. (1996). Maternal effects in animal ecology. *American Zoologist, 36,* 83-105.

Boots, M., & Begon, M. (1994). Resource limitation and the lethal and sublethal effects of a viral pathogen in the Indian meal moth, *Plodia interpunctella*. *Ecological Entomology, 19,* 319-326.

Boots, M., & Roberts, K. E. (2012). Maternal effects in disease resistance: poor maternal environment increases offspring resistance to an insect virus. *Proceedings of the Royal Society. Biological Sciences, 279,* 4009-4014.

Brambell, F. W. R. (1970). The transmission of passive immunity from mother to young. *North Holland Research Monographs Frontiers of Biology, 18,* 324-365.

Dennis, Hasselquist, & Jan-Åke, Nilsson. (2009). Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philosophical Transactions: Biological Sciences, 1513,* 51-60.

Gliwicz, Z. M. & Guisande, C. (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia,* 91, 463-467.

Grindstaff, J. L., Brodie, E. D., & Ketterson, E. D. (2003). Review Paper. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proceedings: Biological Sciences, 270,* 2309-2319.

Hall, M. D., & Ebert, D. (2012). Disentangling the influence of parasite genotype, host genotype and maternal environment on different stages of bacterial infection in *Daphnia magna*. *Proceedings of the Royal Society B-Biological Sciences, 279*, 3176-3183.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., & Ezekowitz, R. A. B. (1999). Phylogenetic perspectives in innate immunity. *Science, 284,* 1313-1318.

Huang, C, & Song, Y. (1999). Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Developmental and Comparative Immunology, 23*, 545-552.

Janeway, C.A., & Medzhitov, R. (2002). Innate immune recognition. *Annual Review Of Immunology, 20,* 197-216.

Jenkins, W. R. (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Reporter, 48,* 692-692.

Kowalczyk, K., Daiss, J., Halpern, J., & Roth, T. F. (1985). Quantitation of maternal-fetal IgG transport in the chicken. *Immunology, 54,* 755-762.

Little, T.J., O'Connor, B., Colegrave, N., Watt, K., & Read, A.F. (2003). Maternal transfer of strain-specific immunity in an invertebrate. *Current Biology, 13*, 489-492.

Mitchell, S. E., & Read, A. F. (2005). Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings: Biological Sciences, 272,* 2601-2607.

Moller, A. P., Christe, Ph, Erritzoe, J., & Mavarez, J. (1998). Condition, disease and immune defence. *Oikos, 83,* 301-306.

Moret, Y. & Schmid-Hempel, P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science, 290,* 1166-1168.

Prasad, N. G., Mallikarjun, S., Rajamani, M., & Amitabh, J. (2003). Interaction between the effects of maternal and larval levels of nutrition on pre-adult survival in *Drosophila melanogaster*. *Evolutionary Ecology Research, 5*, 903-911.

Rao, M. S., Gowen, S. R., Pembroke, B., & Reddy, P. P. (1997). Relationship *of Pasteuria penetrans* spore encumberance on juveniles of *Meloidogyne incognita* and their infection in adults. *Nematologia Mediterranea, 25*, 129-131.

Reeson, A. F., Wilson, K., Gunn, A., Hails, R. S., & Goulson, D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings: Biological Sciences, 265*, 1787-1791.

Rossiter, M. (1996). Incidence and consequences of inherited environmental effects. *Annual Review of Ecology and Systematics. 27*, 451-476.

Sadd, B. M., & Schmid-Hempel, P. (2006). Insect immunity shows specificity in protection upon secondary pathogen exposure. *Current Biology, 16*, 1206-1210.

Sasser, J. N., & Carter, C. C. (1985). *An Advanced treatise on meloidogyne*: Raleigh, N.C., U.S.A. : Dept. of Plant Pathology, North Carolina State University, 1985.

Soumi, J., Mekete, T., Schmidt, L.M., Hewlett, T., Watrin, C. & Timper, P. (2015). Detecting genotypic variation among the single spore isolates of *Pasteuria penetrans* population occurring in Florida using SNP-based markers. *Journal of Nematology, 47*, 269.

Stjernman, M., & Little, T. J. (2011). Genetic variation for maternal effects on parasite susceptibility. *Journal of Evolutionary Biology, 24*, 2357-2363.

Triantaphyllou, A. C. (1985). Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes. *Advanced treatise on Meloidogyne / edited by K.R. Barker, C.C. Carter and J.N. Sasser*: Raleigh, N.C. : Dept. of Plant Pathology, North Carolina State University.

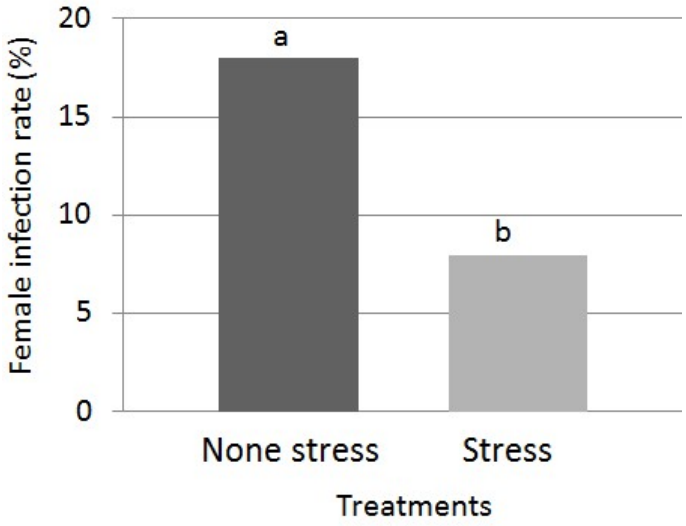


Fig 4.1. Effect of stress and none stress environments experience by female *Meloidogyne arenaria* on the susceptibility of their progeny to *Pasteuria penetrans* infection. Bars with different letters indicate significant differences ($P<0.05$). Each bar represents the mean of two SEM lines, six replicates and two trials (N=24).

# CHAPTER 5

# EVIDENCE FOR NEGATIVE FREQUENCY-DEPENDENT SELECTION IN A FIELD POPULATION OF THE ROOT-KNOT NEMATODE *MELOIDOGYNE ARENARIA* AND ITS PARASITE *PASTEURIA PENETRANS* [1]

[1] Chang Liu, Patricia Timper, Amanda Kyle Gibson and Levi T. Morran. To be submitted to *Evolutionary Applications*.

**Abstract**

Negative frequency-dependent selection is often suggested as a mechanism for diving host-parasite coevolution as well as maintaining genetic diversity. Finding direct evidence has proven to be difficult. In this study, we examined the host-parasite interaction between *Meloidogyne arenaria* and its bacterial parasite *Pasteuria penetrans* in 8 plots over 4 years with two rotations: continuous peanut and peanut rotated with soybean. We hypothesized that the dominant phenotype in a population of *M. arenaria* changes over time, leading to changes in the phenotype of *P. penetrans* in the field. There was no direct way to determine the nematode phenotype in the field; therefore, we used nematode single egg mass (SEM) lines that differ in spore attachment phenotype as probes to test the spore phenotype. Our results showed that there were rapid changes in the dominant spore phenotype in each plot and in each year. Furthermore, such changes were occurring on the local level, indicating that the changes in the *P. penetrans* polulations were driven by effects of the local environment. These observations provide solid evidence that the bacterial population was changing yearly at the local level, which agrees with the theory of negative frequency-dependent selection. Our results indicate that frequency-dependent selection is occurring in a field population of *P. penetrans* and *M. arenaria*, and genetic variation is likely to be promoted. This evidence is important to understand the causes and maintenance of genetic diversity within populations of *P. penetrans* and its host *M. arenaria*.

**Introduction**

Negative frequency- dependent selection (NFDS) is a key process in driving host-parasite coevolution in many natural communities. The fundamental mechanism is the selection imposed on rare genotypes in both host and parasite populations, and the decrease in relative fitness of a

genotype as its frequency increases (Ayala & Campbell, 1974; Clarke, 1976; Hamilton, 1982). Rare host genotypes have an advantage over common genotypes with more chances of escaping parasite infection (Jayakar, 1970; Tellier & Brown, 2007). The common host genotypes tend to decrease in frequency because of parasitism, and will be gradually replaced by rare genotypes that are resistant to the parasite. In turn, the parasite genotypes will change after a time lag in response to the reciprocal selection imposed by the hosts (Hutson & Law, 1981; Bell, 1982; Seger & Antonovics, 1988). For NFDS to occur, there must be genetic variation in both the host and parasite population (Auld, Hall, & Duffy, 2012; Castagnone-Sereno & Danchin, 2014; Ebert, 2008). The parasites and host must have matching virulent and susceptible genotypes, and the host should reciprocally impose resistance to the unmatched parasite genotypes (Hamilton, 1980). NFDS is believed to be important in preserving genetic diversity in both host and parasite populations (Chesson, 2000; Byers, 2005;). Although the mechanisms and processes contributing to the maintenance of genetic polymorphism are not fully understood (Takahashi & Kawata), many studies have proven that systems with higher genetic diversity tend to be more stable (Cadotte, Dinnage & Tilman, 2012). More studies have been done investigating the role of NFDS in plant resistance against pathogens, and there are handful of studies looking at the presence of NFDS in resistance against parasites of animals. Some well-known studies include mollusk - trematode (Lively, 1989; Koskella & Lively, 2009), insect - parasite (Henter & Via, 1995; Hufbauer & Via, 1999), and *Daphnia*- parasite (Ebert et al., 1998; Little et al., 2006). The wide range of biological factors, difficulty in tracking individual genotypes under experimental conditions, and temporal fluctuations have made it more difficult to document NFDS. Direct examination of host – parasite population genetics has been successful with the *Daphnia*-

parasite system (Ebert et al., 1998; Carius et al., 2001; Ebert, 2005; Decaestecker et al., 2007; Luijckx et al., 2011).

*Daphnia* are transparent crustaceans that can be kept in freshwater medium feeding on algae, and they are host to numerous parasites including *Pasteuria ramosa*. *P. ramosa* can sterilize the host and imposes a strong negative impact on the host population. Under natural conditions, both organisms are able to remain viable for a long time, and the layered pond sediments are very helpful in reconstructing the evolution process over decades. The convenient availability of *Daphnia* and *P. ramosa* in both lab and natural conditions has greatly helped in revealing evolutionary changes in this host-parasite system. There is high genetic diversity in both *Daphnia* and *P. ramosa* populations, and Daphnia host clones varied strongly in resistance to the parasite (Carius et al., 2001; Mitchell et al., 2005). Decaestecker et al. (2007) proved that there is NFDS occurring between *Daphnia* and *P. ramosa*. Moreover, Luijckx et al. (2011) revealed that the attachmet of *P. ramosa* to *D. magna* was a gene for gene interaction.

