Sustainability in Plant and Crop Protection

Rosa H. Manzanilla-López
Luis V. Lopez-Llorca *Editors*

# Perspectives in Sustainable Nematode Management Through *Pochonia chlamydosporia* Applications for Root and Rhizosphere Health

Springer

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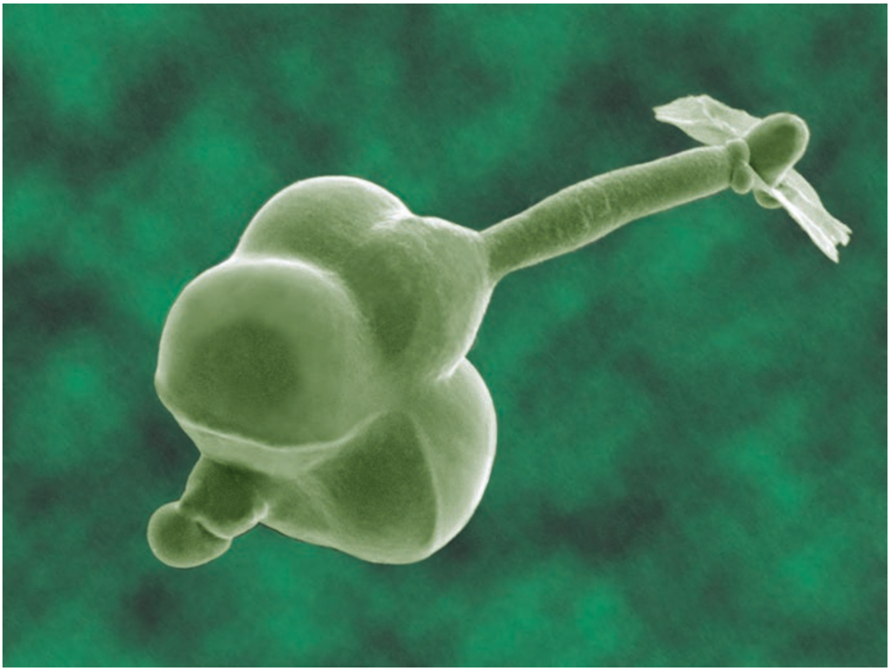

