A challenge: biocontrol strategies for the management of potato cyst and root-knot nematodes

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setembro 2012

UNIVERSIDADE DE COIMBRA

Dissertação apresentada à Universidade de Coimbra para a obtenção do grau de Doutor em Biologia, especialidade em Ecologia, realizada sob a orientação científica da Professora Doutora Isabel Maria de Oliveira Abrantes do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra e co-orientação da Doutora Rosane Hazelman Cunha Curtis do *Department of AgroEcology, Rothamsted Research*, Reino Unido.

This research was supported by *Fundação para a Ciência e a Tecnologia* (FCT) and the European Social Fund through the *Programa Operacional Potencial Humano - POPH* of The National Strategic Reference Framework (SFRH/BD/27317/2006)

The experimental work was also partially funded by *Fundo Europeu de Desenvolvimento Regional-FEDER* through the *Programa Operacional Fatores de Competitividade – COMPETE* and by national funds through FCT – under the Project PTDC/AGR-AAM/74574/2006.

To my father

The Old Walking Song

The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say.[1]

J. R. Tolkien

[1] The Lord of the Rings: The Fellowship of the Ring (1st ed. 1954)

To *Fundação para a Ciência e a Tecnologia* (FCT) for sponsoring a PhD grant (SFRH/BD/27317/2006) by ESF funds through the *Programa Operacional Potencial Humano - POPH* of The National Strategic Reference Framework. This research was also partially supported by FEDER funds through the *Programa Operacional Fatores de Competitividade – COMPETE* and by national funds through FCT under the Project PTDC/AGR-AAM/74574/2006.

This work had also the support of many people to whom I would like to express my gratitude.

To Professor João Carlos Marques of the Department of Life Sciences from the Faculty of Sciences and Technology, University of Coimbra, as Coordinator of the IMAR-Marine and Environmental Research Center (IMAR-CMA) and to Professor John Lucas as Director of the Plant Pathology and Microbiology Department, Rothamsted Research, United Kingdom, for providing the facilities to carry the work.

To my supervisor, Professor Isabel Abrantes, for her continued encouragement, guidance and invaluable suggestions not only during my PhD but also through the past 20 years.

To my co-supervisor, Dr. Rosane Curtis, to whom I am undoubtedly grateful for the thoughtful guidance, helpful suggestions and for the affectionate welcome to Rothamsted Research.

To Professor Brian Kerry, who was greatly responsible for initiating this work. I will always cherish the memories of our rewarding and delightful discussions during the planning stages of this Thesis.

To Professor Maria Susana Almeida Santos, who initiated me in Nematology and taught me the key principles of research.

To the many people in Rothamsted Research, from the greenhouse staff to the Administrative Services, who were really helpful and provided me a wonderful work environment. Special thanks go to Ayano Sasaky-Crawley and Katarzyna Dybal for being the best lab mates/supervisors one could wish for. To everybody I met in the Manor House and Southwood Court who made me feel at home, making my stay in England an extremely enjoyable experience.

To Professor Maria José Cunha, for the facilities provided during the pot trials at the Escola Superior Agrária de Coimbra. To my colleagues at the Nematology laboratory, University of Coimbra, for keeping up the challenge. A special acknowledgement is due to Ivânia Esteves for her collaboration, ideas and hard work. Also to Luci Conceição for her friendship and for supplying many of the soil samples.

To Sofia Costa, for her outstanding support, collaboration, valuable experience and friendship.

To Mum, Jó and Big, for their never-ending support and to my little butterflies: Tita, ZeGui, João Eduardo and AnaTê, for making my world a better place!

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Improved crop protection strategies are required to address the need for efficient use of resources and ensure food security. The soil-dwelling plant-parasitic nematodes are a major pest causing crop disease and yield loss. Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, and the root-knot nematode (RKN) *Meloidogyne chitwoodi* are quarantine plant-parasitic nematodes that can cause significant losses in potato production. Current management practices are not economically attractive and often rely on toxic nematicide applications. Therefore, research on sustainable strategies for nematode control is a priority. The exploitation of natural enemies of nematodes such as the nematophagous fungus *Pochonia chlamydosporia* and of natural defence mechanisms in plants could lead to successful management strategies. The main goals of this work were: to acquire knowledge on the key factors that may determine the efficacy of *P. chlamydosporia* as a biological control agent; to assess the use of plant elicitors to strengthen the endogenous defence of plants; and to evaluate a sustainable integrated management strategy that exploits the combined use of a biological control agent with the use of induced plant defence. The key factors that may determine the efficacy of *P. chlamydosporia* as a biological control agent were evaluated in standard bioassays and pot trials. Three Portuguese isolates (Pc1, Pc2 and Pc3) and two non-native isolates (Vc10 and Pc280) were characterised using ERIC-PCR and screened, by *in vitro* assays, for their ability to produce chlamydospores, colonise the rhizosphere of barley and parasitise *G. rostochiensis* and *M. chitwoodi* eggs. The genetic variability of the isolates assessed by ERIC-PCR profiling could not be related with their *in vitro* bioassays performance. The isolate Pc2 has desirable traits to be become a biological control agent, such as high chlamydospore production (1.15×10^7 spores/g) and rhizosphere competence (>90% root fragments), although egg parasitism was moderate (<43%). The non-native isolates, Vc10 and Pc280, were poor chlamydospore producers and rhizosphere colonizers when compared to Pc2, but the Vc10 isolate was a better parasite of PCN eggs (ca. 55%). Fungal response to abiotic factors (temperature or water availability) and enzymatic activity were studied to characterise and compare isolates as these may influence their biological control potential. All isolates responded similarly to temperature or water stress with growth inhibition in extreme conditions. Growth was similar for all isolates at 25 °C and was inhibited at temperatures of 10 and 33 °C. The isolates were more tolerant to osmotic

stress than to matric stress with growth being inhibited at water potentials of -10 and -7.1 MPa, respectively. Spores/hyphal fragments remained viable for almost one month in limiting growth conditions but, when transferred to favourable conditions, the growth, spore production and egg parasitism were affected. An increase in the number of spores produced was observed after the isolates were submitted to matric water stress. Enzymatic activity of the isolates, grown under different nutrient media, was evaluated using the API ZYM® system and was determined to be the highest in Pc2 in low nutrient media. When grown in liquid media, all isolates produced esterases, but protease activity was only observed in Pc2 and Vc10. Enzymatic activity decreased in the presence of nematode eggs for both Pc2 and Vc10, which may be related to a metabolic switch between saprophytic and parasitic activity. These two isolates were selected for further studies to evaluate their survival and proliferation in soil and rhizosphere and to test their efficacy against *M. chitwoodi* using pot trials simulating filed conditions during crop rotations of potato-maize-potato-potato as the host plants. Establishment of *P. chlamydosporia* in soil was slow and was only obtained at the end of the crop cycle and multiple fungal inoculations, which influenced root colonisation and, as a consequence, nematode eggs parasitism. Nutrient availability and temperature had a strong effect on the efficacy of both isolates and the native isolate (Pc2) seemed to perform better, being established in soil successfully by the end of the experiment. The other alternative strategy evaluated was the use of plant defence activators to strengthen the endogenous defence of plants. The effects of benzothiadiazole (BTH), β-aminobutyric acid (BABA), jasmonates (*cis*-jasmone and methyl-jasmonate) and salicylic acid (SA) in the development and reproduction of the root-knot nematode *M. chitwoodi* in tomato plants cv. Tiny Tim, inoculated with 300 second-stage juveniles (J2), were assessed in pot trials in controlled conditions. The effects of BTH and of the jasmonates were further analysed on potato plants cv. Désirée inoculated with 300 J2. Foliar applications of BABA, *cis*-jasmone or methyl-jasmonate effectively reduced nematode penetration in tomato plant roots, but nematode reproduction was only reduced in the BABA treatment. Treatments with *cis*-jasmone and SA had a negative effect on nematode development in tomato plants but did not affect reproduction. In general, the effects of the plant defence activators were stronger in potato plants, which may be due to different dosage application. Nematode reproduction was more markedly affected by BTH, and only BTH or *cis*-jasmone affected

nematode development significantly. However, all activators produced comparable results in reducing nematode penetration. The results suggest that the SA pathway of systemic acquired resistance has a larger effect on nematode development and reproduction in potato and tomato, and should be considered in the design of integrated pest management programmes. The observed effects on the determent of nematode penetration are a highly desirable feature of the application of plant defence activators that can enhance *P. chlamydosporia* performance, as this egg parasite cannot prevent the initial infestation of roots by the mobile nematode juveniles. The efficacy of the combined application of the selected isolate Pc2 and the plant defence activators BTH and *cis*-jasmone against *M. chitwoodi* in potato was assessed in pot trials, in controlled conditions. Nematode penetration was significantly affected by both plant defence activators. However, only BTH treatments (alone or combined with fungus) and *cis*-jasmone combined with the fungus significantly reduced nematode reproduction when compared to the untreated plants. In this assay, where temperature and humidity were maintained without fluctuations, growth and proliferation of Pc2 in soil and rhizosphere colonization were higher compared to that observed in the earlier outdoor pot trial. Foliar sprays of BTH did not seem to affect fungal parasitism but this was significantly increased in the *cis*-jasmone treatment, suggesting that the biocontrol efficacy of Pc2 was enhanced by the application of this plant defence activator. Activation of the jasmonic acid (JA) pathway may produce changes in the plant and/or the rhizosphere that prompt the fungal transition from the saprophytic to the parasitic trophic phase. Egg deposition was lower in BTH and *cis*-jasmone treatments in the presence of the fungus which may be indicative of an additive effect of both strategies (activator and fungus). A sustainable, natural and effective strategy of nematode control may rely on the activation of the JA pathway by plant defence activators that reduce RKN penetration and promote egg parasitism by a selected *P. chlamydosporia* isolate, with biological control potential, which also acts to improve the efficacy of the plant defence activators.

Key words: β-aminobutyric acid (BABA), benzothiadiazole (BTH), biocontrol alternative management strategies, *cis*-jasmone, egg parasite, matric potential, *Meloidogyne chitwoodi*, osmotic potential, plant defence, plant-parasitic nematodes, *Pochonia chlamydosporia*, rhizosphere interactions, temperature, water stress.

O melhoramento de estratégias de proteção de culturas é fundamental para responder à necessidade crescente do uso eficiente dos recursos agrícolas e garantir a segurança alimentar. Os nemátodes fitoparasitas são um fator limitante da produção agrícola mundial, afetando não só a produção como a qualidade dos produtos. Os nemátodes-de-quisto da batateira, *Globodera rostochiensis* e *G. pallida*, e o nemátode-das-galhas-radiculares (NGR), *Meloidogyne chitwoodi*, são organismos de quarentena responsáveis por perdas significativas na produção de batata. As práticas de controlo atuais não são economicamente atraentes e, muitas vezes, incluem aplicações de nematodicidas tóxicos. Assim, a investigação de estratégias sustentáveis para o controlo de nemátodes fitoparasitas é uma prioridade. O estudo de inimigos naturais dos nemátodes, como o fungo nematófago *Pochonia chlamydosporia,* e de mecanismos naturais de defesa das plantas pode levar ao desenvolvimento de novas estratégias de controlo. Os objetivos principais deste trabalho foram: contribuir para o conhecimento dos fatores-chave que podem influenciar a eficácia de *P. chlamydosporia* como agente de controlo biológico, avaliar o uso de indutores da defesa endógena de plantas contra os nemátodes fitoparasitas e desenvolver uma estratégia integrada de gestão sustentável, baseada na utilização conjunta de um agente de controlo biológico e de ativadores dos mecanismos de defesa das plantas. Os principais fatores que podem determinar a eficácia de *P. chlamydosporia* como agente de controlo biológico foram avaliados em bioensaios e ensaios de vaso. Três isolados portugueses (Pc1, Pc2 e Pc3) e dois isolados não-nativos (Vc10 e Pc280) foram caraterizados por ERIC-PCR, tendo sido avaliada, em ensaios *in vitro*, a sua capacidade para produzir clamidósporos, colonizar a rizosfera da cevada e parasitar ovos de *G. rostochiensis* e de *M. chitwoodi*. A variabilidade genética dos isolados detetada nos perfis obtidos por ERIC-PCR não poderá ser relacionada com o seu comportamento nos bioensaios *in vitro*. O isolado Pc2, apesar de o parasitismo de ovos ter sido moderado (<43%), revelou ser um agente de controlo biológico com potencial, uma vez que a sua capacidade de produzir clamidósporos *in vitro* foi elevada (1,15x107 esporos/g de substrato) e colonizou extensivamente a rizosfera (>90% dos fragmentos de raízes). Os isolados não-nativos, Vc10 e Pc280, mostraram ter uma baixa capacidade para produzir clamidósporos e colonizar a rizosfera relativamente ao Pc2, mas o isolado Vc10 foi o melhor parasita de ovos de *G. rostochiensis* (ca. 55%). A reação dos isolados aos fatores abióticos

(temperatura ou stress hídrico), assim como a sua atividade enzimática, foram também avaliadas, uma vez que estes parâmetros podem influenciar o seu potencial como agentes de controlo biológico. A reação dos isolados à temperatura e ao stress hídrico foi análoga, havendo inibição do crescimento em condições extremas. A 25 °C, o crescimento foi semelhante para todos os isolados, tendo sido inibido às temperaturas de 10 e 33 °C. Os isolados foram mais tolerantes ao stress osmótico do que ao stress mátrico com potenciais limitantes para o crescimento de -10 e -7,1 MPa, respetivamente. Os esporos/fragmentos de hifas permaneceram viáveis por aproximadamente um mês em condições limitantes para o crescimento. No entanto, quando transferidos para condições favoráveis, o crescimento, a produção de esporos e o parasitismo de ovos foram afetados. Após os isolados terem sido submetidos a stress mátrico foi observado um aumento na produção do número de esporos. A atividade enzimática dos isolados, cultivados em meios com diferentes níveis de nutrientes, foi avaliada através do sistema API ZYM® e foi mais elevada no isolado Pc2 em meio pobre em nutrientes. Quando cultivados em meio líquido, todos os isolados produziram esterases, mas a atividade das proteases foi observada somente em Pc2 e Vc10. A actividade enzimática diminuiu na presença de ovos de nemátodes, tanto para Pc2 como para Vc10, o que poderá refletir uma diferença metabólica entre a atividade saprófita e a parasítica do fungo. Estes dois isolados foram selecionados para avaliar não só a sua capacidade de sobrevivência e proliferação no solo e na rizosfera mas também a sua eficácia no controlo de *M. chitwoodi* em ensaios de vasos, em condições semelhantes às naturais, utilizando uma rotação de culturas de batateira-milho-batateira-batateira como plantas hospedeiras. A proliferação e crescimento do fungo no solo foi lenta e só foi obtida no final da rotação de culturas e após múltiplas inoculações, o que influenciou a colonização da raiz e, em consequência, o parasitismo dos ovos do nemátode. A disponibilidade de nutrientes e a temperatura tiveram um grande efeito sobre a eficácia de ambos os isolados, tendo o isolado nativo Pc2 revelado um melhor desempenho, estabelecendo-se no solo, com sucesso, antes do final da experiência. A estratégia alternativa avaliada consistiu na utilização de ativadores da defesa das plantas para reforçar a sua defesa endógena. Os efeitos do benzotiadiazole (BTH), ácido β-aminobutírico (BABA), jasmonatos (*cis*-jasmona e metil-jasmonato) e do ácido salicílico (AS) no desenvolvimento e reprodução do NGR *M. chitwoodi* em tomateiro cv. Tiny Tim, inoculados com 300 jovens do segundo estádio (J2),

foram avaliados em ensaios de vaso, em condições controladas. Os efeitos do BTH e dos jasmonatos foram ainda avaliados em batateiras cv. Désirée inoculadas com 300 J2. As aplicações foliares de BABA, *cis*-jasmona ou metil-jasmonato reduziram efetivamente a penetração dos nemátodes nas raízes de plantas de tomateiro, mas a reprodução só foi reduzida nas plantas tratadas com BABA. Os tratamentos com *cis*-jasmona e AS tiveram um efeito negativo no desenvolvimento de *M. chitwoodi* em tomateiro, mas não afetaram a reprodução. Os efeitos dos ativadores da defesa das plantas foram mais acentuados na batateira, o que pode ser devido à aplicação de uma dosagem superior. A reprodução do nemátode foi claramente afetada pelo tratamento com BTH, e só este tratamento ou o tratamento com *cis*-jasmona afetaram significativamente o desenvolvimento do nemátode. No entanto, a penetração do nemátode foi reduzida em todos os tratamentos com os ativadores. Os resultados sugerem que os mecanismos de resistência sistémica adquirida pela via bioquímica AS têm um maior efeito sobre o desenvolvimento e reprodução do nemátode em batateira e tomateiro e devem ser considerados no desenvolvimento de programas de gestão integrada de pragas. Os efeitos observados na inibição da penetração dos nemátodes nas raízes são uma característica vantajosa da aplicação dos ativadores de defesa de plantas que podem melhorar o desempenho de *P. chlamydosporia*, na medida em que este parasita de ovos não pode prevenir a penetração dos J2 (estádio móvel) nas raízes. A eficácia da aplicação conjunta do isolado Pc2 e dos ativadores da defesa das plantas BTH e *cis*-jasmona no controlo de *M. chitwoodi* em batateira foi também avaliada em ensaios de vaso, em condições controladas. A penetração dos nemátodes foi significativamente afetada por ambos os ativadores. No entanto, apenas as aplicações foliares de BTH, isoladamente ou em combinação com o fungo, ou de *cis*-jasmona combinado com o fungo reduziram significativamente a reprodução do nemátode quando comparado com as plantas testemunha. Neste ensaio, onde a temperatura e a humidade foram mantidas sem flutuações, o crescimento e proliferação do isolado Pc2 no solo e a colonização da rizosfera foram maiores em comparação com o observado nos ensaios de vaso anteriores, em condições não controladas. O tratamento com BTH não pareceu afetar o parasitismo do fungo, mas este foi significativamente superior no tratamento com *cis*-jasmona, o que sugere que a eficácia do isolado Pc2, como agente de controlo biológico, foi aumentada com a aplicação deste ativador da defesa da planta. A ativação da via bioquímica do ácido

jasmónico (AJ) poderá causar alterações na planta e/ou na rizosfera que podem promover rapidamente a transição da fase saprófita do fungo para a fase parasítica. A deposição de ovos foi menor nos tratamentos BTH e *cis*-jasmona na presença do fungo, o que pode ser indicativo de um efeito aditivo das duas estratégias (ativador e fungo). Uma estratégia sustentável, natural e eficaz para o controlo de nemátodes pode ser desenvolvida a partir da ativação da via do AJ por ativadores de defesa da planta que reduzam a penetração dos NGR e promovam o parasitismo de ovos por um isolado de *P. chlamydosporia*, com potencial para ser usado como agente de controlo biológico, que, por sua vez, poderá melhorar a eficácia dos ativadores de defesa da planta.

Palavras-chave: ácido β-aminobutírico (BABA), benzotiadiazole (BTH), *cis*-jasmona, estratégias de biocontrolo alternativas, interações na rizosfera, mecanismos de defesa da planta, *Meloidogyne chitwoodi*, nemátodes fitoparasitas, parasita de ovos, *Pochonia chlamydosporia*, potencial mátrico, potencial osmótico, stress hídrico, temperatura.

This Thesis is divided into General introduction, Chapters 1-5 and a General discussion where key results, highlights and future perspectives are presented and synthesized. The key factors that may determine the efficacy of the fungus *Pochonia chlamydosporia* as a biological control agent are studied in Chapters 1, 2 and 3. The use of plant defence activators to strengthen the endogenous defence of plants is assessed in Chapter 4 and the evaluation of the combined use of a biological control agent with induced plant defence activators is presented in Chapter 5.

A detailed outline of Chapters 1-5 follows:

Chapter 1 - Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolated from potato cyst and root-knot nematodes

Three Portuguese isolates (Pc1, Pc2 and Pc3), and 2 non-native isolates (Pc10 and Pc280) were characterised using ERIC-PCR and screened by standard *in vitro* assays. The isolates were also characterised for their tolerance to temperature, a factor that can have a great impact on their establishment in the field and compromise their efficacy in controlling the target nematodes. Information about the enzymatic activity was collected and compared among isolates grown under different nutrient media for isolates Pc2 and Pc10, in the presence or absence of *G. rostochiensis* and *M. chitwoodi* eggs. The variability detected in the fitness among *P. chlamydosporia* isolates to adapt to specific environmental and nutritional conditions highlights the importance of screening when selecting potential biological control agents.

Chapter 2 - *In vitro* water stress bioassays with the nematophagous fungus *Pochonia chlamydosporia*: Effects on growth and parasitism

The impact of water stress on sporulation, germination and parasitism was evaluated by *in vitro* bioassays for the first time in *P. chlamydosporia* isolates. Spores/hyphal fragments of the fungus can remain viable at water potentials that inhibit growth, for prolonged periods of time, and the osmoregulation mechanisms, used to compensate water stress, appear to

affect *in vitro* sporulation pathogenicity. These findings are discussed in light of their biological control potential.

Chapter 3 - The nematophagous fungus, *Pochonia chlamydosporia*, and *Meloidogyne chitwoodi* interactions in a crop rotation scheme

The ability of two *P. chlamydosporia* isolates to survive and proliferate in soil and in the rhizosphere in a crop rotation including potato-maize-potato-potato and fungal efficacy in the control of *M. chitwoodi* was evaluated in a pot experiment. Subtle differences between isolates were detected in host preference, plant compatibility and tolerance to abiotic conditions that seem to have lead to different outcomes in nematode control. The results are discussed in context with previous research and in the wider perspective of integrating biological control with crop rotation to design a sustainable nematode management programme.

Chapter 4 - Plant elicitors as inducers of plant defence against the root-knot nematode, *Meloidogyne chitwoodi*

The effect of the plant activators β-aminobutyric acid, benzothiadiazole (BTH), jasmonates (*cis*-jasmone and methyl-jasmonate) and salicylic acid (SA) on the development and reproduction of *M. chitwoodi* was determined on tomato and the effects of BTH and jasmonates were further investigated in potato. The results suggest that the SA pathway of systemic acquired resistance has a stronger effect on nematode development and reproduction for both plant species. The results were compared with previous research and in the wider perspective of the potential use of plant activators to strengthen plant defence as part of a sustainable nematode management programme.

Chapter 5 - *Pochonia chlamydosporia* combined with plant activators - a potential sustainable strategy for M*eloidogyne chitwoodi* control

The potential of *P. chlamydosporia* as a biological control agent combined with the use of the plant activators, BTH and *cis*-jasmone against *M. chitwoodi* was assessed for the first time. The presence of the fungus did not affect the efficacy of the plant activators. Furthermore, the application of the plant activator *cis*-jasmone may have created the

conditions for a fungal switch from saprophytic to parasitic, thus increasing nematode parasitism. The effects of plant activators on nematode reproduction were also enhanced by the presence of the fungus. Therefore, an integrated pest management strategy based on inducing plant defence mechanisms and use of the nematophagous fungus *P. chlamydosporia* could be a potential alternative strategy for the management of *M. chitwoodi*.

General Introduction

A major global challenge in the 21st century is to ensure food security and to feed the increasing human population mainly in poor-resource areas of the world. Pests and diseases affect crop yield and quality, and decrease resource-use efficiency. The losses may be substantially greater in subsistence agriculture, where crop protection measures are often not applied. Improved crop protection strategies to prevent such damage and loss can increase production and make a substantial contribution to food security. More efficient environmentally-friendly and durable crop protection measures are therefore a priority. The broad range of crop production limitations needs to be considered. This includes the often overlooked nematode constraints. Plant-parasitic nematodes are important soil-dwelling pests that affect a wide range of crop plants and also facilitate fungal and bacterial plant pathogens, acting as a major cause of preventable crop disease and yield loss. Additionally, several plant-parasitic nematodes are major pests of quarantine importance interfering with free trade of several agriculture products (Chitwood, 2003; Lucas, 2010; Nicol et al., 2011).