Root-knot nematodes (*Meloidogyne* spp.; RKN) are economically important pathogens of numerous crop plants in tropical and subtropical regions of the world. RKN damage plants by acting as metabolic sinks, transferring energy from the plant to the nematode, and by impairing root function (Sasser & Carter, 1985). Infection of roots results in the formation of giant cells leading to the development of root galls. The life cycle of RKN takes 3 to 6 weeks to complete, and begins as eggs deposited into a gelatinous matrix on the surface of the root gall. All nematodes pass through an embryonic stage, four juvenile stages (J1–J4) and an adult stage. The second-stage juvenile (J2) is the mobile and infective stage. The J2 infect root tips of host plants, and the mature female will continue to produce eggs to complete the life cycle. *Pasteuria*

*penetrans* is an obligate parasite of RKN and is frequently involved in natural suppression of these nematodes (Stirling, 1984; Chen, 1996; Timper et al., 2001). The spores of *P. penetrans* adhere to the cuticle of J2 of RKN as they migrate through the soil in search of host plant roots. As the nematode enters the root and establishes a feeding site, the bacterium penetrates the nematode cuticle, and develops vegetatively within the nematode body. The bacterium continues to develop within the nematode body and sterilizes the female. The mature female will be filled with spores of *P. penetrans* instead of eggs. Juveniles encumbered by large number of spores (>15) becomes less mobile, leading to the decrease in ability to locate and penetrate host roots (Davies et al., 1991). When the female cuticle degrades, spores will be released into the soil. The parasite does not kill the host nematode, and in this way, the parasite monopolizes the host resources to complete its life cycle.

The host range of *P. penetrans* is limited to *Meloidogyne* spp. The initial phase of parasitism by *P. penetrans* is determined by the attachment of endospores to the J2 cuticle. Stirling (Stirling, 1985) showed that a population of *P. penetrans* could attach to different species of RKN, but not to every population within a species. Similarly, Trudgill et al. (Trudgill et al., 2000) reported spore attachment differed among populations within a species of RKN were sometimes as great as those between species, indicating that the attachment specificity occurs on a population level instead of species level. The difference in susceptibility of individuals within a RKN population provides opportunities for developing resistance. When repeatedly challenged with a *P. penetrans* population in greenhouse pots, a population of RKN showed decreased spore attachment (Channer, 1992; Tzortzakakis, 1996). It is likely this strong selection pressure on the nematode leading to an increase in resistant genotypes also occurs in the field. Evidence for this scenario was observed in a long-term crop rotation study carried out from 1998 to 2008 in a field

site naturally infested with *P. penetrans* and *M. arenaria* (Timper, 2009). *M. arenaria* reproduces by mitotic parthenogenesis resulting in nearly clonal progeny for an individual female. Five single egg mass (SEM) lines from the field population plus a greenhouse population (GH) of *M. arenaria* were used to assay *P. penetrans* spores in the soil. Four SEM lines acquired numerous spores, while one SEM line and the GH population acquired few spores, indicating that the population of *M. arenaria* is heterogeneous for spore attachment. The GH population had been used to assay the field soil for *P. penetrans* spores from 1997 to 2008 (Timper, 2009). In 1997 and 1998, this population acquired numerous spores; however, spore acquisition declined from 2000 to 2007, only to increase again in 2008. Since GH population was not exposed to *P. penetrans* selection pressure, it is unlikely that the GH population changed. The changes in spore acquisition over time likely reflect the changes in the frequency of *P. penetrans* attachment phenotypes. It is noteworthy that when spore attachment to the GH population was low, several SEM lines acquired numerous spores. This indicates that some pathotypes of *P. penetrans* were abundant in soil, but pathotypes capable of attaching to the GH population were low.

In this study, we looked at the spore attachment of a natural field population of *P. penetrans* to *M. arenaria,* and we tracked the spore attachment over time. We used single egg mass lines of *M. arenaria* differing in spore adhesion as probes to test the attachment pathotypes of *P. penetrans* in each year, and monitored the shifts *P. penetrans* pathotypes from year to year. We hypothesize that the dominant phenotype in the population of *M. arenaria* changes over time leading to changes in the dominant pathotype of *P. penetrans*.

## Material and Methods

### Experiment design and treatments

This study was conducted at Gibbs Farm at Coastal Plain Experiment Station, Tifton, Georgia. The soil was a Tifton loamy sand (fine-loamy, siliceous, thermic Plinthic Kandiudult; pH 6.1), and it is naturally infested with *M. arenaria* race 1 and *P. penetrans*.

The experiment was a randomized complete block design with two crop rotations: peanut (*Arachis hypogaea,* cultivar Georgia green) rotated 1 year with soybean (*Glycine max,* cultivar Pioneer 95Y20) (P-S-P) and continuous peanut (P-P-P). For P-S-P rotation, soybean was planted in 2012, 2014 and 2016. Both peanut and soybean are good hosts for *M. arenaria.* Each rotation was replicated four times. The soil was plowed to a depth of 20 to 25 cm before planting, and beds were shaped into 1.8 m wide and 10 to 15 cm high. Each plot included four beds; soil for determining abundance of *P. penetrans* spores was collected from the center two beds. Soil samples from each of eight plots were collected in early October from 2013 to 2016. For each plot, 10 root-zone soil cores (2.5 cm diam. X 15 cm deep) were collected in each row and mixed thoroughly. All soil was heated at 60 °C for 2h before use to kill the living J2. Both peanut and soybean were planted in May with seeds spaced in two rows, 0.9 m apart on the bed with 20 seed/m for peanut, and 24 seed/m for soybean. Crop management, including fertilization and pest management were conducted based on recommendations for this area (Guillebeau, 2009). Crops were harvested at optimum maturity in mid to late September.

### *Pasteuria penetrans* bioassay

To determine the density of *P. penetrans* spores, a bioassay was conducted using a method previously described by Timper et al. (2001). Briefly, a subsample of 100 $cm^3$ soil was added

along with tap water to a flask and the flask was shaken vigorously to make a slurry before decanting the soil-water suspension into another 250 ml flask. Greenhouse-cultured J2 (1500) were added into the soil-water suspension and shaken on a rotary shaker at 150 rpm. The assay nematodes were extracted after 24 h by centrifugal floatation (Jenkins, 1964) and the number of spores adhering to 25 randomly selected nematodes were determined at 400x magnification with an inverted microscope. Four SEM lines (SEM 3, 6, 8, 40) of *M. arenaria* race 1 and a greenhouse (GH) population (originally obtained from the same field site) were used in this bioassay. The SEM lines were selected for this study based on differential spore attachment when exposed to different single spore lines of *P. penetrans*. We assume that these SEM lines are acquiring different spore pathotypes. These SEM lines and GH population were maintained on eggplant in a greenhouse at 22-30°C. The assay nematodes were obtained by placing roots with egg masses separately in a mist chamber, and the hatched J2 were collected 3-4 d later.

Statistic analysis.

Prior to statistical analysis, spore counts were converted to binary variables, where spore attachment was represented by 1 and no spore attachment was represented by 0. Analysis was conducted with R i386, 3.3.1. Generalized estimating equation (GEE) model was used in evaluating the effect of SEM line, rotation, year, and their interactions on the probability that an individual nematode acquires spores in the bioassay. The GEE model was used to evaluate population level trends; therefore, replicate plots were not included in the model. A second analysis was done using the generalized linear model (GLM) to determine the effect of SEM line, year, replicate plot and their interactions on the probability of spore attachment within each rotation treatment. The GLM analysis was used to focus on the individual plot effects on spore acquisition. Because the total number of *P. penetrans* spores fluctuated from year to year, the

relative mean of an SEM line was calculated as the mean spore attachment in that SEM line divided by the mean spore attachment for all SEM lines in a given year. Data were analyzed using the Standard Least Square Option in JMP Pro (v.13). LS Means Student's t-test was used to determine differences among SEM lines.

**Results**

Effect of treatment on the probability that an individual nematode acquires spores in the bioassay.

Crop rotation, SEM line, year and their interactions all influenced the probability of nematodes acquiring spores ($P<0.001$; Table 5.1). The major effects on spore attachment by rank were Year*SEM, Rotation, Year*SEM*Rotation. Spore acquisition was greater ($P<0.001$) from the continuous peanut plots than from peanut-soybean rotation plots (Fig 5.1). The mean percentage attachment was 47.4% in P-P-P plots, and 25.5% in P-S-P plots.

Spore acquisition by the SEM lines.

There was a strong SEM*Plot* Year effect in both continuous peanut and peanut rotated with soybeans (Table 5.2, Table 5.3), indicating that the probability of the spores being acquired by the different SEM lines was not consistent among plots or years.

The yearly change in spore acquisition by SEM lines in a given plot.

In every plot, there was a significant Year*SEM effect (Table 5.4), which was always either the most important or second most important factor in the model, indicating that the spore acquisition by each SEM line changed over time in each plot. This was true for both continuous peanut and peanut rotated with soybeans. The relative mean was used to track the dominant spore population in each plot and year. We observed fluctuations in the dominant SEM line that

acquired spores in each plot across the years (Fig. 5.2). The SEM line that acquired the dominant spore pathotypes differed among replicate plots within each rotation. However, there was a distinct trend for one or two SEM lines picking up more spores in each plot and year. Within each plot, the SEM line that acquired the dominant spore pathotype also tended to change from one year to the next. In Replicate 3 of continuous peanut for example, the dominant spore pathotype attached to SEM6 in 2013, but changed to attach to SEM40 in 2014. The changes were rapid, often occurring over one year. There were also times when there was no SEM line that acquired more spores than other SEM lines (i.e., no dominant spore pathotype). For example, Replicate 4 of continuous peanut in 2014, no SEM line acquired more spores than the other lines.

**Discussion**

It is difficult to study evolution dynamics in nature because monitoring of both host and parasite over many generations are needed, and it is hard to restructure the evolutionary dynamics of natural populations over time (Decaestecker et al., 2007). No previous study has looked at the coevolutionary dynamics between RKN and *P. penetrans*, and here we report the first observation of the occurrence of NFDS between a parasite and its parthenogenetic host. Our results are consistent with, but do not prove that NFDS occurs in a field population of *M. arenaria* and *P. penetrans*.

Because we do not have a direct way to determine the adhesion phenotypes of either *M. arenaria* or *P. penetrans*, we used SEM lines that appeared to acquire different *P. penetrans* spore pathotypes as probes to evaluate the changes in the spore population. The assumption that the SEM lines were acquiring different spore pathotypes of *P. penetrans* was based on preliminary data showing different patterns of spore acquisition by the four SEM lines. However,

our results over the four years of the study strengthened this assumption. Firstly, spore acquisition by the SEM lines was not consistent within a plot, indicating that the SEM lines vary in their susceptibility to the dominant spore pathotypes present in each plot, and no two SEM lines showed similar patterns of spore acquisition across plots and years. Secondly, spore acquisition by the SEM lines was not consistent over time, indicating that a given SEM line was not inherently more susceptible or resistant to *P. penetrans*.

