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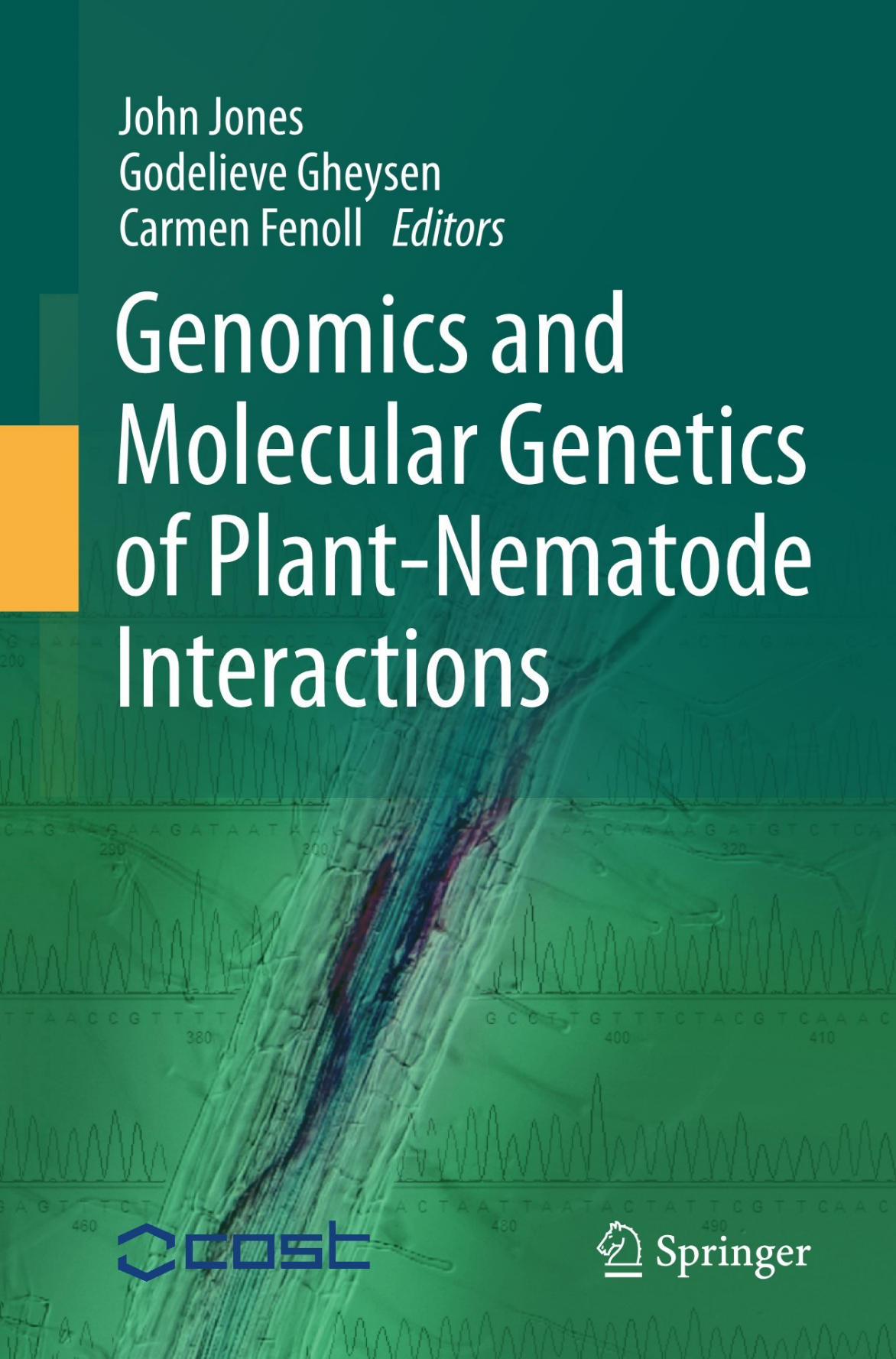
Genomics and Molecular Genetics of Plant-Nematode Interactions



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Editors

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COST Description

COST—the acronym for European Cooperation in Science and Technology—is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds.

The funds provided by COST—less than 1% of the total value of the projects—support the COST cooperation networks (COST Actions) through which, with EUR 30 million per year, more than 30,000 European scientists are involved in research having a total value which exceeds EUR 2 billion per year. This is the financial worth of the European added value which COST achieves.

A "bottom up approach" (the initiative of launching a COST Action comes from the European scientists themselves), "à la carte participation" (only countries interested in the Action participate), "equality of access" (participation is open also to the scientific communities of countries not belonging to the European Union) and "flexible structure" (easy implementation and light management of the research initiatives) are the main characteristics of COST.