1. Plant-parasitic nematodes of economic importance to horticultural crops

The most economically damaging plant-parasitic nematodes to horticultural and field crops are the cyst nematodes, *Heterodera* spp. and *Globodera* spp., and root-knot nematodes (RKN), *Meloidogyne* spp., (Trudgill and Blok, 2001). These obligate endoparasites infect the roots and other belowground plant parts such as tubers or bulbs, interfering with plant water and nutrient uptake (Oka et al., 2000).

Cyst nematodes are a major group of plant parasitic nematodes that include two main genera: *Heterodera*, parasitizing a wide range of plants, mainly from the Fabaceae family, but also economically important crops such as cereals, tomato (*Solanum lycopersicum*), sugarbeet (*Beta vulgaris*), rice (*Oryza sativa* and *O. glaberrima*), potato (*S. tuberosum*) and soybean (*Glycine max*); and *Globodera*, the potato cyst nematodes (PCN) (Turner and Rowe, 2006).

Originally, *Heterodera* was considered a major pest of temperate regions, but many species occur in tropical and subtropical areas, with the soybean cyst nematode, *H. glycines*, being the only species listed as a quarantine organism in Europe (OEPP/EPPO, 2009a). Potato cyst nematodes, *G. pallida* and *G. rostochiensis*, are classed as category A2 quarantine organisms by EPPO (European and Mediterranean Organization for Plant Protection) and are

highly specialized and successful plant-parasitic nematode pests in agricultural systems (OEPP/EPPO n.d.; Turner and Rowe, 2006). These two very closely related species co-evolved with potato in South America, and have later been introduced in most of the potato growing areas of the world through seed potatoes. They are considered the most important constraint to potato production in Europe and contribute to the poor potato yields in Portugal (Cunha et al., 2004). Although potato is by far the most important host crop, other important crops of the Solanaceae family such as tomato and aubergine, *S. melongena*, can also be parasitized (OEPP/EPPO n.d.).

Symptoms of infestation on crops are not specific and resemble those of plants with an inefficient root system. Patches of poor crop growth can be detected in the potato fields, with plants being more prone to wilting during periods of water stress and to early senescence. Also, a reduction in tuber yield up to 50% can be observed upon PCN attack. While most of the nematode life-cycle occurs within the root system, females mature into cysts outside the roots and are released into the soil, making them easily transported within soil attached to tubers, plants or seeds. The eggs can hatch immediately and re-infect the same crop or can remain dormant and act as a source of inoculum for future crops (Nicol et al., 2011).

Specific quarantine requirements related to PCN include mandatory soil sampling surveys and regulations concerning transport of seed potatoes, nursery stock and soil to prevent spread and introduction to areas where they are not already established (OEPP/EPPO, n.d.). Also, restrictions are recommended in imports of soil or rooted plants with soil from countries were *H. glycines* has been detected (OEPP/EPPO, 2009a).

Root-knot nematodes, *Meloidogyne* spp., are important highly adapted obligate plant pathogens and are one of the oldest known nematode diseases of plants. These nematodes can parasitize the roots of thousands of plant species, from monocotyledoneous to dicotyledoneous, herbaceous and woody plants (Eisenback and Hirschmann Triantaphyllou, 1991). They interact with their hosts in a remarkable way by establishing permanent polyploid, multinucleate and enlarged giant feeding cells, leading to the formation of root galls that are the first visible symptom of infection. The subsequent damage to the root system decreases plant nutrient and water uptake and root-shoot translocation, resulting in weak plants, low yields and poor quality produce. The above-ground symptoms are similar to

those produced in plants that have a damaged and malfunctioning root system but are related to infestation levels, becoming more severe in high infestations (Karssen and Moens, 2006). The genus *Meloidogyne* contains the most damaging crop pathogens due to their pathogenic effect, their worldwide distribution and large host. Infestation of potato fields by RKN is becoming widespread in the potato-growing areas in Portugal (Conceição et al., 2009). The four most common species *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* are ubiquitous pests with an enormous host range including potato. Other species such as *M. chitwoodi* have been recently added to the list of polyphagous damaging species (Trudgill and Blok, 2001). *Meloidogyne chitwoodi* was first described from the Pacific Northwest of the USA in 1980 (Golden et al. 1980) and it has been a quarantine organism in Europe notorious for its effects on potato tubers since 1998. This RKN has been reported in Argentina, Belgium, Germany, the Netherlands, Mexico, Portugal and South Africa (Conceição et al., 2009; OEPP/EPPO 2009b). With a wide host range, that includes several economically important crops, such as potato and tomato, this species is also tolerant to low soil temperatures (Santo et al, 1980; Santo and O'Bannon, 1982; O'Bannon et al., 1982; Ferris et al., 1993).

2. Limitations of current control strategies

The management of the plant-parasitic nematodes can be done by chemical nematicides, cultural practices and growing resistant cultivars. Cyst nematode control strategies include planting with resistant or tolerant cultivars, long rotations, and fertilization with high doses of organic matter. Chemical (fumigants or granular systemic compounds) and biological methods, such as solarisation or trap crops, have also been used to reduce the populations or damage caused by these nematodes (Nicol et al., 2011). However, the above-mentioned strategies are not economically attractive approaches. The use of resistant or tolerant cultivars may further increase selection pressure for virulent populations or limit the durability of resistance in some cultivars. Although the efficiency of nematicides against PCN has been demonstrated, their use is being banned or reduced due to environmental concerns (Turner and Rowe, 2006).

Root-knot nematodes are more difficult to control than PCN, because of their wide host ranges, short generation times, and high reproductive rates (Trudgill and Blok, 2001).

Effective control measures generally include the use of chemicals, but for some species, including *M. chitwoodi*, the use of nematicides proved to be less effective than for other nematodes. Thus, control measures rely on the multiple applications of highly toxic nematicidal treatments (Pinkerton et al., 1986; Santo and Wilson, 1990). The withdrawal of many nematicides and soil fumigants further limits control options, and urges the need for alternative control strategies (Chitwood, 2003).

3. Alternative control strategies

The development of new environmentally friendly control approaches, that can be included in integrated pest management programmes, has become an important research subject (Oka et al., 2000). There are several approaches to reduce dependence on crop protection chemicals: the use of organic and inorganic amendments or of naturally occurring nematicides; plant pest- and disease-resistant crop genotypes; exploitation of the natural mechanisms that restrict pest and pathogen populations in ecosystems (biological control); and the exploitation of natural plant defences (Oka et al., 2000; Lucas, 2010). The use of organic and inorganic amendments of naturally occurring nematicides has been effective against nematodes and can lead to increased crop yield. Nevertheless, amendments are not as efficient or long lasting as chemical nematicides, they only produce effects when applied in large quantities and some inconsistent results have been observed (Muller and Gooch, 1982; Oka, 2010; McSorley, 2011). Resistant crop genotypes, obtained by traditional breeding strategies for diversification of host resistance such as crop cultivar mixtures, or by transgenic approaches, have found limited commercial application. Product quality and standardization are strong market drivers and, although genetic modification is a potentially powerful tool, it is also the subject of intense public and political debates (Lucas, 2010).

Biological control of plant-parasitic nematodes can be achieved either by conservation or enhancement of the activity of indigenous antagonists, by introduction of antagonistic organisms or a combination of both strategies (Timper, 2011). The use of biological control agents against nematodes is more inconsistent, less effective and slower acting than nematicides. As these limitations are inherent to most biological control agents, their successful application depends on integration with other control measures. So far, the knowledge of how interactions between a nematode pest and a given antagonist, and also

how the biotic and abiotic factors can influence these interactions, are unknown. Better understanding of these interactions can lead to further improvement of the use of microorganisms as biological control agents (Kerry, 2000).

The enhancement of plant natural defence mechanisms can be accomplished by application of plant defence activators and, when these are integrated into good agricultural practices, both productivity and resistance to disease can be enhanced. On the other hand, the performance of plant activators is often variable: they may not consistently provide the same level of disease control as nematicides and can have side effects on crop growth and development. Nonetheless, there are a number of attractive aspects of using natural plant defence systems for nematode management, with the broad spectrum nature of the induced resistance being the most appealing feature and providing additional options for their use in integrated disease and pest control programmes (Kuć, 2001).

Studies on naturally occurring enemies of nematodes and natural plant defence mechanisms will unquestionably contribute to the development of successful, efficient and non-chemical alternatives for plant-parasitic nematode management.

3.1 Potential biological control agents of plant-parasitic nematodes

Plant-parasitic nematodes have many natural enemies including bacteria, fungi, protozoa, predatory nematodes, insects and mites. The efficient exploitation of these enemies as biological control agents against nematodes depends on the knowledge of their biology as well as on the interactions between them (Kerry, 1987). Natural control has been detected in several soils where the build-up of nematophagous fungi and bacteria, under some perennial crops and under those grown in monocultures, lead to the control of cyst and root-knot nematodes (reviewed in Stirling, 2011). The decline of populations of the cereal-cyst nematode, *H. avenae,* under monocultures of susceptible cereals in many soils throughout northern Europe is one of most studied examples of natural control of plant-parasitic nematodes by fungi (Kerry, 1982). Research on the manipulation of natural control has been restricted, due to difficulties in mass production of biological control agents and manipulation of soil activity, and has mainly been concentrated on the introduction of specific agents to provide more rapid control that might be commercially exploitable (Kerry and Hominick, 2002). The majority of the natural enemies found to parasitize nematode

hosts in suppressive soils, i.e. soils that contain microbial communities able to prevent the increase of nematode populations on susceptible crops, are bacteria and nematophagous fungi (Viaene et al., 2006).

Antagonistic bacteria exhibit diverse modes of action including, production of toxins, antibiotics or enzymes and parasitism. Their action can also be due to nutrient competition, plant health promoting or induction of systemic resistance of plants that do not involve direct parasitism to nematodes (Tian et al., 2007). Several bacteria such as *Burkholderia* spp, *Pseudomonas* spp, *Bacillus* spp. and *Agrobacterium radiobacter* have been found to reduce nematode invasion of roots and interfere with nematode hatching and mobility (Viaene et al., 2006), but the nematode parasitic bacteria *Pasteuria* spp. have been considered as having the greatest potential among bacteria to be exploited as biological control agents. The endospore-forming *P. penetrans and P. nishizawe* are obligate parasites of *Meloidogyne* spp and cyst nematodes, respectively. Though host-specific, all produce adhesive spores that attach to the mobile stages of the nematodes. The infected nematodes, if not heavily burdened with spores, are still able to penetrate the plant roots and develop, but reproduction is compromised. Most *Pasteuria* spp. isolates are highly virulent and the infective spores are highly resistant to drying and to physical and chemical aggressions (Chen and Dickson, 1998). However, the high specificity of isolates and lack of methods for large scale production are important constraints for the development of efficient management control strategies using *Pasteuria* spp. (Davies, 2009).

Fungal antagonists are among the most promising nematode biological control agents (Stirling, 2011). The key features that should be taken into account when selecting potential biological control agents are: growth and proliferation in soil and/or rhizosphere; ability to parasitise nematodes, whether mobile or sedentary stages; host- or non-host specificity; and mass production (Kerry, 1987).

Nematophagous fungi can be obligate parasites that need nematodes to survive, other are either facultative or opportunistic parasites and can survive saprophytically, or have characteristics that are in-between those categories. According to their mode of action, they can be divided into three main groups: endoparasitic fungi, nematode-trapping fungi, and parasites of sedentary nematode eggs and females (Viaene et al., 2006).

Endoparasitic fungi penetrate nematodes after germination of their spores, which can be ingested or attached to the cuticle. They are mainly obligate parasites and generally have a broad nematode host range: *Hirsutella rhossiliensis,* for example, was found to reduce plant invasion by two *Meloidogyne* species, by the cyst nematode *H. glycines* and by the ring nematode *Criconemella xenoplax*. Some endoparasitic fungi form flagellated zoospores that attach to the nematode host cuticle, such as the opportunistic fungi *Catenaria anguillulae* that attack active nematode stages, while others, like *Nematophthora gynophila* and *C. auxiliaries*, are obligate parasites that infect young female cyst nematodes. However, all of them are unsuitable for use as biological control agents due to difficulties in *in vitro* production and in soil establishment (Viaene et al., 2006).

Nematode trapping fungi are characterised by the formation of specialised organs of capture, consisting of constricting and non-constricting rings and adhesive structures such as rings, knobs, branches or networks that immobilize nematodes (Siddiqui and Mahmood, 1996). The ability to capture nematodes varies, since those that produce adhesive network traps may be good saprophytes and grow rapidly *in vitro*, but are less efficient when compared with those that produce adhesive knobs, branches or constricting rings, although these have slow growth and lower saprophytic ability. The fungus *Arthrobotrys oligospora* is probably the most studied trapping fungus and produces an hyphal network to trap soil-dwelling nematodes. It is easily produced *in vitro* and has a wide host range, but the trap formation is affected by numerous factors. However, limited trapping activity and non-host specificity, together with difficulties in the establishment of active mycelium in soil hamper its development as a biological control agent (Kerry, 1987).

The characteristics of facultative fungal parasites of sedentary nematode eggs and females give them the highest potential as biological control agents. They can be rapidly grown *in vitro* and their survival in soil is not dependent on the presence of the nematodes. Among the fungal parasites, *Paecilomyces lilacinus* and *Pochonia chlamydosporia* are perhaps the most studied. *Paecilomyces lilacinus* is abundant and active in subtropical and tropical regions (Stirling, 1991), it has been associated to plant-parasitic nematode suppressive soils and was effective in reducing nematode damage to a range of crops in pots and field trials (reviewed in Siddiqui and Mahmood, 1996). The isolate 251 from the Philippines was registered as a biological control agent against plant-parasitic nematodes in

Australia by the Australian Technologic Innovation Corporation Pty Ltd and Bioact Corporation Pty Ltd. Currently, is being commercialised as BioAct®WP (Prophyta Biologischer Pflanzenschutz GmbH, Germany) (Holland et al., 2003). This biological control agent is promising because some isolates are rhizosphere competent and are not host-specific, attacking the eggs of several nematode species. Although, this fungus has given variable control in a range of conditions, it requires high soil temperatures as well as large numbers of propagules for nematode control, and some isolates may be pathogenic to immuno-compromised humans (Kerry, 1987).

Pochonia chlamydosporia, an ubiquitous facultative egg parasite of cyst and root-knot nematodes, has been successfully developed under the name KlamiC® as a biological control agent against RKN in Cuba (Kerry, 1987; Hérnandez and Hidalgo-Diaz, 2008). The potential of *P. chlamydosporia* isolates to be developed as effective biological control agents is a subject of study in this Thesis (Chapters 1, 2 and 3). Thus, the saprophytic behaviour and permanence in soil, in the absence of plant and nematode hosts, its worldwide distribution, natural occurrence in nematode suppressive soils and effectiveness in nematode control are considered in depth in this chapter.

3.1.1 *Pochonia chlamydosporia*: a potential biological control agent

Pochonia chlamydosporia has been associated with soils that suppress the multiplication of the cereal cyst nematode *H. avenae* (Kerry and Crump, 1977; Kerry et al., 1982) and it has been reported in many countries in almost all continents as an important antagonist in the regulation of *Heterodera* spp. and RKN (Kerry and Hirsch, 2011). The fungus produces two types of spores: conidia and chlamydospores. In most isolates, conidia production can be induced in large amounts without effort and at a low cost, but chlamydospores are slow to be produced *in vitro*, as they cannot be produced in liquid culture, and large amounts need to be added to soil to promote control (Kerry and Hirsch, 2011; Mo et al., 2005). Chlamydospores are resistant structures, able to withstand harsh conditions in soil for long periods without an additional food source and, when in favourable environmental conditions are able to initiate fungal growth. Thus, besides the difficulties mentioned above, they are still considered the preferred source of inoculum (Kerry, 2001).

Although there seems to be limited growth of *P. chlamydosporia* in soil, the fungus is rhizosphere competent and appears to proliferate in this niche by using nutrients released in root exudates (De Leij et al., 1993; Kerry et al., 1993; Bourne and Kerry, 2000). The fungus is mostly confined to the rhizosphere and limited endophytic growth (within the roots) has been detected (De Leij and Kerry, 1991; Lopez-Llorca et al., 2002). When the colonizing *P. chlamydosporia* contacts a nematode egg mass in the rhizosphere, the fungus ceases its saprophytic stage and switches to a parasitic stage. Appressoria formed in response to the contact with the surface of a nematode egg enable both the attachment to and penetration of the nematode egg, which involves mechanical and enzymatic action. The secreted enzymes destroy the outer vitteline membrane of the egg cuticle exposing the chitin layer that is then penetrated by an infection peg (Lopez-Llorca et al, 2002, Mauchline et al., 2004). Though nematode host preference at the infra-specific level may be involved in the switch between trophic phases, nutrient availability either released by plants or available in nematode eggs may play an important role (Kerry, 2000; Mauchline *et al*., 2004; Esteves et al., 2009). Readily metabolised carbon sources and pH could influence the trophic phase switch as they interfere with the production of an alkaline serine protease (VCP1), known to be involved in the early stages of nematode egg infection (Segers et al., 1994, Ward et al., 2012).

The efficacy of *P. chlamydosporia* isolates as biological control agents against RKN and PCN has been demonstrated in pots, microplots and field experiments (De Leij and Kerry, 1991; De Leij et al., 1993; Bourne and Kerry, 1999; Ciancio et al., 2002; Atkins et al., 2003; Sorribas et al., 2003; Tobin et al., 2008; Tzortzakakis, 2007; Puertas and Hidalgo-Díaz, 2009; Yang et al., 2012). Nevertheless, there are also some studies reporting the reduced efficacy of this fungus in controlling nematode populations (Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003; Tzortzakakis, 2000, 2009). Abiotic factors, such as temperature and water availability, have great influence in the survival and proliferation of the fungus in soil (Bourne and Kerry, 2000; Magan, 2001) but the impact on nematode reproduction is more affected by the fungal ability to colonize the rhizosphere, than the ability to proliferate in soil (De Leij and Kerry, 1991; Bourne et al., 1994, 1996; Kerry and Bourne, 1996). Isolates of *P. chlamydosporia* differ markedly in their rhizosphere competence and virulence and the outcome of these variations in the regulation of nematode populations is difficult to predict,

increasing the need for carefully screening and selection of isolates (Abrantes et al., 2002). Plant susceptibility to nematode attack is another key factor that influences the performance of *P. chlamydosporia* isolates. In very susceptible hosts or large nematode population densities, gall size tends to be larger and egg masses remain embedded in such galls thus protected from fungal attack (Bourne et al., 1996).

Various aspects of the toxicity and ecotoxicity of a *P. chlamydosporia* var. *catenulata* isolate have been studied including vertebrate tests with rats and rabbits and quails (Garcia et al, 2004a, 2004b, 2009), non-target invertebrates (Garcia et al., 2008b) and beneficial plants (Garcia et al., 2008a). No toxic, irritant, pathogenic or infective effects were detected. Significantly, harmful effects on human health or environmental risk of contamination by *P. chlamydosporia* have never been reported. In addition, the fungus seems to be compatible with mycorrhizae, *Rhizobium* sp. and other fungi (Puertas et al., 2006).

As an egg parasite, *P. chlamydosporia* is not able to prevent the initial infestation of roots by nematode juveniles. Therefore, its biocontrol efficacy should be enhanced by integration with control measures that prevent initial infestations, such as crop rotation with poor hosts for the nematode or the application of plant defence activators (Kerry and Bourne, 1996; Vieira dos Santos et al., submitted).

3.2 Plant defence activators

The induced systemic resistance (ISR) of plants against pathogens has been widely studied with respect to the basic signalling pathways and to its potential use in plant protection. The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene are major players in the network of defence signalling pathways (Pieterse et al., 2001). Plants are able to differentially activate inducible broad-spectrum defence mechanisms, depending on the type of attacker encountered. This response to local attack by a given pathogen involves production of compounds that reduce or inhibit either its performance or further attack. Responses include an oxidative burst, which can lead to cell death, changes in cell wall composition that can inhibit penetration by the pathogen and *de novo* synthesis of antimicrobial compounds such as phytoalexins and pathogenesis related (PR) proteins. Caused either by or following these local responses, a signal spreads through the plant, inducing subtle changes in gene expression in uninfected plant parts and leading to a

systemic response with the possible involvement of PR proteins and phytoalexins. The functional role of both groups of compounds is still unknown, but two groups of PR proteins have been detected. Acidic PR proteins are predominantly located in the intercellular spaces, whereas basic PR proteins are mainly located intracellularly, the latter being functionally similar to the former but having different molecular weights and amino acid sequences (Heil and Bostock, 2002). Some of the compounds associated with ISR are expressed in uninfected tissue in response to the initial attack, whereas other biochemical changes of ISR-expressing plants become obvious only in plant parts where effective resistance is required and only in response to a further attack. This physiological condition associated with an enhanced ability is also known as priming (Conrath et al., 2001). The molecular mechanisms underlying priming are not fully understood, although chemical ISR activators such as, for example, β-aminobutyric acid (BABA), can elicit priming effects (Jakab et al., 2001).

The discovery of induced resistance pathways in plants opened the possibility of chemically activating one or more of these pathways and the development of protective chemicals mimicking the mode of action of natural elicitors to provide useful tools for the development of new strategies for crop protection (Pieterse et al., 2001). The identification of SA as an essential endogenous signal in ISR led to the synthesis of chemical mimics such as benzothiodiazole (BTH) (Gorlach et al., 1996). This plant activator is commercialized in Europe (Bion®) and in the USA (Actigard® and Boost®). The non-protein amino acid BABA is an effective plant defence inducer not only against nematodes but also against a large number of plant-parasites that revealed post-infectional activity. Unlike other plant activators, BABA has not been formulated as a commercial product due to the lack of toxicological data and biodegradation studies (Cohen 2002). Jasmonic acid and its methyl ester are usually mainly implicated in plant defence responses triggered by wounding and insect feeding. However, involvement in resistance against plant pathogens has also been confirmed (Gozzo, 2003).

Although chemically-induced resistance to plant-parasitic nematodes has thus far not been studied as widely as that of other pathogens, foliar sprays of BABA, BTH, JA and derivatives and SA were effective in reducing invasion, development and reproduction of economically important plant-parasitic nematodes and these studies are further detailed in Chapters 4 and 5. However, the performance of plant defence activators is often variable,

and may not provide the same level of disease control as conventional nematicides. Moreover, time of application is an issue, as they need to be applied ahead of nematode attack (Lucas, 2010). Defence activators act through the physiology of the plant and may, therefore, have side effects on crop growth and development (Heil and Baldwin, 2002). Nonetheless, there are several attractive features of using natural plant defence mechanisms for nematode management. Plant defence activators may require fewer inputs than current management based on chemicals. In addition, the broad-spectrum nature of the induced resistance provides additional options for their use in integrated disease and pest control programmes (Lucas, 2010).

4. Aims of this study

To develop potential non-chemical, sustainable and environmentally friendly management strategies of reducing plant-parasitic nematode population densities exploiting the use of: biological enemies of nematodes such as *P. chlamydosporia* and activators of natural plant defence mechanisms.

The main objectives of this Thesis were: i) to determine the key factors that may influence the efficacy of the fungus *P. chlamydosporia* as a biological control agent; ii) to assess the use of plant elicitors to strengthen the endogenous defence of plants; and iii) to evaluate a sustainable integrated management strategy that exploits the combined use of a biological control agent with the use of activators plant defence.

The specific objectives were:

- To characterise biologically and molecularly *P*. *chlamydosporia* isolates associated with the PCN *Globodera rostochiensis*. To evaluate the ability of three *P*. *chlamydosporia* isolates, collected in different regions of Portugal, and two non-native isolates, to grow, sporulate and parasitize nematode eggs *in vitro* under different water stress regimes;

- To assess the performance of selected *P. chlamydosporia* isolates on the multiplication of *M. chitwoodi* and its survival in soil in a crop rotation scheme;

- To analyse the effects of exogenous activators on host plant defence against *M. chitwoodi* in tomato and potato;

- To evaluate the effect of a Portuguese isolate of *P. chlamydosporia* associated with the application of selected plant activators on *M. chitwoodi* infection of potato.

