Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för landskapsarkitektur, trädgårds- och växtproduktionsvetenskap

Biological control of root-knot nematodes *(Meloidogyne spp.)* by the fungus *Pochonia chlamydosporia*

– A review

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– En litteraturstudie

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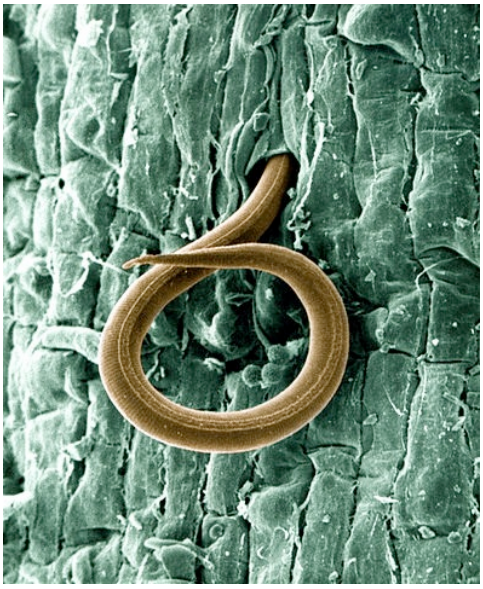

Självständigt arbete • 15 hp
Trädgårdsingenjör:odling – kandidatprogram
Alnarp 2015

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Omfattning: 15 hp
Nivå och fördjupning: G2E
Kurstitel: Kandidatarbete i biologi
Kurskod: EX0493

Program/utbildning: Trädgårdsingenjör: odling – kandidatprogram

Examen: Trädgårdssingenjör, kandidatexamen i biologi
Ämne: Biologi

Utgivningsort: Alnarp
Utgivningsmånad och -år: Mars 2015
Omslagsbild: *Meloidogyne incognita* juvenile penetrates tomato root. Photo by William Wergin and Richard Sayre. Colorized by Stephen Ausmus. US Department of Agriculture.
Elektronisk publicering: http://stud.epsilon.slu.se

Nyckelord: ***Biological control, Root-knot nematodes, Meloidogyne, Pochonia chlamydosporia, Verticillium chlamydosporium, integrated pest management.***

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Table of contents

Abstract

Pochonia chlamydosporia, a facultative parasite of root-knot nematodes is able to provide varying levels of control on populations of root-knot nematodes *(Meloidogyne spp.)*. Experiments in vitro, in greenhouse cultures and in the field have shown both promising and disappointing results. Various factors, such as temperature, host plant species, nematode species, fungal isolate, amount of inoculum, mode of application, time of inoculation and nematode population size affect the ability of the fungus to control. Combining the inoculation with other measures such as crop rotation and the use of resistant cultivars is likely to be needed if sufficient levels of control are to be obtained. Further research based on long-term experiments including combinations with other measures in an economical context is needed to make conclusions of the usefulness of this biological control agent.

Introduction

Root-knot nematodes are responsible for great losses in crop production systems across the globe (Moens et al, 2009). To reduce these yearly losses of plant products, researchers around the world are currently investigating new alternatives for control. Many different techniques are being investigated to provide necessary information for development of future management programs for nematode pests. One technique of interest is biological control of the nematodes using various organisms and tactics. This work explores the possibilities and restrictions of one such organism: the fungus *Pochonia chlamydosporia.*

The purpose of this review is to explore the ability of the fungus *Pochonia chlamydosporia* to control root-knot nematodes in horticutural and agricultural crop production systems. This is done in order to gain understanding of one of many alternative methods to reduce yield losses caused by root-knot nematodes. The goal is to analyse and present current knowledge of the interaction between the control agent and the pest and to describe the factors affecting the performance of the control agent. If possible, suggestions for further research and optimisation of the practices involved in application of the technique will be proposed.

In order to achieve the goal of this review, the following questions will be discussed: Can *Pochonia chlamydosporia* be used to control root-knot nematodes in a crop production system? Which factors determine the performance of the control agent? How can scientists and growers optimize the fungus' performance as a biological control agent?

Introduction to root-knot nematodes *(Meloidogyne)*

The *Meloidogyne* genus, commonly referred to as root-knot nematodes is a group of exclusively plant parasitic nematodes (Moens et al, 2009). The taxon has a cosmopolitan distribution and it is the most important group of plant parasitic nematodes responsible for crop damage estimated to 5% of global yield losses. The *Meloidogyne* species are considered generalists with an extraordinary wide host range covering nearly all higher plants. Some species are confined to a narrower

group of plants such as a subclass or a family while others can attack a very wide range of plants. *M. hapla,* for example, is able to reproduce on most dicotyledons. By June 2009, 97 valid species had been identified in the genus. Most species are found in tropical and subtropical climates. In a horticultural context *M. arenaria, M. javanica, M. incognita and M. hapla* are the four most important species. *M. arenaria, M. javanica and M. incognita* are mostly found in tropical and subtropical climates but also in greenhouses in temperate climates. *M. hapla* is the species of greatest economic importance in agriculture in Nordic countries. Most knowledge of *Meloidogyne* comes from research of these four species. There are, however, a few species that might have been overlooked in the past and whose importance is probably underestimated. Moens et al (2009) lists *M. fallax, M. chitwoodi, M. enterolobi, M. minor and M. paranaensis* as species of emerging importance.