As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a "bridge" towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of "Networks of Excellence" in many key scientific domains such as: Biomedicine and Molecular Biosciences; Food and Agriculture; Forests, their Products and Services; Materials, Physical and Nanosciences; Chemistry and Molecular Sciences and Technologies; Earth System Science and Environmental Management; Information and Communication Technologies; Transport and Urban Development; Individuals, Societies, Cultures and Health. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

Web: http://www.cost.eu

Foreword

This book reflects two decades of collaborative research on plant nematode interactions with a core of European teams that were brought together in the early nineties. When asked to write the foreword for this book, I wanted to document the origin of this group as it demonstrates that chance happenings can shape the future.

In the pioneering years of genetic engineering, I was working as a plant physiologist at the first plant biotech company in the Netherlands, Mogen International. In 1988 the Dutch potato processing industry approached us to solve their number one headache; potato cyst nematodes. It was a 4 year program with a team of 3–4 scientists to engineer resistance into their main cultivars. For a small biotech company that had no income except from investors, this was a big contract and we were keen to sign it in 1989.

To put this into historic perspective, these were the days where consumers had no clue yet about genetically modified food and we could routinely express a viral coat protein in tobacco and show systemic virus resistance… but anything else was far from routine or could even be classified as science fiction. We had just barely demonstrated transgenic potato plantlets and the first frail GM plants were cultivated in our high containment space age growth chambers, dazzling every visitor. We thought we were the new masters of the universe, carrying an unbelievable new toolbox that was growing every month with breathtaking inventions like PCR, reporter genes, genome sequencing, DNA synthesizers, etc. So, the fact that no one in the company had ever seen a live nematode before (my main background was in root physiology, but all the others were top-ranking molecular biologists) was considered a minor issue by management and by our contractors. They had faith in the scientists and the new toolbox. In retrospect, the naivety with which we entered this project was laughable, but without dreams there is no progress. It turned out to become the most fascinating period of my scientific career.

Parasitic nematodes are a problem in every country but are notoriously difficult to study. As a research subject they present many obstacles, delaying scientific progress to a pace that is no longer acceptable in the competitive world of grants and careers. On the other hand, the economic damage is significant everywhere and each country had at least one group of specialists to study their local threats and maintain a level of expertise, with relatively secure national funding. Therefore

the European landscape was scattered with small but dedicated research groups, looking for ways to reduce chemical treatments, running breeding programs or just fascinated by the complex interactions between multicellular organisms. With one or two exceptions, molecular techniques were not widely used by these groups.

In the beginning of the project at Mogen, I visited many of these groups to get a better feeling for the state-of-the-art. Mogen in those days was just about 25 scientists, but being an "industry representative", I was met with scepticism and also with curiosity, as we were definitely a new kid-on-the-block. There was no budget for collaborations so I had very little to offer and my hosts kept their cards close to their chests. Seeing the work done by these groups, it gradually began to dawn on me that studying the life cycle of cyst nematodes on potato plants *in vitro* would already be a challenge, let alone interfering with its life cycle. A turning point in those visits was a trip to Kiel in Germany to meet Professor Urs Wyss. His enthusiasm was inspiring to say the least. After a crash course on nematode behaviour with his magnificent videos, I noticed that the Kiel group was able to grow cyst nematodes routinely on rapeseed *in vitro*, and from a root physiologist's point of view, these roots looked excellent, a far cry from the stunted potato roots we were growing at Mogen. The Kiel group offered me a few of their Petri dishes to bring back to Holland to use as a starter culture and to allow me get hands-on experience growing nematodes. No paperwork, no lawyers, no signatures, this was mutual trust only.

For other projects, I was growing *Arabidopsis* plants. At this time *Arabidopsis* was rapidly becoming the gold standard for plant molecular biologists, attracting the best and the brightest in plant science across the globe. One of the few areas where this model was not considered seriously was Phytopathology; *Arabidopsis* appeared to be resistant to most pathogens. Deviating way off from my project (I wouldn't dare tell my industrial partners that I was working on anything other than potato and I didn't dare tell my colleagues I was infecting *Arabidopsis* with a pathogen, which would have been considered a rather stupid venture in those days), I set up several experiments with *Arabidopsis* to see if I could get juveniles harvested from rapeseed cultures to infect the roots. I checked progress outside lab hours or during coffee breaks when I had the lab for myself. On several occasions I noticed behaviour similar to that which I had seen on the videos from Urs Wyss and realized that the worms recognized the presence of roots. Over the next few days I could even see movement within the translucent roots, indicating that the nematodes had managed to penetrate. I did not dare tell anyone yet. I remember vividly the first day I saw syncytia developing, the most prominent syncytia I had ever seen as they were developing in these really tiny roots. It was obvious that the nematodes were changing root growth in a way I had only seen with nitrogen fixing Rhizobia. But more astonishing, this was a pathogen infecting the model-plant *Arabidopsis*! Even before informing my colleagues, I phoned Urs to tell him what I had done. The message didn't really sink in and I took the next plane to Kiel, a bunch of *Arabidopsis* reviews in my bag, preparing a lecture on the model plant during the flight, it was my turn to inspire Urs and his group. The message did get through this time. We could jump on the fast train of *Arabidopsis* research. I left them with seeds and detailed protocols to repeat this in their lab.