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Chapter 1

Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolated from potato cyst and root-knot nematodes

Written as a journal article:
Vieira dos Santos, M.C., Esteves, I., Kerry, B. and Abrantes, I. (2012). Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolated from potato cyst and root-knot nematodes. Nematology (*accepted*)

Abstract - *Pochonia chlamydosporia*, a widespread fungal parasite of potato cyst (PCN), *Globodera* spp., and root-knot nematodes (RKN), *Meloidogyne* spp., has been studied as a biological control agent. Three Portuguese isolates (Pc1, Pc2, Pc3), obtained from PCN eggs, and 2 non-native isolates (Vc10, Pc280) were characterised using ERIC-PCR and screened, by *in vitro* assays, for their ability to produce chlamydospores, parasitise *G. rostochiensis* and *M. chitwoodi* eggs and colonise the rhizosphere of barley. The effects of temperature on growth, sporulation, parasitism and enzymatic activity were also evaluated. Isolates Pc1 and Pc3, despite their different geographical origins, had identical molecular profiles. Pc2 produced the higher numbers of chlamydospores in solid medium ($1.15x10^7$ chlamydospores/g) whereas Pc3 produced the least ($3x10^5$ chlamydospores/g). These isolates colonised the rhizosphere of barley extensively (>90% root fragments) and the proportion of parasitized eggs, detected on agar plates, was low (<60% for RKN and <55% for PCN), Pc1 being the best parasite against both nematode species. The influence of temperature was similar for all isolates: no growth was observed at 10, 33 and 35 °C. Spores/hyphal fragments remained viable for nearly one month at 10 and 33 °C and isolates resumed growth after incubation at 25 °C, although chlamydospores and conidia production, viability and nematode egg parasitism were affected. Exposure to 35 °C was lethal for isolates Pc1, Pc2 and Pc280. When grown in liquid media, all isolates produced esterases, but protease activity was only observed in Pc2 and Vc10. The highest enzymatic activity was detected in isolate Pc2 in low nutrient media. Enzymatic activity decreased in the presence of nematode eggs for both Pc2 and Vc10. Molecular, biochemical and biological analyses, including biotic and abiotic factors, are determinant in the screening of potential biological control agents, particularly in the case of *P. chlamydosporia*, due to the high variability among isolates.

Key words: biological control, egg parasite, enzyme production, plant-parasitic nematodes, temperature.

1.1. Introduction

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are one of the most specialized and successful plant-parasitic nematode pests in agricultural systems. Depending on biotic and abiotic conditions, these nematodes can reduce yield by 50% (Nicol et al., 2011). Along with yield reduction, the costs of control measures and reduction of marketable products turn these nematodes into the most important constraint to potato production in Europe and contribute to the poor potato yields in Portugal (Cunha et al., 2004). Root-knot nematodes (RKN), *Meloidogyne* spp., are important soil-dwelling pests that affect a wide range of crop plants (Trudgill and Blok, 2001). Although they are not yet recognised as serious nematode pests in the potato-growing areas in Portugal, it is increasingly acknowledged that infestation of potato fields by RKN is becoming widespread (Conceição et al., 2009). Management practices usually include crop rotation, which does not allow intensive production, and nematicide application, that not only is expensive but also involves the handling and use of highly toxic materials. Furthermore, their use is being banned or significantly reduced over increasing environmental and health concerns. Biological control is a non-chemical alternative, as well as a sustainable strategy, and fungal antagonists are among the most promising nematode biological control agents (Stirling, 1991). A thorough understanding of the interactions between nematode pests and their parasites, and of the influence of biotic and abiotic factors on these interactions, is essential in the development of biological control agents (Kerry, 2000).

Pochonia chlamydosporia (Goddard) Gams and Zare is a facultative nematophagous fungus with parasitic activity against eggs and females of PCN and RKN. The biocontrol potential of *P. chlamydosporia* has been widely studied and its efficacy increases in combination with other traditional control measures (Kerry and Hirsch, 2011). *Pochonia chlamydosporia* isolates differ in their ability to colonise the root surface, virulence and chlamydospore production, these being considered desirable features for its use as a biological control agent (Abrantes et al., 2002). The ability of a given isolate to adapt to specific environmental and nutrient sources may be related with genetic variation (Rosso et al., 2011). Enterobacterial repetitive intergenus consensus sequence (ERIC)-PCR profiling showed that isolates can be grouped according to both their geographical and host nematode origin (Morton et al., 2003). The importance of this intraspecific variation in the

regulation of host populations is unknown, but it suggests that potential biocontrol agents need careful selection. *In vitro* bioassays are normally rapid high-throughput analyses that allow many isolates to be eliminated before more time and resource consuming screens are conducted in pot and field experiments (Abrantes et al., 2002). Studies on a facultative fungal egg parasite, such as *P. chlamydosporia*, rely on the use of *in vitro* bioassays to evaluate the two main trophic stages of the fungus. Saprophytic growth is generally measured by studying the ability to colonise the rhizosphere of a host plant, whereas parasitic growth is studied by measuring the ability to parasitize nematode eggs. *In vitro* bioassays have also been developed to screen and compare differences in saprophytic and parasitic growth among fungal isolates (Abrantes et al., 2002). *Pochonia chlamydosporia* produces two types of spores: conidia and chlamydospores. In most isolates, conidia production can be induced in large amounts without effort and at a low cost. Chlamydospores are slow to be produced *in vitro*, but are resistant structures able to withstand harsh conditions in soil for long periods of time, without an additional food source, and hold the capacity to initiate fungal growth when in favourable conditions. Although large amounts need to be added to soil to promote control, they are widely seen as the preferred source of inoculum (Kerry and Hirsch, 2011).

When released in the environment, a biocontrol agent is exposed to fluctuating abiotic factors, which can have a great impact on the establishment of the organism in the field and compromise its efficacy in controlling the target pest. The optimum temperature for *P. chlamydosporia* growth is between 25 and 30 °C. However, different isolates can differ in their optima temperature (Bourne and Kerry, 2000; Olivares-Bernabeu and Lopez-Llorca, 2002). Spore production is also influenced by temperature (Nagesh et al., 2007). Tolerance to environmental-stress conditions, such as high or low temperatures, can determine the success of an ecologically competent biological control agent in the field (Magan, 2001).

Enzymes play an important role in egg infection by *P. chlamydosporia*. Specific proteases and chitinases have been isolated from the fungus and have shown activity against the nematode eggshell (Segers et al., 1994; Tikhonov et al., 2002). Areas of low electron density were found around appressoria, suggesting that enzymes are secreted when appressoria are formed and, therefore, are important in the infection process (Morgan-Jones et al., 1983; Lopez-Llorca and Robertson, 1992). Isolates vary in the production of extracellular enzymes

but proteolytic activity is usually high *in vitro* (Esteves et al., 2009; Olivares-Bernabeu and Lopez-Llorca, 2002). The implications for infection and virulence of different saprophytic/parasitic performances, as well as enzymatic differences between the two stages, are still incompletely understood.

The main objective of this study was to characterise and compare three *P. chlamydosporia* isolates, collected from PCN eggs in different regions of Portugal, and two non-native reference isolates, using standard *in vitro* bioassays to assess their ability to produce chlamydospores, colonise the rhizosphere and parasitise eggs. The effects of temperature on *in vitro* growth, sporulation and parasitism and enzyme activity were also evaluated, as these factors may determine the efficacy of an isolate as a biological control agent.

1.2. Material and Methods

1.2.1. Isolation, identification and molecular characterisation

Eggs from 45 PCN populations, originating from potato fields in the major regions of potato production in Portugal (Aveiro, Coimbra, Faro, Guarda, Setúbal and Vila Real) and from the island of Madeira (Cunha et al., 2004), were screened for the presence of *P. chlamydosporia*. Cysts extracted from soil were crushed in sterile distilled water and examined for the presence of fungi. Eggs that were half empty, containing destroyed nematodes or with detectable fungal material, i.e. growing hyphae, were plated onto *P. chlamydosporia* selective medium (De Leij and Kerry, 1991). After 14 days incubation at 25 °C, colonies growing out of eggs and morphologically similar to *P. chlamydosporia* were transferred onto 1.7% corn meal agar (CMA) (Oxoid, UK) to look for characteristic diagnostic features (Abrantes et al., 2002).

The identities of three *P. chlamydosporia* isolates (Pc1, Pc2 and Pc3) and two non-native isolates: Vc10 (IMI 331547), originally obtained from *M. incognita* eggs (Brazil); and Pc280 (IMI 380407), isolated from *G. pallida* eggs (UK), kindly supplied by Rothamsted Research, UK, were confirmed by PCR using specific diagnostic primers derived from the *ß*-tubulin gene (Hirsch et al., 2000). The Portuguese isolates are maintained in the Nematology Laboratory, University of Coimbra, in 1.7% CMA at 25 °C. The isolates were also characterised by ERIC-PCR (Arora et al., 1996). DNA was extracted from mycelium grown for 10 days in 1.7% CMA

with the REDExtract-N-Amp Tissue PCR Kit according to the instructions of the manufacturer (Sigma, USA).

1.2.2. Bioassays

1.2.2.1. Inocula production

Standard bioassays are usually performed using agar plugs colonised with fungus (Abrantes et al., 2002). However, the amount of chlamydospores, conidia and hyphal fragments in each plug may vary with the isolate, depending on individual growth rates and sporulation. Therefore, in order to assess differences among the *P. chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280), an equal amount of spores (chlamydospores and conidia) was used as inoculum. Ten days-old colonies of each isolate, growing on 1.7% CMA, were washed with 10 ml of sterile distilled water and the resulting suspension was sieved through ethanol rinsed and UV sterilised plastic sieves (20 µm) to separate spores from mycelium. The numbers of chlamydospores and conidia/ml were quantified using a haemocytometer.

1.2.2.2. Chlamydospore production

The production and viability of chlamydospores was assessed using barley:sand substrate (1:1) in 250 ml flasks following the method described by De Leij and Kerry (1991) with some modifications regarding inoculation. Briefly, conical flasks (250 ml) were filled with 60 g of substrate medium, prepared with milled barley washed trough a 53 µm sieve and mixed with sterilised coarse river sand in equal parts. Five flasks per isolate were prepared and autoclaved prior to inoculation with 1500 spores (chlamydopores and conidia) each. After 21 days incubation at 25 °C, the number of chlamydospores per 1 g sub-sample of the colonised medium was estimated using a haemocytometer. Chlamydospore viability was assessed in three plates per isolate by plating 0.2 ml of a chlamydospore suspension onto sorbose agar with antibiotics (12 g/ l technical agar, 2 g/l of sorbose and 50 mg/l of each of streptomycin sulphate, chlortetracycline and chloramphenicol). After 2 days at 25 °C, the percentage of germinated chlamydospores was calculated using a stereomicroscope.

1.2.2.3. Rhizosphere colonisation

Rhizosphere colonisation was assessed using barley plants as described by Hidalgo-Diaz et al. (2000) with some modifications regarding inoculation. Barley seeds were surfaced sterilised with 7% calcium hypochlorite and plated in 1.2 % agar amended with 10 g/l glucose

(Panreac, Spain), 0.1 g/l peptone (Sigma, USA) and 0.1 g/l yeast extract (Oxoid, UK). Glass tubes 2/3 filled with autoclaved vermiculite were inoculated with 10^5 spores each and planted with a 2 days-old single germinated barley seed. Five replicates per isolate were prepared and tubes without fungal inoculum were used as controls. After incubation for 10 days at 25 °C, 12 h photoperiod, the roots were shaken free of vermiculite, cut into 1 cm fragments, placed onto 0.8% water agar and incubated for a further three days. Root fragments with evident hyphal material were considered colonised by the fungus and the percentage of colonised root fragments was estimated.

1.2.2.4. Nematode egg parasitism

A PCN population of *G. rostochiensis*, obtained from field soil collected in Guarda, Portugal (Cunha et al., 2004), was propagated on roots of potato, *Solanum tuberosum*, cv. Désirée grown in 900 cm^3 plastic pots with sterilised sandy loam soil and sand (1:2). Pots were inoculated with a small polyester bag containing cysts, to give approximately 5 eggs/g of soil. The number of eggs/cyst was assessed by counting five replicates of a suspension of eggs obtained by crushing 50 cysts in water. Pots were placed in a glasshouse at 20 °C, 16 h photoperiod and watered regularly. After three months, new cysts were extracted from the soil using a modified Fenwick can (Shepherd, 1986). Eggs within cysts were released using forceps and suspended in water.

A RKN population of *M. chitwoodi*, obtained from infected potato tubers collected in Porto, Portugal (Conceição et al., 2009), was propagated on roots of tomato, *S. lycopersicum*, cv. Easypeel grown in 800 cm^3 plastic pots with sterilized sandy loam soil and sand (1:1). Pots, inoculated with 10 egg masses/pot, were placed in a glasshouse at 25 °C, 12 h photoperiod and watered regularly. After two months, plants were uprooted, egg masses hand-picked and mechanically disrupted, and eggs suspended in water by agitation.

Plates containing 0.8 % technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg/l of each) were inoculated with a 0.2 ml of a 10^5 spores/ml suspension of *P. chlamydosporia*. After two days incubating at 25 °C, 250 eggs of each nematode species were spread on each plate colonised by the fungus. Plates were further incubated for three days, at 25 °C and, after this time, the number of parasitized eggs was counted using standard methods (De Leij and Kerry, 1991). Controls consisted of plates inoculated with nematode eggs, without the fungus.

1.2.2.5. Conidia production

Conical flasks (250 ml), containing 100 ml of Czapek Dox broth (Oxoid, UK), were inoculated with 1000 spores each. Four flasks were set up per each fungal isolate. After incubation, at 22 °C with shaking at 125 rpm for 7 days, the numbers of conidia in 5 ml aliquots from each flask were counted using a haemocytometer.

1.2.3. Effects of temperature on growth, sporulation and parasitism

Plates were inoculated centrally with 5 mm diameter agar plugs taken from the edge of 10-days old colonies growing on 1.7% CMA. Five plates per isolate were prepared for each temperature (10, 25, 33 and 35 °C). The growing colonies were measured in two directions at right angles to each other, every two days, for 25 days. Data were used to assess the temporal radial extension for each replicate and the growth rates (mm/d) were calculated for the linear phase of the growth curve. Plates where no growth was observed were incubated for another 25 days at 25 °C and growth was assessed as described.

Colonies were suspended in 5 ml of sterilised distilled water and chlamydospores and conidia were counted using a haemocytometer. To estimate conidial germination, 1 ml of suspension (10^5 conidia/ml) was incubated at 25 °C, for 7 h with shaking at 950 rpm in a Thermomixer (Eppendorf AG, Germany), and the percentage of germination was measured in a haemocytometer. Conidia were considered to have germinated when the forming germ tube was longer than the diameter of the spore (Marín et al., 1998). Chlamydospore viability was assessed by plating 0.2 ml of suspension onto sorbose agar with antibiotics as described.

Suspensions of spores were also used to evaluate the nematode egg parasitism as described.

1.2.4. Enzymatic activity

Conical flasks (250 ml), containing 100 ml Czapek Dox broth (Czpk), were inoculated with 10^3 conidia/ml of each isolate and incubated (25 °C, 120 rpm). After 7 days, half of the cultures were transferred to weak Czapek Dox broth (wCzpk) (0.001 g/l sucrose, 0.014 g/l $NaNO_3$, 0.25 g/l KCl, 0.25 g/l $MgSO_4$, 0.5 g/l K_2HPO_4, 0.01 g/l $FeSO_4$) and grown for 3 days. Cultures were centrifuged, hyphae discarded and supernatants tested for enzymatic activity with the API ZYM® enzymatic quantification test according to the manufacturer instructions

(Biomérieux SA, France), using 65 µl of supernatant in each well incubated for 4 hours at 37 °C. Enzymatic activity was scored for each of the 19 enzymes tested (acid phosphatase, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, alkaline phosphatase, β-galactosidase, β-glucuronidase, β-glucosidase, chymotrypsin, cystine arylamidase, esterase C4, esterase lipase C8, leucine arylamidase, lipase C14, N-acetil-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase), using a colourimetric scale (0-5) provided by the manufacturer (http://biomerieux.com). Isolates Pc 2 and Vc10 were further evaluated for enzymatic activity in the presence of nematode eggs. The isolates were inoculated in Czpk as described. After 7 days, half of the cultures were transferred to wCzpk or wCzpk with 100 nematode eggs/ml, either PCN or RKN. The cultures were grown for 7 days before enzymatic activity was assessed. In both experiments, controls were set up without fungal inoculation, incubated as described and tested for enzymatic activity.

1.2.5. Data analysis

Data on chlamydospores and conidia production and viability, rhizosphere colonization and parasitism were compared among isolates by one-way ANOVA, using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA).

A two-way ANOVA (isolate × temperature) was applied to compare differences among isolates in growth, production of chlamydospore and conidia, viability and egg parasitism in control treatments (25 °C) and in colonies which were transferred and incubated at 25 °C after being submitted to limiting growth temperatures (10 and 33 °C).

Whenever the ANOVAs returned statistically significant effects ($P<0.05$), differences among treatments were further assessed using the LSD test at the 5% level.

Possible interactions among temperature treatments, growth, spore production and egg parasitism were analysed for each isolate using Pearson correlations for quantitative data. The non-parametric Spearman ranked correlations were used for the assessment of possible interactions between temperature treatments and the biological parameters for each isolate. Symmetric matrices of correlation values and significance were obtained in SPSS for all possible pair-wise combinations of parameters.

The values representing the level of activity of expressed enzymes for each isolate, in a given medium, were compared and dendrograms were constructed by group average linkage

in the hierarchical clustering based on Pearson correlation using STATISTICA version 10 (StatSoft Inc., USA).

1.3. Results

1.3.1. Identification and molecular characterisation

Pochonia chlamydosporia was detected in three *G. rostochiensis* populations collected in Aveiro (Pc1), Guarda (Pc2) and Setúbal (Pc3), representing 10% of the total of fungi found associated with cysts (data not shown). After electrophoresis separation of products obtained through PCR with specific *β*-tubulin primers, all the five isolates (Vc10, Pc280, Pc1, Pc2, Pc3) presented a specific band (ca. 270 bp) characteristic of *P. chlamydosporia* var. *chlamydosporia* (Fig. 1.1a). Molecular characterization of the isolates showed that Pc1 and Pc3 shared an identical ERIC-PCR profile (Fig. 1.1b).

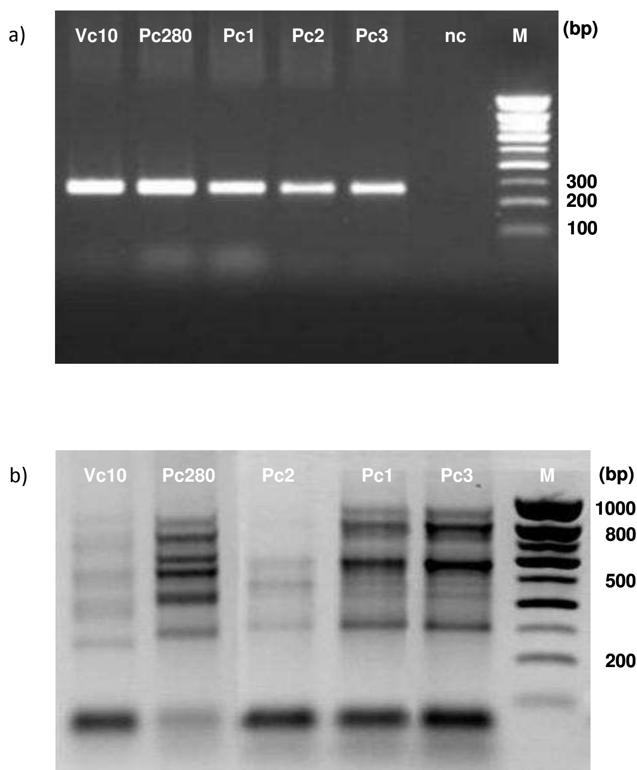


Figure 1.1 - Molecular characterization of five *Pochonia chlamydosporia* isolates (Vc10 and Pc280 Pc1, Pc2, Pc3,). PCR product of DNA amplified with the *β*-tubulin primer set (a) and ERIC-PCR (b) profiles. nc: negative control without template DNA; M: DNA size marker Smart Ladder SF, 100 bp ladder (Eurogentec, Belgium). Empty lanes have been excised from the pictures to bring the marker and profiles together.

1.3.2. Bioassays

1.3.2.1. Chlamydospore production

Differences were detected among isolates in the ability to produce chlamydospores except for isolates Vc10 and Pc280. Isolate Pc2 produced the most spores whereas Pc3 was the least producer (Fig. 1.2a). The viability of chlamydospores was higher than 78% for all isolates, being 98% for isolate Vc10 (Fig. 1.2b).

1.3.2.2. Rhizosphere colonisation

No differences were detected between isolates Pc2 and Pc3, both colonising the rhizosphere of barley extensively (> 90%). Pc1 was a poor rhizosphere coloniser and invaded less than 40% of root sections after 10 days, and, for Vc10 and Pc280, the percentage of colonisation varied between 55 – 73% (Fig. 1.2c).

1.3.2.3. Egg parasitism

After three days at 25 °C, the proportion of parasitized eggs was low for all isolates (Fig. 1.3a). Although no statistically significant differences were detected, isolates Pc1 and Pc280 were the best parasites against RKN. The proportion of PCN eggs parasitized by Pc1 and Vc10 was significantly higher when compared to the other isolates.

1.3.2.4. Conidia production on liquid media

The numbers of conidia produced by Pc3 were greater than those produced by isolates Pc1, Pc2 and Vc10 but the greatest number of conidia/ml medium was produced by Pc280 (Fig. 1.3b).

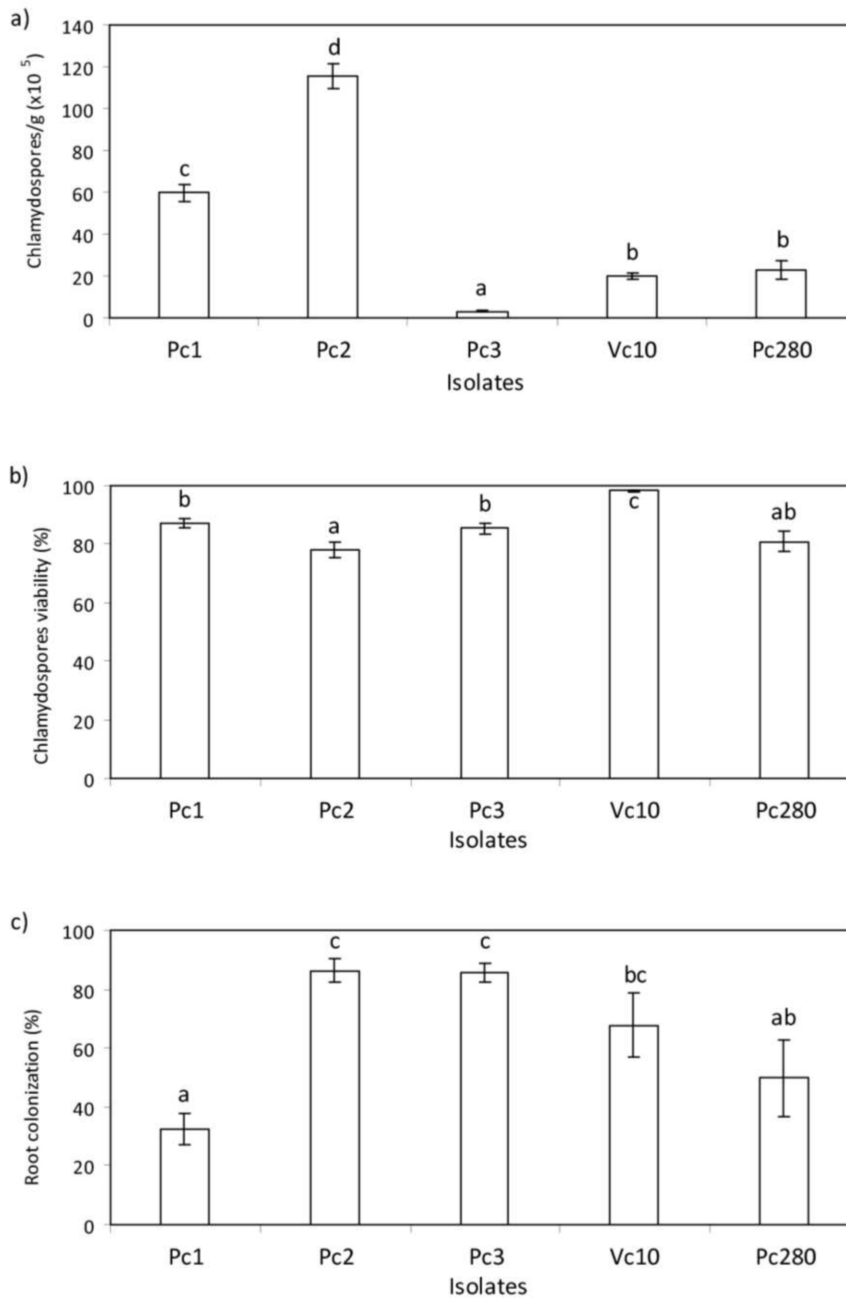
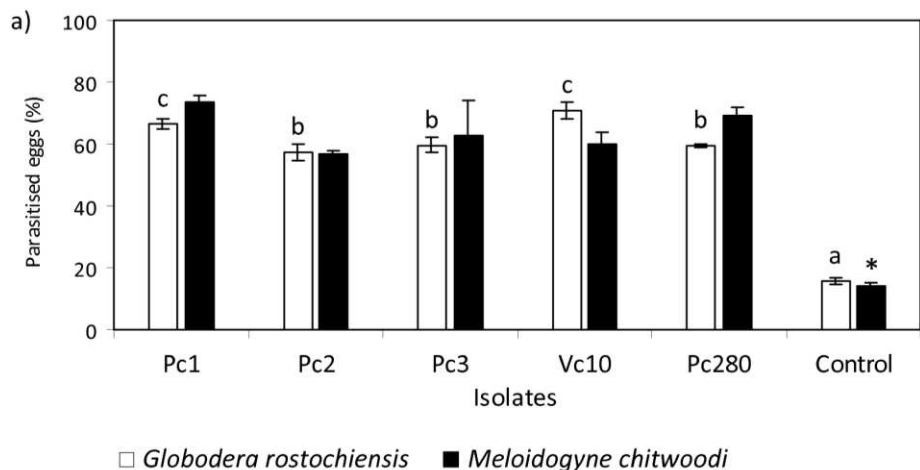


Figure 1.2 - Number of chlamydospores (a), percentage of germinated chlamydospores (b), and percentage of colonized roots (c) of five *Pochonia chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for 25 days on 1.7% corn meal agar (CMA). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).