Within the different species variation in host range, host preference and virulence is commonly found. The genetic variation within species is not represented by distinct races with fixed hosts as with many other groups of pests but rather a variation among larger or local populations (Trudgill and Blok, 2001). In many cases the genetic variation of host preference is not clearly tied to a defined set of other traits. Some of the most economically important crops such as potato, tomato, carrot, soybean, sugar beet and tobacco are common hosts of various *Meloidogyne* species (Moens et al, 2009). In the apomictic polyphagous *Meloidogyne* species the full host range is normally present relatively intact in most populations rather than divided between populations of narrower host range (Trudgill and Blok, 2001). For biotrophic organisms such as *Meloidogyne*, an intimate relationship with the host is required and biotrophs are therefore often monophagous as a result of coevolution with a single or a few hosts. How some *Meloidogyne* species have managed to acquire such a broad and large host range and maintain it is quite extraordinary from an ecological point of view. Few other organisms display similar properties. In the infection process *Meloidogyne* nematodes alter the genetic expression in the host tissue during formation of giant cells. The nematodes are also known to trigger susceptible response in the host by manipulating signaling pathways. To conserve this ability in many hosts over time requires conservation of the involved parts of the genomes in both nematode and host. A possible explanation for this relationship to persist is that the plant genes involved in the infection process are crucial for plant

growth and survival and therefore pose a barrier in the fitness landscape for plants difficult to overcome by the mechanisms of evolution. The origin of the apomictic Meloidogyne species is also a question for debate. The most common species are globally widespread and either have to have existed for a very long time or are recently spread by humans. A common hypothesis is that these nematode species has their origin in repeated hybridisations of closely related species.

Meloidogyne nematodes parasitise plants by infecting roots where they become sedentary until they finish their life cycle (Moens et al, 2009). The nematodes feed from specialized sites in the root called giant cells. The giant cells are plant cells modified by the nematode to reach an abnormal size and function. Around the giant cells, galls consisting of swollen root tissue are often formed. Galls vary in size and can often be almost undetectable. The shape and size of galls depends on both the nematode and host species. In some interactions galls are entirely missing and the egg masses and developing nematodes are exposed on the root surface. Plants with succulent roots such as tomato and cucumber often form big and clearly visible galls while galls on hosts with woody fibrous roots are often indistinct. Graminatious hosts rarely form galls at all. Heavily infected roots of susceptible hosts can form massive amounts of gall tissue that completely takes over the root system. The galls receive nutrients and photosynthates from the plant, which the developing nematodes consume. Infected susceptible plants generally get stunted and produce less yield of lesser quality.

The life cycle of *Meloidogyne* species

The adult female nematode lays her eggs in a gelatinous glycoprotein matrix excreted from rectal glands (Moens et al, 2009). The glycoprotein holds the eggs together and provides protection against physical stress, predation and microbial infections (Orion and Kritzman, 1991). The eggs are located on the root surface or inside galls (Moens et al, 2009). Embryogenesis results in a first stage juvenile (J1) that continues to develop inside the egg. When the juvenile has reached the second stage of development (J2) it is ready to hatch. Eggs hatch depending on various environmental factors such as moisture, temperature and concentration of plant exudates. After hatching the J2-stage individuals moves within the root of the host plant or through the soil to find a new site to infect. The J2 locates a new host by

following gradients of root exudates or reinfects the host plant on which it was born. In the J2-stage the nematode is relatively exposed to environmental conditions and vulnerable to predation. A new host needs to be located as quickly as possible. When arriving at a root of a suitable host the J2 penetrates the root, often near the tip just behind the root cap. After breaching the root epidermis, nematode juveniles move through the plant tissue to find a feeding site in the proto-xylem or proto-phloem of the root. The J2 start feeding and chemical components excreted by the nematode in the feeding process causes the plant cell to enlarge and become a giant cell. A single nematode often induces several adjacent cells to enlarge (Abad et al, 2009). Giant cells are multinucleate (up to 80 per cell) polyploid plant cells induced by the nematode (Huang and Maggenti, 1969; Wiggers et al, 1990). Giant cells differ from syncytia which are similar feeding structures induced by other sedentary plant parasitic nematodes in that they derive from a single plant cell instead of a number of neighboring cells fused together as in syncytia. The giant cell reaches a final volume nearly 100 times greater than a normal root cell. The formation of giant cells in the host is unique to *Meloidogyne* and exists in all species of the genus. After feeding has started the J2 transforms into a 'sausage-shape' and if conditions are right develops into the J3-stage in about two weeks (Moens et al, 2009). The 'sausage-shape' consists of the hardened swollen J2 cuticle in which the nematode continues to develop, resembling an insect imago developing in its pupa. Sex differentiation occurs in the development of the J3. Males and females now display distinguishable morphological traits. The J3 individuals no longer feed but continue to develop through the J4 stage into adults, typically in 4-6 days. The life cycle is completed when the adult female lays her own eggs and the process is repeated several times during a cropping period depending on host species, environmental conditions and, in an agricultural setting, the duration of the culture.