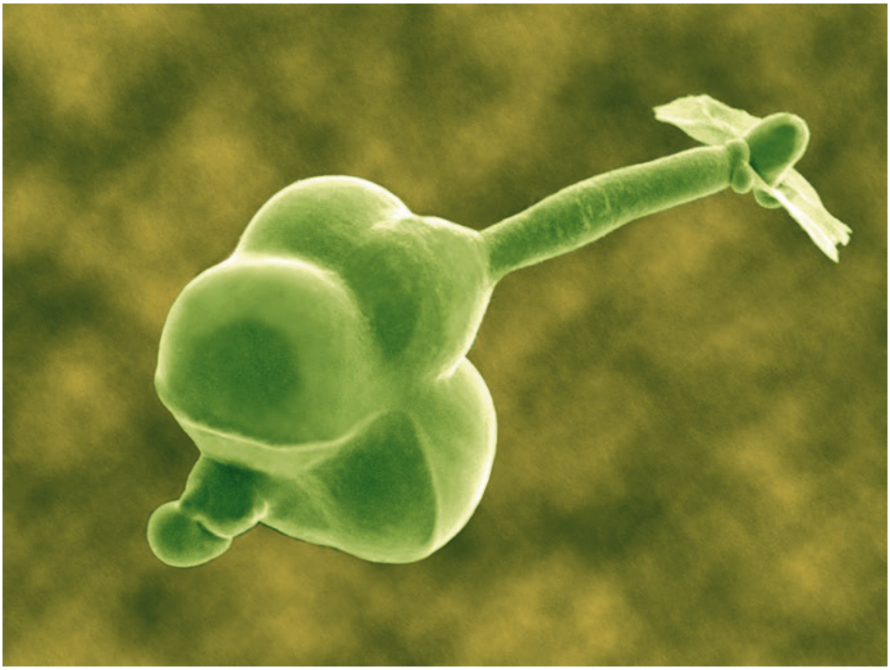

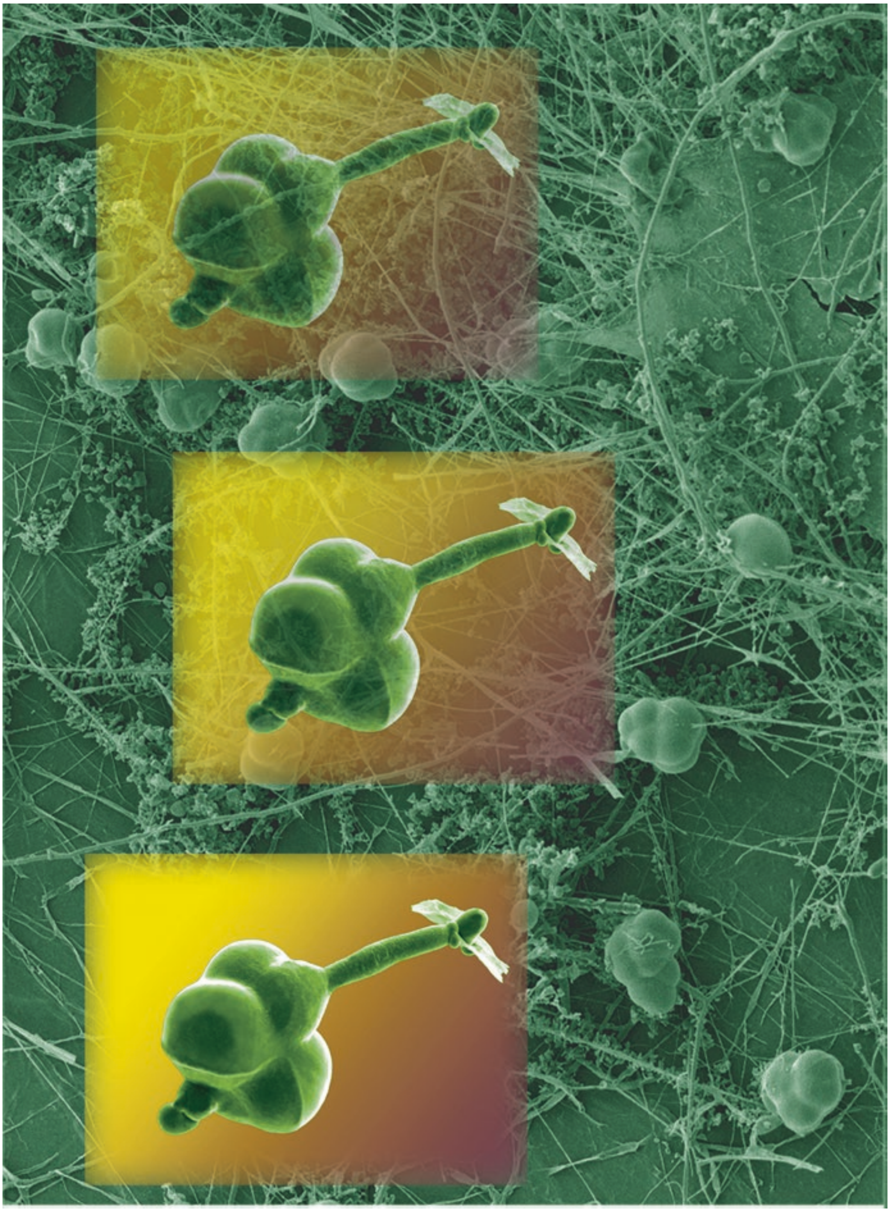

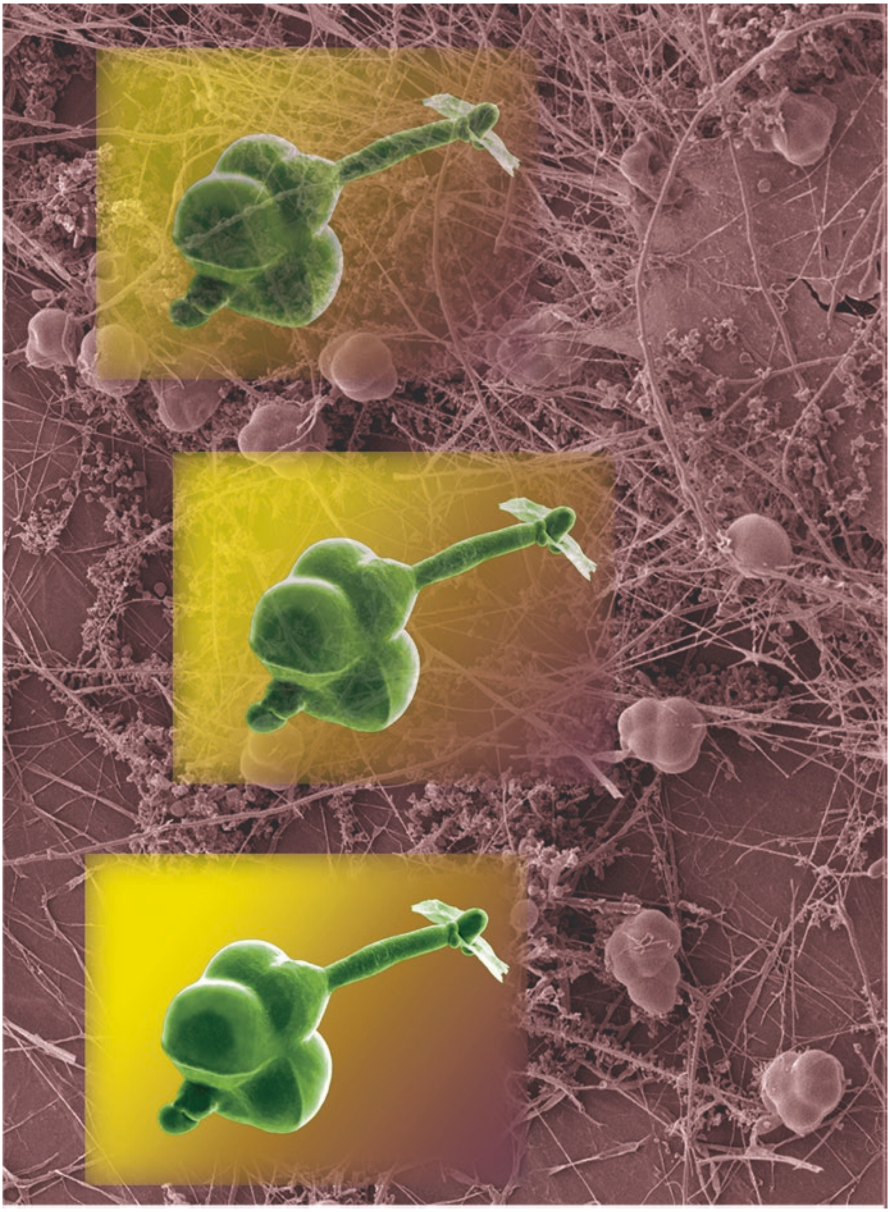

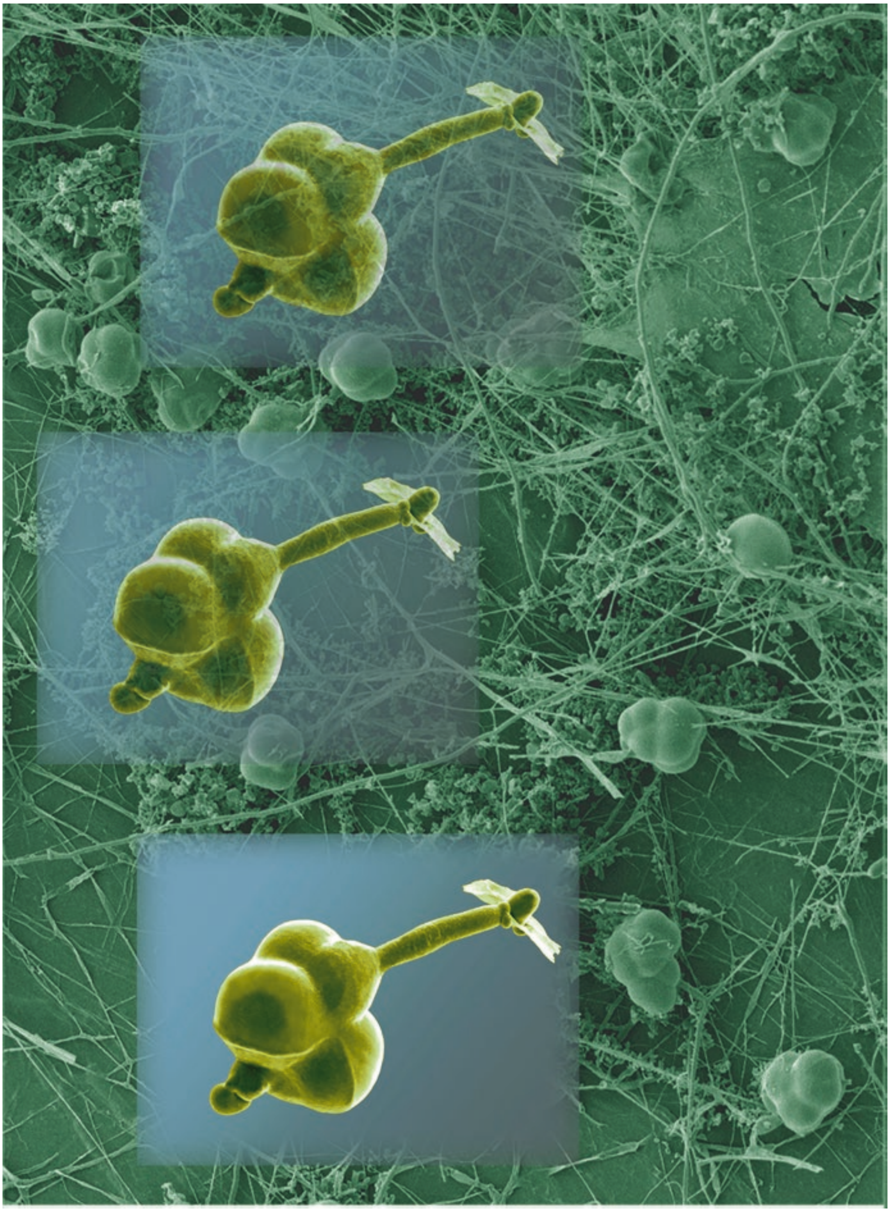

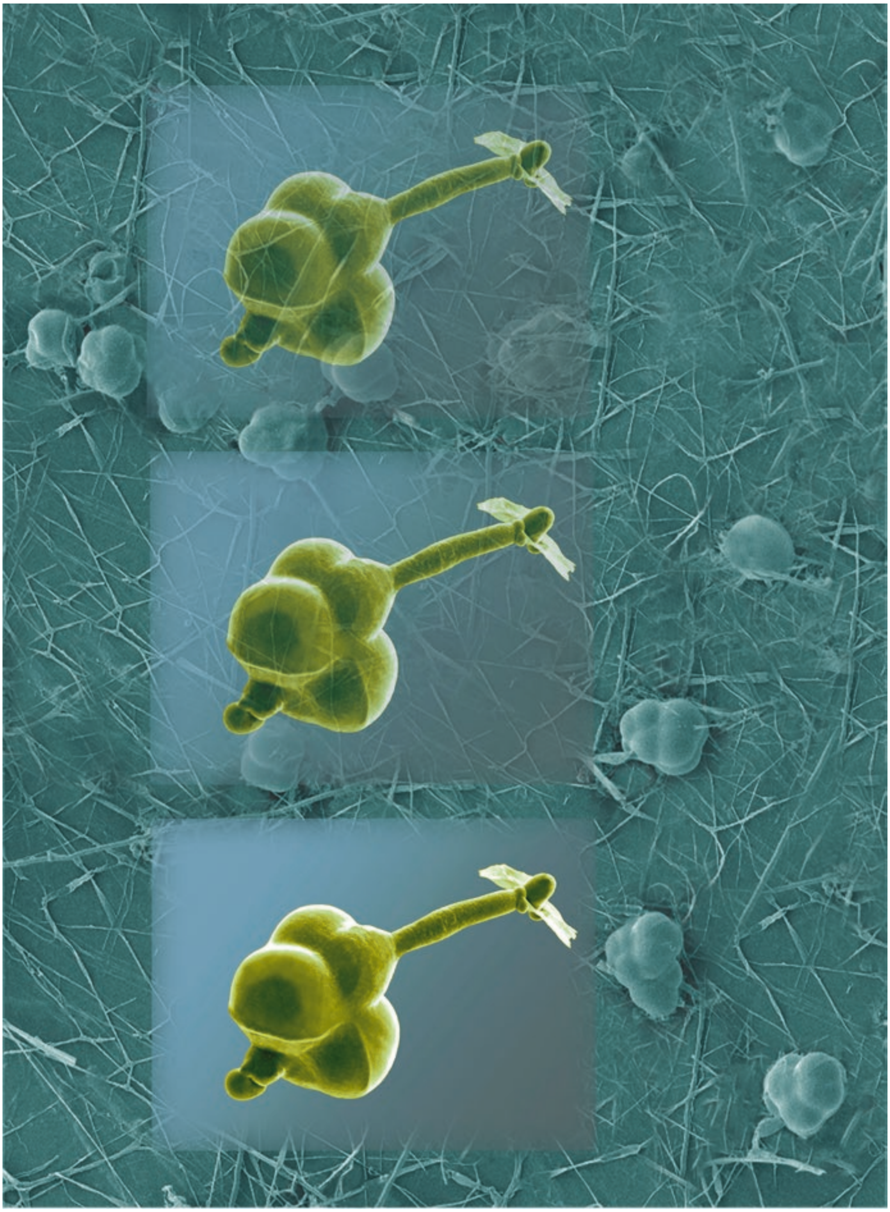

**Figure legend**

Cryo-SEM of a chlamydospore of *Pochonia chlamydosporia* isolate Pc60. Image design Jean Devonshire (Original image Copyright Rothamsted Research Ltd.)

# Foreword

Plant-parasitic nematodes pose a serious and continuing threat to crop production worldwide. The diseases they cause are difficult to diagnose and often overlooked by comparison with other biotic constraints such as insect pests, microbial pathogens and weeds. Nonetheless, their impact, either alone or as members of disease complexes, is substantial, with losses running into many billions of dollars. In resource-poor regions, such losses can have a direct impact on food security.