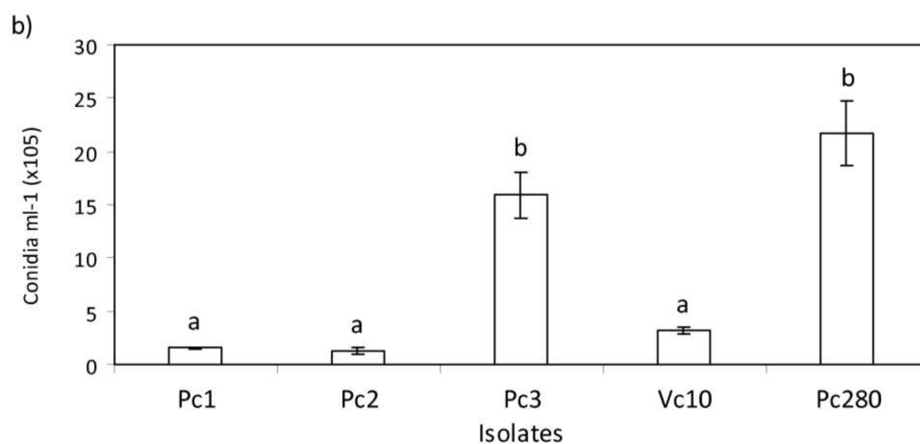


Figure 1.3 - Percentage of parasitized eggs (a) and number of conidia (b) of five *Pochonia chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for 25 days on 1.7% corn meal agar (CMA). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$). * significantly different from same pattern columns according to LSD test ($p<0.01$).

1.3.3. Effects of temperature on growth, sporulation and parasitism

At 25 °C, all isolates reached the edge of the plates after 25 days and similar growth rates among isolates were observed except for Vc10 that showed a higher growth rate (Table 1.1). No growth was observed at 10, 33 and 35 °C, but hyphae, conidia and chlamydospores could be observed on the surface of inoculated plugs. Therefore, these plates were transferred to 25 °C and colony diameters were measured for another 25 days. After this time, growth on plates inoculated with colonised plugs, maintained at 35 °C, was observed only in 1 replicate of isolate Vc10 and in two replicates of Pc3 (data not shown). Growth rates at 25 °C, after previous incubation at 10 and 33 °C, varied according to the isolate (Table 1.1). Results revealed that spores/hyphal fragments remained viable for almost one month at 10 and 33 °C and the isolates recovered their growth after incubation at 25 °C (Table 1.1).

The number of chlamydospores produced in colonies growing out of colonised plugs submitted to limiting growth temperatures also differed among isolates (Table 1.1). The numbers of chlamydospores in colonies of isolate Pc3, growing out of colonised plugs submitted to 10 °C for 25 days, had increased ca. 5-fold. Viability of chlamydospores was high for all isolates (>83%) and variability was found in all isolates except for Vc10 (Table 1.1). Regarding conidia production, although some variability was found in isolates Pc1, Pc2, Pc3 and Pc280, no significant differences were detected for Vc10. Colonies growing from colonised plugs submitted to limiting temperatures for growth produced smaller numbers of conidia except for Pc2 and Vc10 and the conidia viability in colonies, inoculated with colonised plugs submitted to different temperatures, decreased except for Pc1 (Table 1.1).

Correlations between pairs of biological parameters as influenced by exposure to temperatures limiting for growth gave inconclusive results. For isolates Vc10 and Pc280 this was negatively and significantly correlated with the growth rate (r=-0.60 for both). The opposite effect was observed in Pc2 (r=0.81). For this isolate, exposure to the different limiting temperatures was significantly correlated with sporulation parameters such as the production of chlamydospores (r=-0.98) and conidia (r=-0.78) and also with the viability of conidia (r=-0.76), whereas for isolate Pc3 this was only significantly correlated with chlamydospore production (r=-0.93). Other significant correlations found were not considered because they could not be interpreted biologically.

Table 1.1 – Growth rate, number of chlamydospores, conidia and percentage of germinated conidia of *Pochonia chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for 25 days on 1.7% corn meal agar (CMA) at 25 °C.[a)]

Isolate	Growth conditions[b)]	Growth rate (mm/day)	Nº chlamydospores/cm^2 (x10^3)	Chlamydospores germination (%)	Nº conidia/cm^2 (x10^3)	Conidia germination (%)
Pc1	A	1.99 ± 0.02 bcde	9.43 ± 2.30 cd	87.06 ± 4.40 ab	58.95 ± 4.35 f	87.17 ± 2.52 f
	B	1.89 ± 0.03 abc	8.12 ± 0.79 bcd	95.41 ± 1.00 d	136.58 ± 33.02 gh	66.54 ± 5.13 bcd
	C	2.09 ± 0.03 de	25.33 ± 6.71 e	94.76 ± 1.63 d	19.47 ± 3.32 de	77.46 ± 0.83 e
Pc2	A	1.75 ± 0.03 a	30.39 ± 0.92 e	95.91 ± 0.81 d	20.57 ± 2.71 de	92.63 ± 0.59 f
	B	1.88 ± 0.04 abc	10.57 ± 0.86 cd	87.46 ± 0.57 abc	11.18 ± 3.38 bc	92.33 ± 0.88 f
	C	1.96 ± 0.02 bcd	5.76 ± 0.00 abc	95.40 ± 0.83 d	8.38 ± 2.58 ab	58.60 ± 2.91 ab
Pc3	A	2.02 ± 0.04 cde	63.40 ± 7.56 f	87.28 ± 2.47 ab	50.04 ± 1.83 f	63.49 ± 2.02 abc
	B	1.96 ± 0.07 bcd	13.62 ± 5.29 cd	85. 67 ± 0.67 a	85.14 ± 19.56 fg	75.39 ± 3.44 de
	C	1.99 ± 0.15 bcde	7.34 ± 1.09 bc	91.92 ± 1.94 bcd	17.38 ± 2.09 cde	62.55 ± 6.84 abc
Vc10	A	2.14 ± 0.03 e	2.23 ± 0.73 a	95.13 ± 3.38 d	225.31 ± 85.20 h	66.71 ± 4.06 bcd
	B	2.14 ± 0.03 e	2.88 ± 1.06 ab	93.50 ± 0.76 cd	212.82 ± 32.22 h	71.09 ± 1.09 cde
	C	1.97 ± 0.03 bcd	9.17 ± 5.38 bcd	97.48 ± 0.43 d	130.21 ± 15.10 gh	70.00 ± 2.31 cde
Pc280	A	2.04 ± 0.03 cde	10.09 ± 1.70 cd	83.41 ± 4.35 a	11.27 ± 0.92 bcd	56.60 ± 1.10 a
	B	1.85 ± 0.05 ab	15.37 ± 0.31 de	93.38 ± 1.19 cd	24.71 ± 3.93 e	72.35 ± 3.16 de
	C	1.87 ± 0.05 ab	1.92 ± 0.63 a	94.56 ± 0.57 d	5.07 ± 0.44 a	70.23 ± 2.82 cde

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column for a specific isolate are not significantly different according to LSD test ($p<0.05$).

[b)] Growth conditions: A – Colonies inoculated with colonised plugs submitted to 10 °C for 25 days; B – Colonies inoculated with colonised plugs from 10-days old colonies grown at 25 °C; and C - Colonies inoculated with colonised plugs submitted to 33 °C for 25 days.

Nematode egg parasitism revealed by fungal colonies growing out of colonised plugs submitted to unfavourable temperatures was similar to that obtained in colonies growing at 25 °C for isolates Pc1, Pc2 and Vc10 (data not shown). For Pc3, however, parasitism of PCN eggs increased by 10% ($p<0.01$) but no significant differences were detected on parasitism of RKN eggs. Parasitism of isolate Pc280 on PCN eggs was 7% higher by spores produced by colonies growing out of colonised plugs submitted to 33 °C ($p=0.01$), but a decrease was detected in RKN eggs parasitism: 12% when spores were produced by colonies growing out of colonised plugs submitted to 10 °C ($p=0.02$) and 5% when spores were produced by colonies growing out of colonised plugs submitted to 33 °C ($p=0.05$).

1.3.4. Enzymatic activity

From the 19 enzymes assayed, only 8 were produced by the isolates (esterase C4, esterase lipase C8, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, N-acetyl-B-glucosaminidase). Acid phosphatase and naphthol-AS-BI-phosphohydrolase were the only enzymes detected in all the isolates. The highest enzymatic activity was detected in Pc2 in wCzpk. All isolates produced esterases but protease activity was detected only in isolates Pc2 and Vc10. Most of the proteases tested were not secreted by any of the isolates. Only Pc2 and Vc10 showed strong activity in producing the valine arylamidase in wCzpk but none of the isolates produced the serine proteases trypsin and chymotrypsin. Lipase activity was not detected. Enzymatic activity decreased in the presence of nematode eggs for both species. The reproducibility of the assay gave consistent results although the supernatants were only tested twice.

Cluster analysis revealed that the isolates were grouped according to their similarities on the ability to produce enzymes in different media. The dendrogram confirmed the existence of different groups of isolates according to growth media, except isolate Pc2 in wCzpk, which was clustered apart from the other isolates, and isolate Pc280 in wCzpk (Fig. 1.4a). Regarding enzyme activity of Pc2 and Vc10, in the presence or absence of nematode eggs, these isolates clustered apart from each other except Pc2 in the presence of PCN eggs (Fig. 1.4b).

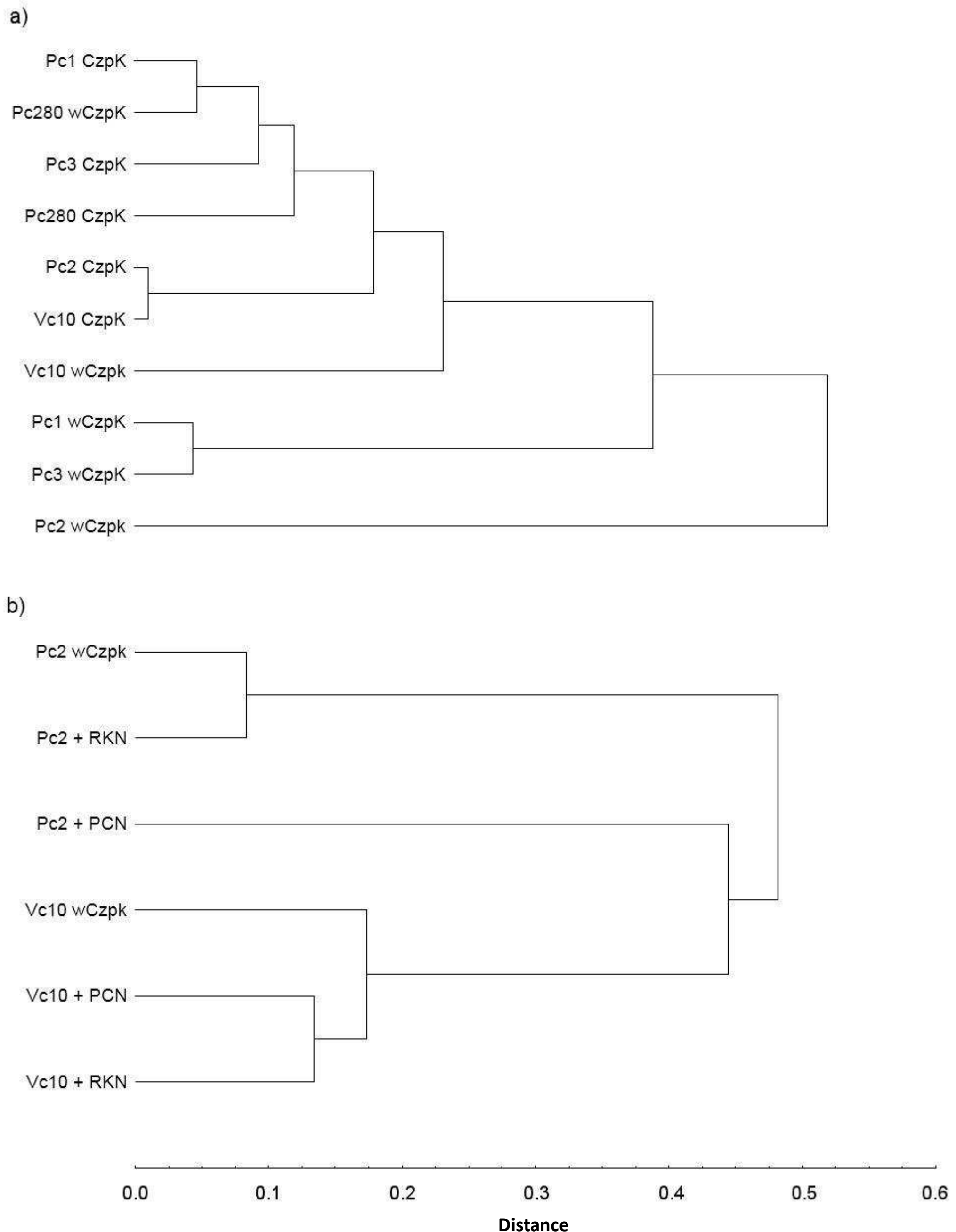


Figure 1.4 - Dendrograms showing similarities between groups based on extracellular enzymatic activity of five *Pochonia chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for: a) 10 days in Czapek Dox broth (Czpk) and 7 days in Czpk followed by 3 days in weak Czapek Dox broth (wCzpk); b) 7 days in Czpk followed by a transfer to wczpk inoculated with 100 eggs/ml either of *Globodera rostochiensis* (wCzpk+PCN) or *Meloidogyne chitwoodi* (wCzpk+RKN). Results are the average values of enzymatic activity between two replicates of each isolate for the enzymes: α-galactosidase, acid phosphatise, esterase (C4), esterase lipase (C8), leucine arylamidase, N-acetyl-B-glucosaminidase, naphthol-AS-BI-phosphohydrolase, and valine arylamidase. The dendrograms were constructed using cluster analysis (group average).

1.4. Discussion

Pochonia chlamydosporia isolates associated with PCN eggs collected in Portugal were isolated and identified for the first time, using molecular and standard methods. Isolates Pc1 and Pc3 shared an identical profile, although they were isolated from cysts originating from two different regions. Identical ERIC-PCR profiles from isolates with different geographical origins had already been reported and may indicate that this fungus is less ubiquitous and diverse in different agricultural systems than many other micro-organisms (Flores-Camacho et al., 2008, Manzanilla-Lopez et al., 2009).

The pathogenicity of the isolates varied between 41-55% against *G. rostochiensis* eggs and 42-60% against *M. chitwoodi* eggs. The low percentage of egg infection obtained through the standard bioassay was also observed by other authors. The mucilage surrounding the eggs may be an important source of nutrition for the fungus that ensures a high rate of egg colonisation (Irving and Kerry, 1986). This mucilage may have been altered after re-suspending the eggs in distilled water before plating. Spontaneous hatching was also detected in both nematode species during the bioassay. Maturity of the eggs is related with parasitism as, in general, isolates parasitise immature eggs more actively than mature eggs containing second-stage juveniles (Irving and Kerry, 1986). Unexpectedly, isolate Vc10 was a better parasite against PCN when compared with RKN, whereas all the other isolates presented a reverse response. *Pochonia chlamydosporia* isolates have shown nematode-host preferences at the sub-specific level (Kerry, 2000). Host-related genetic variation in the alkaline serine protease VCP1, produced during infection of the nematode egg, grouped more strongly on the basis of their original host rather than by their geographic origin (Morton et al., 2003). Therefore, pathogenicity towards nematode eggs *in vitro* should be validated in more realistic environments such as greenhouse and field conditions.

The isolates were characterised for their tolerance to temperature, which can have a great impact on the establishment of the organism in the field and compromise its efficacy in controlling the target nematodes (Magan, 2001). High temperature (35 °C) was lethal to mycelium of some isolates and growth was inhibited at 33 and 10 °C. These results were similar to those obtained with Spanish and Cuban isolates (Hidalgo-Diaz et al., 2000, Olivares-Bernabeu and Lopez-Llorca, 2002). However, studies with isolates associated with *Heterodera avenae* from soils, in England, showed that the optimum temperature for growth

was 18 °C and that growth was slower at 10 °C (Kerry et al., 1986). In addition, isolates from soils in India had an optimum growth at 30 °C, with sporulation and growth occurring at 35 °C (Nagesh et al., 2007). Therefore, it seems likely that the temperature tolerance of each isolate is related to the climatic conditions of its geographic origin. This adaptation to the original conditions seems to be conserved even after years of sub-culturing in laboratory conditions, which reveal an immense potential variability of the fungus: each isolate could be adapted to micro-scale parameters that could limit its biocontrol potential in some conditions and not others. Such variability may provide an opportunity to be exploited in selecting isolates tailored for application in specific field climatic conditions.

After being submitted to growth-limiting temperatures of 10 and 33 °C, for 25 days, all isolates were able to grow, sporulate and parasitise nematode eggs when later transferred onto 25 °C. However, chlamydospore and conidia production, viability and parasitism of nematode eggs were affected. A significant increase in chlamydospore production was observed in Pc3 in colonies growing out of colonised plugs submitted to 10 °C for 25 days, indicating that this isolate is probably more fit to withstand lower temperatures, as growth rate in these colonies was also higher than at 25 °C and parasitism against PCN eggs also increased. Several significant correlations were found between the exposure temperature, growth rate and sporulation, suggesting that these processes may be biologically related. Nonetheless, a pattern of correlations that permits any inferences on the effects of the exposure to limiting temperatures on the biological parameters assessed or among those parameters could not be perceived. This is probably due to the high variability among *P. chlamydosporia* isolates. The isolates were able to survive and to resist at temperatures limiting for growth which may be an advantage for the establishment of the fungus in the field and adaptation to variable environmental conditions, but further experiments should be conducted in soil, in order to validate this hypothesis.

Activity was observed in only a few enzymes in all isolates. It is unclear whether all the tested enzymes are in fact produced by the fungus above the level of detection in a non-inductive media such as Czapek-Dox broth, or if they are produced by the fungus at all (Carder et al, 1993). Nevertheless, variation in enzymatic activity was detected among the isolates, and it was possible to perceive a degree of relation with nutrient availability. Variability of fungal enzymatic activity is thought to be dependent on nutrient availability

(Mendoza de Gives et al., 2003). Only isolate Pc2 had a strong activity of esterases, proteases and oxidases when grown in a low nutrient medium. Enzymatic activity shown by this isolate decreased in high nutrient medium. It is unclear whether such enzymes are up-regulated in nutrient-limiting conditions, as it was expected that a larger vegetative growth in high nutrient medium would elicit a higher enzymatic activity, but there seems to be a clear metabolic difference in the fungus. All isolates produced esterases but protease activity was detected only in Pc2 and Vc10. Specific extracellular enzymes such as proteases are secreted by *P. chlamydosporia* and thought to play an important role in the infection process of eggs, as they enable the fungus to degrade the nematode egg shell (St. Leger et al., 1986; Segers et al., 1994; Tikhonov et al., 2002). However, the generalist enzyme kit used may not be able to recognise specific enzymes produced by *P. chlamydosporia*. Isolates vary in the production of extracellular enzymes, but detection of proteolytic activity is usually high *in vitro* in solid media (Esteves et al., 2009; Olivares-Bernabeu and Lopez-Llorca, 2002). Enzymatic activity decreased in the presence of nematode eggs of both species, which points to the down-regulation of the housekeeping enzymes in the parasitic stage and a potential larger investment in parasitism-related enzymes. Studies on the transcriptome analysis of *P. chlamydosporia* in the presence or absence of nematode eggs have revealed significant transcriptional reprogramming with differences in expression of up-regulated specific genes involved in metabolic functions, cellular transport and detoxification (Rosso et al., 2011).

In the standard *in vitro* bioassays, it was apparent that isolate Pc1 had the least potential as a biological control agent as it was less able to colonise the rhizosphere than the other isolates and rhizosphere colonisation is considered to be a key factor for nematode control by *P. chlamydosporia* (Bourne et al., 1996). Nonetheless, this isolate had been associated with a PCN suppressive soil (Conceição, pers. comm.). On the other hand, isolate Pc3 produced a significantly smaller number of chlamydospores in solid medium, which would make mass production difficult. The performance of the two non-native isolates, Vc10 and Pc280, was not indicative of potential biological control agents, as they were poor rhizosphere colonizers and chlamydospore producers when compared to Pc2. However, isolate Vc10 was the best parasite of PCN eggs. The variability detected in the fitness among *P. chlamydosporia* isolates to adapt to specific environmental and nutritional conditions highlights the importance of screening when selecting potential biological control agents.

1.5. Acknowledgements

This research was supported by FEDER funds through the "Programa Operacional Fatores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the Project PTDC/AGR-AAM/74574/2006. The first author was supported by ESF funds through the "Programa Operacional Potencial Humano - POPH" of "The National Strategic Reference Framework" (SFRH/BD/27317/2006).

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Chapter 2

In vitro water stress bioassays with the nematophagous fungus *Pochonia chlamydosporia*: Effects on growth and parasitism

Written as a journal article:

Vieira dos Santos, M.C., Esteves, I., Kerry, B. and Abrantes, I. (2012). *In vitro* water stress bioassays with the nematophagous fungus *Pochonia chlamydosporia*: Effects on growth and parasitism. Biological control DOI: 10.1016/j.biocontrol.2012.09.001.