Facultative or obligate apomixis is common in *Meloidogyne* species and in important pest species in particular. Most amphimichtic species are polyploid or aneuploid with n=18 and apomictic species are usually diploid with varying chromosome number. Males of *Meloidogyne* are vermiform and in most cases only develop when food is scarce. The males have not been seen to feed and are therefore not believed to be directly causing any plant injury. Facultative apomixis is common within the genus

and within the species that function as agricultural and horticultural pests sexual reproduction is especially rare and relatively unimportant.

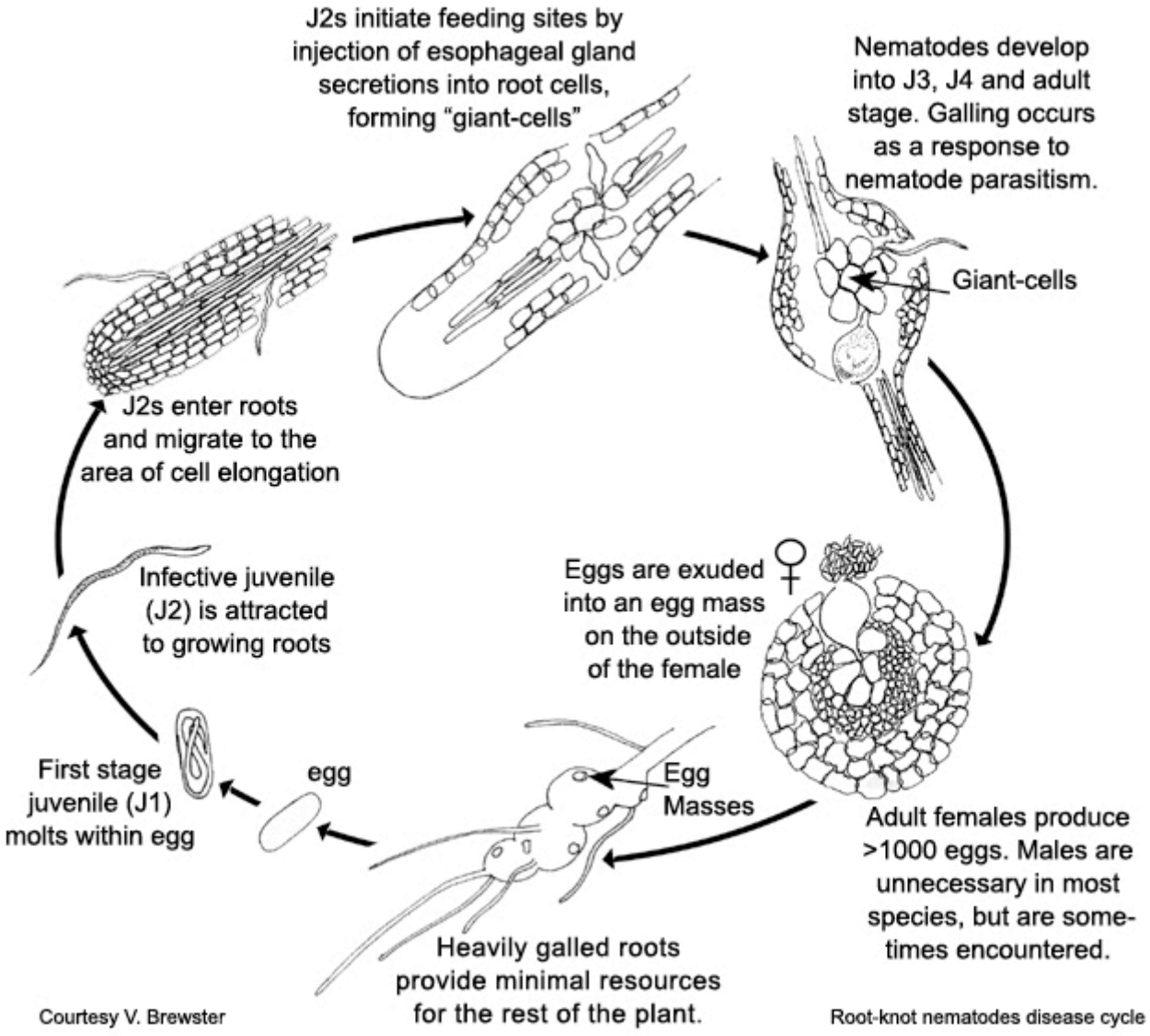

Figure 1. Meloidogyne life cycle. (American phytopathological society, 2003)