A few more groups got involved and they all got the protocols to grow nematodes on *Arabidopsis*. Preparations were made to get European funding through a Concerted Action, bringing together 16 groups from all over Europe. All groups had basic funding already and we only applied for money to increase collaborations. *Arabidopsis* would be the common theme, a worthless weed so there were no issues about valuable crop species, exclusive fields and other potential roadblocks for such a large project. I could convince our industry partners that this was definitely a faster track to reach useful results that, at a later stage, we could transfer to potatoes. So fortunately, they stayed on board and I was allowed to continue. Brussels approved the program in 1992. For such a large group, it was a modest amount of money but just for travelling expenses, it was a staggering figure. With all expenses paid, any scientist from any of those groups could travel to any other group for the next 4 years and we organized large annual meetings where even the most junior members were able to attend. Obviously, collaborations flourished and gathered momentum with hundreds of exchange visits across the continent. The group had reached a critical mass that was unheard of in this field, resulting in excellent scientific publications in high ranking journals, patent applications, newspaper coverage, professorships, and last but not least, it attracted new scientists and students.

There were times where we thought that breakthroughs were close, as we were able to target gene expression directly in the syncytia and could beat the parasite using its own tricks; triggering plant promoters that were now coupled to toxic genes. But nature proved to be far more complex and within the time span of the Concerted Action, nobody came close to showing resistance even though the first field tests were done in 1995. The final annual meeting was staged in Toledo, Spain, and although we did not reach our ambitious milestones, it was clear that research on plant-nematode interactions had made a great leap forward. It was no longer a completely black box. The irony now is that to date, not one *Arabidopsis* ecotype could be identified with natural resistance against nematodes and this line of work still solely relies on crop species.

Even though I moved on to another job at that time, the momentum of this group remained and follow-up EU projects were prepared, submitted and granted throughout the following 15 years. People come and go and move on with their lives, but this book demonstrates that the backbone of our first Concerted Action is still prominently visible. No less then 21 of the 24 chapters include labs or scientists from the original group and the critical mass has been kept together for all these years. This is a vital ingredient for a niche in science that involves so many disciplines and focuses on such a complex biological interaction.

The book reviews progress that is impressive. Whole genome sequences of important plant-parasitic nematodes, application of new molecular tools for *Arabidopsis,* microanalysis of feeding cells, unravelling (suppression of) the host immune reaction, hormone regulation, cell cycle- and cell wall interference, cytoskeleton design, new breeding strategies and a series of field trials with GM-crops are all milestones within their specialized areas. Of course, *Arabidopsis* can not claim all the credit for this progress, but to have a non-commercial common interest was

essential to start the initial collaboration and became the basis for the long term collaborations of which this book is the concrete proof.

It has been a privilege and a pleasure to work with this group of dedicated and enthusiastic scientists.

Peter C. Sijmons
Szienz

Preface

These are extraordinary times to be a biologist. The advent of new DNA and RNA sequencing technologies that allow massive amounts of sequence to be generated at a very low cost means that the opportunities offered by application of genomics tools are now available to researchers working with almost any organism. This is in stark contrast to the situation just a few years ago where almost the only genome sequences that were available were those of a few carefully chosen model organisms. With so much data available, the best way to drive biological discovery forward and ensure that practical developments emerge is to work in teams rather as individuals.

The potential benefits of closer co-operation between researchers seeking to exploit this new genome information were recognised by COST who, in 2006, approved funding for COST Action 872 "Exploiting genomics to understand plant-nematode interactions". The aim of this Action (as lifted from the original proposal) was "to develop a co-ordinated approach to exploitation of genomics information that is appearing for plant parasitic nematodes and host crops".

Plant parasitic nematodes cause economic losses to crops throughout the world. The need for new control strategies for plant nematodes has become more pressing in recent years as many of the most effective nematicides have been withdrawn from use, or scheduled for withdrawal, on environmental grounds. In addition, increased international trade and movement of materials means pressure on quarantine organizations to keep new pests and diseases out of new areas. The difficulties faced by workers in this sector are reflected by the introduction and apparent establishment since 1999 of the pine wilt nematode, *Bursaphelenchus xylophilus* into the EU.