This study was first designed with four plots in each rotation as replicates, but results showed that each plot was independent in their population dynamics. To exclude the possibility that the temporal changes in the field may be driven by environmental factors, we compared SEM*Year effect and SEM*Plot*Year effect. If there was a dominant SEM*Year effect and no SEM*Plot*Year effect, it might indicate that all the plots were changing similarly between years. However, in both the continuous peanut and the rotation treatments, there was a significant SEM*Plot*Year interaction demonstrating that the *P. penetrans* population was changing differently among the plots and years. In peanut-soybean rotation treatment, SEM*Plot*Year effect was slightly weaker than Year*SEM, but it was still a dominant effect. The spore acquisition was lower in peanut-soybean rotation plots; therefore, there was less variation overall, and this may explain the weaker SEM*Plot*Year effect. Our observation that the spore dynamics were occurring on the local level supports the concept of local adaptation in which changes in populations are driven by effects of the local environment on genotypes within the population (Kawecki & Ebert, 2004). In a study of the snail *Potamopygus antipodarum* and its parasite trematode *Microphallus* sp., local adaptation was observed in individual lakes even when the lakes were very close to each other (Lively, 1989; Dybdahl & Lively, 1996). Ebert et al. (1994) showed that virulence and spore production by a microsporidial parasite of *D. magna* declined

with increasing distance between the host and parasite collection site. In other words, local hosts were more susceptible to local parasite isolates than to distant parasite isolates (Ebert, 1994). Gene flow disrupts local adaptation (Kawecki & Ebert, 2004). In our study, very little gene flow is expected between plots for either *P. penetrans* or *M. arenaria* because both organisms have limited dispersal capabilities and the plots were distantly spaced (> 12 m). Different environmental conditions (e.g., soil moisture, pH, plant health) among the plots may interact with host or parasite genotypes leading to varied outcomes for each plot (Wolinska & King, 2009), or small differences in the relative abundance of host or parasite genotypes may lead to independent evolutionary trajectories among the plots.

Within each plot, spore acquisition among SEM lines varied among years (SEM*Year interaction). This observation provides solid evidence that the bacterial population was changing yearly at the local level. The relative mean provides a direct view of this yearly change. There were always one or two SEM lines that acquired dominant spore pathotype in each plot in each year, indicateing that the parasite population were adapting to attach the most common host phenotypes. Moreover, the SEM lines that acquired the dominant spore pathotypes changed from one year to the next, consistent with fluctuating selection (Bell, 2010). No SEM line consistently acquired more spores than other SEM lines demonstrating that there was no case of universal resistance against the parasite. There were years where there were no dominant SEM lines that acquired spores. For example, in 2015 in P-P-P Replicate 2, all SEM lines acquired the same level of spores. The reason could be that, since we were only using four SEM lines, we may not have an SEM line that the dominant pathotype spores can adhere to. Or, the decrease of the old dominant spore pathotype and the rise of the new dominant spore pathotype reached balance at the time we sampled.

We anticipated a slower selective process, but our results showed that shifts in spore pathotypes can happen rapidly, and this may because RKN have three to five generations in one growing season. Several aspects of the *P. penetrans*-RKN interaction may contribute to the rapid change in spore pathotypes. Not only does *P. penetrans* sterilize infected females, thus reducing the recruitment rate of new hosts, it also reduces root penetration by J2 that are heavily encumbered with spores. The failure of these J2 to infect a root depletes the number of viable spores in the soil and also limits the production of new spores. Although spores can remain viable for years in storage (Giannakou et al., 1997), they may leach through the soil during rain and irrigation events (Mateille et al., 1996; Timper et al., 2016). The leaching of the spores may contribute to the rapid change in spore population as well.

Spore acquisition was always greater in continuous peanut plots than in peanut-soybean rotation plots. Both soybean and peanuts are hosts for *M. arenaria*; however, nematode reproduction is more than 10-fold greater on peanut than on soybean (Noe, 1991). Because of the density-dependent relationship between *P. penetrans* and its host, the larger nematode population in the continuous peanut compared to the peanut-soybean rotation likely resulted in greater spore densities. Another explanation is that since peanuts were inverted at harvest, it may have distributed the spores in the field by bringing the infected females to the soil surface and releasing spores over a greater area.

For NFDS to occur, both the host and parasite populations require high polymorphism which is promoted by sexual reproduction (Ayala, 1974; Bell, 1982; Takahashi & Kawata, 2013). *M. arenaria* reproduce by mitotic parthenogenesis, which would seem to be at odds with the theory. *M. arenaria* have a wide variation in their chromosome number (n=36-56) and contains both diploids and triploids (Sasser & Carter, 1985). Such cytogenetics bestow them long-term

evolutionary flexibility (Sasser & Carter, 1985, Triantaphyllou, 1980). Because they reproduce asexually and without meiosis, they do not undergo gene reduction to become a haploid. Mutations and gene rearrangements can be accumulated in the chromosomes, which lead to the substantial variation (Castagnone-Sereno & Danchin, 2014). One of the advantages of being a polyploid is that gene redundancy, which prevents the deleterious effects of mutations (Comai, 2005). In this case, mutations that influence spore attachment by different spore pathotypes may be maintained in the *M. arenaria* population, and the dominant spore pathotypes impose a selective effect on these genes. Migration from sympatric populations may also contribute to high polymorphism within the *M. arenaria* population.

It is difficult to show direct evidence of NFDS in the field because we could not distinguish the genotypes of either *M. arenaria* or *P. penetrans*, except for our indirect method. In general, there is little known about the the genetic mechanisms underlying *M. arenaria* and *P. penetrans* interaction. Developing genetic or immunological tools that can distinguish *M. arenaria* and *P. penetrans* adhesion phenotype may provide conclusive evidence for NFDS in this system.

**References**

Auld, S. K. J. R., Hall, Spencer R., Duffy, & Meghan A. (2012). Epidemiology of a *Daphnia*-multiparasite system and its implications for the red queen. *PLoS One, 7*, 6p.

Ayala, F. J., & Campbell, Cathryn A. (1974). Frequency-dependent selection. *Annual Review of Ecology and Systematics, 5*, 115-138.

Bell, G. (1982). The masterpiece of nature : the evolution and genetics of sexuality. Berkeley : University of California Press.

Bell, G. (2010). Fluctuating selection: the perpetual renewal of adaptation in variable environments. *Philosophical Transactions: Biological Schiences. 365,* 87-97.

Byers, D. L. (2005). Evolution in heterogeneous environments and the potential of maintenance of genetic variation in traits of adaptive significance. *Genetica, 123,* 107-124.

Cadotte, M.W., Dinnage, R., Tilman, D. (2012). Phylogenetic diversity promotes ecosystem stability. *Ecology, 93*, 223-233.

Castagnone-Sereno, P., & Danchin, E. G. J. (2014). Parasitic success without sex – the nematode experience. *Journal Of Evolutionary Biology, 27*(7), 1323-1333.

Carius, H. J., Little, T. J., & Ebert, D. (2001). Genetic variation in a host-parasite association: Potential for coevolution and frequency-dependent selection. *Evolution, 55,* 1136-1145.

Channer, A.G., & Gowen, S.R. (1992). Selection for increased host resistance and increased pathogen specificity in the *Meloidogyne* spp and *Pasteuria penetrans* interaction. *Fundamentals of Applied Nematology, 15*, 331-339.

Chen. (1996). Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. *Journal of Nematology, 28*, 159-168.

Chesson, P. (2000). Mechanisms of maintenance of species diversity. *Annual Review of Ecology and Systematics, 31,* 343-366.

Clarke, B. (1976). The ecological genetics of host-parasite relationships. *Genetic aspects of host-parasite relationships.* Oxford, London, Edinburgh, Melbourne: Blackwell Scientific Publications.

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature Reviews Genetics, 6*, 836-846.

Davies, K. G., Laird, V., & Kerry, B. R. (1991). The motility, development and infection of *Meloidogyne incognita* encumbered with spores of the obligate hyperparasite *Pasteuria penetrans*. *Revue de Nematologie*, 14, 611-618.

Decaestecker, E., Gaba, S., Raeymaekers, J. A., Stoks, R., Van Kerckhoven, L., Ebert, D., & De Meester, L. (2007). Host-parasite 'Red Queen' dynamics archived in pond sediment. *Nature, 450,* 870-873.

Dybdahl, M.F., & Lively, C.M. (1996). The geography of coevolution: comparative population structures for a snail and its trematode parasite. *Evolution, 50,* 2264-2275.

Ebert, D. (2005). Ecology, epidemiology, and evolution of parasitism in *Daphnia*. National Library of Medicine National Library of Medicine.

Ebert, D. (1994). Virulence and local adaptation of a horizontally transmitted parasite. *Science, 265,* 1084-1086.

Ebert, D. (2008). Host-parasite coevolution: insights from the *Daphnia*-parasite model system. *Current Opinion in Microbiology, 11,* 290-301.

Ebert, D., Zschokke-Rohringer, C. D., & Carius, H. J. (1998). Within and between population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society of London, 265,* 2127-2134.

Ebert, D. (2008). Host-parasite coevolution: insights from the *Daphnia*-parasite model system. *Current Opinion in Microbiology, 11,* 290-301.

Giannakou, I.O., Pembroke, B., Gowen, S.R., & Davies, K.G. (1997). Effects of long term storage and above normal temperatures on spore adhesion of *Pasteuria penetrans* and infection of the root-knot nematode Meloidogyne javanica. *Nematologica, 43,* 185-192.

Guillebeau, P. (2009). *Georgia pest control handbook*: Athens, Ga. : Cooperative Extension Service, The University of Georgia, College of Agricultural and Environmental Sciences.

Hamilton, W. D. (1982). Pathogens as causes of genetic diversity in their host populations. *Population biology of infectious diseases, 25,* 269-296.

Hamilton, W. D. (1980). Sex versus non-sex versus parasite, *Oikos, 35,* 282-290.

Henter, H. J., & Via, S. (1995). The potential for coevolution in a host-parasitoid system. I. genetic variation within an aphid population in susceptibility to a parasitic wasp. *Evolution, 49,* 427-438.

Hufbauer, R. A., & Via, S. (1999). Evolution of an aphid-parasitoid interaction: variation in resistance to parasitism among aphid populations specialized on different plants. *Evolution, 53,* 1435-1445.

Hutson, V., & Law, R. (1981). Evolution of recombination in populations experiencing frequency-dependent selection with time delay. *Proceedings of the Royal Society of London, 213,* 345-359.

Jayakar, S. D. (1970). A mathematical model for interaction of gene frequencies in a parasite and its host. *Theoretical Population Biology, 1,* 140-164.

Jenkins, W. R. (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Reporter, 48,* 692-692.

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters, 12,* 1225-1241.

Koskella, B, & Lively, C. M. (2009). Evidence for negative frequency-dependent selection during experimental coevolution of a freshwater snail and a sterilizing trematode. *Evolution, 63,* 2213-2221.

Little, T. J., Watt, K., & Ebert, D. (2006). Parasite-host specificity: experimental studies on the basis of parasite adaptation. *Evolution, 60,* 31-38.

Lively, C. M. (1989). Adaptation by a parasitic trematode to local populations of its snail host. *Society for the Study of Evolution, 43,* 1663-1671.

Luijckx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., & Ebert, D. (2011). Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions. *Ecology Letters, 14*, 125-131.

Mateille, T., Duponnois, R., Dabire, K., Ndiaye, S., & Diop, M. T. (1996). Influence of the soil on the transport of the spores of *Pasteuria penetrans*, parasite of nematodes of the genus *Meloidogyne*. *European Journal of Soil Biology, 32*, 81-88.

Mitchell, S. E., Rogers, E. S., Little, T. J., & Read, A. F. (2005). Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. *Evolution; International Journal Of Organic Evolution, 59*, 70-80.

Mitchell, S. E., Read, A. F., Little, T. J., & Koella, J. (2004). The effect of a pathogen epidemic on the genetic structure and reproductive strategy of the crustacean *Daphnia magna.* *Ecology Letters, 7*, 848-858.