Integrated pest management and biological control

Prohibition of previously commercially available chemical control agents such as methyl bromide in many countries has made control of *Meloidogyne* populations in the field very difficult (Nyczepir and Thomas, 2009). Since the ban of some important nematicides, research on alternative control methods has been aimed at resistance breeding, crop rotation with trap crops or non hosts, application of biofumigants, soil management including application of organic soil amendments, and biological control using various groups of organisms. Due to the generalist nature of some common pest species of *Meloidogyne*, the nematodes often reproduce well on many weeds and weed management is therefore an important part of reducing populations in the field (Moens et al, 2009).

Integrated pest management (IPM) is a concept proposed as a strategy for sustainable and effective plant protection (Abrol and Shankar, 2012). The strategy is to apply ecological theory in the design of management programs for agricultural and horticultural cropping systems to avoid yield losses without adverse effects on human health and the environment. The strategy is generally applied by using combinations of various cultural practices and other control measures, such as mechanical weed management, crop rotation, resistant cultivars and biological control. A combination of such efforts is intended to reduce the need for extensive use of herbicides and pesticides harmful to humans or other components of ecosystems such as birds or beneficial insect species. To apply pesticides more accurately in lesser quantities with the aid of epidemiological models of disease development is another important component of IPM. Strategies based on the IPM concept will be necessary in the development of future management programs for *Meloidogyne* (Stirling, 2014).

Biological control can be described as the practice of introducing or managing populations of natural enemies of a pest or pathogen in a crop in order to reduce populations or at least slow down the reproductive rate (Stirling, 2014). Within the concept of biological control three main strategies are often described. Inundative biological control refers to the strategy of releasing a large number of commercially produced individuals of a predator or other antagonist to the pest. The aim of inundative biological control is for the released individuals to quickly reduce the

population of the pest. Inoculative or classical biological control refers to the strategy of releasing an exotic antagonistic species not already present in the ecosystem in hope that it will reproduce and build up a relatively stable population and in that way provide a long-term control of the pest. Conservation biological control is a strategy, based on the idea that a naturally occurring antagonist species can be favoured by enhancing its habitat. The design of many modern intensive cropping systems often lack important components required by the naturally occurring antagonists of plant pests and pathogens (Abrol and Shankar, 2012). Habitat enhancement often includes the introduction of appropriate protection, food sources and mating sites of antagonists by planting host species of the antagonists in vicinity of the crop.

Nematophagous fungi

Nematophagous fungi function either as obligate parasites or facultative parasites (Godoy et al, 1983; Morgan-Jones and Rodriguez-Kabana, 1988; Stirling and West, 1991). The obligate parasites attack the host as a spore (Hallman et al, 2009). The fungal spore adheres to a migrating nematode or is ingested by a feeding nematode. An infectious hypha grows directly from the spore and penetrates the host through the cuticle or the gastrointestinal tract. The facultative parasites can grow in soil or rhizosphere as saprotrophs and from that state develop specialized spores, conidia or hypha that trap or adhere to nematodes and infect them (Barron, 1977). Many fungal species are known to infect nematodes but only a few are considered suitable as biological control agents (Siddiqui and Mahmood, 1996). The most studied species in the context of biological control are *Pochonia chlamydosporia* and *Paecilomyces lilacinus.*

Pochonia chlamydosporia (syn. *Verticillium chlamydosporium*)

Pochonia chlamydosporia is a member of the Clavicipitaceae family in the order Hypocreales in the division of Ascomycota (Zare et al, 2001). The genus *Pochonia* was formerly included in the genus *Verticillium,* but *P. chlamydosporia* was removed in 2001 together with a few other species to form the genus *Pochonia*. The fungus is a facultative parasite capable of functioning both as a saprotroph in soil (Siddiqui et al, 2009), and as a parasite of eggs and females of root knot nematodes (Morgan-Jones et al, 1982, 1983; Rodriguez-Kabana et al, 1984; Freire and Bridge, 1985; De Leij and Kerry,1991). *P. chlamydosporia* is also able to colonize roots of a broad range of both monocotyledon and dicotyledon plants as an endophyte (Bordallo et al,

2002; Lopez-Llorca et al, 2002). In some plants, like barley (*Hordeum vulgare*) for example, growth promotion and modulation of plant defense mechanisms by the fungus has been documented (Macia-Vicente et al, 2009).