Abstract - The biocontrol potential of *Pochonia chlamydosporia*, a fungus with parasitic activity against economically important plant-parasitic nematodes, can be influenced by abiotic factors such as water availability. The objective of this study was to evaluate the effects of different water stress regimes on *in vitro* growth, sporulation, germination and parasitism of *P*. *chlamydosporia* isolates*.* The osmotic water potential of 1.7% corn meal agar (CMA) was modified by addition of potassium chloride (KCl) or glycerol, and the matric water potential was modified using polyethylene glycol (PEG 8000). The fungus was able to grow over a range of potentials, but radial growth rates decreased with the increase of osmotic and matric stress. No growth was observed at -10 MPa on 1.7% CMA amended with glycerol and at -7.1 MPa on medium with PEG 8000, but all isolates were able to resume growth when transferred onto unmodified 1.7% CMA. The production of chlamydospores was repressed in both osmotic and matric modified media. Although the production of conidia increased in medium modified with KCl, the germination rate was lower. Spores/hyphal fragments remained viable in all isolates that were previously inoculated onto media with growth-limiting water potential (-10 MPa on 1.7% CMA amended with glycerol and -10MPa on medium with PEG 8000). The percentage of viable conidia produced on 1.7% CMA, after inoculation under osmotic or matric stress conditions for 25 days, was over 74.5% in all isolates (osmotic stress) and ranged from 1% (Pc1) to 65.8% (Pc280) (matric stress). The *in vitro* infection of potato cyst nematodes, *Globodera rostochiensis*, eggs by *P. chlamydosporia* isolates, grown under these limiting conditions, was studied using a standard bioassay. The percentage of parasitized eggs was significantly higher under osmotic stress except for isolates Pc2 and Pc3. *Pochonia chlamydosporia* spores/hyphal fragments can remain viable at water potentials limiting for growth, for prolonged periods of time, suggesting that the osmoregulation mechanisms, used to compensate water stress, affect *in vitro* sporulation and increased pathogenicity. Knowledge on water requirements of *P*. *chlamydosporia* enables a better understanding of its survival and growth strategies in the soil environment and could aid the development of effective strategies to increase the production and quality of inoculum, thus contributing to the implementation of biosafe, sustainable management strategies against plant-parasitic nematodes.

Key words: biological control, matric potential, osmotic potential, plant-parasitic nematodes, *Pochonia chlamydosporia*.

2.1. Introduction

Plant-parasitic nematodes, such as *Heterodera* and *Globodera* spp. (cyst-forming nematodes) and *Meloidogyne* spp. (root-knot nematodes), are important soil-dwelling pests that affect a wide range of crop plants. In addition to direct root damage, these nematodes facilitate fungal and bacterial plant pathogens and therefore act as a major cause of preventable crop disease and yield loss. The magnitude of their damage to crops varies according to the agricultural system conditions: cyst nematodes can be one of the main nematode pests in Northern Europe but, in East Africa, root-knot nematodes parasitize over 50% of all plant cultivars (Trudgill and Blok, 2001). Depending on biotic and abiotic conditions, these nematodes can reduce yield by 40-60% and some cultures can simply be abandoned in nematode infested areas (Sikora et al., 2005). Current management practices of these nematodes include crop rotation and nematicide application. However, crop rotation does not allow intensive production and the use of nematicides, in addition to being expensive, involves the handling and application of highly toxic materials. Also, continuing concerns over the use of nematicides has led to their use being banned or significantly reduced. There is an increasing demand for alternative, non-chemical, sustainable strategies of nematode control. Biological control may have an important role and fungal antagonists are among the most promising nematode biological control agents (Stirling, 1991).

Pochonia chlamydosporia (Goddard) Gams and Zare is a facultative nematophagous fungus with parasitic activity against eggs and females of economically important plant-parasitic nematodes. This fungus has been associated with soils that suppress the multiplication of cyst nematode populations (Kerry et al., 1982). The biocontrol potential of *P*. *chlamydosporia* has been widely studied and its effectiveness increases when applied in combination with other traditional control measures such as organic amendments and crop rotation (Atkins et al., 2003, Kerry and Bourne, 1996; Hidalgo-Diaz and Kerry, 2008; Kerry and Hirsch, 2011). Importantly, its biosafety has been evaluated and it has been considered as low health risk for humans, animals and the ecosystem (García et al, 2004a, 2004b; 2008a; 2008b; 2009; Puertas et al. 2006). Isolates of this fungus differ in their virulence, ability to colonize the root surface, and chlamydospore production, these being considered desirable features for its use as a biological control agent (Abrantes et al., 2002). Although the efficacy of selected isolates has been demonstrated, there are some reports on failure to establish

the fungus in soil and inconsistent nematode control in pot and field tests (Bourne and Kerry, 1999; Ciancio et al., 2002; Atkins et al., 2003; Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003; Tobin et al., 2008; Tzortzakakis, 2000; 2007; 2009; Puertas and Hidalgo-Díaz, 2009). The results of these studies suggest that the successful establishment of the fungus in soil relies on the provision of an energy source to help overcome competition from the resident soil microbial community. In most isolates of *P. chlamydosporia* conidia production can be induced in large amounts without effort and at a low cost. The fungus also produces chlamydospores, resistant structures that are slow to be produced *in vitro*, as they are not produced in liquid culture, and large amounts need to be added to soil to promote control (Mo et al., 2005; Kerry and Hirsch, 2011). Although their production is more laborious, they are able to withstand harsh conditions in soil for long periods of time without an additional food source, and hold the capacity to initiate fungal growth when in favourable conditions. Therefore, chlamydospores are widely seen as having a better biocontrol potential and are a preferred source of inoculum (Kerry, 2001).

Isolates of *P. chlamydosporia* differ in their ability to grow in soil and tend to be more abundant in organic rather than in mineral soils but parasitic ability could not be related with fungal numbers (Atkins et al., 2003). The survival and proliferation of this fungus in soil is also affected by abiotic factors that can play an important role in establishing a fungus with biocontrol potential in the field, and these include fluctuations in temperature and water availability (Bourne and Kerry, 2000; Magan, 2001).Water availability in soil can be expressed by the total water potential that is the sum of three forces affecting its energy state : i) osmotic or solute potential, due to the presence of ions or other solutes; ii) matric potential, due directly to forces required to remove water bound to the matrix, an indicator of the capacity for active growth and survival in the soil; and iii) turgor potential (Jurado et al., 2008). Osmotic and matric water potentials, components of the total water potential, are important in determining whether fungal sporulation can occur, how and when spores are released, and may also affect growth and morphology, possibly affecting the viability of the spores to be dispersed (Lacey, 1986). Some information is available on the water relations on hyphal growth and production of chlamydospores of *P. chlamydosporia* (Kerry et al., 1986, Bourne and Kerry, 1999). However, little is known regarding tolerance to solute and matric stress. Previously, *P. chlamydosporia* isolates have shown differences in growth and their

tolerance to osmotic and matric water stress (Olivares-Bernabeu and Lopez-Llorca, 2002; Esteves et al., 2009). Under water stress, isolates accumulated a combination of different polyols important in osmoregulation, which depended on the solute used to generate the stress (Esteves et al., 2009), but the impact of water stress on the numbers and viability of the spores produced was not assessed. Low water potentials can increase spore germination rates (Hallsworth and Magan, 1995) and affect virulence of biocontrol fungi such as *Metarhizium anisopliae* (Andersen et al., 2006). Although virulence of *P. chlamydosporia* is usually related to host preference, the ability to parasitize nematode eggs is also dependent on mechanical and enzymatic mechanisms that allow infection of the eggs (Lopez-Llorca et al., 2002; Mauchline et al., 2004). Egg parasitic fungi must adhere to the eggshell, then penetrate the egg cuticle and often appressoria are formed in response to the contact with the surface of a nematode egg. Appressoria enable both the attachment to and penetration of the nematode egg, which involves mechanical (depending on turgor) and enzymatic action. Secreted enzymes destroy the outer vitteline membrane of the egg cuticle and expose the chitin layer that is then penetrated by an infection peg, a specialised hypha (Lopez-Llorca et al, 2002). Studies on the plant pathogenic fungus *Magnaporthe grisea* established that osmotic stress plays an important role in parasitism as it decreases mechanical penetration due to changes in appressoria turgor pressure (Howard et al., 1991; Dixon et al., 1999). Therefore, the responses of *P. chlamydosporia* to water stress induced by increased matric or osmotic potentials may not only influence its establishment and proliferation in soil but can also play an important role in sporulation and parasitic ability of this potential biological control agent.

The objective of this study was to evaluate the ability of three *P. chlamydosporia* isolates, collected in different regions of Portugal, and two non-native isolates to grow, sporulate and parasitize nematode eggs *in vitro* under different water stress regimes.

2.2. Material and Methods

2.2.1. *Pochonia chlamydosporia* isolates

Five isolates of *P. chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc 280) were used in this study. Isolates Pc1, Pc2 and Pc3 were obtained from *Globodera* spp. eggs extracted from cysts in soil samples collected in Portuguese potato fields. Isolate Vc10, originally obtained

from *M. incognita* eggs (Brazil), and Pc280, isolated from *G. pallida* eggs (UK), were kindly supplied by Rothamsted Research, UK. All isolates were maintained in 1.7 % corn meal agar (CMA) (Oxoid, Basingstoke, UK), at 25 °C, and their identities were confirmed using PCR with specific diagnostic primers derived from the ß-tubulin gene (Hirsch et al., 2000) (data not shown).

2.2.2. Effects of osmotic and matric potential on growth

Plates were inoculated centrally with 5 mm diameter agar plugs taken from the edge of 10-days old colonies growing on 1.7% CMA. Six plates/treatment (solute x water potential) were inoculated and incubated at 25 °C. The growing colonies were measured in two directions at right angles to each other for 25 days. Data were used to assess the temporal radial extension for each replicate by linear regression and the growth rates (mm/day) in osmotic and matric water stress treatments were calculated. Plugs from plates where no growth was observed were transferred onto non-amended 1.7% CMA plates and growth was assessed for further 25 days.

2.2.2.1. Modification of the osmotic potential

The osmotic potential of 1.7% CMA (-0.7 MPa) was modified to -2.8, -7.1 and -10 MPa (Magan, 1997) by addition of the ionic solute potassium chloride (KCl) or the non-ionic solute glycerol (Dallyn and Fox, 1980). Control plates, where water was not restricted, were made using non-amended 1.7% CMA.

2.2.2.2. Modification of the matric potential

For modification of the matric potential, known amounts of polyethylene glycol (PEG 8000) (Sigma Aldrich, USA) were used to reduce the water potential to -1.4, -2.8 and -7.1 MPa (Magan, 1997). The water potential generated by PEG 8000 is mainly (99%) due to matric forces (Steuter et al., 1981). PEG 8000 medium was enriched with peptone (10 g/l) and glucose (40 g/l) and was autoclaved prior to use. Control plates (-1.0 MPa) were prepared as treatment plates but lacked PEG 8000. Round make-up cotton disks (8.5 cm diameter, 4 mm thick) sterilised by autoclaving were placed in sterile 9 cm plates to which 20 ml of the cooled liquid medium were added. The matting cotton disk was overlaid with a

sterile disk of black polyester lining cloth and a cellophane disk, to provide support for fungal growth in the liquid medium (Ramos et al., 1999). Since water potentials, generated by PEG, are more limiting for growth than those generated from KCl or glycerol, water stress at -10 MPa was not tested (Esteves et al., 2009).

2.2.3. Effects of osmotic and matric potential on spore production

Numbers of chlamydospores and conidia/plate and the percentage of spore viability were assessed on 1.7% CMA, osmotic and matric-modified media, prior colonies reached the edge of plates. For chlamydospore and conidia counts, colonies were suspended in 5 ml of 0.05% Tween 80 and spores were counted using a haemocytometer. To estimate conidial germination, 1 ml of spore suspension in water (10^5 spores/ml) was incubated at 25 °C, for 7 h with shaking at 950 rpm in a Thermomixer (Eppendorf AG, Hamburg, Germany), and the percentage of germination was counted at 400x magnification in a haemocytometer. Spores were considered to have germinated when the forming germ tube was longer than the diameter of the spore (Marín et al., 1998).

2.2.4. Effects of osmotic and matric potential on nematode egg parasitism

To obtain nematode inoculum for this experiment, potato cyst nematodes, *G. rostochiensis*, were propagated on roots of *Solanum tuberosum* (potato cv. Désirée) grown in plastic pots containing 1L of sterile sandy soil. The pots were inoculated with cysts contained in a small polyester bag, to give approximately 5 eggs/g of soil. The number of eggs/cyst was assessed by counting five replicates of a suspension of eggs obtained by crushing 50 cysts in water. Pots were placed in a glasshouse at 20 °C, 16 h photoperiod and were watered regularly. After three months, new cysts were extracted from soil using a modified Fenwick can (Shepherd, 1986). Eggs within cysts were released using forceps and suspended in water.

Conidia from Pc1, Pc2, Pc3, Vc10 and Pc280 colonies, grown in 1.7% CMA and under different water potentials, were suspended in sterile distilled water and the concentration adjusted to 10^5 spores/ml and 0.2 ml of suspension were spread onto plates containing 0.8% technical agar and antibiotics (50 mg/l streptomycin sulphate, 50 mg/l chloramphenicol, 50 mg/l chlortetracycline). After two days incubating at 25 °C, 500 eggs of *G. rostochiensis*

were spread on each plate colonised by the fungus. Plates were further incubated for three days at 25 °C, and, after this time, the number of parasitized eggs was counted using standard methods (Irving and Kerry, 1986; De Leij and Kerry, 1991). Controls consisted of plates, without the fungus, inoculated with nematode eggs.

2.2.5. Data analysis

Analysis of variance (ANOVA) was applied to compare differences between growth on the control treatment (1.7% CMA) and on media with low water potentials (-10 MPa, glycerol and -7.1 MPa, PEG 8000), after transference onto 1.7% CMA.

Data on spore production, viability and parasitism were compared separately for osmotic and matric stress using ANOVA. To ensure normality, data were transformed when required using log (x+1).

Statistically significant differences were assessed with Statistica 7.0 (StatSoft, Inc., USA) using the Fisher LSD test ($P<0.05$).

2.3. Results

2.3.1 Effects of osmotic and matric potential on growth

P. chlamydosporia radial growth on agar plates was found to be linear over time and decreased with the increase in osmotic water stress (Fig 2.1). Growth rates on 1.7% CMA, where water was freely available (-0.7 MPa), averaged 1.9 ± 0.5 mm/day, after 25 days at 25 °C. Colonies grown on this medium were white and became translucent with a sparse mycelium with time. All the five isolates were able to grow at -10 MPa in medium amended with the ionic solute KCl (Fig. 2.1b, d, f, h and j), whereas no growth was observed in medium amended with glycerol for this potential (Fig. 2.1a, c, e, g and i). In isolate Pc2, growth was also inhibited in medium amended with glycerol at -7.1 MPa (Fig. 2.1c). Analysis of variance revealed no significant differences between growth rates in the control and on 1.7% CMA after water stress at -10 MPa, for 25 days, in medium amended with glycerol (Fig. 2.2a).

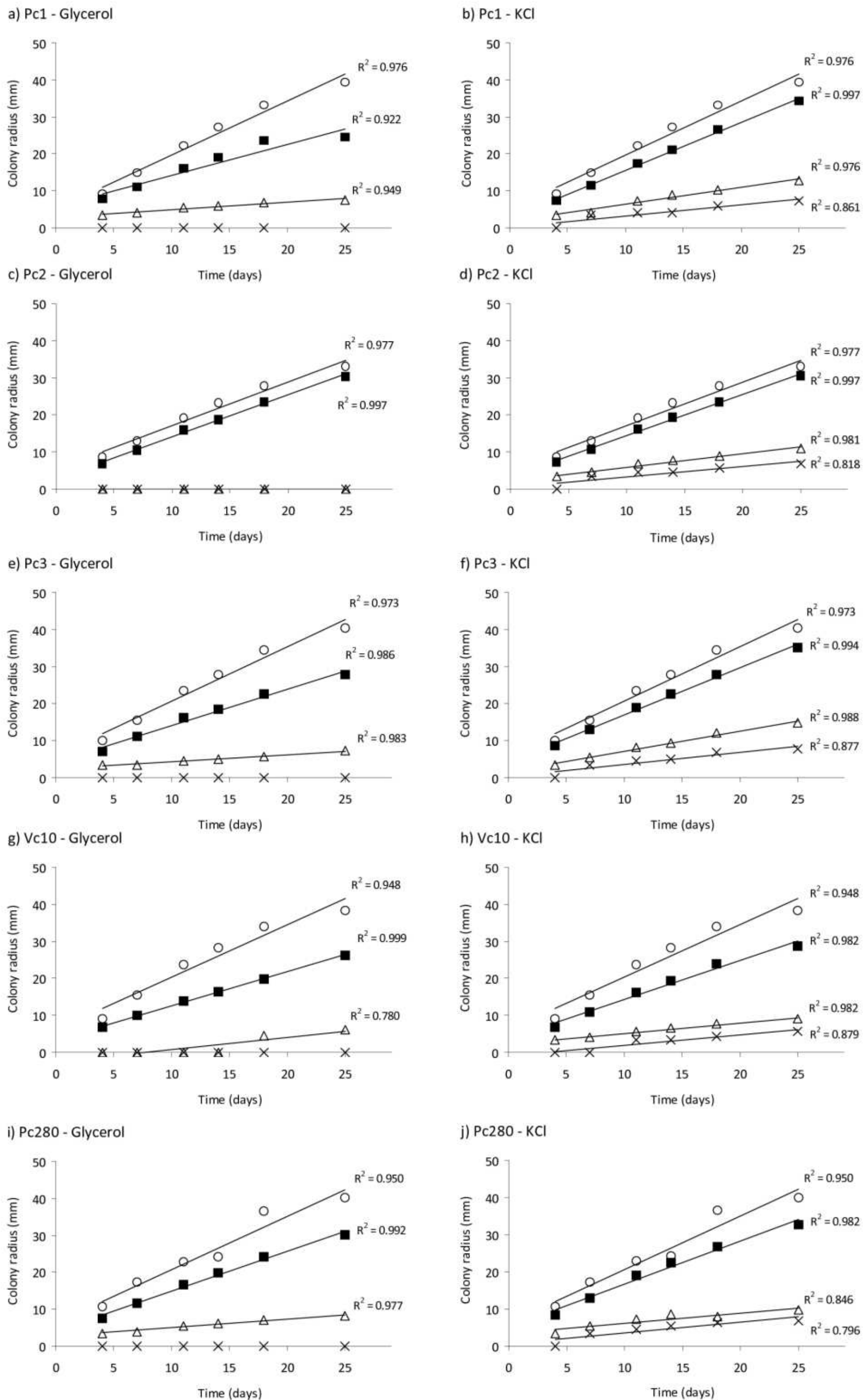

Figure 2.1 - Effect of osmotic potential, glycerol and potassium chloride (KCl) modified media, on the colony radial diameter (mm) over time (days) in five isolates of *Pochonia chlamydosporia*; Pc1 (a, b), Pc2 (c, d), Pc3 (e, f), Vc10 (g, h) and Pc280 (i, j), at 25 °C (x, -10 MPa; Δ, -7.1 MPa; ■, -2.8 MPa and ○, -0.7 MPa). Lines show the fitted regressions through the means. Bars represent standard error of means. Where the bars are not shown they were smaller than the symbol size.

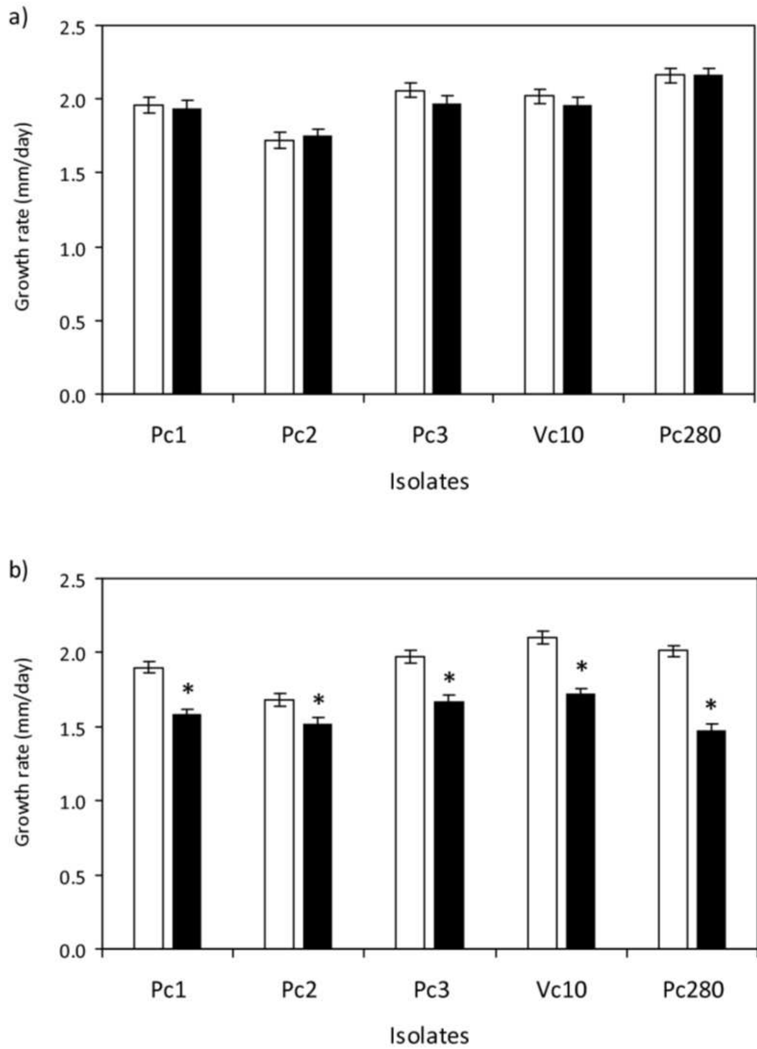

Figure 2.2 – Growth rates (mm/day) of *Pochonia chlamydosporia* isolates (Pc1, Pc2, Pc3, Vc10 and Pc280) grown on 1.7% corn meal agar (CMA) for 25 days: □, Control and ■, After water stress for 25 days: a) at -10 MPa, on medium amended with glycerol and b) at -7.1 MPa, on medium amended with PEG 8000. Bars represent standard error of means. * - significantly different according to Fisher LSD test (P<0.05).

Colonies grown on medium amended with PEG 8000 presented a white to ochre velvety and slender mycelium. The mean radial growth of colonies, in response to matric stress, increased linearly over time (Fig. 2.3). Growth was lower in medium modified with PEG at -1.4 and -2.8 MPa when compared to growth observed in control plates (-1.0 MPa) and none of the isolates were able to grow at -7.1 MPa (Fig. 2.3). Analysis of variance revealed that radial growth rates were significantly lower in all isolates, on 1.7% CMA after water stress at -7.1 MPa, for 25 days, than those observed in the control plates (Fig. 2.2b).