Noe, J. P. (1991). Development of *Meloidogyne arenaria* on peanut and soybean under two temperature cycles. *Journal of Nematology, 23*, 468-476.

Sasser, J. N., & Carter, C. C. (1985). An Advanced treatise on meloidogyne. Raleigh, N.C., U.S.A. : Dept. of Plant Pathology, North Carolina State University.

Seger, J., & Antonovics, J. (1988). Dynamics of Some Simple Host-Parasite Models with More than Two Genotypes in Each Species. *Philosophical Transactions of the Royal Society of London, 319,* 541-555.

Stirling, G. R. (1984). Biological control of *Meloidogyne javanica* and *Bacillus penetrans*. *Phytopathology, 74*, 55-60.

Stirling, G. R. (1985). Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne.* *Nematologica, 31*, 203-209.

Tellier, A, & Brown, J. K. M. (2007). Stability of genetic polymorphism in host-parasite interactions. *Proceedings: Biological Sciences, 274,* 809-817.

Timper, P., Minton, N. A., Johnson, A. W., Brenneman, T. B., Culbreath, A. K., Burton, G. W., & Gascho, G. J. (2001). Influence of cropping systems on stem rot (*Sclerotium rolfsii*), *Meloidogyne arenaria*, and the nematode antagonist *Pasteuria penetrans* in peanut. *Plant Disease, 85,* 767-772.

Timper, P. (2009). Population Dynamics of *Meloidogyne arenaria* and *Pasteuria penetrans* in a long-term crop rotation study. *Journal of Nematology, 41,* 291-299.

Timper, P, Liu, C, Davis, R. F., & Wu, T. (2016). Influence of crop production practices on Pasteuria penetrans and suppression of Meloidogyne incognita. *Biological Control, 99,* 64-71.

Takahashi, U. & Kawata, M. (2013). A comprehensive test for negative frequency-dependent selection. *Population Ecology, 55,* 499-509.

Triantaphyllou, A.C. (1991). Further studies on the role of polyploidy in the evolution of *Meloidogyne. Journal of Nematology, 23,* 249-253.

Trudgill, D. L., Bala, G., Blok, V. C., Daudi, A., Davies, K. G., Gowen, S. R., & Voyoukallou, E. (2000). The importance of tropical root-knot nematodes (*Meloidogyne* spp.) and factors affecting the utility of *Pasteuria penetrans* as a biocontrol agent. *Nematology, 2,* 823-845.

Tzortzakakis, E. A., Channer, A., Gowen, S. R. & Ahmed, R. (1997) Studies on the potential use of *Pasteuria penetrans* as a biocontrol agent of root-knot nematodes (*Meloidogyne* spp). *Plant Pathology, 46,* 44-55.

William, O. H. H., & Boomsma, J. J. (2004). Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution, 58,* 1251-1260.

Wolinska, J. & King, K. C. (2009). Review: Environment can alter selection in host–parasite interactions. *Trends in Parasitology, 25*, 236-244.

Table 5.1. Effect of Year, SEM line, Rotation and their interactions on the probability of an individual *Meloidogyne arenaria* second-stage juvenile acquiring a *Pasteuria penetrans* spore.

| **Factor** | **DF*** | **$\chi 2$** | **P-value** |
|---|---|---|---|
| Year | 3 | 24.9 | <0.001 |
| SEM line | 4 | 40.5 | <0.001 |
| Rotation | 1 | 65.6 | <0.001 |
| Year*SEM | 12 | 165.2 | <0.001 |
| Year*Rotation | 3 | 12.5 | 0.006 |
| SEM*Rotation | 4 | 33.7 | <0.001 |
| Year*SEM*Rotation | 12 | 43.6 | <0.001 |

*Degree of freedom

Table 5.2. Effect of factors on the probability of an individual *Meloidogyne arenaria* second-stage juvenile acquiring a *Pasteuria penetrans* spore in the continuous peanut plots.

| **Factor** | **DF**[a] | **D**[b] | **P-value** |
|---|---|---|---|
| Year | 3 | 44.7 | <0.001 |
| SEM | 4 | 8.9 | 0.064 |
| Plot | 3 | 38.9 | <0.001 |
| Year*SEM | 12 | 72.4 | <0.001 |
| Year*Plot | 9 | 107.7 | <0.001 |
| SEM*Plot | 12 | 58.9 | <0.001 |
| Year*SEM*Plot | 36 | 122.4 | <0.001 |

[a] Degree of freedom

[b] Deviance

Table 5.3. Effect of factors on the probability of an individual *Meloidogyne arenaria* second-stage juvenile acquiring a *Pasteuria penetrans* spore in the peanut- soybean rotation plots.

| **Factor** | **DF**[a] | **D**[b] | **P-value** |
|---|---|---|---|
| Year | 3 | 26.7 | <0.001 |
| SEM | 4 | 41.0 | <0.001 |
| Plot | 3 | 18.9 | <0.001 |
| Year*SEM | 12 | 99.4 | <0.001 |
| Year*Plot | 9 | 110.3 | <0.001 |
| SEM*Plot | 12 | 30.3 | <0.001 |
| Year*SEM*Plot | 36 | 88.5 | <0.001 |

[a] Degree of freedom

[b] Deviance

Table 5.4. Effect of year and single egg mass (SEM) line on the probability of an individual *Meloidogyne arenaria* second-stage juvenile acquiring a spore in each of 8 plots.

| | Factor | DF[a] | D[b] | P-value |
|---|---|---|---|---|
| Plot 1[c] | Year | 3 | 51.4 | <0.001 |
| | SEM | 4 | 23.1 | 0.001 |
| | Year*SEM | 12 | 55.1 | <0.001 |
| Plot 2 | Year | 3 | 67.4 | <0.001 |
| | SEM | 4 | 19.0 | <0.001 |
| | Year*SEM | 12 | 41.7 | <0.001 |
| Plot 3 | Year | 3 | 21.2 | <0.001 |
| | SEM | 4 | 4.5 | 0.327 |
| | Year*SEM | 12 | 55.8 | <0.001 |
| Plot 4 | Year | 3 | 9.5 | 0.023 |
| | SEM | 4 | 21.9 | <0.001 |
| | Year*SEM | 12 | 45.3 | <0.001 |
| Plot 5 | Year | 3 | 14.1 | 0.003 |
| | SEM | 4 | 31.1 | <0.001 |
| | Year*SEM | 12 | 72.2 | <0.001 |
| Plot 6 | Year | 3 | 53.8 | <0.001 |
| | SEM | 4 | 2.0 | 0.729 |
| | Year*SEM | 12 | 26.1 | 0.01 |
| Plot 7 | Year | 3 | 6.8 | 0.079 |

| | | | | |
|---|---|---|---|---|
| | SEM | 4 | 17.6 | 0.001 |
| | Year*SEM | 12 | 40.1 | <0.001 |
| | Year | 3 | 56.2 | <0.001 |
| Plot 8 | SEM | 4 | 23.8 | <0.001 |
| | Year*SEM | 12 | 51.1 | <0.001 |

[a] Degree of freedom

[b]Deviance

[c] Plot 1-4: continuous peanut plots; Plot 5-8: peanut rotated with soybean plots.

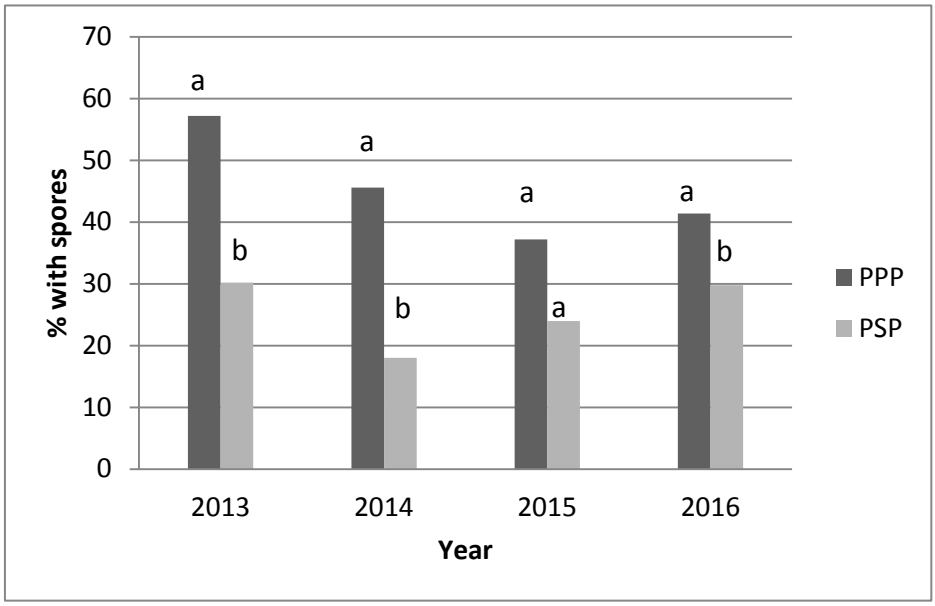


Fig 5.1. Comparision of continuous peanut and peanut rotated with soybean on the mean percentage of nematodes with *Pasteuria penetrans* spore attachment in four years. PPP: continuous peanut plots; PSP: peanut rotated with soybean plots. Each bar represents the mean of four replicate plots.