When infecting its host, *P. chlamydosporia* grows a branched network of hyphae that covers egg masses and form appressoria that penetrate the eggs (Morgan-Jones et al, 1983; Lopez-Llorca and Duncan, 1988; Lopez-Llorca and Claugher, 1990). Various enzymes are involved in breaching the eggshell and dissolving its contents (Segers et al,1996; Morton et al, 2004). Segers et al (1996) showed that the enzyme VCP1, an alkaline serine protease produced by *P. chlamydosporia,* can break down the protein layer in the *Meloidogyne* egg shell and expose the inner chitin layer. The authors conclude that this enzyme is an important component for parasitizing *Meloidogyne* egg masses. They also speculate that VCP1 could be used by the fungus to break down various food sources also in a saprotrophic state. Larriba et al (2014) sequenced and assembled ~41 Mb of *P. chlamydosporia* genomic DNA. In the analysis the conclusion was that the *P. chlamydosporia* genome "is highly enriched in genes encoding hydrolytic enzymes, such as proteases, glycoside hydrolases and carbohydrate esterases". *P. chlamydosporia* can easily be cultivated in vitro (Bourne et al, 1999), which is an important property for a fungus to be considered as a biocontrol agent since it otherwise would be difficult to produce commercially (Kerry, 2000). The fungus produces chlamydospores; resting structures that make it able to survive stressful environmental conditions such as drought or cold (Hallman et al, 2009). The chlamydospores are also useful for commercialising the fungus as they can be stored, shipped and then used for inoculating a field or a volume of substrate.

P. chlamydosporia grows well in pH 4.0-7.0 and optimum temperature for growth is 25-30°C (Karakas et al, 2012). Optimum temperature for growth according to Kerry et al (1986) is 25°C but varies between isolates. The optimum temperature for infection of *Meloidogyne* eggs and females can differ from the optimum temperature for hyphal growth (Irving and Kerry, 1986). Irving and Kerry (1986) measured the optimum temperature for growth of strain II of *P. chlamydosporia* to be 25°C while 12°C was the optimum for parasitism.

Luambano et al (2015) found that pre-decomposed organic materials (sawdust from mixed trees, sunn hemp and maize cobs) added to soil can increase saprotrophic growth of *P. chlamydosporia*, but high C:N ratios did not promote parasitism on *M. incognita* eggs. Higher N values (from 5 to 100 mM) at a constant C concentration of 10 mM promoted parasitism. Pre-decomposing of the organic amendments was done for three weeks at temperatures of 15°C, 20°C and 25°C. Sunn hemp decomposed at 20°C was the most effective amendment in increasing egg parasitism. The same authors also tested the effect of varying pH which did not show a clear pattern, but highest levels of parasitism was recorded at pH 4.7. More research is needed to make accurate conclusions on the optimal soil conditions for *P. chlamydosporia* parasitic behaviour. Optimum conditions can differ between isolates (Kerry, 1986). This makes the question more specific, but might also provide future opportunities to match specific cropping systems with a suitable fungal isolate.

Materials and Methods

To summarize and analyse current scientific knowledge on a subject, a literature review is a helpful tool. It is likely that the questions posed in this work are too broad to be answered by a single or a few experiments and since many studies have been made by different scientists and are available in scientific journals, a literature review seems to be the most suitable method for this work. A literature review was made using the databases Primo, Web of Science and Google Scholar. The search strategy was to use the names of the genera involved (*Meloidogyne, Pochonia and Verticillium*) and the trivial name: root-knot nematode, combined with ”biological control”. For specific information, other words were added in combination with the names of the organisms such as: effect, temperature, pH, host range, virulence, growth, etcetera. Many different combinations were tried to avoid missing useful information. The focus of the search was experiments aimed at determining the effect of *Pochonia chlamydosporia* on populations of root knot nematodes parasitising a plant host. The details of various biological mechanisms involved in the process such as activation of certain genes in fungus or nematode and the specific chemical components involved in infection and other related processes was largely left out due to lack of time.

Results and Discussion

P. chlamydosporia as a control agent of root knot nematodes

In combination with other control methods such as crop rotation, trap crops and green manure, the general opinion among researchers of the subject seems to be that the use of *P. clamydosporia* as a biological control agent has a good potential of becoming an economically feasible and environmentally sustainable alternative for control of *Meloidogyne* populations (Carneiro et al, 1998; Ferraz and Freitas, 2004; Inomoto et al, 2006). Kerry (2000) lists 10 key factors affecting the performance of *Pochonia chlamydosporia* as a biological control agent of root-knot nematodes: 1. Fungal isolate, 2. Fungal colonization of the rhizosphere, 3. Plant species, 4. Plant susceptibility to nematode, 5. Application rate, 6. Gall size, 7. Plant tolerance to nematode, 8. Egg maturity, 9. Soil temperature, and 10. Nematode species.