Although they are damaging pests, many plant parasitic nematodes have fascinating interactions with their hosts. Plant nematodes can be ectoparasites, browsing on cells at the root surface, or can be endoparasites that invade the host plant and migrate through host tissues. The most complex interactions are those between the sedentary endoparasites and their hosts, including the most economically important nematodes—the root knot and cyst forming nematodes. These induce feeding structures (giant cells or syncytia) which are kept alive for several weeks in order to supply the nematodes with the nutrients they need to reach maturity. This is a degree of biotrophy that is almost unparalleled by any plant pathogen. In order to induce the formation of the feeding site the nematodes induce huge changes in plant gene

expression including changes in the cell cycle and other fundamentally important developmental processes. Uncovering the mechanisms behind feeding site induction and suppression of host defences offers huge scientific opportunities.

Nematodes, of course, do not have it all their own way. Natural resistance against many nematode species is available and there is much work ongoing aimed at understanding resistance mechanisms and identifying resistance genes. One of the immediate outputs of genomics programmes is a full list of potential targets for new control strategies against nematodes using chemical or GM approaches. Much progress has been made—particularly in the latter area.

The purpose of this book is to showcase the developments in plant-nematode interactions over the last few years and to summarise the impact that genomics has had on our field. We have also tried to include sufficient background information in Part I to make the book accessible to relative newcomers to the field. We hope that this will make it useful to new students and postdocs entering this area for the first time as well as to more established researchers.

We would like to acknowledge the impact that COST funding has had on plant nematology in Europe over the last four years. Funds from COST have allowed researchers to meet each year and forge new partnerships that will tackle important areas in this field. Funding has been made available to early career stage researchers to attend these meetings, undertake exchange visits and attend training events. COST funding has therefore had an impact on the skill development of many young plant nematologists.

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About the Authors

John Jones did a first degree in Zoology at the University of Newcastle upon Tyne and then a PhD in nematode sense organ structure and function at University College of Wales Aberystwyth and Rothamsted Research. After a brief diversion working on moths and zebrafish in the USA, John moved to SCRI in 1993 and has worked on plant-nematode interactions here ever since. Current interests include PCN genomics and suppression of host defences by biotrophic nematodes. John also has a guest professor position at Ghent University. John contributes to the teaching of plant nematology at Ghent, Dundee and St Andrews Universities and is Chair of COST Action 872.

Godelieve Gheysen received an M.Sc. degree in zoology and a PhD degree in plant molecular biology from Ghent University, Belgium. Her PhD research was on plant transformation by *A. tumefaciens*. In 1991 she started her own research group on the molecular analysis of plant-nematode interactions. This research focuses on (1) the plant response in the compatible interaction at the molecular and cytological level (2) characterisation of the parasitism genes of nematodes (3) using this knowledge to engineer nematode resistance in plants. Since 1993, she has been a professor in molecular genetics at Ghent University and teaches several classes including molecular biology, plant biotechnology and plant-nematode relations.

Carmen Fenoll obtained her PhD in Biological Sciences at the Universidad Autónoma de Madrid, working on photosynthetic bacteria. She moved to plant molecular biology as a Fulbright postdoc at the University of California-San Diego and established her group at the Universidad Autónoma de Madrid in 1990. She has been Visiting Scientist at the Salk Institute in La Jolla and Tinker Professor at the University of Wisconsin, Madison. Since 2000, she is Professor of Plant Biology at the Universidad de Castilla-la Mancha (Spain), where she teaches molecular biology, genomics, plant physiology and biotechnology. Her research group works on the regulation of gene expression and cell differentiation using molecular genetics and genomics in several biological systems, one of which is nematode feeding sites.

Part I
Introductory Chapters

Chapter 1
Introduction to Plant-Parasitic Nematodes; Modes of Parasitism

Roland N. Perry and Maurice Moens

1.1 Introduction to Nematodes

Nematodes are astonishing organisms. Despite their deceptively simple morphology and the fact that they are essentially aquatic, requiring at least a film of liquid for active life, they have been successful in colonising an enormous range of environments. Irrespective of their habitat, nematodes have a similar external morphology, with a worm shaped, bilaterally symmetrical, unsegmented body. The phylum Nematoda comprises >25,000 described species and the importance of nematodes should never be underestimated. Species parasitic on plants and animals have a massive deleterious social and economic impact on man. As will be discussed below, one major attribute that contributes to the undoubted success of nematodes is the amazing ability of some of the life cycle stages to survive adverse environmental conditions. Free-living nematodes of the species *Caenorhabditis elegans*, carried as part of the experimental payload on the Columbia spacecraft, even survived when the spacecraft broke up on re-entry in 2003 (Szewczyk and Lamb 2005).