Management of plant-parasitic nematodes also poses major challenges. Natural resistance is present in some crops, but may not be durable or effective against all strains of a nematode. In other crop species, few natural sources of genetic resistance have been found, and engineering via biotechnological approaches may be the only potential solution. Most plant-parasitic nematodes live in soil or association with plant roots and are difficult to target with chemicals. Many of the most effective nematicides originally available for use have now been withdrawn due to their more general biocidal effects, and concerns over their environmental impact. Alternative sustainable strategies for nematode control are urgently needed. Biological approaches, exploiting the natural enemies of these pests, are therefore of particular interest.

The topic of this book starts with a detective story, as pioneering scientists searched for a biological explanation for the observation that on some hosts in certain soils the severity of infestation by nematodes is naturally suppressed. Amongst the suspects was a fungus with the capability to attack some stages in the nematode life cycle, in particular female worms and eggs. The fungus, originally named *Verticillium chlamydosporium*, was later reclassified as a single species in the related genus *Pochonia*. But this was not a simple case. The main suspect, *P. chlamydosporia,* turned out to have several identities, as a dormant survivor, soil saprophyte, facultative parasite, and root endophyte, with the ability to switch between these different states. To exploit the fungus as a biological control agent required more research and a greater understanding of its ecology and interactions with plant roots and nematode parasites.

The editors of this book have assembled an international team of authors expert in all aspects of the biology of *P. chlamydosporia* and its use as a biocontrol agent in horticulture and agriculture. The chapters cover a wide range of topics, from laboratory culture and identification, through physiology and biochemistry, to state of the art studies of the recently assembled genome sequence, proteomics and secondary metabolism. Examples of current and potential applications in the control of both plant and animal parasitic nematodes are covered, as well as recent findings on the beneficial effects on plant development of colonisation as an endophyte. While the focus is on a single fungal taxon, research on *P. chlamydosporia* has raised questions of much wider biological significance in soil ecology, fungal biology, and host-pathogen interactions. The genetic and molecular basis of the multi-trophic lifestyle is a recurrent theme relevant to other fungi that undergo physiological transitions at different stages of their life cycle. Improved understanding of the factors regulating such processes should aid effective production, formulation and delivery of the fungus as a bio-inoculant.

The impetus for all this scientific investigation is the prospect of developing the fungus as an important ally in the sustainable management of parasitic nematodes. Much progress has been made, especially in the local production and use of the fungus, but challenges remain in achieving consistently high levels of control, and scaling up application to broad-acre staple crops. There are no quick fixes in the business of sustainable crop protection. In the longer term, however, the knowledge and experience gained should be invaluable in developing integrated pest management systems that will stand the test of time.

Former Head of Plant Pathology and Microbiology John A. Lucas,
Rothamsted Research
Herts, UK

# Series Preface

This is the third Volume of the Series *Sustainability in Plant and Crop Protection* (SUPP). Its specific focus is on the beneficial hyphomycete *Pochonia chlamydosporia*, treated and examined in relation to its biology and potential for crop protection and sustainable management. The Volume considers the fungus biochemistry, taxonomy, ecology and application, providing actualised data on its genome, gene expression, and metabolism. It has been produced thanks to the endless efforts and enthusiastic endeavours of the Editors, Dr Rosa Manzanilla Lopez and Prof. Luis Vicente Lopez Llorca, and all the contributing Authors.

Management of nematode pests is a challenging task, due to the widespread distribution of species that attack the most important food and industrial crops or livestock, the peculiar ecology of parasitism including the environments in which nematodes live and multiply, and the complexity of the rhizosphere interactions. Although the use of synthetic pesticides against parasitic nematodes is still applied in many agroecosystems, concerns about the environment and human safety issues progressively increased the demand and use of organic products and technologies. Several studies and research efforts thus focused on more sustainable control practices, oriented towards non-chemical management approaches. Biological control and plant-microorganisms interactions are now established research fields within plant protection and nematology disciplines. However, detailed knowledge on the communities of microorganisms and their interactions are fundamental, finalized at switching from pesticide-based to information-based, and more resource-conservative, sustainable approaches.

Studies on soil microorganisms such as *P. chlamydosporia* are, therefore, welcome. This fungus has been the object of intense research work by taxonomists and nematologists since the early 70's, through laboratory and field-based studies, including the pioneering research work developed by Brian Kerry at Rothamsted Research. Brian first identified and reported the potential of *P. chlamydosporia* in cyst nematode management in the cereal crops in Northern Europe, and led a fruitful research activity in this field for many years, until his premature loss.

Many of the researchers involved in this Volume had the priviledge of his friendship and worked with him, following up with many studies with innovative and advanced research approaches, including the exploration of molecular and genomic dimensions. It is hence with interest that the reader may look at his Volume. It provides comprehensive reviews of many aspects of the *P. chlamydosporia* interactions with plants and soil, both as an endophyte and biological control agent, which are presented, described and illustrated in several chapters.