Isolates of *P. chlamydosporia* differ in their ability to control *Meloidogyne* (Siddiqui et al, 2009; Medina-Canales et al, 2014; Dackman and Nordbring-Hertz, 1985; Dallemole-Giaretta et al, 2012). Populations of *P. chlamydosporia* are isolated, often from nematode egg-masses in naturally infested soils and taken to laboratory for testing. Some isolates are better adapted than others to the environmental conditions in experiments and commercial cropping systems. Kerry (2000) states that “some isolates are able to proliferate on organic matter in soil but nematode control is poor if they are not able to colonise the rhizosphere”. *P. chlamydosporia* has a worldwide distribution (Larriba et al, 2014) and fungal populations might be specifically adapted to certain soil types, nematode hosts, plant hosts and climatic conditions (Siddiqui et al, 2009, Medina-Canales et al, 2014, Dackman and Nordbring-Hertz, 1985).

Siddiqui et al (2009) compared eight biotypes of *P. chlamydosporia* isolated from either *Globodera pallida* or *Meloidogyne spp.* egg masses. The tested isolates were more effective at infecting the host that they had been isolated from and the different fungal isolates also showed individual differences in virulence. Soil sterilization prior to inoculation improved saprotrophic growth in general but saprotrophic growth had a linear inverse relationship to virulence of the preferred host. Host preference at the intraspecific level was also found in experiment by Mauchline et al (2004). An isolate of *P. Chlamydosporia* from egg masses of potato cyst nematode *Globodera pallida*

and another isolate from egg masses of *Meloidogyne spp.* were cultured in soil with nematode infected potato and tomato plants. Both isolates were found to exploit the host species they had been isolated from better than the other isolate. The results of Siddiqui et al (2009), Medina-Canales et al (2014) Dallemole-Giaretta et al (2012) and Dackman and Nordbring-Hertz (1985) might be considered as snap-shots of a complex reality with a great variety of adaptations in *P. chlamydosporia* populations of ecosystems around the world.

For the fungus to be able to control a plant parasitic nematode population it has to colonise the rhizosphere of the nematodes' host plant (Bourne et al, 1996). Plant species differ greatly in their capability to support colonisation of the rhizosphere by the fungus. Plant susceptibility to nematodes also affects this relationship. A very susceptible plant will, if infected, host a larger nematode population, which will favor the fungus. However, the fungal proliferation in such cases is not fast enough to effectively control a fast growing nematode population. Bourne et al (1996) also speculate that exposure of the egg masses on the root as occurs in some plant species might favor control by the fungus compared to roots forming big galls where the egg masses are better protected.

In crop rotation, a cover crop suitable to the fungus can increase the population of *P. chlamydosporia* in the soil and thereby increase its efficacy in controlling the nematode population in the main crop (Bourne et al, 1994, 1996; Kerry and Bourne, 1996). Several cover crops have been tested for compatibility with *P. chlamydosporia* in the rhizosphere with varying results depending on the species of cover crop. Ferraz et al (2011) tested the effect of *P. chlamydosporia* (isolate Pc-10) on the development of *M. javanica* in tomato (*Solanum lycopersicum* cv. Santa Clara) grown after winter cover crops, summer cover crops, continuous tomato culture and fallow respectively. The winter cover crops tested in the experiment were oil radish (*Raphanus sativus* cv. AL 1000) and black oat (*Avena strigosa* cv. IAPAR 61) and the summer cover crops were pearl millet (*Pennisetum glaucum* cv. BN2) and surinam grass (*Brachiaria decumbens* cv. Basilisk). Black oat, surinam grass, and pearl millet were the most effective at reducing the nematode populations without the aid of the fungus, by >90% respectively. In the presence of *P. chlamydosporia* in combination with the cover crops the effect was further significantly increased only on surinam

grass, which was also the most effective cover crop on its own. In the surinam grass the fungus decreased root galling by 71% compared to the cover crop alone. This particular combination of fungus and plant illustrates the idea of combining *P. chlamydosporia* with other measures to gain an acceptable level of control. Although the cover crop itself can provide most of the effect, the application of the fungus might reduce the nematode population further and provide nematode control in the following main crop. Number of galls was highest in the fallow and continuous tomato treatments without fungus, with means of 1078 and 1741 galls per root system respectively. The presence of *P. chlamydosporia* in fallow and continuous tomato treatments resulted in means of 824 and 1352 galls per root system.

These levels of galling seem likely to produce nematode populations well above the damage threshold for tomato (Gharabadiyan et al, 2013; Greco and Di Vito, 2009). Control of *Meloidogyne* could therefore be expected to be insufficient if *P. chlamydosporia* was to be used as a single measure in a continuous susceptible main crop. To develop a practical strategy for the use of *P. chlamydosporia,* a highly compatible nematode resistant cover crop should be coupled with a suitable fungal isolate to be introduced together in a crop rotation (Ferraz et al, 2011; Kerry, 2000). In Nordic climate, barley (*Hordeum vulgare*) might be a good candidate for this since it is a good host for the fungus (Macia-Vicente et al, 2009) and has also been shown to control *M. hapla* in crop rotation for carrot production increasing the yield in the following carrot culture (Bélair and Parent,1996). If a resistant cultivar of the main crop is added to such a system the chance of eliminating significant yield losses would improve even more (Gharabadiyan et al, 2013). Gharabadiyan et al (2013) showed that some tomato cultivars display a varying degree of quantitative resistance, which they measured as “effective population of nematodes, reducing 10% of maximum yield or shoot fresh weight respectively” (EP10). The population size refers to number of eggs present in the soil at the start of the culture. The tomato cultivars in this study were 'Rutgers', 'Efialto', 'Falat111' and 'GinaVF'. 'Rutgers' had an EP10 value of 500 eggs per kg soil while the other three cultivars displayed EP10 values around 3000 eggs per kg soil. At a level around 5000 eggs per kg soil the cultivar 'GinaVF' showed yield reductions of 40% compared to 20% in 'Efialto’ and 'Falat111'.