Free-living species, which make up the bulk of the phylum Nematoda, feed primarily on bacteria and fungi, and are found in soil, marine and freshwater habitats; species have been thawed out of Antarctic ice (Cobb 1914) and others have been found in hot water springs in New Zealand (Rahm 1937). The free-living forms in the soil are beneficial as they are involved in nutrient turnover; in addition, they may be of use as indicator species for pollution monitoring (Wilson and Khakouli-Duarte 2009). The parasitic forms have devastating effects on man, his crops and his livestock as well as infecting wild plants and animals. Nematodes infecting plants are also known as ‘eelworms’ and some species infecting animals are colloquially called ‘roundworms’.

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Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been commercialised as environmentally acceptable control agents for several insect pests (Ehlers 2001; Gaugler and Han 2002). Like many free-living nematodes, individuals of these genera feed on bacteria but they have become specialised in using insect larval stages as a basis for culturing their food supply. The infective juvenile nematodes invade the target insects and release symbiotic bacteria in the haemocoel of the host. The bacteria multiply and kill the insect by septicaemia, usually within 48h, and the nematodes feed on the proliferating bacteria, develop and reproduce. The research information on these nematodes is extensive (Gaugler 2002).

Although the majority of nematodes are microscopic in size (less than 1 mm in length and between 15 and 20 μm in diameter; Fig. 1.1), the animal-parasitic species are often considerably larger. The largest nematode is *Placentonema gigantisma*, discovered in the placenta of a sperm whale; the adult nematode can grow to 8 m in length. In humans, nematodes are of great medical importance and it has been estimated that a quarter of the world's population suffer from a nematode infection of some sort. One of the most familiar diseases caused by nematodes is elephantiasis, caused by *Wuchereria bancrofti*. *Ascaris lumbricoides* is a major parasite of the intestine as is *Enterobius vermicularis*, which is probably familiar to many mothers as the 'pin worm' parasite of children. Dogs and cats are infected by several nematodes, among which are the microscopic *Toxocara cati* (in cats) and *T. canis* (in dogs). If the animals are not wormed the eggs voided in the faeces in parks and play areas, for example, can attach to fingers and, if ingested by humans, the juvenile nematode will hatch. The juvenile does not develop further, but not being in the proper host will wander around the body causing serious damage to organs. If the nematodes enter the cerebrospinal fluid and migrate to the brain, the victim can suffer brain damage and blindness. Deaths, usually of small children, have been reported. These are only a few examples of animal-parasitic nematodes; for further reading illustrating these pests and the horrific diseases they cause see Matthews (1998) and Lee (2001).

The subjects of this book, plant-parasitic nematodes, do not have such obvious and unpleasant effects. However, their economic and social impacts are no less severe,

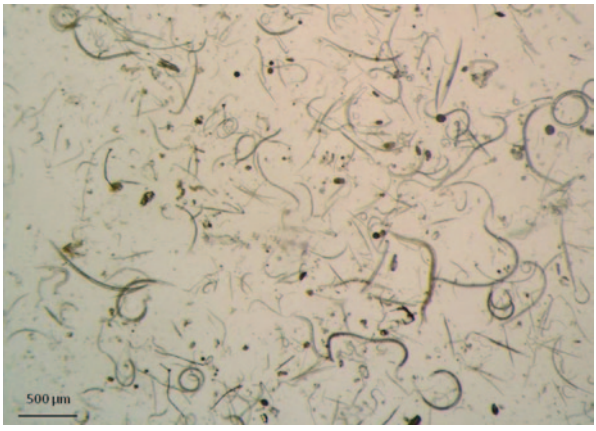

Fig. 1.1 Free-living nematodes and free-living stages of plant-parasitic nematodes obtained from a field soil extraction. (Courtesy Wim ML Wesemael, Institute for Agricultural and Fisheries Research, Belgium)

especially in developing countries where crop loss due to nematodes may be disastrous. All crop plants have one or more species of nematodes that feed on the roots as ectoparasites or invade the roots and feed internally as endoparasites. Some species are migratory endoparasitic, moving in and out of the plants and there are also species that feed on the aerial parts of plants (stems, leaves, buds and seeds). As well as the detrimental effects on the growth of the plants, causing stunting, early senescence and in severe cases total crop loss, the damage caused, especially to root crops such as carrots, can render the produce unmarketable and eliminate income. A major difficulty in controlling the plant-parasitic species is convincing farmers, growers and advisors that the crop problems are actually caused by these microscopic pests. With good reason, plant-parasitic nematodes have been called 'The Invisible Enemy'.