The production of data on the ecology, taxonomy and genetics of biological control agents, antagonists and root endophytes is indeed a challenge for the years to come. This is true because of the large number of species present in soil, in their vast majority still unclassified, and of the many interactions taking place on the root and soil interfaces.

This Volume provides a first comprehensive contribution to key aspects of a single species, within the context of its support to sustainable farming. The information provided is impressive, flanked by a rich bibliography, updating actual knowledge through the Authors' personal experience and work. It is desirable that it could be followed by many other similar treatises, dealing with the functions and services produced by other members of the soil microbial communities. Scientific data will provide us a clearer view on underground complex food webs, on their role in sustaining crops health and, ultimately, food production.

Aurelio Ciancio

# Preface

The idea of preparing a book written by world experts on *Pochonia chlamydosporia* had been maturing for several years due to an increasing interest in *Pochonia chlamydosporia* (Fungi: *Ascomycota*), one of the most promising microorganisms for the biological control of plant-parasitic nematodes such as cyst (*Heterodera* spp., *Globodera* spp.), root-knot (*Meloidogyne* spp.) and false root-knot nematodes (*Nacobbus aberrans sensu lato*). This book is the result of colleagues willing to share their expertise and knowledge with others working on *Pochonia*. We hope that you may enjoy and find the knowledge compiled between the covers of this book useful.

The book contains 18 chapters divided into six sections, starting with a historical background chapter, followed by 16 chapters, each contributed by experts, concerning those key aspects necessary to work with this biocontrol agent in a multidisciplinary treatise. Topics covered include systematics, biology, nematode-fungus interactions, nematode management strategies, secondary metabolites and other methods including more novel research areas such as molecular, -*omics*, plant growth enhancement and endophytic abilities of *P. chlamydosporia*. The final chapter deals with the future perspectives of *P. chlamydosporia* research.

The book is aimed at researchers, professionals, practitioners and graduate or undergraduate students involved in both basic and applied studies, as well as in activities dealing with biocontrol agents of plant-parasitic nematodes, helminths, crop protection and integrated pest management. It concentrates on fundamental aspects and on the evolution of nematophagous fungi.

The focus of the book is based on *P. chlamydosporia* varieties. The authors and editors have tried to put together most of the information available on the fungus up to March 2017. Scientific names of fungi, as they appear cited in the book, have been revised using the *Index Fungorum* (http://www.indexfungorum.org/names/names.asp) to provide their updated, valid names. Readers wishing to know more on the research made on other biological control agents of nematodes are encouraged to consult specialised books on the subject, such as those written by Stirling (1991, 2014).

Finally, we would like to express our sincere gratitude to our families, authors and friends and colleagues, whose enthusiasm, support and hard work have made the completion of this task possible and also for sharing their images with us to illustrate the book.

Harpenden, Herts, UK Rosa H. Manzanilla-López
Alicante, Spain Luis V. Lopez-Llorca
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# Part I
# Introduction and Systematics

# Chapter 1
# Introduction (Historical and Overview)

**Ken Evans, Rosa H. Manzanilla-López, and Luis V. Lopez-Llorca**

**Abstract** Biological control is an alternative to chemical control of plant-parasitic nematodes. This is largely due to public demand for biologically-based and environment-friendly management options for safer pest control. Such demands have had an important impact on biological control research expansion and funding. However, the development of any strain of a biological control agent for nematode control requires many years of research, experimentation, validation and safe-use tests before the biological control agent becomes available to farmers or is further developed by industry as a commercial biopesticide or bionematicide. Biological control potential can be unconstrained when biological control agents are used in combination with compatible integrated pest management tactics, which may include some chemical products and other biological control agent-based products that are currently available on the biopesticide market. This chapter presents part of the history behind some of the initial studies that help to illustrate the scientific work carried out by the many scientists who laid the foundations and helped to develop *Pochonia chlamydosporia* as a viable, sustainable alternative to chemical control in the integrated management of plant-parasitic nematodes.

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## 1.1 Introduction

*Pochonia chlamydosporia* is one of the most studied biological control agents (BCA) of major nematode pests such as cyst nematodes (*Globodera* spp., *Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.). The number of publications on this fungus is increasing continuously, not only as a result of the demand for research on new environmentally friendly strategies to manage plant-parasitic nematodes (PPN), but also due to the availability of new molecular technologies and sophisticated equipment at more accessible costs. Researchers worldwide have taken advantage of such factors to explore and understand this BCA, not only at the macro level but also at the molecular level. This chapter presents part of the history behind the development of this microorganism as a sustainable alternative to chemical management of PPN as one of the available tactics of Integrated Pest Management (IPM). The present account is largely based on UK experience but may also apply to other countries.