Bontempo et al (2014) tested *P. chlamydosporia* against *M. incognita* in field-grown carrot (*Daucus carota subsp. Sativus* cv. Juliana) in a naturally infested field in Minas Gerais, Brazil. Two modes of application were tested. Mechanical mixing of the top soil after spraying a suspension of the fungus improved the effect of the control agent compared to only spraying on top. Marketable yield was increased by 53.03% and the nematode population in the soil decreased during the growing season by roughly 2/3. Carrot cultivars display varying degrees of tolerance to M. incognita (Siddiqui et al, 2011). Depending on the level of susceptibility of the cultivar Juliana, this result seem promising considering that the nematode population decreased within the crop cycle. Mixing the top soil after spraying the fungus probably reduces the risk of damage to the inoculum caused by dehydration and solar radiation (Bontempo et al, 2014).

Sharf et al (2014) tested *P. chlamydosporia* against *M. incognita* in a greenhouse experiment with common bean (*Phaseolus vulgaris)* for 90 days. Six different fungal treatments were made. Three treatments were inoculated with 2g, 4g or 8g of *P. chlamydosporia* mycelium 15 days prior to nematode infection with 1000 J2 per 2 kg soil. Three additional treatments where inoculated with equal amounts of mycelium as in as the others but 15 days after nematode infection. All treatments reduced number of galls significantly compared to a nematode positive control without fungal inoculum but fungal inoculation prior to nematode exposure was more effective. Treatments with fungal inoculation prior to nematode infection reduced galling per root system from to 35.74, 32.99 and 30.00 compared to 80.49 in the control. Inoculation 15 days after nematode infection only reduced galling slightly to 77.23, 75.39 and 73.46. Growth parameters such as leaf area and number of seedpods of the plants responded proportionally. This result leads to the conclusion that *P. chlamydosporia* should if possible be inoculated in advance of nematode exposure of the plant. In other cases, combining the control agent with other measures might be necessary if sufficient control is to be obtained. Doubling the amount of inoculum resulted in slightly higher levels of control, which the authors speculate might be explained by the fungus being close to reaching its carrying capacity in this experiment.

Vianene and Abawi (2000) applied *P. chlamydosporia* to lettuce plants (*Lactuca sativa* cv. Montello) grown in soil containing up to 75% organic material and pH of 4.7 from a commercial lettuce field. The experiment was conducted in a greenhouse with temperatures varying between 24°C and 27°C. Chlamydospores were applied at rates of 500, 5000 and 10.000 spores per gram soil and two weeks later the plants were infected with *M. hapla* eggs at rates of 2, 4 and 8 eggs per cm^3 soil. Control of *M. hapla* was more effective at higher rates of chlamydospores. *P. chlamydosporia* colonized eggs of *M. hapla* and reduced population growth at the lower levels of nematode infection but failed to reduce population growth at 8 eggs per cm^3. This resulted in higher growth reduction of lettuce plants. Verdejo-Lucas et al (2003) reported similar rates of root galling in *P. chlamydosporia* treated and non-treated lettuce plants. Even though the fungus seemed unable to reduce galling in lettuce, nematode population was lower after the culture. Verdejo-Lucas et al (2003) explain this decrease as a result of the short duration of the lettuce crop, which when grown at low temperatures can act as a trap crop by attracting the nematodes, who are unable to finish their reproduction cycle during the short cropping period. If the fungus is allowed to proliferate on a seedling prior to nematode exposure the critical amount of inoculum needed for an acceptable level of control might be lower (Sharf et al, 2014). Rhizospheric compatibility between fungus and plant should, however, be expected to affect the outcome of such applications (Kerry, 2000) . To conclude whether lettuce is a compatible host to *P. chlamydosporia* more research is needed (Vianene and Abawi, 2000).