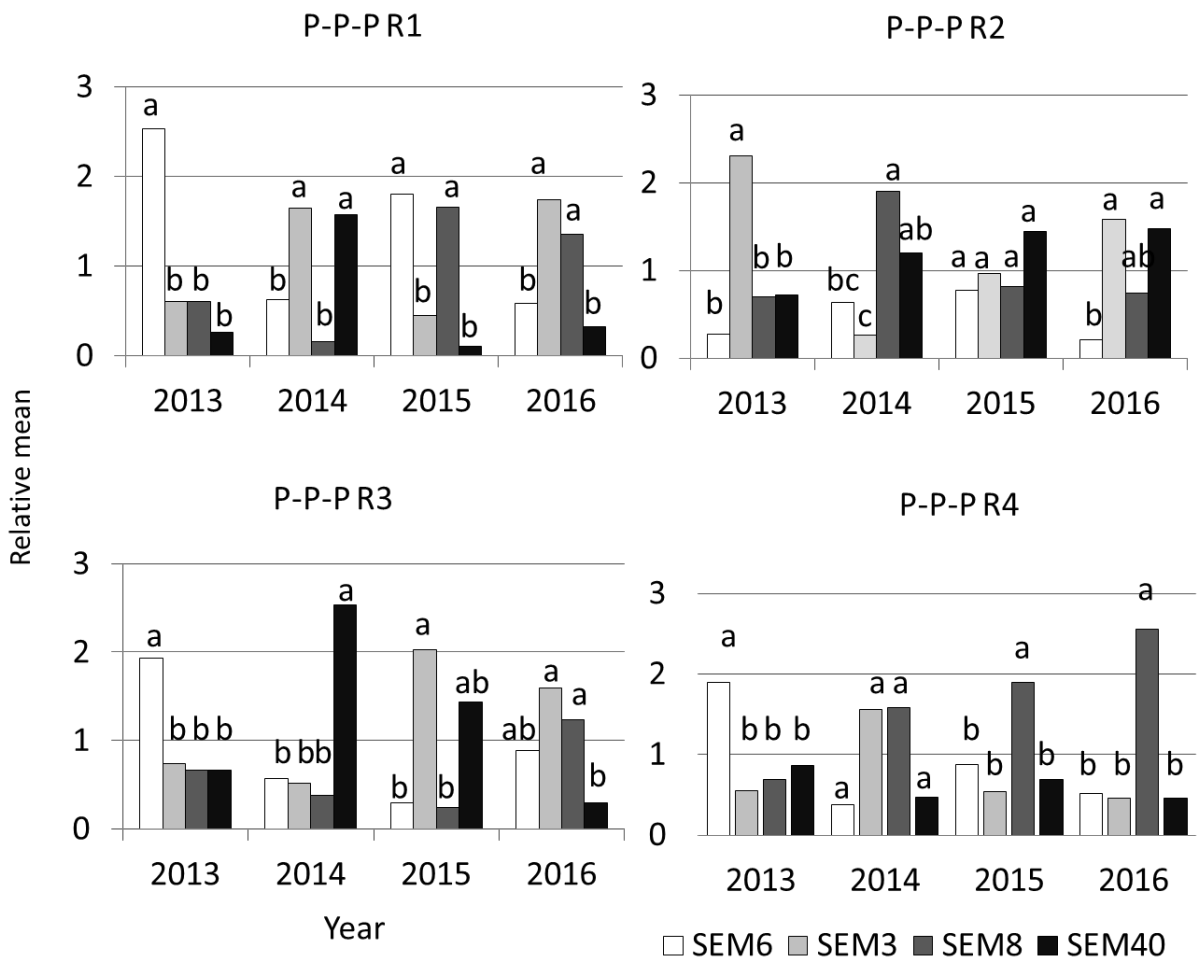


Fig 5.2. Comparison of the relative mean of *Pasteuria penetrans* spore attachment of four single egg mass (SEM) lines in each replicate plot (R1-R4) in each year for continuous peanut (P-P-P). Letters indicate significant difference ($P < 0.05$) within a year. Relative mean was calculated within each year = Spore per J2 in one SEM line / Spore per J2 for all SEM lines.

# CHAPTER 6

# PHENOTYPIC AND IMMUNOLOGICAL CHARASTERISTICS OF SINGLE SPORE ISOLATES OF A *PASTEURIA PENETRANS* POPULATION [1]

[1] Chang Liu and Patricia Timper. Not submitted to a journal.

**Abstract**

*Pasteuria penetrans* is a parasite of root-knot nematodes (*Meloidogyne* spp.). Endospores of P. penetrans attach to the surface coat of second-stage juveniles (J2) and subsequently sterilize infected females. This study tested the spore attachment specificity by using single spore lines of a *P. penetrans* population from Florida and single egg mass lines of *M. arenaria* and *M. incognita* from Georgia. We hypothesized that the spore attachment between the single spore lines and single egg mass lines would be an all or nothing response. However, we failed to see a binary pattern in spore attachment. Our results showed that different single spore lines of *P. penetrans* showed differential attachment to the single egg mass lines, but the pattern of the spore attachment were not repeatable. It is possible that instead of being controlled by a single gene, there are several genes involved in host specificity. Only after beginning this study, we learnt that all the single spore lines were derived from one single egg mass line of *M. arenaria*, therefore they may be the same attachment phenotype. The variation observed within each trial was likely due to environmental factors instead of genotypic interaction between the host and parasite. Three monoclonal antibodies (Mab) were produced from one single spore line, and were used in western blot and immune-blocking experiments. With the western blot, all single spore lines had an antigen that could be recognized by the Mabs, and with immune-blocking, there was no difference in reducing spore attachment between any of the Mab combinations. Such results support the view that the three single spore lines were the same attachment phenotype. Additional attempts with generating single spore lines derived from genetically diverse single egg mass lines of root-knot nematodes are needed in determining the spore attachment pattern occurs within *P. penetrans*.

## Introduction

The degree of specificity in host-parasite interactions is an important factor in developing an effective biological control agent. A successful biological control agent should be highly specific to a certain pathogen, would not damage non-target organisms, and would not disturb the native environment (Brodeur, 2012; Parker & Gilbert, 2004). Ideally, a successful biological control agent should have a very restricted host range, and should not switch in host preferences (Greathead, 1995). Beside effectiveness, the durability of a biological control agent is also essential if developed into a commercial product. High specificity may impair the effectiveness of a biological control agent due to the pathogen adaptation via co-evolution (Brodeur, 2012). The durability of a biological control agent is related to several traits of the host, such as the genetic diversity and the ability to evolve in response to a selection pressure exerted by the biological control agent (Bardin et al., 2015). The occurrence of resistance to pesticides in plant pathogens has been widely studied (Das, 2013) and such risk also exists for biological control agents. For example, resistance was found shortly after the widely used bio-insecticide *Bacillus thuringiensis* was commercialized (McGaughey, 1985). A commercialized biocontrol formulation will soon fail as a product if it does not contain enough genetic diversity. A successful biological control agent should have high genetic diversity to overcome the resistance developed in the host population. The understanding of the host-parasite specificity is essential in this perspective.

Host-parasite specificity is controlled genetically (Wilfert & Schmid-Hempel, 2008). High specificity has been found in multiple plant-pathogen interactions, involving a gene-for-gene relationship (Siegrist, 1998; Thompson & Burdon, 1992). In plants, there is a resistance (*R*) gene, and it produces a specific R gene product. Correspondingly, in the parasite, there is an avirulence

(*Avr*) gene that produces an *Avr* gene product. The R gene is resistant towards the *Avr* gene (Flor, 1971). In animal systems, a gene-for-gene interaction has been shown in the *Daphnia magna-Pasteuria ramosa* system. Studies have shown that there was significant genetic variation among both *D. magna* and *P. ramosa* isolates (Carius et al, 2001; Ebert et al, 1998). Clonal genotypes of *P. ramosa* exhibited specificity against host genotypes, and the host was found to be either fully susceptible or fully resistant to a particular parasite clone (Luijckx et al, 2011). Such specific resistance indicates that the interaction between *D. magna* and *P. ramosa* is controlled by a single gene.

Root-knot nematodes (*Meloidogyne* spp.; RKN) are economically important parasites of plants. They invade host root systems, act as metabolic sinks, cause wounds in roots, and impair root function. *Pasteuria penetrans* is an endospore-forming, obligate parasite of RKN. Spores of *P. penetrans* attach to the surface coat of second-stage juveniles as they migrate through the soil in search of host plants. Spore attachment to the nematode surface coat is a fundamental step for future infection. When a nematode with attached spores enters a host plant root, the bacterium develops within the nematode body and sterilizes the female. Suppression of RKN by *P. penetrans* has been documented in many field studies (Stirling, 1984; Chen, 1996; Trudgill, 2000), demonstrating the potential of this bacterium as a biocontrol product. *Pasteuria penetrans* is an obligate parasite of *Meloidogyne* spp., and as such, does not affect other organisms or the environment. Such traits make it ideal candidate for development into a commercialized biocontrol product.

Within a population of *P. penetrans* spores, polyclonal antibodies recognized 100% of the tested spores, while different monoclonal antibodies (Mabs) recognized different subsets of the spores, indicating that different subpopulations of the spores were present in a population.

Moreover, the subpopulation of spores showed differential adhesion (Davies, 1994). Stirling (1985) tested four *P. penetrans* isolates for attachment to 15 RKN populations. Some populations of *P. penetrans* could attach to different species of RKN, but not to every population within a species. Differences in spore attachment among populations within a species of RKN were sometimes as great as those between species (Trudgill et al., 2000). Therefore, specificity in attachment is at the population level and perhaps even at the individual level. Populations of RKN have been shown to be heterogeneous for *P. penetrans* spore attachment. When repeatedly challenged with a *P. penetrans* isolates, a population of RKN showed decreased spore attachment over time, indicating the occurrence of a shift to genotypes resistant to attachment (Channer & Gowen, 1992; Tzortzakakis & Gowen, 1994; Tzortzakakis et al, 1996). Evidence for this scenario was also observed in a long-term field study carried out from 2000 to 2008 (Timper, 2009). Five single-egg mass (SEM) lines from the field population plus a Greenhouse population (originally from Gibbs farm) of *M. arenaria* were used to assay soil containing *P. penetrans* spores. Four SEM lines acquired numerous spores, while one SEM and the Greenhouse population acquired few spores. Occurrence of negative frequency-dependent selection is reported in this field (Chapter 3), indicating that there is variation in pathogenicity in *P. penetrans* population and resistance in the *M. arenaria* population.

Both molecular and immunological methods have been used to test the heterogeneity of *P. penetrans* populations. But due to the lack of single spore isolation, no further research toward the immunological heterogeneity of *P. penetrans* has been reported so far. When the 16S rRNA of two populations of *P. penetrans* was compared, no variation was found (Bekal et al., 2001). However, nucleotide polymorphisms (SNPs) in the *spoII*AB gene between two isolates of *P.*

*penetrans* were detected, and a single individual could be infected by multiple *P. penetrans* genotypes (Nong et al., 2007).

Due to selection pressure, if the RKN population is diverse enough, the nematodes will develop resistance to a commercial formulation of *P. penetrans*. When we use *P. penetrans* as a biocontrol agent, if the spore population is not diverse enough, it will fail to adapt to the development of RKN resistance, which would lead to the failure of biocontrol. So it is important to understand the host-parasite specificity to make *P. penetrans* a more durable biocontrol agent. In this study, we carried out an adhesion bioassay using single spore lines of *P. penetrans* and single egg mass (SEM) lines of RKN. We hypothesized that the spore attachment between SEM lines and single spore lines would be an all or nothing response. We also developed monoclonal antibodies (Mab) from a single spore line and evaluated them against multiple single spore lines to differentiate *P. penetrans* adhesion phenotypes.

**Material and Methods**

General methods

Seventeen SEM lines of *M. arenaria* (Ma 3, 6, 8, 12, 14, 19, 23, 25, 26, 27, 28, 30, 32, 34, 36, 40, and 43) and four SEM lines of *M. incognita* ( Mi 15, 19, 31 and 40) were obtained from field population in Tifton, GA. Single egg mass lines were established by picking a single egg mass and transferring to an eggplant seedling in a greenhouse at 22-30 °C. Eggplants were given 4 mon to grow, and eggs were separated from egg masses using 0.5 % NaOCl (Hussey & Barker, 1973). Eggs were placed on tissue paper supported by a screen on top of a hatching dish. Water was added to the hatching dish to just submerge the tissue paper. Hatched second-stage juvenile

(J2) were collected by sieving the water in the hatching dish. The hatched J2 were collected every two days.

Three single spore lines (SS16, 17 and 25) of a population of *P. penetrans* were obtained from University of Florida (Joseph et al.). To produce *P. penetrans* spores, about 200 J2s attached with an average of 2-5 spores/J2 were inoculated onto 4-week-old eggplant seedlings to produce *P. penetrans* spores. Eggplant seedlings were grown in a greenhouse for 4 mon before root harvest. Harvested roots were washed with running water and placed in a beaker containing 100 ml of 10 g Lallzyme EX-V (Lallemand, Montreal, Canada) to digest root tissue for 1 d on a shaker (100 rpm). Fully opaque infected females were freed from roots and hand-picked into a glass petri dish containing deionized water. Females were crushed with a dissecting needle to free endospores into dH2O. The spore solution was vacuum filtered (8-12 um) to remove the female cuticle. The spores that passed through the filter were enumerated on a hemocytometer at 1000x magnification. Spore concentration was adjusted to $10^6$ spores/ml for each single spore line and kept frozen.