Among the most economically important nematodes are those endoparasitic species that form complex feeding structures in the roots of their host plants. The most damaging are the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes. The root-knot nematodes cause most damage worldwide (Moens et al. 2009). In general, species of *Meloidogyne* have broad host ranges,

Fig. 1.2 Galls of the false root-knot nematode, *Nacobbus bolivianus,* on potato roots. (Courtesy Rosa H Manzanilla-López, Rothamsted Research, UK; from Manzanilla-López 2010)

and are able to infect almost all species of flowering plants. The world-wide spread of root-knot and cyst nematodes and their enormous economic impact has formed the justification for much research; information on these two groups is extensive and this bias is, of necessity, reflected in the following sections. However, there are other groups that have a major agricultural impact, especially species of the genus *Nacobbus* (Manzanilla-López et al. 2002; Manzanilla-López 2010; Fig. 1.2) and the migratory endoparasitic root lesion nematodes of the genus *Pratylenchus* (Castillo and Vovlas 2007).

With the decline in use or banning of many chemicals because of adverse environmental impacts, it is imperative that new strategies for nematode control and management are developed and implemented. In this context, understanding plant-nematode interactions will be vital, and the ability to exploit genomics will not only indicate novel control targets, but also justify re-examination of some older suggestions for control based on interrupting certain phases of the life cycle.

1.2 Evolution of Plant Parasitism

The conserved morphology of nematodes and the absence of extensive fossil records make discussion of the evolution of parasitism in nematodes problematic (Poinar 2011). Several hypotheses about the origins of plant parasitism have been put forward by nematode taxonomists (for example, Maggenti 1971; Poinar 1983; Siddiqi 1983) with little agreement. However, more recent studies using molecular phylogenies based on the small subunit ribosomal RNA (SSU RNA) demonstrate that parasitism of plants by nematodes has arisen independently on at least three separate occasions (reviewed by Baldwin et al. 2004). The rRNA phylogenies also support convergent evolution of sedentary endoparasitism and feeding site establishment by root-knot and cyst nematodes, rather than the theory that the two groups shared a common ancestor.

The co-evolution of plants and plant-parasitic nematodes has resulted in remarkable synchrony of the host and parasite life cycles that enhances the chances of the nematode infection and, thus, survival and reproduction. This integration between host and nematode has progressed furthest in cyst nematodes and the dependency of some species on stimulation from host plants to cause hatch is one aspect of this integration (see Sect. 1.3).

Comparative genomics have been used to provide insights into the evolution of parasitism in the phylum Nematoda, especially the acquisition of novel genes associated with parasitic lifestyles (Rosso et al. 2009). Several genes have been identified in the transcriptomes of plant-parasitic nematodes that are most similar to microbial genes, and these may have been acquired by horizontal gene transfer (HGT) from microbes associated with ancestral nematodes. Jones et al. (2005) argued that acquisition of such genes via HGT has played a critical role in the evolution of plant parasitism. For example, several genes coding for enzymes such as cellulase, pectate lyase and chorismate mutase have been identified in cyst and root-knot nema-

todes with bacteria as the likely origin (Jones et al. 2005). Fungi are the probable origin for the gene coding for GHF45 cellulase, which is vital for the parasitic phase of the life cycle of *Bursaphelenchus xylophilus* (Kikuchi et al. 2004).

The use of RNAi enables loss-of-function phenotypes to be analysed and will provide information on the evolution of nematode parasitism. Rosso et al. (2009) consider that RNAi will facilitate the elucidation of the molecular determinants of parasitism. Information from this approach, together with functional and behavioural data, may provide pointers to new control targets centred on perturbing aspects of the parasitic life cycle such as hatching, host location and survival.

1.3 Hatching

The embryo in each nematode egg develops through embryogenesis to a first-stage juvenile (J1), which, in some longidorids and trichodorids, hatches. However, in most species of plant-parasitic nematodes, the J1 moults within the egg to the second-stage juvenile (J2). It is this invasive J2 that hatches and then feeds on a host plant. There is little variation in the average size of nematode eggs, irrespective of the size of the adult, and the eggshell of plant-parasitic nematodes typically consists of three layers, an outer vitelline layer, a middle chitinous layer and an inner lipid layer. The lipid layer is the main permeability barrier of the eggshell and makes the egg resistant to chemicals, including non-fumigant nematicides. Physiological adaptations, such as different states of dormancy, are an essential component of the survival of nematodes in the absence of a host and are frequently associated with the unhatched juveniles (Perry 1989, 2002). In the majority of species of plant-parasitic nematodes, the juvenile hatches provided environmental conditions, including temperature and moisture content of the soil, are favourable. However, in some species, co-evolution of host and parasite has resulted in a sophisticated relationship whereby the nematode does not hatch unless stimulated by chemicals emanating from the host roots. These emanations have been termed root diffusates, root leachates or root exudates. Root diffusates is the preferred term of the present authors because ‘diffusate’ conveys the idea of volatile and non-volatile components diffusing through the soil and establishing a concentration gradient; thus, it is an especially apposite term in relation to hatching and attraction of nematodes.