The nematology research carried out at Rothamsted Research (RRes) in the UK, including biological control of PPN, during a period that spanned more than six decades (1948 to 2014), has been reviewed (Evans and Manzanilla-López 2017). Part of that history is presented in this chapter. Many references to seminal papers on *P. chlamydosporia* that were produced during this period at Rothamsted and outside the UK can be found within the chapters in this book, covering the basic, pre-molecular beginnings through to the molecular, high tech and '*-omics*' studies currently in vogue.

## 1.2 The Beginnings (Pre-molecular)

From the late 1990s and well into the new century, increasing worldwide pressure was put on growers to reduce their reliance on chemical nematicides. The availability of fewer chemicals registered for such use, strict supermarket protocols and extended harvest intervals (i.e., the minimum time permitted between chemical application and crop harvest) were some of the factors responsible for this pressure. In response, the then department of Entomology and Nematology of Rothamsted became involved in a number of collaborative projects looking into alternative technologies as components of IPM.

In 1950, the Rothamsted Nematology department reported observation of hyperparasites on *Ditylenchus dipsaci*, mentioning the genera *Harposporium* and *Arthrobotrys*. Interest in these relationships was taken up, thus paving the way for future biological control research. The idea of using one species to limit the numbers of a second species that causes economic damage (biological control) has been around for a long time, and it was natural to apply this concept to the control of PPN. Terry Williams reported, in 1972, that formalin treatments in experiments from 1964 and onwards increased the numbers of *Heterodera avenae* females found on cereal roots and thereby caused a large increase in soil populations of the nematodes. A fungus was suspected of infecting the females and the improved nematode reproduction was put down to control of the fungus by formalin. In 1973, Brian Kerry found an *Entomophthora*-like fungus (ELF) on *H. avenae* females at Rothamsted and proposed that it was at least partly responsible for the natural control of this nematode. Despite continuous cropping with cereals, the number of *H. avenae* rarely exceeds 10–20/g soil in the UK. In 1974, Kerry found the ELF and *Verticillium chlamydosporium* (= *Pochonia chlamydosporia*[1]) (71%) and *Cylindrocarpon destructans* (= *Ilyonectria destructans*) (52%) in many of the examined field populations of *H. avenae*. In 1975, Kerry confirmed that the ELF killed *H. avenae* females whilst *V. chlamydosporium* parasitized eggs (Chaps. 3 and 4). All *Heterodera* species tested were infected by the ELF (later on described as *Nematophthora gynophila*) and *V. chlamydosporium* but the potato cyst nematodes (*Globodera* spp.) were only infected by *V. chlamydosporium*. Kerry went on to point out that the cereal monoculture practised after World War II had provided the opportunity for the fungi to reach an equilibrium with *H. avenae* that held the nematode population density below the economic damage threshold. The realisation that this equilibrium existed between the nematode and its parasites and that soils can become suppressive to nematodes was the stimulus for Brian Kerry to go on to develop an understanding and subsequently exploit this relationship.

Finding soil suppressiveness to PPN coincided with the withdrawal of some nematicides from the market due to their toxicity to humans (Kerry 1991). Surveys showed *N. gynophila* to occur in fields in Denmark, Poland, Holland, California and Tennessee (USA), while *V. chlamydosporium* occurred in Denmark, Holland and California (see also Pyrowolakis et al. 2002; Westphal and Becker 1999, 2000, 2001). Morgan-Jones et al. (1981, a, b) encountered *V. chlamydosporium* parasitizing *Meloidogyne arenaria* females and cysts of *Heterodera glycines* (see also Gintis et al. 1982, 1983; Godoy et al. 1982; Morgan-Jones et al. 1983). Some specialization enabled these opportunistic fungi to exploit a unique ecological niche. Their frequency of occurrence among cyst nematode populations indicated a capacity to compete successfully in agricultural soils (Rodríguez-Kábana et al. 1984; Morgan-Jones and Rodriguez-Kabana 1987).

The biological control (BC) approach later followed at Rothamsted would largely correspond to that defined by Stirling in 1991: “A reduction of nematode populations which is accomplished through the action of living organisms other than nematode-resistant host plants, which occurs naturally or through the manipulation of the environment or the introduction of antagonists”.

[1] *Verticillium chlamydosporium* is a synonym of *Pochonia chlamydosporia*, the current valid name of the fungus.

## 1.3 Honing Skills and Harmonizing Protocols

In 1976, the ‘pathogens of nematodes subgroup’, also known as the ‘Working Group on the integrated Control of Soil Pests’, was formed as part of the International Organization for Biological and Integrated Control of Noxious Animals and Plants/West Palaearctic Regional Section (IOBC/WPRS) (Kerry 1991). At that time, some of the research activities were focused on the control of PPN by the application to soil of selected fungal agents, including those that had already been developed commercially. However, the levels of control were variable and too little was known about these fungi to obtain predictable results (Kerry 1991). Biological control research was not only concentrated on targeting PPN but also on the use of nematode-trapping fungi for the control of animal parasitic nematodes (e.g. in Denmark and France), as some fungal isolates had shown considerable potential as control agents of nematodes that infect ruminants (Kerry 1991), a line of research that will extend in future to other latitudes (see Chap. 17).