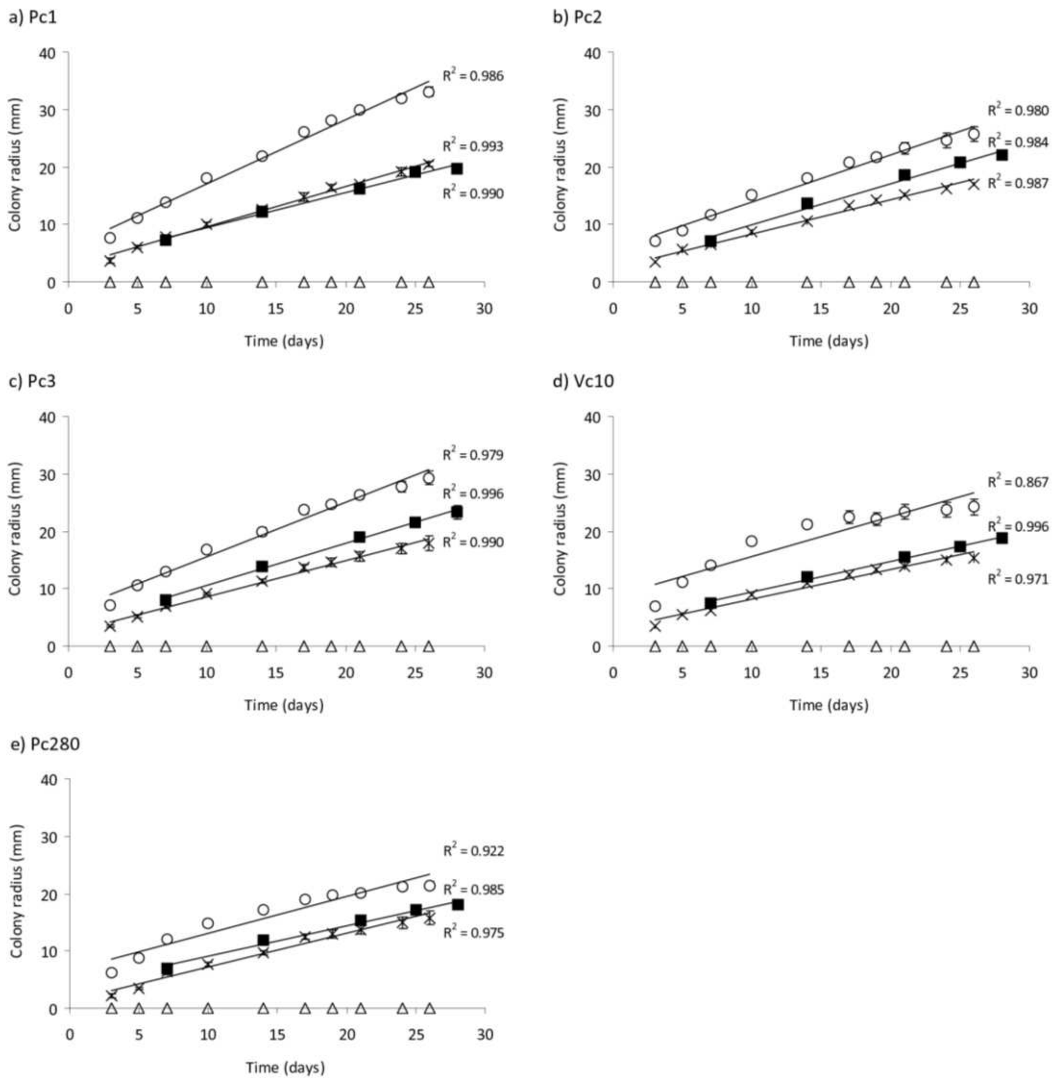

Figure 2.3 - Effect of matric water stress on the colony radial diameter (mm) over time (days) in five isolates of *Pochonia chlamydosporia*; Pc1 (a), Pc2 (b), Pc3 (c), Vc10 (d) and Pc280 (e), at 25 °C (Δ, -7.1 MPa; ✕, -1.4MPa; ■, -2.8 MPa and ○, -1.0 MPa). Lines show the fitted regressions through the means. Bars represent standard error of means. Where the bars are not shown they were smaller than the symbol size.

2.3.2. Effects of osmotic and matric potential on spore production

On plates where KCl was used to modify the osmotic potential of CMA, colonies presented a white and dense mycelium and the average number of chlamydospores produced per plate decreased with the increase in water stress, except in Pc2 grown at -2.8 MPa, in which there was no significant decrease in the numbers of chlamydospores produced (Table 2.1). In general, conidia numbers increased when isolates were grown

under osmotic water stress. Numbers of conidia were particularly increased in isolates Pc1, Pc2, Pc3 and Pc280 grown at -2.8 MPa. However, the viability of the conidia obtained in water stress medium was significantly lower than the control, except for isolate Vc10 (Table 2.1).

Table 2.1 – Number of chlamydospores, conidia and percentage of germinated conidia of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown on 1.7% corn meal agar (CMA) amended with potassium chloride at -0.7, -2.8, -7.1 and -10 MPa. [a)]

Isolate	Water potential (MPa)	Chlamydospores/cm^2 (10^3)	Conidia/cm^2(10^3)	Germinated conidia (%)
Pc1	-0.7	5.9 ± 0.8 d	32.4 ± 5.2 ijkl	89.7 ± 0.0 bcdefgh
	-2.8	0.0 ± 0.0 fg	499.9 ± 28.6 b	24.9 ± 2.0 klmn
	-7.1	0.0 ± 0.0 fg	96.7 ± 11.8 fghi	13.8 ± 1.0 mn
	-10.0	0.0 ± 0.0 fg	104.7 ± 16.7 fghi	31.9 ± 4.0 jklm
Pc2	-0.7	42.4 ± 4.9 ab	5.1 ± 0.9 lmn	96.0 ± 0.0 abcde
	-2.8	45.6 ± 6.1 ab	360.0 ± 65.4 c	49.7 ± 5.0 ijkl
	-7.1	1.0 ± 0.3 ef	222.2 ± 24.4 de	31.1 ± 1.0 jklm
	-10.0	0.0 ± 0.0 fg	144.0 ± 12.1 efg	76.1 ± 2.0 efghi
Pc3	-0.7	39.7 ± 2.2 ab	10.2 ± 0.9 klmn	90.8 ± 2.0 bcdefg
	-2.8	33.4 ± 2.8 b	662.2 ± 53.1 a	43.6 ± 3.0 ijklm
	-7.1	0.6 ± 0.3 efg	184.1 ± 37.7 defg	5.2 ± 3.0 o
	-10.0	0.0 ± 0.0 fg	204.0± 26.6 defg	37.1 ± 2.0 ijklm
Vc10	-0.7	5.3 ± 0.6 d	59.9± 4.2 ghijk	86.5 ± 6.0 cdefgh
	-2.8	0.0 ± 0.0 fg	104.5 ± 28.5 fghi	97.1 ± 2.0 abc
	-7.1	0.0 ± 0.0 fg	89.0 ± 30.0 ghij	94.6 ± 1.0 abcdef
	-10.0	0.2 ± 0.1 fg	137.4 ± 23.4 efg	95.3 ± 2.0 abcde
Pc280	-0.7	12.2 ± 1.7 c	9.6 ± 3.3 jklmn	90.8 ± 3.0 bcdefg
	-2.8	0.0 ± 0.0 fg	181.9 ± 27.2 defg	58.2 ± 3.0 hijk
	-7.1	0.0 ± 0.0 fg	48.7 ± 6.3 hijk	82.6 ± 4.0 defgh
	-10.0	0.0 ± 0.0 fg	27.7 ± 3.1 ijklm	79.8 ± 9.0 defgh

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column are not significantly different according to Fisher LSD test ($P<0.05$).

Mycelia from colonies, grown on medium amended with glycerol at -2.8 MPa, produced white deeply cottony and thick mycelia in which it was not possible to quantify or extract spores. At -7.1 MPa, only isolate Pc2 produced chlamydospores, but all isolates were able to produce conidia (Table 2.2). At this potential, the numbers of conidia produced per plate were significantly higher in isolates Pc2, Pc3 and Pc280 when compared to the control although their viability decreased with water stress (Table 2.2). When fungi did not grow out of the plugs on plates, containing glycerol modified-medium at -10 MPa, they were transferred onto fresh non-amended 1.7% CMA and colonies, with increased numbers of chlamydospores and conidia were produced, with the exception of isolates Pc1 and Pc280. For these, the conidial production decreased and no effect was observed on the viability of the spores (Fig. 2.4).

Table 2.2 - Number of chlamydospores, conidia and percentage of germinated conidia of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown on 1.7% corn meal agar (CMA) (-0.7 MPa) and 1.7% CMA amended with glycerol (-7.1 MPa).[a)]

Isolate	Water potential (MPa))	Chlamydospores/cm^2 (10^3)	Conidia/cm^2 (10^3)	Germinated conidia (%)
Pc1	-0.7	5.9 ± 0.8 d	32.4 ± 5.2 de	89.7 ± 0.0 bc
	-7.1	0.0 ± 0.0 e	16.9 ± 1.6 bc	3.6 ± 0.0 e
Pc2	-0.7	42.4 ± 4.9 b	5.1 ± 0.9 hi	96.0 ± 0.0 ab
	-7.1	128.7 ± 10.4 a	86.3 ± 4.4 def	20.1 ± 5.0 d
Pc3	-0.7	39.7 ± 2.2 b	10.2 ± 0.9 gh	90.8 ± 2.0 abc
	-7.1	0.0 ± 0.0 e	137.4 ± 25.9 ab	27.1 ± 3.0 d
Vc10	-0.7	5.3 ± 0.6 d	59.9 ± 4.2 bc	86.5 ± 6.0 bc
	-7.1	0.0 ± 0.0 e	21.2 ± 2.0 abc	4.8 ± 2.0 e
Pc280	-0.7	12.2 ± 1.7 c	9.6 ± 3.3 ghi	90.8 ± 3.0 abc
	-7.1	0.0 ± 0.0 e	95.7 ± 15.4 ef	4.5 ± 3.0 e

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column are not significantly different according to Fisher LSD test (P<0.05).

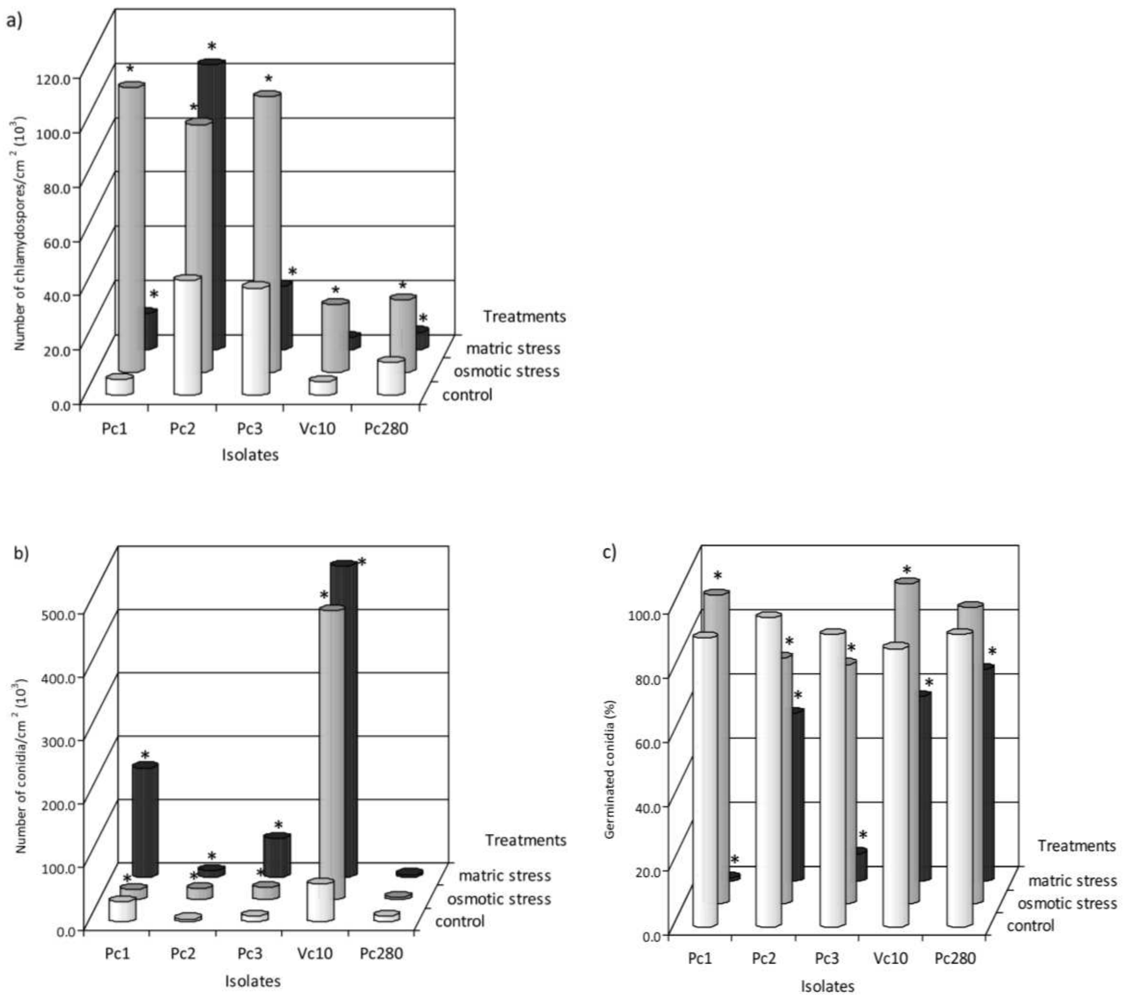

Figure 2.4 – Number of chlamydospores (a), conidia (b) and percentage of germinated conidia (c) of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for 25 days on 1.7% corn meal agar (CMA). □, Control; ■, After osmotic water stress for 25 days: at -10 MPa, on medium amended with glycerol; and ■, After matric water stress for 25 days at -7.1 MPa, on medium amended with PEG 8000. Bars represent standard error of means. *-significantly different to control according to Fisher LSD test (P<0.05).

Few or no chlamydospores (Pc3 and Vc10) were produced at -1.0 MPa in PEG 8000 modified medium (Table 2.3). The numbers of chlamydospores produced per plate decreased significantly for all isolates with the decrease in water potential (Table 2.3). Numbers of conidia increased at -1.0 and -1.4 MPa but decreased at -2.8 MPa. Although the

percentage of germinated conidia was significantly lower at -1.4 MPa it increased significantly in all isolates at –2.8 MPa (Table 2.3).

Table 2.3 - Number of chlamydospores, conidia and percentage of germinated conidia of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown on 1.7% corn meal agar (CMA) and 1.7% CMA amended with PEG 8000 (-1,0, -1.4 and -2.8 MPa).[a)]

Isolate	Water potential (MPa)	Chlamydospores/cm^2 (10^3)	Conidia/cm^2 (10^3)	Germinated conidia (%)
Pc1	-0.7	5.9 ± 0.8 cd	32.4 ± 5.2 fghijk	89.7 ± 0.0 a
	-1.0	0.8 ± 0.3 fghi	901.3 ± 84.4 ab	31.4 ± 2.0 d
	-1.4	0.6 ± 0.3 fghij	233.2 ± 28.9 cd	85.0 ± 5.0 ab
	-2.8	1.2 ± 0.5 fgh	89.4 ± 11.2 ef	77.0 ± 13.0 ab
Pc2	-0.7	42.4 ± 4.9 a	5.1 ± 0.9 mno	96.0 ± 0.0 a
	-1.0	10.6 ± 2.0 b	766.1 ± 24.1 abc	78.3 ± 3.0 ab
	-1.4	0.6 ± 0.3 fghij	86.3 ± 19.0 efg	50.5 ± 1.0 c
	-2.8	1.4 ± 0.6 fgh	44.4 ± 1.9 efghi	95.0 ± 2.0 a
Pc3	-0.7	39.7 ± 2.2 a	10.2 ± 0.9 jklmno	90.8 ± 2.0 a
	-1.0	0.0 ± 0.0 hij	870.4 ± 179.7 abc	16.6 ± 7.0 f
	-1.4	0.0 ± 0.0 hij	296.7 ± 81.3 cd	21.9 ± 11.0 de
	-2.8	0.2 ± 0.0 ghij	16.1 ± 6.6 ijklmn	97.0 ± 1.0 a
Vc10	-0.7	5.3 ± 0.6 cde	59.9 ± 4.2 efg	86.5 ± 6.0 ab
	-1.0	0.0 ± 0.0 hij	527.6 ± 183.1 bcd	27.4 ± 3.0 de
	-1.4	0.6 ± 0.2 fghi	32.0 ± 12.3 ghijk	19.1 ± 5.0 f
	-2.8	0.0 ± 0.0 hij	1.0 ± 0.6 p	90.0 ± 2.0 a
Pc280	-0.7	12.2 ± 1.7 b	9.6 ± 3.3 klmno	90.8 ± 3.0 a
	-1.0	3.3 ± 0.2 de	255.8 ± 46.5 cd	18.4 ± 1.0 f
	-1.4	0.0 ± 0.0 hij	21.0 ± 3.1 hijklm	4.0 ± 0.0 g
	-2.8	0.0 ± 0.0 hij	20.0 ± 7.9 hijklmn	87.0 ± 6.0 ab

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column are not significantly different according to Fisher LSD test (P<0.05).

Plugs inoculated onto PEG modified-medium at -7.1 MPa, and later transferred onto non-amended 1.7% CMA, produced greater numbers of chlamydospores in isolates Pc1 and Pc2 when compared to non-amended 1.7% CMA (Fig. 2.4a). Conidia numbers increased in all isolates except Pc280. Isolate Pc2 produced the most numbers of chlamydospores whereas Vc10 produced the least. However, Vc10 produced the highest numbers of conidia in both situations (Fig. 2.4b). The viability of the spores of all isolates was significantly decreased

after exposure to water potentials limiting for growth when compared to the control (Fig. 2.4c).

2.3.3. Effects of osmotic and matric potential on nematode egg parasitism

Viable conidia, obtained from colonies grown under osmotic water stress, were able to parasitize nematode eggs, although the percentage of egg parasitism was less than 22% in all isolates. A decrease on egg parasitism was observed when compared with control plates (-0.7 MPa), except for isolate Vc10 on KCl modified medium (Table 2.4).

Table 2.4 – Parasitism of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown on 1.7% corn meal agar amended with potassium chloride at -0.7, -2.8, -7.1 and -10 MPa against *Globodera rostochiensis* eggs. [a)]

Isolate	Water potential (MPa)	Parasitized eggs (%)	
Pc1	-0.7	15.7 ± 4.0	abcdefgh
	-2.8	5.8 ± 5.0	fghijkl
	-7.1	5.9 ± 1.0	efghijkl
	-10.0	1.7 ± 1.0	hijkl
Pc2	-0.7	20.5 ± 1.0	abcde
	-2.8	6.5 ± 1.0	efghijkl
	-7.1	6.8 ± 1.0	efghijkl
	-10.0	6.7 ± 2.0	efghijkl
Pc3	-0.7	15.1 ± 5.0	abcdefgh
	-2.8	9.9 ± 1.0	cdefghijk
	-7.1	13.1 ± 1.0	abcdefghi
	-10.0	12.8 ± 3.0	bcdefghij
Vc10	-0.7	16.8 ± 1.0	abcdefg
	-2.8	4.2 ± 1.0	ghijkl
	-7.1	12.2 ± 1.0	bcdefghij
	-10.0	18.9 ± 5.0	abcdef
Pc280	-0.7	9.7 ± 1.0	cdefghijk
	-2.8	5.7 ± 2.0	fghijkl
	-7.1	4.8 ± 1.0	ghijkl
	-10.0	9.1 ± 2.0	defghijk

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column are not significantly different according to Fisher LSD test ($P<0.05$).

A significant increase on the percentage of egg parasitism was observed in all isolates which had been previously inoculated onto media with water potentials limiting for growth (-10 MPa, glycerol and -7.1 MPa, PEG 8000), except Pc2 and Pc3, in which a significant decrease on egg parasitism was detected after osmotic stress when compared to the control (-0.7 MPa) (Table 2.5).

Table 2.5 – Parasitism of five isolates of *Pochonia chlamydosporia* (Pc1, Pc2, Pc3, Vc10 and Pc280) grown for 25 days on 1.7% corn meal agar (CMA), after 25 days on 1.7% CMA amended with glycerol or with polyethylene glycol (PEG 8000) against *Globodera rostochiensis* eggs. [a)]

Isolate		Parasitized eggs (%)	
Pc1	Control[b)]	15.7 ± 4.0	fgh
	Osmotic stress[c)]	29.4 ± 3.0	de
	Matric stress[d)]	42.5 ± 1.0	c
Pc2	Control[b)]	20.5 ± 5.0	efg
	Osmotic stress[c)]	12.0 ± 1.0	ghi
	Matric stress[d)]	28.1 ± 1.0	def
Pc3	Control[b)]	15.1 ± 5.0	fgh
	Osmotic stress[c)]	5.4 ± 2.0	hi
	Matric stress[d)]	53.1 ± 2.0	b
Vc10	Control[b)]	16.8 ± 5.0	fgh
	Osmotic stress[c)]	20.9 ± 0.0	efg
	Matric stress[d)]	64.9 ± 0.0	a
Pc280	Control[b)]	9.7 ± 5.0	ghi
	Osmotic stress[c)]	25.6 ± 7.0	def
	Matric stress[d)]	41.8 ± 0.0	c

[a)] Values are means of six replicates ± standard error. Means followed by the same letter within a column are not significantly different according to Fisher LSD test (P<0.05).
[b)] Control – grown for 25 days on 1.7% CMA (-0.7 MPa).
[c)] Osmotic stress – grown for 25 days on 1.7% CMA (-0.7MPa) after 25 days on 1.7% CMA amended with glycerol (-10MPa).
[d)] Matric stress – grown for 25 days on 1.7% CMA (-0.7MPa) after 25 days on 1.7% CMA amended with PEG 8000 (-7.1MPa).

2.4. Discussion

Plant-parasitic nematodes are a major cause of crop disease and yield loss all over the world leading to an increasing demand of alternative, non-chemical, sustainable management strategies for control (Trudgill and Blok, 2001). Along with the key intrinsic biological factors, considered important for the use of *P. chlamydosporia* as a biological control agent (ability to parasitize nematode eggs, to colonize the root surface, and chlamydospore production), their modulation by abiotic factors such as water availability can play an important role in establishing a fungus with biocontrol potential in natural environments (Bourne and Kerry, 2000; Magan, 2001; Abrantes et al., 2002).

In this study, the impact of water stress on sporulation, germination and parasitism was evaluated for the first time in isolates of *P*. *chlamydosporia*. As found in earlier studies, the fungus was able to grow over a range of potentials; radial growth rates decreased with the increase of osmotic or matric stress and isolates were more tolerant to the ionic solute KCl than to glycerol or PEG 8000. A slight non-significant increase in the growth rate was observed in isolates Pc2 and Pc3 at -2.8 MPa in PEG 8000 medium and has been previously reported for this fungus (Kerry et al., 1986; Olivares-Bernabeu and Lopez-Llorca, 2002; Esteves et al., 2009). In soil and crop residues, matric potential is the major component of total water potential (Magan and Lynch, 1986) and, therefore, matric stress is thought to have a greater effect on fungal growth in soil and spore germination than osmotic stress (Brownell and Schneider, 1985). In *in vitro* experiments using sodium chloride to modify soil water activity, the fungus was able to survive water potentials of -9.7 MPa at temperatures of 25-30 °C (Bourne and Kerry, 2000). When using 30% Sabouraud dextrose agar with increased content in PEG, limiting growth potentials were about -16.5 MPa (Olivares-Bernabeu and Lopez-Llorca, 2002). In the present study, no growth was observed at -10 and -7.1 MPa, in medium modified with glycerol and PEG 8000, respectively, showing that water stress effects depend on solute and nutrient availability. After being submitted to growth-limiting water potentials for 25 days, all isolates were able to grow when later transferred onto 1.7% CMA, where water was not restricted. These results revealed that *P*. *chlamydosporia* spores/hyphal fragments can remain viable at water potentials limiting for growth, for prolonged periods of time. The ability to grow under a range of water potentials and withstand relatively long-term osmotic shocks may be an advantage when competing

with other soil organisms, but further experiments need to be conducted in soil, in order to validate this hypothesis.

Apart from changes in growth, the quantity and viability of spores produced under water stress also varied. The production of chlamydospores was severely repressed in both osmotic and matric modified medium, except for isolate Pc2 under osmotic stress conditions. Previous studies with this isolate showed that it has the ability to produce a higher number of chlamydospores in solid media compared to the other isolates used in this study (Vieira dos Santos et al., submitted). High variability in chlamydospore production has been found among isolates, one of the key features in the screening of isolates with biological control potential (Abrantes et al., 2002). However, as chlamydospores act as survival stages in soil and contain reserves that allow the fungus to survive in hostile environments, their production was expected to increase under water stress conditions. Studies on growth and sporulation with 1.7% CMA proved that isolates produce few chlamydospores in the standard medium (Kerry et al., 1986), which may explain the low numbers of chlamydospore recovered under osmotic stress conditions in 1.7% CMA amended with KCl. Colonies grown on medium amended with PEG 8000 presented a white to ochre velvety and slender mycelium with large hyphal development and few spores. It is unknown whether this indicates a strong investment in hyphal growth, due to more favourable conditions, or an inhibition of sporulation in this modified medium. It has been suggested that fungi may be able to metabolise PEG when growing in media amended with low amounts of this compound (Olivares-Bernabeu and Lopez-Llorca, 2002). While it is unlikely that the fungus could use PEG 8000 as a food source, growth may have been increased in the presence of the added peptone and glucose. This also attests, to the earlier indication, that 1.7 % CMA is a limiting medium for fungal vegetative growth encouraging spore production instead (Smith and Onions, 1994).