The hatching process can be divided into three phases: changes in the eggshell permeability, metabolic activation of the juvenile, and eclosion (or hatch from the egg). The chronological order of the first two phases differs between genera. For example, in *Meloidogyne* spp., activation of the juvenile appears to occur first and causes eggshell changes; in others, such as *Globodera* spp., alteration of eggshell permeability characteristics appears a necessary pre-requisite for activation of the juvenile (Perry 2002). The agents for initiation of these responses vary between species and genera of nematodes but have been studied most extensively in species of root-knot and cyst nematodes. Hatching and survival attributes of these species are associated with the ‘packaging’ of eggs into ecological units (Perry and Moens

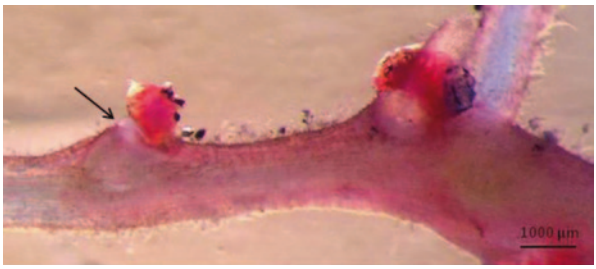

Fig. 1.3 Egg masses of *Meloidogyne chitwoodi* stained with Phloxine B; the posterior end of the adult female (*arrowed*) is visible outside the root. (Courtesy Wim ML Wesemael, Institute for Agricultural and Fisheries Research, Belgium)

2011). Females of root-knot nematodes lay eggs into a gelatinous matrix, which comprises an irregular meshwork of glycoprotein material (Sharon and Spiegel 1993). The gelatinous matrix surrounds the eggs and retains them in a package termed an egg mass (Fig. 1.3). With cyst nematodes, the death of the mature females is followed by polyphenol oxidase tanning of the cuticle resulting in a hard, brown cyst. Egg masses and cysts can each contain several hundred eggs. Egg packaging units similar to cysts and egg masses are not found in animal-parasitic or free-living nematodes.

Hatching of *Meloidogyne* spp. is, in general, temperature dependent and hatching occurs when temperatures are favourable without the need for stimulus from root diffusates. However, there are exceptions and a proportion of the unhatched juveniles of *M. hapla, M. triticoryzae* and *M. chitwoodi,* for example, have been shown to be dependent on root diffusates for hatch, especially in later generations during a host growing season (Gaur et al. 2000; Perry and Wesemael 2008). Although a few other species from other groups (e.g. *Rotylenchulus reniformis* and *Hypsoperine ottersoni*) hatch in response to host root diffusates, this phenomenon is most common among the cyst nematodes but even in this group reliance on host stimulation for hatch varies. *Globodera rostochiensis* and *G. pallida*, have a very restricted host range and are almost completely dependent on host diffusates for hatch, whereas *H. schachtii*, for example, has a very wide host range (some 218 plant species, including many weeds) and hatches well in water (Perry 2002). *Heterodera avenae* has a large hatch in water but a relatively narrow host range; however, the hosts are very common (Turner and Rowe 2006). The dependence of *G. rostochiensis* and *G. pallida* on a plant-derived hatching stimulus is an obvious control target, with the aim of inducing hatch in the absence of a host plant and thus causing the nematodes to die of starvation. However, although much research effort has been expended in elucidating the chemicals, termed hatching factors, in root diffusates, there has been no successful control strategy using analogues of the hatching factors to induce hatch in the field.

Host root diffusates induce a cascade of inter-related changes leading to eclosion, and the sequence of events has been discussed in detail by Jones et al. (1998) and Perry (2002). Unhatched J2 of *Globodera* and *Heterodera* spp. are surrounded by perivitelline fluid, which contains trehalose. Trehalose generates an osmotic pressure that reduces the water content of the J2 and inhibits movement because the turgor pressure is insufficient to antagonise the longitudinal musculature. For

hatching to occur, the pressure needs to be removed. In *G. rostochiensis* and some other species, this is achieved by a change in permeability of the inner lipoprotein membranes of the eggshell via HF binding or displacing internal Ca^{2+} (Clarke et al. 1978). In both *G. rostochiensis* and *G. pallida*, a 5 min exposure to host diffusate is sufficient to stimulate hatch (Perry and Beane 1982), suggesting the involvement of a receptor-ligand interaction between the HF and the eggshell lipoprotein membrane. The change in eggshell permeability enables trehalose to leave the egg, with a concomitant influx of water and subsequent rehydration of the J2 to a water content commensurate with movement. The eggshell of *G. rostochiensis* remains rigid during the hatching process and there is no evidence of enzyme involvement. Devine et al. (1996) demonstrated that the potato steroidal glycoalkaloids, α-solanine and α-chaconine, induce hatch of *G. rostochiensis*; glycoalkaloids are known to destabilise lipid membranes during which leakage of trehalose is possible. However, enzymes have been implicated in softening of the eggshell prior to eclosion in other species, including *Xiphinema diversicaudatum*, *Aphelenchus avenae* and *M. incognita*; in *M. incognita* lipase activity has been positively correlated with hatch (Perry et al. 1992). Rehydration of the J2 of *G. rostochiensis* is accompanied by increased metabolic activity due in part to removal of osmotic pressure and hydration and in part to direct stimulation of the J2 by root diffusate. Changes in gene expression of *G. rostochiensis* J2 appear to occur during or immediately after the hatching process (Jones et al. 1997), but more work is needed on the molecular aspects of the hatching response.