Attachment Bioassay

In the first trial, all 15 SEM lines of *M. arenaria* and all 4 SEM lines of *M. incognita* were tested for adhesion by the three single spore lines. For the endospore attachment bioassay, 200 infective J2 were incubated in 4 ml of 1x PBS in a small (150 mm × 25 mm) glass Petri dish with 0.1 ml of $10^6$ spores/ml of *P. penetrans*. Dishes were incubated for 6 hr in incubater at 24 °C. The number of spores attached to 25 randomly-selected J2 was determined using 400x magnification. Based on preliminary data in Trial 1, SEM lines (Ma 6, 8, 23, 25, 26, 27, 40, 43 and Mi 31, 40) were re-evaluated in Trial 2.

## Monoclonal Antibody Production

Anti-*P penetrans* Mab IgM 1F2,IgG 4E8 and IgM 1G11 were produced by Monoclonal Antibody Facility of University of Georgia by immunizing mice (Balb/C) with suspensions of $10^7$ spores of *P. penetrans* SS16. The antibodies were raised to the surface of *P. penetrans* spores. Mice that exhibited strong immunological responses to the bacterium were boosted with another $10^7$ spores before prior to fusion. Antibodies were isotyped using a mouse monoclonal antibody isotyping kit (Sigma).

## Immunological characterization of spore antigens

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) was performed using a 4-20% precast gel (Bio-Rad). About $10^6$ spores each of SS16, SS17 and SS25 were suspended in 200 ul sample buffer (50 mmol/l Tris/HCL, pH 6.8, 2% SDS, 10% glycerol , 0.002% bromophenol blue and 2% β-mercaptoethanol) and heated for 2 min at 100 °C before 20 ul sample were loaded on the gel. Prestained SDS-PAGE molecular weight markers were run on the gel. Gels were transferred to nitrocellulose membranes (Bio Rad) using a Mini Trans-Blot cell at 50 V voltage for 1.5 h. Membranes were blocked with 5% non-fat dried milk in 10 mM phosphate buffer, pH 7.2, 150 mM NaCl containing 0.2 % Tween 20 (PBST) over night at 4 °C . Membranes were washed three times in PBST before incubation with Mab supernatant diluted 1:50 for 1 h. Fowlling incubation, the membranes were washed three times in PBST and incubated in a 1:500 dilution of anti-mouse IgM alkaline phosphatase conjugate (Bio Rad). The blots were washed three times in PBST and incubated in alkaline phosphate substrate (0.03 % nitro blue tetrazolium, 0.02 % 5-bromo-4-chloro-3indolyl phosphate in Tris/HCl, pH 9.5, substrate buffer).

## Immunoblocking assay

To determine wether the Mabs recognized different epitopes on the spore surface, spores were incubated in various combination of Mabs. We hypothesized that incubating in two Mabs would reduce spore attachment to RKN more than one Mab. The Mab combinations used in this experiment were as follows: IgM 1F2, IgG 4E8, IgM 1G11, IgM1F2 + IgG 4E8, IgM 1F2 +IgM 1G11, IgG 4E8 + IgM 1G11 and IgM 1F2 + IgG 4E8 + IgM 1G11. For all combinations, equal amounts of each Mab tissue culture supernatant were used to make up to a 200 ul volume. About $2x10^5$ spores of SS 25 were added to 2 ml microcentrifuge tubes, and 200ul of each of the Mab combination was added to the tube to incubate with the spores. For the control, spores were incubated with 200 ul PBS. The solutions were incubated for 1 hr at 37°C. Following incubation, the spores were washed three times by repeated spinning and re-suspension in PBS. After the final spin, the spores were re-suspended in 4 ml of PBS to which were added 200 freshly hatched SEM 6 J2 for the spore attachment assay. This experiment was repeated twice.

## Statistical Analysis

Data were analyzed using SAS 9.3. Spore attachment in individual nematodes (N=25) were used as data points. All data were log-transformed and followed a Gaussian distribution after transformation. SEM lines, SS lines, Trial and their interactions were used to construct the model effects. Least Squares Means was used to test the SEM*SS interaction sliced by each SEM line. For immunoblocking assay, all data were also log-transformed and followed a Gaussian distribution after transformation. Mabs, Trial and their interactions were used to construct the model effect, and Least Squares Means was used to test the Mab*Trial interaction.

**Results**

We did not observe an all or nothing response in any of the spore attachment assays. Spore attachment was found in all SEM-SS line combinations, including both *M. arenaria* and *M. incognita*. There was SEM*SS*Trial, SEM*SS and SEM*Trial interaction ($P<0.05$), so the data were analyzed separately by each trial and by each SEM line.

For *M. arenaria* in trial 1, the three SS lines showed differences ($P< 0.05$) in spore attachment to 11 Ma lines, and there was no difference among the three SS lines for Ma 3, 14, 25, 27, 28, 40. For *M. incognita*, SS lines were significantly different in spore attachment for Mi 31, but not for Mi 15, 20 and 40 (Table 6.1). Based on this preliminary data and the availability of SEM lines when we repeated the experiment, we chose Ma 6, 8, 23, 25, 26, 27, 40, 43 and Mi 31, 40 to be re-tested in trial 2. The results of only these SEM lines will be reported in the following analysis.

In trial 2, the three SS lines showed differences ($P<0.05$) in spore attachment for all SEM lines, including both *M. arenaria* and *M. incognita* (Table 6.1). When we compared SS lines within the Ma lines, SS25 still showed the lowest spore attachment, which is consistent with Trial 1. For *M. incognita*, SS 25 showed the highest spore attachment to both of Mi 31 and 40. In trial 2, SS 16 and 17 did not show similarity in spore attachment as in trial 1; two spore lines were different ($P<0.05$) in 7 (Ma 8, 23, 26, 27, 43, Mi 31 and 40) out of 10 SEM lines. Among the two trials, only Ma 25 and Mi 40 showed consistent trend in spore attachment to the three SS lines.

SDS-PAGE followed by Western blotting with the mice monoclonal antibody showed that all three SS lines had an antigen that could be recognized by the Mabs, which means that our Mabs could not distinguish those three SS lines. There was no interaction between two trials for the immunoblocking assay, so the data was combined. There was no difference between any of the

Mab combinations (*P*=0.68), and all Mabs showed reduction in spore attachment compared to the control (*P*=0.0006), with a mean of 12.6 spores per J2 in the control and 7.9 spores per J2 in the Mab treatments (Fig. 6.1).

**Discussion**

In this study, we examined the host-parasite specificity between *Meloidogyne* spp. and *P. penetrans* in terms of spore attachment. We did not observe an all or nothing spore attachment response between the *Meloidogyne* spp. SEM lines and *P. penetrans* single spore lines as we predicted. Different single spore lines of *P. penetrans* showed differences in their ability to attach to *M. arenaria* single egg mass lines. However, patterns of the spore attachment were not repeatable, making the results difficult to interpret. Joseph et al. (2016) analyzed SNPs in individual 16S rRNA gene sequences of 5 SS lines by cluster analysis, and the result showed that SS16 and SS25 clustered, while SS17 formed a sister clade. However, our spore attachment assay showed that SS16 and SS17 were more similar in spore attachment means, whereas SS25 is more distinct from the other two (Table 1). Such results indicate that spore attachment phenotype does not align with 16S rRNA sequence results. Even for SS lines showing high similarity at 16S rRNA sequence level, they can differ in spore attachment.

There was an extreme genotype-genotype specificity between *D. magna* and *P. ramosa.* When clones of *D. magna* were treated with single isolates of *P. ramasa,* the isolates attached to some clones, and not at all to other clones (Luijckx et al., 2011; Duneau et al., 2011). In our study, the spore attachment was not in a binary pattern. One possible reason is that we may not have a nematode genotype that is resistant to the SS lines we tested. Since we were using *M. arenaria* and *M. incognita* from Georgia populations, they may not have the corresponding

resistant gene against the Florida *P. penetrans* spores. The Florida *P. penetrans* populations may have gone through local adaptation, where the bacterium adapted to the host population from which it was obtained (Kawecki & Ebert, 2004). Another possible reason is that instead of being controlled by a single gene, there are several genes involved in host specificity. Metzger et al (2016) proved that with *D. magna*, infection caused by *P. ramosa* depends on the epistasis between linked resistant loci on three genes in the host. They proposed a genetic architecture of resistance, where one locus masks the resistance in two other loci, and the interaction of these three loci determined the resistance of the host to parasite. In our study, we may not have the right loci combination in our SEM lines resulting in the absence of resistance.

Only after beginning this study, we learnt that all the SS lines we used in this study were derived from one *M. arenaria* single egg mass lines; therefore, the three SS lines may be the same attachment phenotype. We observed variation within each trial, but there was no consistency between trials. As similar situation was observed when progeny from a single egg mass line was treated with the same population of *P. penetrans*, there was variation in spore attachment (Davies et al., 2008). The variation may be due to environmental factors instead of a genotypic interaction between the host and parasite. With *P. ramosa*, spore attachment was not influenced by environment effects, including temperature and density level (Duneau et al., 2011). However, with *P. penetrans*, temperature, pH, moisture and root exudates have all been reported to influence spore attachment (R. Ahmed & Gowen, 1991; Riaz Ahmed, 1990; Brown & Smart, 1984; Hatz & Dickson, 1992; Liu et al., 2017). Though this study was conducted under strictly controlled environment, there may be other subtle differences in the environment which influenced spore attachment. It is more likely that the environment influenced the nematodes. Liu (Chapter maternal) showed that maternal environment can slightly influence spore

attachment to progeny, so the variations in spore attachment may be related to the environmental conditions experienced by the mother.

The immunological data also supports the hypothesis that the SS lines are the same attachment phenotype. Both the western blot and the immunological experiments indicated that the same epitope was recognized on all these SS lines. If the Mabs recognized different epitopes, we should see an additive effect when different Mabs were combined. We did not observe a fully blocking of spore attachment in this study, indicating that there may be more epitopes on the spore surface that are involved in the spore attachment.

Attachment specificity may be related to variation in collagen-like proteins on the spore surface. Two isolates of *P. ramosa* that differed in their attachment had different patterns of a collagen-like protein present on the surface of the endospore (Mouton et al., 2009). In *P. ramosa*, 37 collagen-like protein genes have been detected, and these genes were highly polymorphic and matched known *Daphnia/P. ramosa* specificity (McElroy et al., 2011). With bioinformatics analysis of the amino acid sequences coded by the 37 genes, two were identified to be putative proteins believed to be involved in host-parasite interactions. The function of these collagen-like proteins in *P. ramosa* are not clear, but such collagen-like proteins have been found in *Streptococcus equi* and *Bacillus anthracis* (Karlstrom et al, 2006; Steichen et al, 2003) Collagen-like proteins have been described in *P. penetrans*, and they were believed to be involved in the interaction between endospore and nematode surface coat through a Velcro-like attachment process (Davies, 2009). It is possible that for *P. penetrans*, there are multiple collagen-like protein genes involved in the spore attachment process. In depth understanding of the gene sequence of these proteins, as well as the protein pattern, are important in understanding the spore attachment specificity.