Van Damme et al (2005) conducted an experiment for two years in a greenhouse with a crop rotation of tomato and lettuce infected with *M. javanica* at rates of 25 and 50 second stage juveniles (J2) per 100 cm^3 of soil to study long-term effects of *P. chlamydosporia* inoculation. The authors found that *P. chlamydosporia* reduced the rate of increase of the nematodes and significantly fewer egg masses and J2 were found in the treatments with the lighter nematode infection but in the more heavily nematode infected treatment the initial number of J2 was too high for the fungus to successfully control the nematode population and plant growth was very limited. Verdejo-Lucas et al (2003) made a similar two-season experiment with crop rotation of lettuce and tomato infected with M. javanica and compared the effects of methyl bromide with the chemical pesticide oxamyl and *P. chlamydosporia* alone or in

combination on nematode populations. The authors found that only methyl bromide treatment increased the yield of tomato and that a combination of *P. chlamydosporia* inoculation and oxamyl reduced nematode infection but not enough to improve yield of tomato. Since methyl bromide as a control measure is prohibited in most countries (Stirling, 2014), using it to give the fungus a head start is generally not an option. Other chemical methods might however, if legally available and economically feasible be a rational way of reducing already large nematode populations (Nyczepir and Thomas, 2009). Biological control in combination with crop rotation might in such cases prolong the effect of chemical control. This sort of approach fits perfectly with the concept of integrated pest management and is therefore highly interesting as a subject for further research, especially if economical and environmental aspects are considered.

Viggiano et al (2014) applied a commercial control agent (Rizotec®) based on isolate Pc-10 of *P. chlamydosporia* to seedlings of cucumber in doses of various concentration before planting in *M. javanica* infected soil. Application by dipping the seedling in a solution of spores was compared with mixing the control agent into the soil or both. The authors found the best results were obtained by dipping the seedling in a solution of 18 g/L of Rizotec®. This reduced galling per gram of root by about 48%. Additional application to the soil did not improve the effect. The authors suggest the technique of dipping seedlings as an effective and economic way of inoculating the fungus in a commercial context. This conclusion seems accurate as the dipping of the seedlings might be done manually with many plants at a time and the fungus is administered directly onto the rhizosphere and at an early stage of the plants development. An interesting alternative to this technique would be to inoculate the fungal suspension to a nematode-disinfected substrate for sowing and cultivating the seedlings until transplantation. In that case the fungus might already be established in the rhizosphere before contact with nematode infested soil. Seed treatments would also work in this way but not if seeds are sown in infested soil, since the rate of inoculum would be small and mycelium not developed before contact with nematodes.

Conclusions

The usefulness of *Pochonia chlamydosporia* as a biological control agent is hard to determine. Biological factors affecting the outcome of experiments such as host plant species, cultivar, nematode species, nematode intraspecific variation, and fungal intraspecific variation make results of different experiments hard to compare. Despite the stunning complexity of the interactions between nematode, fungus and plant, some conclusions can be drawn.

Pochonia chlamydosporia parasitise eggs and females of root-knot nematodes (Morgan-Jones et al, 1982, 1983; Rodriguez-Kabana et al, 1984; Freire and Bridge, 1985; De Leij and Kerry,1991). To which degree a nematode population is reduced depends on various environmental and biological factors such as fungal isolate, fungal colonization of the rhizosphere, plant species, plant susceptibility to nematode, application rate, gall size, plant tolerance to nematode, egg maturity, soil temperature, nematode species (Kerry, 2000), timing of inoculation in relation to nematode exposure (Sharf et al, 2014), mode of application (Viggiano et al, 2014; Bontempo et al, 2014), combination with other control measures (Ferraz et al, 2011; Verdejo-Lucas et al, 2003), soil pH, moisture and C/N-ratio (Luambano et al, 2015) and crop rotation (Ferraz et al, 2011). It seems like in many cases, *P. chlamydosporia* inoculated in a nematode infested mono-culture would not provide enough control to maintain high yields or good plant health (Van Damme et al, 2005; Verdejo-Lucas et al, 2003; Ferraz et al, 2011). However, combined with other measures, *P. chlamydosporia* might be an option for a grower to consider (Ferraz et al, 2011; Carneiro et al, 1998; Ferraz and Freitas, 2004; Inomoto et al, 2006). If measures already applied in a cropping system still result in significant yield losses the addition of *P. chlamydosporia* might contribute to an extent that makes it economically feasible. To conclude in which situations the fungus is likely to make a significant contribution to a grower's economy and/or the environment, more research is needed. To manage populations of *P. chlamydosporia* in a field for long-term survival and continuous control of nematode populations, better understanding of the ecology of the fungus, with a focus on soil conditions in particular, is necessary (Hallman et al, 2009; Luambano et al, 2015).

A personal suggestion for further research is to evaluate the effect of the fungus together with suitable cover crops in crop rotations including nematode tolerant cultivars in the main crop. Before such applied research projects are made, better understanding of the intraspecific variation of the fungus might be needed in order to avoid that the effect of the fungus is underestimated due to poor choice of fungal isolate in relation to soil and climate conditions (Siddiqui et al, 2009; Medina-Canales et al, 2014; Dackman and Nordbring-Hertz, 1985; Dallemole-Giaretta et al, 2012).

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