The J2 of *Globodera* spp. uses its stylet to cut a regular series of perforations in the subpolar region of the eggshell, and the J2 hatches through the resulting slit. J2 of *D. dipsaci* use a similar approach, except that the stylet thrusts are more random and the J2 uses its head to force open the slit in the eggshell. In *Pratylenchus penetrans* and *H. avenae*, a single stylet thrust penetrates the eggshell and the head extends this into a tear.

Once hatched, nematodes are vulnerable to environmental extremes and have to locate a host to start feeding. For example, under optimal conditions for movement, J2 of *G. rostochiensis* must locate a host root and set up a feeding site within 6–11 days of hatching otherwise it will exhaust its energy reserves and die (Robinson et al. 1987). Hatching in response to host root diffusates has the advantage of ensuring that the nematodes hatch and leave the protection of the egg and cyst when host roots are close by; thus, synchrony of host availability and nematode hatch is advantageous for nematode survival.

1.4 Attraction to Plants

Around actively growing roots there exist several gradients of volatile and non-volatile compounds, including amino acids, ions, pH, temperature and CO_2. It is evident that nematodes use their chemosensory sensilla, the amphids, to orientate towards the roots using at least some of these gradients. The ability to orientate

towards stimuli from plant roots enhances the chances of host location and reduces the time without food (Perry 1997). Evaluating the reality of the attractiveness or otherwise of an individual compound is difficult. Information is usually based on *in vitro* behavioural studies, often using agar plate movement bioassays, which bear little if any resemblance to the situation in the soil; care must therefore be exercised in extrapolating from such assays to the field situation (Spence et al. 2009). It will be especially important in the future for nematologists to link with plant physiologists to determine the temporal and special attributes of putative attractants in the soil. However, some generalisations can be made and certain compounds are strongly implicated in orientating nematodes to the roots.

Perry (2005) separated gradients into three types: 'long distance attractants' that enable nematodes to move to the root area, 'short distance attractants' that enable the nematode to orientate to individual roots, and 'local attractants' that are used by endoparasitic nematodes to locate the preferred invasion site. There is clear experimental evidence that CO_2 is a long distance attractant (Robinson and Perry 2006). With cyst nematodes, such as *Globodera* spp., it is apparent that the J2 responds to host root diffusate and the evidence is persuasive that diffusate contains chemicals that constitute short distance attractants (Perry 1997; Rolfe et al. 2000). Diffusates from the roots of the host plant, potato, increased the activity of the infective J2 of *G. rostochiensis* and also attracted them to the roots. As detailed in Sect. 1.3 potato root diffusate (PRD) is required to stimulate hatching of the majority of J2 of the potato cyst nematodes *G. rostochiensis* and *G. pallida* but work by Devine and Jones (2002) has shown that the chemicals in PRD responsible for hatching differ from those responsible for attracting the J2 to the root. Electrophysiological analysis of sensory responses (Perry 2001) demonstrated that spike activity of J2 of *G. rostochiensis* increased on exposure to PRD but not to root diffusate from the non-host sugar beet, thus indicating that responses to diffusates may be host specific. Pudasaini et al. (2007) found that the migration of *P. penetrans* towards a host depends on both the initial distance between the nematode and the host and the nature of the host. These authors considered that the attractiveness of the host to *P. penetrans* seems to be correlated with its efficiency as a host; the attractiveness of hosts also declines with age.

The orientation of J2 of cyst and root-knot nematodes to the preferred invasion site, the root tip, is well established but the active factors that constitute the 'local attractants' are unknown. The nematodes may orient to an electrical potential gradient at the elongation zone of the root tip but the relative importance of electrical and chemical attractants for root tip location has not been evaluated; in addition the elevated temperature at the zone of root elongation may influence nematode perception.

Blocking sensory perception so that the nematodes are unable to orientate to roots and thus exhaust their food reserves and die is an attractive control option but may be difficult to achieve. Exposure of J2 of *M. javanica* and *G. rostochiensis* to antibodies to amphidial secretions blocked the response to host root allelochemicals (Stewart et al. 1993; Perry and Maule 2004) but responses were not permanently blocked as, after a period of between 0.5 and 1.5 h, turnover of sensilla secretions