The production of conidia increased in medium modified with KCl particularly on the Portuguese isolates at -2.8 MPa. Low concentrations of ionic solutes, such as KCl, may stimulate growth in some fungi (Larsen, 1986), as K ions aid transport across the mycelial cell walls to enable better intracellular osmotic adjustment. Consequently, it is possible that KCl from the medium may have been used to enhance conidial sporulation. Conidial viability

was, in general, lower in water restricted than in unmodified medium, except for isolate Vc10.

Colonies in plates containing PEG produced less conidia than the control (-1.0 MPa) and spore viability either decreased or increased, depending on the isolate, although all revealed a similar percentage of viable conidia at -2.8 MPa. The formation of appressoria was not observed in any isolate, probably due to the length of observation time (7 h). Formation of appressoria *in vitro* has been observed in experiments after 20-24h. However, long incubation times allowed conidial germination of all isolates tested (Esteves, unpublished data; Lopez-Llorca et al., 2002).

The methodology used in this study to assess conidia germination was useful to assess differences among isolates. It is apparent, for instance, that Vc10 copes better than the other isolates to water stress and this could either reflect a genetic variation that allows this isolate to adapt to water stress putatively present in its original conditions or it could simply be indicative of a higher phenotypic plasticity in this isolate. Variation among isolates was expected since isolates can differ in terms of morphology and at the molecular level, and also in their biocontrol potential (Irving and Kerry, 1986; Kerry et al., 1986; Zare et al., 2001). Thus, it is possible that some isolates are more likely to change or adapt to certain culture conditions than others. Much variation has also been found within *P. chlamydosporia* populations at a given site (Hidalgo-Díaz et al., 2000; Morton et al., 2003a; Flores-Camacho et al., 2008). Molecular studies on *P. chlamydosporia*, to discriminate isolates from different geographical regions and nematode hosts, confirmed that isolates can be grouped according to both their geographical and the host nematode origin (Morton et al., 2003b). The isolates used in this study have been collected in different geographical origins and parasitizing different hosts. However, conclusions cannot be drawn on the effects of their original conditions on the encountered variations in response to water stress. Also, to our knowledge, there is no data available on the collection sites regarding water availability and on the amplitude of its variation throughout the year.

Restricting the water availability to *P*. *chlamydosporia*, by increasing the amounts of KCl in the medium, affected the percentage of parasitized eggs (Table 2.4). The percentage of parasitized eggs was low in all treatments including the controls (<20%). This low percentage of egg infection, obtained through the standard bioassay, was also observed by other

authors. The mucilage surrounding the eggs may be an important source of nutrition for the fungus that ensures a high rate of egg colonisation (Irving and Kerry, 1986). This mucilage may have been altered after re-suspending the eggs in distilled water before plating, which could explain the low levels of egg parasitism observed. In non-restricting conditions, growth of fungi out of plugs, which had been previously inoculated onto plates with water potentials limiting for germination (-10 MPa and -7.1 MPa, glycerol and PEG 8000, respectively), produced colonies with significantly larger numbers of chlamydospores and the percentage of parasitism increased for the majority of the isolates (Tables 2.2, 2.3 and 2.5 and Fig.2.4).

The results obtained suggest that the osmoregulation mechanisms, used to compensate osmotic and matric stresses, may have affected the pathways of sporulation and pathogenicity to a certain extent. Although low levels of osmotic and matric water stress did not have a clear effect on virulence, it is possible that exposure to extreme stress conditions may affect the expression of genes that regulate parasitism and other genes responsible for growth and sporulation. Recent studies on the transcriptome analysis of *P. chlamydosporia* have revealed specific metabolic pathways for parasitic behaviour. The expression of certain genes were activated only in the presence of nematode eggs, but the analysis of expression profiles of *P. chlamydosporia*, using a cDNA-amplified fragment length polymorphism approach, proved that genes expressed during egg parasitism are involved in metabolic functions, cellular signal expression and DNA repair, among others (Rosso et al., 2011). Further molecular work should investigate the role of abiotic factors, such as water and temperature, in the expression genes important in parasitism and saprophytic growth. Moreover, the effects of water stress in the production of certain *P. chlamydosporia* secondary metabolites, which may be important in infection, such as monorden (syn. radicicol) and aurovertin-type metabolites (Leinhos and Buchenauer, 1992; Khambay et al., 2000; Stadler et al., 2003; Niu et al., 2010), should also be investigated, as mycotoxins produced by several fungi are known to be affected by changes in water potential (Jurado et al., 2008; Marín et al., 2010).

This study provided new information about the influence of water stress culture conditions on sporulation as well as its potential effects on fungus parasitic ability. Knowledge on water requirements enable a better understanding of the survival and growth strategy employed by *P. chlamydosporia* isolates in natural environments and could lead to

the development of effective strategies in order to increase the production and quality of inoculum, contributing to the implementation of biosafe sustainable management strategies against plant-parasitic nematodes.

2.5. Acknowledgements

This research was supported by FEDER funds through the "Programa Operacional Fatores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the Project PTDC/AGR-AAM/74574/2006. The first author was supported by ESF funds through the "Programa Operacional Potencial Humano - POPH" of "The National Strategic Reference Framework" (SFRH/BD/27317/2006).

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Chapter 3

The nematophagous fungus, Pochonia chlamydosporia, and Meloidogyne chitwoodi interactions in a crop rotation scheme

Written as a journal article:

Vieira dos Santos, M.C., Esteves, I., Kerry, B. and Abrantes, I. (2012). The nematophagous fungus, *Pochonia chlamydosporia*, and *Meloidogyne chitwoodi* interactions in a crop rotation scheme. Phytopathologia Mediterranea (*submitted*).

Abstract - The potential of *Pochonia chlamydosporia* isolates as biological control agents against root-knot nematodes (RKN) *Meloidogyne* spp. has been widely studied and their efficacy relies on selected key factors such as proliferation in soil, establishment in the rhizosphere, and ability to parasitize RKN eggs. A pot experiment was conducted to evaluate the performance of a Portuguese *P. chlamydosporia* isolate (Pc2) and of a non-native isolate (Vc10) against *M. chitwoodi* in a potato-maize-potato-potato crop rotation. During spring and summer 2009, pots were placed under shading net in a field located in Coimbra, Portugal. In the winter, due to the low temperatures and high rainfall (unsuitable for the potato crop), pots were moved indoors to a shelter without controlled temperature. The growth of *P. chlamydosporia* in soil and roots was monitored throughout the experiment by assessing the numbers of colony forming units (cfu). The percentage of *M. chitwoodi* infected eggs, nematode reproduction and the numbers of non-target nematodes were measured at the end of each crop. The numbers of cfu/g of soil varied over time and were particularly low for both isolates during the spring and summer 2009, when high temperatures were registered. However, *P. chlamydosporia* spores remained viable in soil until the next inoculation. The isolate Pc2 was more effective in establishing in soil than Vc10. The increased population density of Pc2 in soil could be related with an increase in rhizosphere colonization. Growth and survival of the fungus did not seem to depend on the presence of nematode or the host plant species. Establishment of *P. chlamydosporia* in soil was slow and could only be achieved after the full crop cycle, affecting then nematode parasitism. Differences in rhizosphere colonization by both isolates in the presence or absence of the nematode pointed to nematode-host preferences. *Meloidogyne chitwoodi* was not detected in the roots of maize. Abiotic conditions such as high temperatures may have led to the decline of soil biota, including nematodes and the fungus. The two *P. chlamydosporia* isolates revealed subtle differences in host preference, plant compatibility or tolerance to abiotic conditions that may cause large discrepancies in their efficacy as control agents in a long-lasting management programme. Additionally, factors affecting establishment, performance and biocontrol efficacy in field-like conditions need to be further studied.

Key words: biological control, integrated pest management, potato, rhizosphere colonization, root-knot nematodes

3.1. Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are ubiquitous plant-parasitic pests that affect a wide range of crops. In addition to direct root damage, these gall-forming sedentary endoparasites elicit infection by secondary pathogens such as fungi and bacteria, thus acting as a major cause of preventable crop disease and yield loss (Nicol et al., 2011). Among RKN, *M. chitwoodi* is a quarantine organism recognized by its effects on potato. This nematode has been reported from Argentina, Belgium, Germany, Netherlands, USA, Mexico, Portugal and South Africa (OEPP/EPPO, 2009). Management practices include the use of highly toxic nematicides and their potential negative impact on the environment led to a total ban or restricted use of most chemical nematicides (Chitwood, 2003). There is an urgent need for safe and more effective alternatives and fungal antagonists are among the most promising nematode biological control agents (Stirling, 1991).

Pochonia chlamydosporia (Goddard) Gams and Zare is a facultative nematophagous fungus, with parasitic activity against eggs and sedentary females of economically important plant-parasitic nematodes. It has been associated with soils that suppress the multiplication of cyst nematode populations (Kerry and Crump, 1977; Kerry et al., 1982). The biocontrol potential of *P. chlamydosporia* has been widely studied in pots and field experiments. Although the efficacy of selected isolates has been demonstrated, it may not be consistently achieved, as the fungus may fail to establish in the soil (Bourne and Kerry, 1999; Atkins et al., 2003b; Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003; Tobin et al., 2008; Tzortzakakis, 2000, 2007, 2009; Puertas and Hidalgo-Díaz, 2009). This fungus is a relatively poor competitor in soil and the ability to produce chlamydospores, rich in endogenous nutrient reserves, represent an important survival strategy. Furthermore, isolates of *P. chlamydosporia* differ in their ability to grow in soil and tend to be more abundant in organic than in mineral soils (De Leij et al., 1993; Kerry et al., 1993; Bourne and Kerry, 2000).

In the rhizosphere, *P. chlamydosporia* seems to be able to proliferate by using nutrients released in root exudates. In addition to the high variability in rhizosphere colonization among isolates, their pathogenicity can also differ significantly, and the outcome of these variations in the regulation of nematode populations is not fully understood. Nonetheless, a number of key factors that influence the plant-nematode-fungus interactions and affect *P. chlamydosporia* activity as a biological control agent have been identified (Abrantes et al.,

2002; Kerry and Hirsch, 2011). The fungal ability to colonize the rhizosphere, rather than the ability to proliferate in soil, significantly affects its impact on nematode reproduction (De Leij and Kerry, 1991; Bourne et al., 1994; 1996; Kerry and Bourne, 1996). Rhizosphere colonization is essential for nematode control and is mediated by the plant species since they differ in their ability to support the fungus (Bourne et al., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999). When the colonizing *P. chlamydosporia* contacts a nematode egg mass in the rhizosphere, the fungus ceases its saprophytic stage and switches to a parasitic stage. The factors that affect this switch are not well understood, but nematode host preference at the infra-specific level may be involved (Kerry, 2000; Mauchline et al., 2004). Fungal abundance is not always related to the extent of parasitism of nematode eggs, although isolates of *P. chlamydosporia* were more abundant on nematode infected than healthy roots (Bourne et al., 1996; Bourne and Kerry, 1999; Atkins et al., 2003b).

Even though, *P. chlamydosporia* does not prevent the initial infestation of roots by nematode juveniles, it affects nematode reproduction. Thus, the biocontrol efficacy should be enhanced by integration with control measures that prevent initial infestations, such as crop rotation with poor hosts for the nematode (Kerry and Bourne, 1996), a frequent traditional control method that prevents, to some extent, the build-up of large plant parasitic nematode populations. A successful combination of crop rotation with the application of *P. chlamydosporia* could lead to an environmental friendly sustainable RKN management. Therefore, understanding how a crop rotation sequence can affect the establishment and biological control efficacy of isolates of the fungus is of key importance. The objective of this study was to evaluate the performance of two *P. chlamydosporia* isolates, from different nematode hosts, on the multiplication of *M. chitwoodi* and survival in soil in a crop rotation including potato-maize-potato-potato in a pot experiment, in conditions similar to those observed in the field.

3.2. Material and Methods

3.2.1. *Meloidogyne chitwoodi* isolate

An isolate of the RKN *M. chitwoodi*, obtained from infected potato tubers collected in Porto, Portugal (Conceição et al., 2009), was maintained on susceptible host tomato plants

cv. Easypeel, in pots with sterilised sandy loam soil and sand (1:1). Eggs were extracted from infected roots using 0.52% sodium hypochlorite (Hussey and Barker, 1973).

3.2.2. *Pochonia chlamydosporia* isolates

Two isolates of *P. chlamydosporia* (Pc2 and Vc10) were used in this study: Pc2 was isolated from *Globodera rostochiensis* eggs extracted from cysts obtained from soil samples collected in Portuguese potato fields; and Vc10, originally isolated from *M. incognita* eggs (Brazil) was kindly supplied by Rothamsted Research, UK. Both isolates were maintained in 1.7 % corn meal agar (CMA) (Oxoid, UK), at 25 °C, and their identities were checked using specific diagnostic primers derived from the ß-tubulin gene (Hirsch et al., 2000), and confirmed to be *P. chlamydosporia* using PCR (data not shown). Chlamydospores were produced using a barley:sand substrate (1:1) and extracted following the method described by De Leij and Kerry (1991). Chlamydospore viability and germination were assessed on sorbose agar with antibiotics (Abrantes et al. 2002).

3.2.3. Plant material

Potato, *Solanum tuberosum*, cv. Désirée plants were grown from a single sprout per pot. Maize, *Zea mays*, cv. Belgrano plants were grown from seeds, in plastic containers, for three weeks, in a greenhouse at 25 °C and a 16 h photoperiod, before being transplanted to the pots. The host suitability of maize cv. Belgrano to *M. chitwoodi*, under greenhouse conditions (25 °C, 16 h photoperiod), was assessed 60 days after inoculation with 5000 eggs on the basis of gall index and reproduction factor (Rf), being considered as a susceptible host (Rf = 10.8±1.2) (data not shown).

3.2.4. Experimental design

Pots (5 l) were filled with non-sterilized sandy soil collected from a field in Carapinheira, Coimbra, Portugal, where potato had been the primary crop for a number of years. The soil was previously screened, using a modified Whitehead and Hemming method (Abrantes et al., 1976), to confirm the absence of RKN nematodes. The use of a soil dilution plating technique on a semi selective medium (De Leij and Kerry, 1991) confirmed the absence of *P. chlamydosporia*. Each pot was partially buried in a larger pot (40 l) containing sand to

avoid rapid temperature fluctuations. Pots were placed under shading net in a field located in “Escola Superior Agrária” of Coimbra, Portugal, during spring and summer 2009. In the winter, due to the low temperatures and high rainfall in the region, unsuitable for the potato crop, pots were transferred to a shelter without controlled temperature. The experiment was conducted from April 2009 to May 2010 and included the following treatments: *P. chlamydosporia* + *M. chitwoodi* + plant; *P. chlamydosporia* + no nematode + plant; *P. chlamydosporia* + no nematode + no plant. Controls were made for each treatment without the application of the fungus. Fungal inoculum was applied three times at a rate of: 5000 chlamydospores/g of soil at the time of planting the first crop (potato); 2500 chlamydospores/g of soil at the time of planting the second crop (maize); and 5000 chlamydospores/g of soil at the time of planting the third crop (potato). One week after the first inoculation, the pots were inoculated with 4000 *M. chitwoodi* eggs (Pi). Five replicates of each treatment were set up using a completely randomised design and the pots were watered as needed.

3.2.5. *Pochonia chlamydosporia* survival, proliferation and parasitism

In order to monitor the survival and proliferation of the fungus in the soil, samples were collected from each pot at the beginning of each crop, one week after planting, then every four weeks, and at harvest. The number cfu/g of soil was assessed by dilution plating (De Leij and Kerry, 1991). Root samples were collected at harvest and processed to estimate the number of cfu/cm of root by a similar dilution plating (De Leij and Kerry, 1991). Egg parasitism was assessed by plating *M. chitwoodi* eggs (ca. 200 eggs/plate), extracted from infected roots, on 0.8 % technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg/l of each) for three days, at 25 °C. The number of parasitized eggs was counted using standard methods (Kerry and Crump, 1977).

3.2.6. *Meloidogyne chitwoodi* reproduction

At the end of each crop, roots were collected to evaluate the nematode reproduction. The numbers of galls and egg masses per root system were counted and eggs, extracted from each root system using 0.52% sodium hypochlorite (Hussey and Barker, 1973), were counted to estimate the final population density (Pf). The numbers of eggs/egg mass and

eggs/g of root were calculated for each plant. Also, population densities in soil were assessed by counting the number of J2 extracted from soil samples (100 cm^3) collected at harvest using a modified Whitehead and Hemming tray method (Abrantes et al., 1976).

3.2.7. Nematode diversity in soil

Nematode diversity in soil was assessed in the samples (100 cm^3) collected from each pot before planting the first crop and at harvest of each crop after extraction using a modified Whitehead and Hemming tray method (Abrantes et al., 1976). Nematodes were identified on the basis of their morphology and quantified.

3.2.8. Data analysis

Data on cfu/g of soil, cfu/cm^2 of root, egg parasitism and nematode reproduction were compared by ANOVA using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA). A square root transformation was used when needed to ensure a Normal distribution and constant variance of the data that, otherwise, would not meet the ANOVA assumptions. Statistically significant differences between treatments were computed using the LSD test ($P<0.05$).

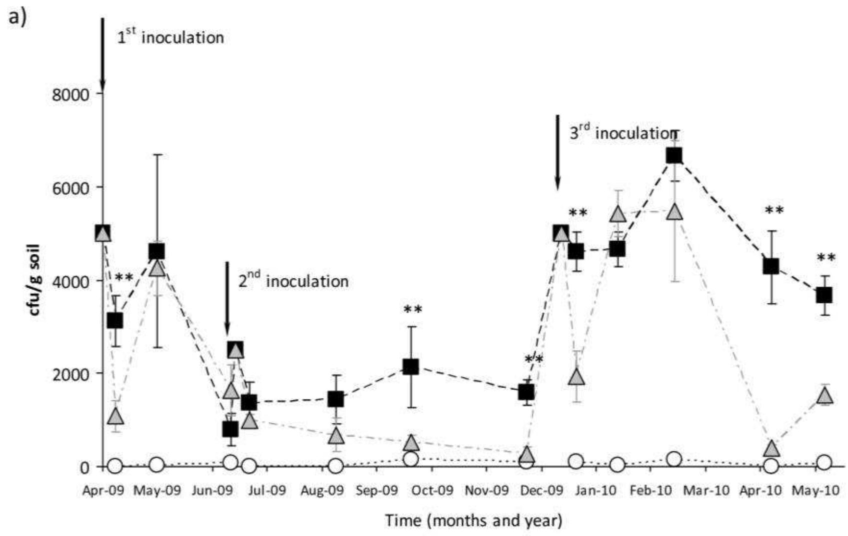
3.3. Results

3.3.1. *Pochonia chlamydosporia* survival, proliferation and parasitism

The numbers of cfu found in soil varied over time, and were particularly low between the second and third inoculations for both isolates, though higher for Pc2. However, *P. chlamydosporia* spores remained viable in soil between inoculations and survived during the high temperatures registered in the spring and summer 2009 (Fig. 3.1). No significant differences were observed one week after each inoculation and after harvest of the first crop in any of the isolates, except after the first and third inoculations, where the number of cfu/g of soil of Pc2 was significantly higher in pots inoculated with *M. chitwoodi* (Fig. 3.1a). During the second crop, although differences were detected between Pc2 and Vc10 (Fig. 3.1a, b and c), the growth and survival of the fungus did not seem to be influenced by the presence of nematodes or by the plant host. After the third inoculation, both isolates were able to grow above the initial fungal inoculation rate (5000 chlamydospores/g soil) in all treatments. After

planting of the last potato crop (March 2010), there was a decrease in the numbers of cfu/g soil, which was significantly sharper for isolate Vc10 in the presence of the nematode (Fig. 3.1a). Proliferation in soil was similar in all treatments. High variability was observed among replicates of the same treatment and, in some pots, the isolates could not be detected. Furthermore, the fungus was randomly detected over time in some pots of the control non-inoculated with *P. chlamydosporia*, although in very low numbers (< 150 cfu/g).

Figure 3.1 (next page) - Numbers of colony forming units (cfus) of *Pochonia chlamydosporia* Pc2 (■) and Vc10 (▲) isolates and of control (no fungus) (O) in soil in pot experiments in a crop rotation (potato cv. Désirée, maize cv. Belgrano, potato, potato). Treatments: a) *P. chlamydosporia* + *M. chitwoodi* + plant; b) *P. chlamydosporia* + no nematode + plant; and c) *P. chlamydosporia* + no nematode + no plant. Fungus was applied at a rate of 5000 chlamydospores/g of soil in the first and third crops and 2500 chlamydospores/g at the second crop. Bars represent standard error of means. Where the bars are not shown they were smaller than the symbol size. Arrows show dates of fungal inoculum application. * and ** significantly different according to Fisher LSD test (P<0.05 and P<0.01, respectively).


a)
1st inoculation
2nd inoculation
3rd inoculation
cfu/g soil
8000
6000
4000
2000
0
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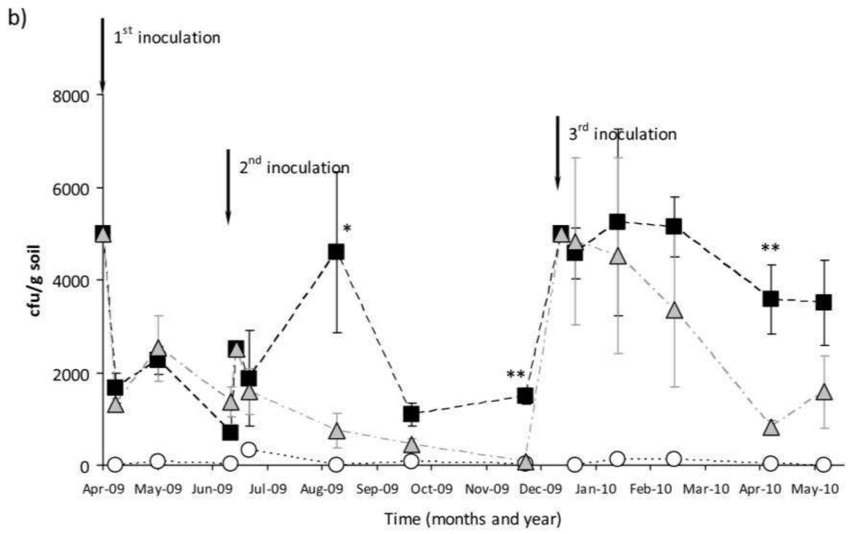
Apr-09 May-09 Jun-09 Jul-09 Aug-09 Sep-09 Oct-09 Nov-09 Dec-09 Jan-10 Feb-10 Mar-10 Apr-10 May-10
Time (months and year)


b)
1st inoculation
2nd inoculation
3rd inoculation
cfu/g soil
8000
6000
4000
2000
0
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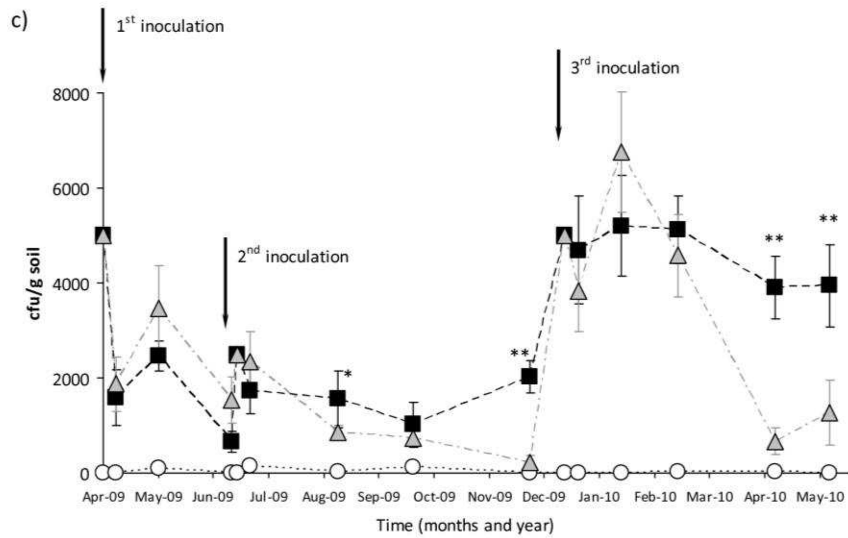
Apr-09 May-09 Jun-09 Jul-09 Aug-09 Sep-09 Oct-09 Nov-09 Dec-09 Jan-10 Feb-10 Mar-10 Apr-10 May-10
Time (months and year)


c)
1st inoculation
2nd inoculation
3rd inoculation
cfu/g soil
8000
6000
4000
2000
0
*
**
**
**
Apr-09 May-09 Jun-09 Jul-09 Aug-09 Sep-09 Oct-09 Nov-09 Dec-09 Jan-10 Feb-10 Mar-10 Apr-10 May-10
Time (months and year)

Similar results were also observed in the numbers of cfu/cm^2 root: low numbers of cfus were recovered from roots; a high variability among pots of the same treatment was detected; and in some roots, the fungus was not found. In the last two potato crops, Pc2 was present in higher rates than Vc10 (Fig. 3.2). Significant differences among crops were only detected for isolate Pc2. The numbers of cfu/cm^2 root were higher in the presence of the nematode for isolate Vc10 in all crops, except the first one, whereas for Pc2 the reverse was observed except for the fourth crop (Fig. 3.2).