We failed to show that the spore attachment between *Meloidogyne* spp. and *P. penetrans* was an all or nothing response. The most likely reason is that the three SS lines used in this study were the same attachment phenotype. Additional attempts with generating single spore lines derived from different genetically diverse SEM lines of RKN would be helpful to determine of a binary spore attachment pattern occurs within *P. penetrans*. Moreover, Mabs raised against collagen-like proteins are worth trying to differentiate spore attachment phenotypes. SEM lines in our field study showed large differences in spore attachment, and the patterns of spore attachment varied over time and location. For example, SEM 6 had the highest spore attachment in plot 1 in 2013, but SEM 3 had highest spore attachment in plot 2 in the same year (Chapter NFDS). In addition, the SEM line that acquired most spores was changing from year to year in each plot, indicating that there was a higher level specificity between SEM line and the spores in the field than the result of this study. The failure in maintaining highly diverse spore genotypes within a biological control product could lead to the resistance to the product through selection on the host population, and would lead to the failure of nematode suppression. Thus, the understanding of spore diversity is urgent for preventing RKN from developing resistance to *P. penetrans* biological control agents. To understand the interaction specificity between *P. penetrans* and RKN, more insight into the genetic, immunological and environmental factors are required.

**References**

Ahmed, R., & Gowen, S. R. (1991). Studies on the infection of *Meloidogyne* spp. with isolates of Pasteuria penetrans. *Nematologia mediterranea, 19*, 229-233.

Ahmed, Riaz. (1990). Studies on the efficacy of *Pasteuria penetrans* for the biological control of Meloidogyne species: University of Reading.

Channer, A. G. D. R., & Gowen, S. R. (1992). Selection for increased host resistance and increased pathogen specificity in the *Meloidogyne* spp *Pasteuria penetrans* interaction. *Fundamentals of Applied Nematology, 15*, 331-339.

Barrett, L.G., Kniskern, J.M., Bodenhausen, N, Zhang, W., & Bergelson,J. (2009). Continua of specificity and virulence in plant host-pathogen interactions: causes and consequences. *The New Phytologist, 183,* 513-529.

Bekal, S., Giblin-Davis, R.M., Becker, J.O., Borneman, J., & Springer, M.S. (2001). Phenotypic and molecular analysis of a *Pasteuria* strain parasitic to the sting nematode. *Journal of Nematology*m 33, 110-115.

Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillot, B., Siegwart, M. &Nicot, P.C. (2015) Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Frontiers in Plant Science*, 26, 566.

Blank-Mathieu, R., Perfus-Barbeoch, L., Aury, J., Da Rocha, M. Gouzy, Jérôme

Sallet, E., Martin-Jimenez, C., Bailly-Bechet, M., Castagnone-Sereno, P., Flot, J., Kozlowski, D. K., Cazareth, J., Couloux, A., Da Silva, C., Guy, J., Kim, J., Yu, J., Rancurel, C., Schiex, T., Abad, P., Wincker, P. (2017). Hybridization and polyploidy enable genomic plasticity without sex in the most devastating plant-parasitic nematodes. *PLos Genetics, 13*, 1-36.

Brodeur, J. (2012). Host specificity in biological control: insights from opportunistic pathogens. *Evolutionary Applications, 5*, 470-480.

Brown, S. M., & Smart, G. C., Jr. (1984). Attachment of Bacillus penetrans to *Meloidogyne incognita*. *Nematropica, 14*, 171-172.

Carius, H. J., Little, T. J., & Ebert, D. (2001). Genetic variation in a host-parasite association: Potential for coevolution and frequency-dependent selection. *Evolution, 55,* 1136-1145.

Chen. (1996). Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. *Journal of Nematology, 28,* 159-168.

Das., S.K. (2013). Mode of action of pesticides and the novel trends - a critical review. *International Research Journal of Agricultural Science and Soil Science, 3,* 393-401.

Davies, K.G., Laird, V.& Kerry,B.R. The motility, development and infection of *Meloidogyne incognita* encumbered with spores of the obligate hyperparasite *Pasteuria penetrans. Revue Nematol, 14,* 611-618.

Davies, K. G., Redden, M., & Pearson, T. K. (1994). Endospore heterogeneity in *Pasteuria penetrans* related to adhesion to plant-parasitic nematodes. *Letters in Applied Microbiology, 19,* 370-373.

Davies, K. G., Rowe, J. A., & Williamson, V. M. (2008). Inter- and intra-specific cuticle variation between amphimictic and parthenogenetic species of root-knot nematode (*Meloidogyne* spp.) as revealed by a bacterial parasite (*Pasteuria penetrans*). *International Journal For Parasitology, 38,* 851-859.

Davies, K. G. (2009). Understanding the interaction between an obligate hyperparasitic bacterium, *Pasteuria penetrans* and its obligate plant-parasitic nematode host, *Meloidogyne* spp. *Advances in Parasitology*, 68, 211-245.

Duneau, D., Luijckx, P., Ben-Ami, F., Laforsch, C., & Ebert, D. (2011). Resolving the infection process reveals striking differences in the contribution of environment, genetics and phylogeny to host-parasite interactions. *BMC Biology, 9*:11.

Dunn, A. M., & Hatcher, M. J. (2015). Review: Parasites and biological invasions: parallels, interactions, and control. *Trends in Parasitology, 31*, 189-199.

Ebert, D., Zschokke-Rohringer, C. D., & Carius, H. J. (1998). Within- and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society of London Series B-Biological Sciences, 265*, 2127-2134.

Flor, H.H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology, 9,* 275-296.

Greathead, D. J. (1995). Benefits and Risks of Classical Biological Control. *Plant and Microbial Biotechnology, 4,* 53-63.

Hamilton, W. D. (1982). Pathogens as causes of genetic diversity in their host populations. In R. M. Anderson & R. M. May (Eds.), *Population biology of infectious diseases.* Berlin; Germany: Springer Verlag.

Hatz, B., & Dickson, D. W. (1992). Effect of temperature on attachment, development, and interactions of Pasteuria penetrans on *Meloidogyne arenaria*. *Journal of Nematology, 24,* 512-521.

Hussey, R. S., & Barker, K. R. (1973). A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. *Plant Disease Reporter, 57*, 1025-1028.

Karlström, A., Jacobsson, K., & Guss, B. (2006). SclC is a member of a novel family of collagen-like proteins in *Streptococcus equi* subspecies *equi* that are recognised by antibodies against SclC. *Veterinary Microbiology, 114*, 72-81.

Kawecki, T.J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters, 7,* 1225-1241.

Levin, Bruce R., & Cornejo, Omar E. (2009). The population and evolutionary dynamics of homologous gene recombination in bacteria. *PLoS Genetics, 5,* 1-16.

Liu, C., Timper, P., Ji, P., Joseph, S. & Mengistu, T. (2017). Influence of root exudates and soil on attachment of *Pasteuria penetrans* to *Meloidogyne arenaria*. *Journal of Nematology, 49*, 304-310.

Luijckx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., & Ebert, D. (2011). Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions. *Ecology Letters, 14*, 125-131.

McElroy, K., Mouton, L., Du Pasquier, L., Qi, W. & Ebert, D. (2011). Characterisation of a large family of polymorphic collagen-like proteins in the endospore-forming bacterium *Pasteuria ramosa*. *Research in Microbiology, 162*, 701-714.

McGaughey, W.H (1985). Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science*, 4709, 193-195.

Mirończuk, A. M., Kovács, Á. T., & Kuipers, O. P. (2008). Induction of natural competence in *Bacillus cereus* ATCC14579. *Microbial Biotechnology, 1*, 226-235.

Mouton, L., Traunecker, E., McElroy, K., Du Pasquier, L., & Ebert, D. (2009). Identification of a polymorphic collagen-like protein in the crustacean bacteria *Pasteuria ramosa*. *Research in Microbiology, 160*, 792-799.

Nong, G., Chow, V., Schmidt, L. M., Dickson, D. W., & Preston, J. F. (2007). Multiple-strand displacement and identification of single nucleotide polymorphisms as markers of genotypic variation of Pasteuria penetrans biotypes infecting root-knot nematodes. *Fems Microbiology Ecology, 61*, 327-336.

Parker, I.M. & Gilbert, G.S. (2004). The evolutionary ecology of novel plant-pathogen interactions. *Annual Review of Ecology, Evolution, and Systematics*, 35, 675-7006.

Siegrist, J. (1998). The gene-for-gene relationship in plant-parasite. *Journal of Plant Diseases and Protection*. 105, 550.

Joseph. S., Schmidt, T., Danquah, W.B., Timper, P. & Mekete, T. Genotyping of single spore isolates of a *Pasteuria penetrans* population occurring in Florida using SNP-based markers.*Journal of Applied Microbiology, 122,* 389-401.

Steichen, C., Chen, P., Kearney, J. F., & Turnbough, C. L., Jr. (2003). Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *Journal Of Bacteriology, 185,* 1903-1910.

Stirling, G. R. (1984). Biological control of *Meloidogyne javanica* and *Bacillus penetrans*. *Phytopathology,* 1, 55.

Stirling, G. R. (1985). Host specificity of Pasteuria penetrans within the genus *Meloidogyne*. *Nematologica, 31*, 203-209.

Taraschewski, H. (2006). Hosts and parasites as aliens. *Journal of Helminthology, 80,* 99-128.

Thompson, J. N., & Burdon, J. J. (1992). Gene-for-gene coevolution between plants and parasites. *Nature, 360,* 121-125.

Timper, P. (2009). Population dynamics of *Meloidogyne arenaria* and *Pasteuria penetrans* in a long-term crop rotation study. *Journal of Nematology, 41*, 291-299.

Trudgill, D. L., Bala, G., Blok, V. C., Daudi, A., Davies, K. G., Gowen, S. R. &Voyoukallou, E. (2000). The importance of tropical root-knot nematodes (*Meloidogyne* spp.) and factors affecting the utility of *Pasteuria penetrans* as a biocontrol agent. *Nematology, 2,* 823-845.

Trudgill, D.L., & Block, V.C. (2001). apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annual Review of Phytopathology, *39,* 53-78.

Tzortzakakis, E. A., & Gowen, S. R. (1994). Resistance of population of *Meloidogyne* spp. to parasitism by the obligate parasite *Pasteuria penetrans*. *Nematologica, 40,* 258-266.

Tzortzakakis, E. A., Gowen, S. R., & Goumas, D. E. (1996). Decreased ability of *Pasteuria penetrans* spores to attach to successive generations of *Meloidogyne javanica*. *Fundamental and Applied Nematology, 19,* 201-204.

Wilfert, Lena, & Schmid-Hempel, Paul. (2008). The genetic architecture of susceptibility to parasites. *BMC Evolutionary Biology, 8,* 187-195.

Woolhouse, M.E.J., Taylor, L. H.& Haydon, D.T. (2001). Population biology of multihost pathogens. *Science*, 229, 1109-1112.

William H. McGaughey, author. (1985). Insect Resistance to the Biological Insecticide Bacillus thuringiensis. *Science*(4709), 193.