There was a time (1970s–1980s) when the training of mycologists was moving from morphology and taxonomy as main subjects to include fungal biology. In 1977, the publication of the book *The nematode-destroying fungi* by G.L. Barron showed that nematode-destroying fungi were taxonomically diverse, as shown by morphological adaptations to the nematophagous habit. However, little was known about the morphogenesis of trapping/infection devices and the physiology of these fungi (Barron 1977). The book also included methods and techniques to recover and culture predatory and nematode-parasitic fungi.

Research on biological control of nematodes had also begun to move from being a predominantly observational to a more experimental science (Kerry 1991). Several laboratories from different countries in Europe (e.g., Belgium, England, France, Germany, The Netherlands) were already involved by 1988 in the development of nematophagous fungi or products containing them for the control of nematodes, and in the development of a range of methods for the isolation, selection and testing of nematophagous fungi. These methods were brought together and made available to the public in a special IOBC bulletin (Kerry and Crump 1991). However, at the time it was acknowledged that techniques still needed to be standardised (Kerry 1991). Therefore, fundamental knowledge was required and research was done on several aspects of the biology, ecology, epidemiology, mode of action and suitable methods of study of the fungi. In 1991, much of the information that had become available on biological control was put together by Graham Stirling in a book (Stirling 1991) on biological control of plant-parasitic nematodes, an important source of information that became a bench mark, and which was followed by a second edition in 2014 (Stirling 2014).

The nematophagous fungus *Verticillium chlamydosporium* was considered easier to culture and handle than other fungi isolated from UK fields. Attention became focused on tests with formulations of carrier materials for application to soil and mutants were selected for their ability to tolerate carbendazim (see Chap. 11) so that this fungicide could be incorporated into inoculum granules and thereby allow the chosen isolate to multiply after application to soil, competing fungi being selectively held back. Various media were tested as carriers for such introductions but could not provide the population densities of 1000 propagules per g of soil that *V. chlamydosporium* was found to reach naturally. Another concern was the possibility of loss of infectivity after repeated subculturing, so original isolates were carefully preserved (see Chap. 12). As a result, more than 100 isolates of the fungus were held in culture at RRes for long term storage.

The publication of a set of improved and standardised methods and protocols was achieved in 2002 thanks to the many contributions made by scientists to the *Manual for research on* Verticillium chlamydosporium*, a potential biological control for root-knot nematodes* (Kerry and Bourne 2002). Many of these protocols are still in use worldwide (see Chap. 12).

## 1.4 Understanding the Fungus

Although fungi such as *N. gynophila* had been shown to have a significant impact on the multiplication of *H. avenae*, the fungus biology, environmental requirements and mode of nutrition hindered its use as a BCA (Stirling 1991). Progress was also made on understanding the infection process in cereal cyst nematodes (CCN). Nematode defences to fungal infection such as egg shell thickness (Lopez-Llorca and Robertson 1992a) and cross-linking of nematode proteins were identified (Lopez-Llorca and Fry 1989). A collaboration with Mr. D. Claugher from The Natural History Museum, London, led to the study of *Pochonia* appressoria differentiation. This research discovered adhesive production by the fungus infecting nematode eggs (Lopez-Llorca and Claugher 1990). This was possible using high resolution (Field Emission) Scanning Electron Microscopy. A serine protease (P32) from *Pochonia rubescens* [= *Metapochonia rubescens*], isolated from CCN suppressive soils in Scotland, was purified and characterized (Lopez-Llorca 1990). It was later immunolocalized in appressoria of the fungus infecting nematode eggs (Lopez-Llorca and Robertson 1992b). This provided the first evidence of the involvement of *Pochonia* spp. extracellular enzymes in nematode egg parasitism (see Chap. 4).

*Verticillium chlamydosporium* (= *Pochonia chlamydosporia*) grows saprophytically in soil and will survive when no nematodes are available if it is added to soil together with an energy source. When commercially available preparations of parasitic fungi were stringently tested, they failed to control *Meloidogyne incognita*, one of the most important PPN species in the world. Therefore, one of the principal targets of the fungus studies became the root-knot nematodes *Meloidogyne* spp. and isolates able to control them were selected in RRes from the early 1990s. Key features to improve fungus performance focused on selection of isolates capable of: i) colonizing the rhizosphere of plants; ii) production *in vitro* of chlamydospores; and iii) infecting nematode eggs (Kerry 2000). Chlamydospores produced in large numbers on solid media could be added as aqueous suspensions, thereby rapidly