Table 6.1. Mean spore attachment of three *Pasteuria penetrans* single spore (SS) lines to eight *Meloidogyne arenaria* (Ma) single egg mass lines and two *Meloidogyne incognita* (Mi) single egg mass lines in two trials.

| | Trial 1 Mean Spore/J2 | | | | Trial 2 Mean Spore/J2 | | | |
|---|---|---|---|---|---|---|---|---|
| | SS 16 | SS 17 | SS 25 | Pr>F | SS 16 | SS 17 | SS25 | Pr>F |
| Ma 3 | 1.7$^{ab*}$ | 1.97$^{a}$ | 1.13$^{b}$ | 0.13 | --* | -- | -- | |
| Ma 6 | 4.76$^{b}$ | 6.52$^{a}$ | 3.08$^{bc}$ | 0.0004 | 10.76$^{a}$ | 10.8$^{a}$ | 4.16$^{b}$ | <.0001 |
| Ma 8 | 7.32$^{a}$ | 5.36$^{b}$ | 0.56$^{c}$ | <.0001 | 12.2$^{b}$ | 19.48$^{a}$ | 4.6$^{c}$ | <.0001 |
| Ma 12 | 1.7$^{b}$ | 2.1$^{a}$ | 1$^{b}$ | 0.03 | -- | -- | -- | |
| Ma 14 | 1.7$^{a}$ | 1.3$^{a}$ | 1.27$^{a}$ | 0.63 | -- | -- | -- | |
| Ma 19 | 7.67$^{a}$ | 4.2$^{b}$ | 4.73$^{b}$ | 0.02 | -- | -- | -- | |
| Ma 23 | 4.08$^{a}$ | 1.72$^{ab}$ | 0.52$^{c}$ | 0.0004 | 13.84$^{a}$ | 5.6$^{bc}$ | 8.2$^{b}$ | 0.0001 |
| Ma 25 | 3.28$^{ab}$ | 3.72$^{a}$ | 1.71$^{b}$ | 0.0599 | 8$^{a}$ | 6.4$^{a}$ | 2.72$^{b}$ | 0.0009 |
| Ma 26 | 1.6$^{b}$ | 1.68$^{b}$ | 4.32$^{a}$ | <.0001 | 4.08$^{b}$ | 15.32$^{a}$ | 4.24$^{b}$ | < .0001 |
| Ma 27 | 3.48$^{a}$ | 1.96$^{ab}$ | 0.68$^{ab}$ | 0.2311 | 4.6$^{bc}$ | 6.76$^{a}$ | 5$^{ab}$ | 0.0124 |
| Ma 28 | 2.03$^{a}$ | 1.67$^{a}$ | 2.57$^{a}$ | 0.72 | -- | -- | -- | |
| Ma 30 | 6.27$^{a}$ | 6.1$^{a}$ | 0.84$^{b}$ | <.0001 | -- | -- | -- | |
| Ma 32 | 3.83$^{a}$ | 6.3$^{a}$ | 2.1$^{b}$ | 0.005 | -- | -- | -- | |
| Ma 34 | 3.93$^{a}$ | 0.77$^{b}$ | 3.93$^{a}$ | 0.0002 | -- | -- | -- | |
| Ma 36 | 1.47$^{a}$ | 1.7$^{a}$ | 3.7$^{b}$ | 0.009 | -- | -- | -- | |
| Ma 40 | 3.16$^{ab}$ | 5.04$^{a}$ | 1.92$^{ab}$ | 0.2454 | 15.2$^{ab}$ | 20$^{a}$ | 5.44$^{c}$ | <.0001 |
| Ma 43 | 2.36$^{b}$ | 7.56$^{a}$ | 2.32$^{b}$ | <.0001 | 5$^{b}$ | 7.6$^{a}$ | 1.8$^{c}$ | < .0001 |
| Mi 31 | 8.06$^{a}$ | 1.3$^{c}$ | 6.16$^{ab}$ | <.0001 | 3.1$^{c}$ | 5.03$^{b}$ | 10.1$^{a}$ | <.0001 |
| Mi 40 | 1.9$^{ab}$ | 1.43$^{bc}$ | 2.43$^{a}$ | 0.54 | 2.1$^{b}$ | 0.9$^{c}$ | 7.06$^{a}$ | <.0001 |
| Mi 15 | 1.44$^{a}$ | 1.75$^{a}$ | 1$^{a}$ | 0.82 | -- | -- | -- | |
| Mi 20 | 2.1$^{a}$ | 2.46$^{a}$ | 2.24$^{a}$ | 0.29 | -- | -- | -- | |

*Means followed by the same letter within a row are not significantly different ($P \geq 0.05$).

*SEM lines with dashed lines were not re-tested in trial 2.

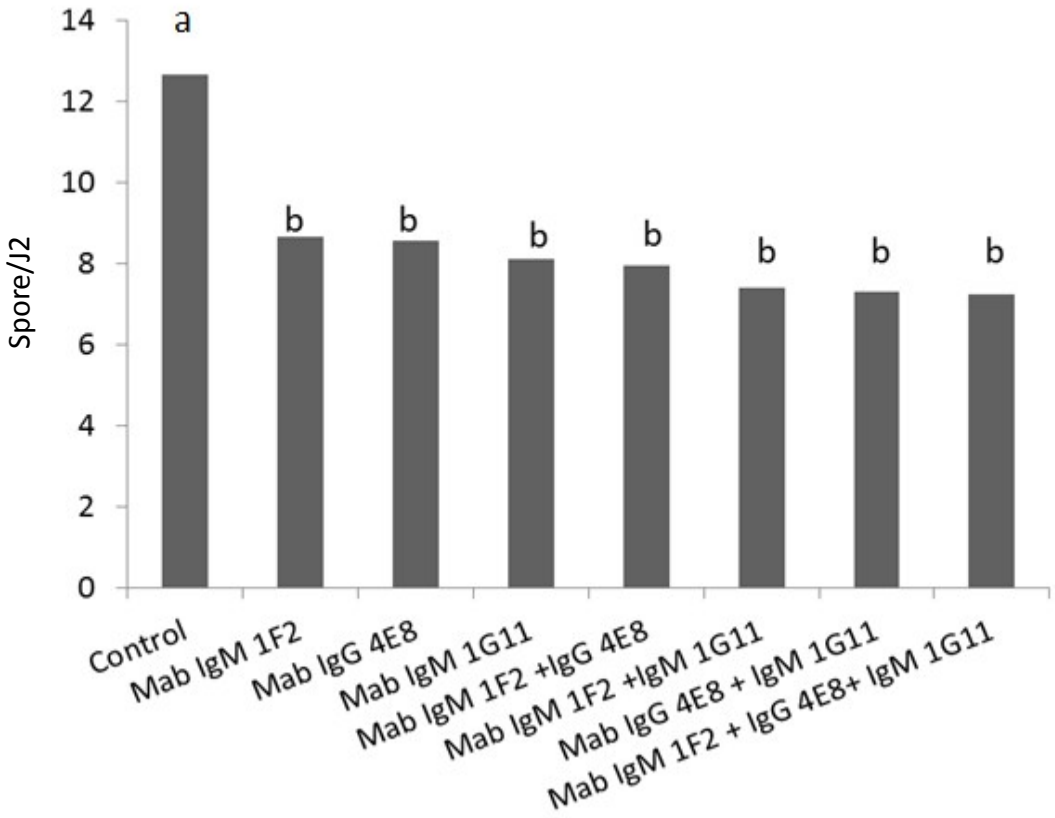


Fig.6.1 Mean *Pasteuria penetrans* spore attachment after incubating *Meloidogyne arenaria* second-stage juvenile with different monoclonal antibody (Mab) combinations. Bars with different letters indicate significant difference ($P<0.05$). Each bar represents the mean of two trials.

# CHAPTER 7

# CONCLUSION

In this project, both genetic and environmental factors on influencing the interaction between *P. penetrans* and *Meloidogyne* spp were investigated. Multiple environmental factors, including root exudates and maternal stress, were shown to influence the host-parasite interaction. I found that the incubation of J2 with root exudates decreased spore attachment, indicating the nematode surface coat was altered or the spore recognition domains on the nematode surface were blocked. Spore attachment was equally reduced following exposure to root exudates from both host and non-host plants for *M. arenaria*, indicating that there is a common signal that affects spore attachment. The signal that caused this surface coat change was unknown. Root exudates reduced spore attachment more in sterilized soil than in natural soil. Sterilization may have eliminated microbes that consume root exudates, or altered the chemical components of the soil solution or root exudates. In the field, the first generation nematodes that migrate to the roots are likely to be more susceptible to *Pasteuria* spore attachment because of lower exposure to root exudates than future generations that will be hatching around the roots. The effect of root exudates in reducing spore attachment also influenced the effectiveness of *P. penetrans* as a seed treatment compared to in furrow application. Applying *P. penetrans* spores in furrow reduced egg production more than as a seed treatment. When applying *P. penetrans* as a seed treatment, the spores would distribute along the roots as the seed germinates. Second stage juveniles hatching from eggs in the soil will be exposed to root exudates as they enter the root zone and may, thereby, become more resistant to spore attachment. However, when *P. penetrans* were

applied in furrow, the spores may have a wider distribution than just along the roots. The nematodes, therefore, may have more chances to encounter the spores before their surface coat is influenced by root exudates. The results of this study indicate that it is important to apply *P. penetrans* spores early in the season, and application in furrow is more effective than as a seed treatment in reducing nematode egg production. Maternal stress also influenced the susceptibility of *M. arenaria* progeny to *P. penetrnas*. When mothers were exposed to low food supply, no difference in spore attachment to progeny was observed between the stressed and non-stressed treatment. However, infection rate of progeny from the stressed treatment was significantly lower than that in the non-stressed treatment. This study indicates that *M. arenaria* can transfer immunity maternally to provide better protection to the progeny. In the field, nematodes often reach the carry capacity of their host plant by the end of the season, and their progeny may become more resistant to the infection by *P. penetrans*. In addition to environment factors, the spore attachment phenotype specificity was tested by using three single spore lines of *P. penetrans* and single egg mass (SEM) lines of *Meloidogyne* spp. The single spore lines showed differential attachment to the SEM lines; however, there was no all-or-nothing response and the attachment patterns were not repeatable. The most likely reason for the lack of differential attachment might be that the single spore lines were derived from one *M. arenaria* single egg mass line, therefore they may be the same attachment phenotype. In a field experiment, there was evidence for negative frequency-dependent selection between *P. penetrans* and *M. arenaria* in a field, indicating there was a higher level specificity between the host and parasite than the result of the attachment study. More attempts with generating single spore lines derived from different single egg mass lines of root-knot nematodes would be helpful to determine of whether a spore attachment pattern occurs within *P. penetrans*. The failure in maintaining highly diverse spore

genotypes within a biological control product could lead to the resistance to the product through selection on the host population, and would lead to the failure of nematode suppression. In a field study with 8 plots, the host parasite interaction between *M. arenaria* and *P. penetrans* supported the model of negative frequency-dependent selection. There were rapid changes in the dominant spore pathotype in each plot in each year, and such changes occurred on the local level, indicating the changes in the *P. penetrans* polulations were driven by effects of the local environment. This evidence is important to understand the causes and maintenance of genetic diversity within populations of *P. penetrans* and its host *M. arenaria*. In conclusion, the host-parasite interaction between *P. penetrans* and root-knot nematodes is complicated. Both genotypic and environmental factors need to be taken into consideration when developing it into a biological control product.