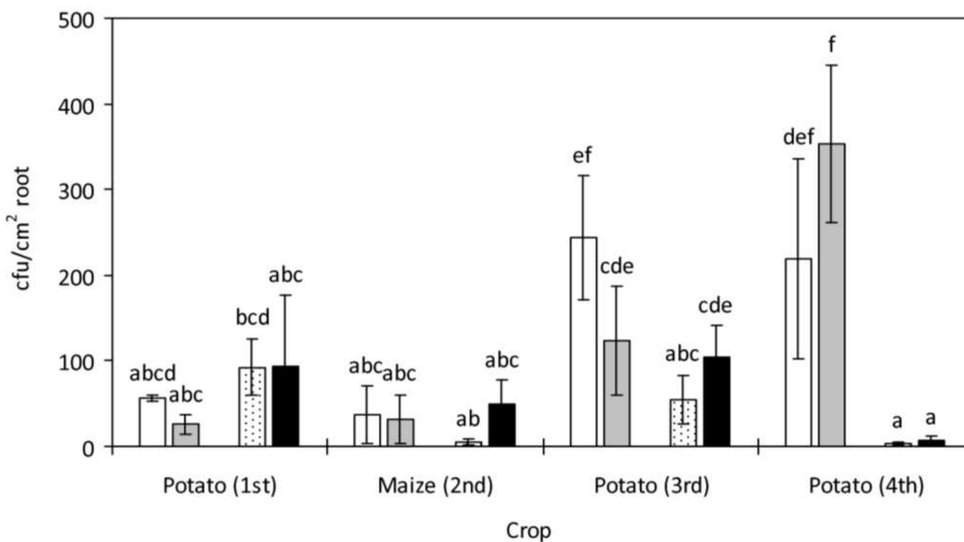


Figure 3.2 – Numbers of colony forming units (cfus) of *Pochonia chlamydosporia* Pc2 and Vc10 isolates in roots at the end of each crop in pot experiments in a crop rotation (potato cv. Désirée, maize cv. Belgrano, potato, potato) inoculated with 4000 *Meloidogyne chitwoodi* eggs/ pot one week after planting the first crop. Fungus was applied at a rate of 5000 chlamydospores/g of soil in the first and third crops and 2500 chlamydospores/g at the second crop. (□) Pc2 + no nematode; (■) Pc2 + *M. chitwoodi*; (⚃) Vc10 + no nematode; (■) Vc10 + *M.chitwoodi*. Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).

After the first crop, the percentage of egg parasitism (< 4%) was low in all pots and treatments (Fig. 3.3) and after the third and fourth crops, egg parasitism was still low (< 3% and 12%, respectively). Significant differences in egg parasitism were only detected after the last potato crop, when a ca. 10-fold difference was detected between the percentage of eggs parasitized by Pc2 and Vc10 isolates.

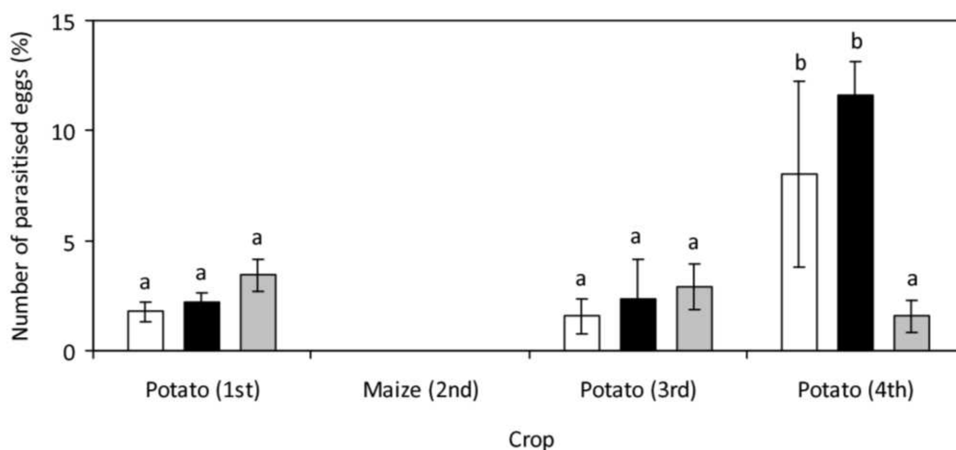


Figure 3.3 – Numbers of *Meloidogyne chitwoodi* eggs parasitized by *Pochonia chlamydosporia* Pc2 and Vc10 isolates in roots at the end of each crop in pot experiments in a crop rotation (potato cv. Désirée, maize cv. Belgrano, potato, potato) inoculated with 4000 *M. chitwoodi* eggs/pot one week after planting the first crop. *Pochonia chlamydosporia* isolates were applied at a rate of 5000 chlamydospores/g of soil at planting of first and third crops and 2500 chlamydospores/g at the second crop. (□) no fungus; (■) Pc2; (■) Vc10. Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test (p<0.05).

3.3.2. *Meloidogyne chitwoodi* reproduction

After the first crop, *M. chitwoodi* soil population densities were higher in pots treated with Pc2 but no significant differences were found between fungal isolates. A decrease was observed after the second crop followed by an increase after the third crop except for Pc2. At the end of the fourth crop, soil population densities were low in all treatments. Also, the numbers of galls and eggs/g of root and eggs/egg mass were lower in pots treated with Pc2 particularly after the fourth crop. The RKN *M. chitwoodi* was not detected in plant roots after the second crop (maize). The numbers of eggs per egg mass were also lower in pots treated with the fungus when compared with controls (Table 3.1).

Table 3.1 – Effect of *Pochonia chlamydosporia* isolates (Pc2 and Vc10) on the root-knot nematode, *Meloidogyne chitwoodi*, final population densities in soil and roots in a pot experiment crop rotation (potato cv. Désirée-maize cv. Belgrano-potato-potato).[a)]

Treatment	J2[d)]/100 cm^3 of soil (Nº)	Galls/g of root (Nº)	Egg masses/g of root (Nº)	Eggs/g of root (Nº)	Eggs/egg mass (Nº)
Pc2[b)] + Potato (1st)[c)]	606.4±286.5 e	51.6±4.0 a	51.7±4.4 ab	18477.8±978.3 b	369.5±25.9 c
Pc2[b)] + Maize (2nd)	12.0±22.4 abc	--- -	--- -	--- -	--- -
Pc2[b)] + Potato (3rd)	9.0±89.0 abcd	46.8±11.6 a	47.5±11.9 ab	2016.0±939.3 a	36.0±9.3 a
Pc2[b)] + Potato (4th)	0.8±1.8 a	55.4±26.9 a	47.2±22.2 ab	2832.4±1485.4 a	41.2±14.7 a
Vc10[b)] + Potato (1st)[c)]	528.0±141.9 e	40.0±11.7 a	54.3±19.5 ab	15387.5±6535.1 ab	270.6±61.5 c
Vc10[b)] + Maize (2nd)	3.2±5.2 ab	--- -	--- -	--- -	--- -
Vc10[b)] + Potato (3rd)	119.0±173.0 d	58.7±45.7 a	60.7±47.7 ab	5268.2±4346.9 a	97.4±33.7 ab
Vc10[b)] + Potato (4th)	7.0±8.4 ab	150.4±28.9 b	143.3±29.5 c	6496.0±1306.7 ab	49.9±8.9 a
Potato (1st)[c)]	104.4±43.3 cd	38.9±10.7 a	43.0±11.8 a	13843.1±6353.4 ab	250.4±45.1 c
Maize (2nd)	2.8±3.9 ab	--- -	--- -	--- -	--- -
Potato (3rd)	88.0±169.6 bcd	61.8±23.3 a	61.3±23.1 ab	1108.2±6863.8 ab	131.0±36.7 b
Potato (4th)	0±0 a	149.9±32.3 b	119.3±25.8 bc	8005.6±2429.5 ab	57.8±17.1 ab

[a)] Values are replicates of 5 pots/treatment±standard error; columns follow by the same letter within a treatment are not significantly different according to LSD test ($p<0.05$).
[b)] inoculated at planting in 1st, 2nd and 3rd crops (5000, 2500 and 5000 chlamydospores/g of soil, respectively).
[c)] soil was inoculated with 4000 *M. chitwoodi* eggs/pot.
[d)] J2 – second-stage juveniles.

3.3.3. Nematode diversity in soil

At the beginning of the first potato crop, nematodes of the families Dorylaimidae, Mononchidae, and Rhabditidae and of the genera *Aphelenchus, Helicotylenchus*, *Pratylenchus*, and *Tylenchorhynchus s.l.* were detected. Plant-parasitic nematodes were present in small numbers (<7%) and bacterial feeders represented the main nematode group in soil (68-85%). After the first crop, there was a significant increase in the population densities of nematodes of the genera *Tylenchorhynchus* s.l and *Tylenchus s.l.*. This increase was observed only for generalist plant-parasitic nematodes of the genus *Tylenchorhynchus s.l.* after the following crops. Overall, there was a decrease in the numbers of nematodes in soil after the first crop which was sharper in pots without the plant. The lowest numbers of nematodes were recovered from soil after the second crop (maize) and a general increase was detected in the following crops except for pots without plant. Otherwise, only small non-significant fluctuations were detected in the populations of other plant parasitic nematodes (data not shown).

3.4. Discussion

Establishment in soil, rhizosphere and the ability to parasitize nematode eggs are key factors that greatly influence the potential of *P. chlamydosporia* isolates as biological control agents against RKN. In this study, the ability of a Portuguese isolate of *P. chlamydosporia*, from potato cyst nematode eggs, and of a non-native isolate, from RKN eggs, to survive and proliferate in soil and rhizosphere and their efficacy against *M. chitwoodi* during a crop rotation were evaluated.

Fungal population densities in soil varied over time and isolates were only able to surpass the initial fungal inoculum rate after the third inoculation. Following their incorporation in soil, a decrease was observed for both isolates. The poor-nutrient sandy soil used in the pot experiments may have limited the fungus proliferation, as it has been suggested that growth and proliferation, after chlamydospore inoculation, depend to a certain extent on externally available nutrients (De Leij et al., 1992). Also, suppressive soils develop in stable, nutrient rich, monoculture conditions and the fungus may struggle to establish in disturbed nutrient poor soils (Costa et al., 2012). Therefore, successful establishment in soil may require several applications and may take longer than the cropping rotation cycle performed.

The survival of both fungal isolates, during the spring and summer 2009, when high temperatures were registered, reflect the importance of the temperature on the performance of this fungus. *In vitro* studies revealed that both isolates were able to survive and resist temperatures limiting for growth (Vieira dos Santos et al., submitted). Tolerance to temperature can have a great impact on the establishment of an organism in the field and can compromise its efficacy as a biological control agent (Magan, 2001). The Portuguese isolate Pc2 was more effective in establishing in soil than the non-native Vc10. Although isolates of the fungus differ in their ability to grow in soil, the receptivity of the soil to an antagonist may influence proliferation. When applied to the soil, growth of non-native isolates is lower compared to native isolates as in natural environmental conditions a fungus may be better suited to overcome antagonism when adapted to its indigenous microbiota (Monfort et al., 2006). This may also explain the more successful establishment of both isolates following inoculation after the summer 2009, when high temperatures may have caused the collapse of established microbiota.

The high variability observed in soil among replicates of the same treatment could be attributed to an aggregated distribution, stressing the need for adequate methods to monitor fungal development and establishment in soil (Atkins et al 2003a; Tzortzakakis 2009).

Similar to what was observed in soil, low numbers of cfus were recovered from roots, which may explain the low levels of egg parasitism observed. Effective control against RKN can only be achieved after root colonization (De Leij and Kerry, 1991). Nematode presence increased fungal colonization in roots as expected but only for Vc10, which may indicate nematode host preferences (Mauchline et al., 2004). Accordingly, the percentage of parasitized eggs by Vc10 was higher than by Pc2 in the second and third crops, although not significantly different. *Pochonia chlamydosporia* isolates have shown nematode-host preferences at the sub-specific level (Kerry, 2000; Mauchline et al., 2004). Moreover, host-related genetic variation assessed PCR-based DNA fingerprinting and detected in the alkaline serine protease VCP1, which is an enzyme produced during infection of the nematode egg, grouped more strongly on the basis of their original host rather than by their geographic origin (Morton et al., 2003a, 2003b). On the other hand, when in contact with the nematode egg mass, the fungus switches from the saprophytic phase to parasitic stage and

parasitizes nematode eggs. Although the set of conditions causing the switch is not well understood, it may be related with the regulation of genes encoding enzymes involved in egg infection (Kerry and Hirsch, 2011).

In the last crop, the increase in egg parasitism detected in pots treated with Pc2 and not with Vc10 was clearly related with rhizosphere colonization (Figs. 3.2 and 3.3), as the nematode reproduction is significantly affected by the ability to colonize the rhizosphere (De Leij and Kerry, 1991; Bourne et al., 1994; 1996; Kerry and Bourne, 1996). In the last crop, in two of the control pots, egg parasitism was also high. The fungus was not found in plant roots but was recovered from soil. Initially, *P. chlamydosporia* was not detected in the control pots and it is unclear whether this represents a contamination from the surrounding environment or the multiplication of a native fungus that eluded the initial detection efforts. Nevertheless, it seems that the conditions promoting the development of some form of fungal-based soil suppressiveness have been favoured by the chosen crop rotation sequence in this pot experiment.

Growth and survival of the fungus in soil did not seem to be influenced by the presence of the host nematodes or by the plant species, but the increased population density of isolate Pc2 in soil could be related with an increase in rhizosphere colonization. Primary establishment in the rhizosphere is interrelated to fungal abundance in soil (De Leij et al., 1993).

As referred above, maize cv. Belgrano was considered a susceptible host for *M. chitwoodi* but, in this experiment, it was not detected on the roots of any of the treatments. The adverse atmospheric conditions observed during the maize crop were probably responsible for the adverse effect detected on the nematode development and reproduction, as it was observed for fungal saprotrophic growth.

The harsh abiotic conditions such as high temperatures, registered from June to September 2009, may have lead to the decline of soil biota, including nematodes and the fungus. Therefore, an integrated pest management of summer crops that includes the application of *P. chlamydosporia* may not be viable in Mediterranean climates, because of their long dry warm summers and extreme temperature events (Verdejo-Lucas et al., 2003; Tzortzakakis, 2009). In addition to the study-target *Meloidogyne* population, during the summer of 2009, under the maize crop, populations of other nematodes crashed, with the

exception of nematode families Dorylaimidae and Mononchidae. These belong to persister guilds, with a colonizer-persister value of 4 in a scale of 1-5, and may, therefore, resist unfavourable conditions (Bongers, 1990).

Although isolate Vc10 did not reveal potential as a biological control agent against *M. chitwoodi*, it has been widely studied and proven to be effective against *Meloidogyne* spp. populations in pot and microplot trials (De Leij and Kerry, 1991; De Leij et al., 1993). On the other hand, there are also reports of its failure in controlling RKN. Thus the ability of a given isolate could vary in experiments conducted under different conditions or when different nematode populations are involved (Tzortzakakis, 2000, 2007, 2009; Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003). Nonetheless, the two *P. chlamydosporia* isolates revealed subtle differences in host preference, plant compatibility or tolerance to abiotic conditions that may cause large discrepancies in their efficacy as control agents in a long-lasting management programme. Under very similar conditions and exact same experimental design, different outcomes were obtained with Pc2 and Vc10. Laboratory standard screening of isolates may not be sufficient to distinguish the most appropriate biological control agent for a given crop.

Factors affecting establishment, performance and biocontrol efficacy in field-like conditions need further research. A greater understanding of the interactions between the various biotic and abiotic components of the system that combines population dynamics observations with molecular biology tools may help to determine the optimal conditions for fungal performance and help to design efficient and sustainable management strategies for RKN control.

3.5. Acknowledgements

This research was supported by FEDER funds through the “Programa Operacional Fatores de Competitividade – COMPETE” and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the Project PTDC/AGR-AAM/74574/2006. The first author was supported by ESF funds through the "Programa Operacional Potencial Humano - POPH” of “The National Strategic Reference Framework” (SFRH/BD/27317/2006).

This paper is dedicated to the memory of Brian Kerry who was largely responsible for initiating this work.

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Chapter 4

Plant elicitors as inducers of plant defence against the root-knot nematode, *Meloidogyne chitwoodi*

Written as a journal article:

Vieira dos Santos, M.C., Curtis, R.H.C., and Abrantes, I. (2012). Plant elicitors as inducers of plant defence against the root-knot nematode, *Meloidogyne chitwoodi*. European Journal of Plant Pathology (*submitted*).

Abstract - Sustainable strategies for plant-parasitic nematode control are required to reduce dependence on chemical nematicides. Foliar application of various compounds can induce a systemic defence response that reduces nematode infestation. The effects of benzothiadiazole (BTH), β-aminobutyric acid (BABA), jasmonates (*cis*-jasmone and methyl-jasmonate) and salicylic acid (SA) in the development and reproduction of the root-knot nematode *Meloidogyne chitwoodi* in tomato plants were assessed. The effects of BTH and of the jasmonates were further tested on potato plants. Pot assays were conducted using tomato plants cv. Tiny Tim or potato cv. Désirée treated with foliar sprays and inoculated with 300 second stage juveniles. Nematode development and reproduction were assessed 21 and 45 days post-inoculation. Treatments with *cis*-jasmone and SA had a negative effect on nematode development in tomato plants but did not affect reproduction and BABA and methyl-jasmonate treatments were the most effective in reducing nematode penetration (45 and 58% respectively). In plants sprayed with BABA, the reproduction factor (Rf) was lower (8.3) when compared to the control (Rf=13.5). Nematode development was significantly affected in potato plants sprayed with BTH and *cis*-jasmone. Nematode penetration was reduced by 90, 67 and 81% in plants treated with BTH, *cis*-jasmone and methyl-jasmonate respectively, although Rf was only significantly lower in the BTH treatment (Rf=7.6) when compared to the control (Rf=18.1). Our results suggest that the SA pathway of systemic acquired resistance has a larger effect on nematode development and reproduction for both plants, and should be considered in the design of integrated pest management approaches.

Key words: β-aminobutyric acid (BABA); benzothiadiazole (BTH); *cis*-jasmone, foliar sprays; methyl-jasmonate; systemic acquired resistance

4.1. Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are one of the most economically damaging genera of plant-parasitic nematodes on horticultural and field crops (Trudgill and Blok, 2001). *Meloidogyne chitwoodi*, first described from the Pacific Northwest of the USA in 1980, is a quarantine organism recognised by its effects on potato tubers (Golden et al., 1980; OEPP/EPPO, 2009). This nematode has been reported in Argentina, Belgium, Germany, the Netherlands, Mexico, Portugal and South Africa (Conceição et al., 2009; OEPP/EPPO, 2009) and has a wide host range, including several economically important crops such as potato, *Solanum tuberosum*, and tomato, *S. lycopersicum* (Santo et al., 1980; Santo and O'Bannon, 1982; O'Bannon et al., 1982; Ferris et al., 1993).

Current management strategies are based on agricultural practices like crop rotation or the use of nematicides. However, crop rotation does not allow intensive agricultural practices and the use of nematicides has been banned or significantly reduced due to health and environment concerns. There is an increasing demand to reduce dependence on chemical nematicides for the control of RKN. The exploitation of natural defence mechanisms in plants could lead to alternative, sustainable and environmental friendly management strategies.

Plants can react rapidly to the aggression of plant pathogens and are able to recognize compounds produced or released by the aggressor (elicitors). The term 'induced resistance' is used to describe plant defences that are triggered by pathogen attack and involves activation of defence signalling pathways, in which salicylic acid (SA), jasmonic acid (JA) and ethylene play important roles. The nature of induced resistance is regulated by two essential mechanisms: systemic acquired resistance and induced systemic resistance. The former is mediated by accumulation of endogenous SA and correlates with the expression of pathogenesis-related protein genes, providing resistance even against unrelated pathogens, for a relatively long-lasting period. Induced systemic resistance, on the other hand, is linked with the sequential action of JA and ethylene and involves recognition, of a pathogen-expressed gene, by the plant, inducing an array of biochemical reactions that target the pathogen in the site of attempted invasion (Pieterse and Van Loon, 1999; Kuć, 2001; Gozzo, 2003; Lucas, 2010).

The close co-evolution of plants and pathogens has shaped how microbes and animals exploit plants and how plants resist, so that a chemical communication warfare takes place as soon as a nematode penetrates the plant root. Thus, plants are able to differentially activate defence responses to potential pathogens. However, the challenging nematodes, on the other hand, have to break these barriers in order to be successful and they secrete effector molecules into plant cells which target key cell mechanisms to suppress or degrade host defence (Smant and Jones, 2011). The SA and JA pathways play complex roles in plant defence response promoting resistance in some plant-pathogen interactions and increasing susceptibility in others (Gutjahr and Paszkowski, 2009; Walling, 2001).

In addition to pathogen attack, plant defences can be activated by exogenous application by various compounds, including SA and its synthetic mimics such as benzothiadiazole (BTH), JA and its derivatives, and also by the non-protein amino acid β-aminobutyric acid (BABA) (Cohen, 2002; Gozzo, 2003; Lucas, 2010). Chemically-induced resistance to plant-parasitic nematodes has not been studied as widely as that of other pathogens, but the reduction of nematode invasion, development and reproduction, using foliar sprays of BABA, BTH, JA and derivatives and SA, has been demonstrated. Exogenous applications of BABA were effective against RKN and cyst nematodes, *Heterodera* spp., in barley, pineapple, tomato and wheat (Chinnasri et al., 2006; Oka et al., 1999; Oka and Cohen, 2001). The plant defence activator BTH, commercialized in Europe (Bion®) and in the USA (Actigard® and Boost®), had a negative effect on plant-parasitic nematodes in pot experiments, with soybean and cowpea, potato fields and glasshouse-grown Cabernet Sauvignon grapevines (Chinnasri et al., 2003; Collins et al., 2006; Owen et al., 2002). Jasmonates actively induced systemic defence against RKN parasitism in tomato and rice (Curtis et al., 2009; Cooper et al., 2005; Fujimoto et al., 2011; Nahar et al., 2011). Foliar sprays of SA to okra and cowpea reduced nematode infestation and had no effect on plant growth (Nandi et al., 2003). Additionally, when applied to sugarcane, foliar sprays of BABA, Bion® and SA reduced *M. incognita* infection with no effect on yield (Berry et al., 2011).

The above studies also produced different conclusions on the effects of the various plant defence activators, even when reportedly applied to the same organisms under similar conditions. Nevertheless, subtle differences in otherwise comparable methodologies may have caused the apparent idiosyncratic results. Plant defence activators may differ on their

ability to induce plant defence against nematodes and their efficacy could be dependent on the amount of compound absorbed by the plants. Though dependent on the quantity and method of application, levels of root uptake, obtained through soil drench, for example, can be influenced by a range of factors, such as cultivar, age and health of treated plants, and the environmental conditions under which the trials are set (Vallad and Goodman, 2004). The aim of this study was to assess the effects of exogenous activators on host plant defence against *M. chitwoodi*, using a standard assay that allows a direct comparison among treatments, to infer on their potential application in the control of this damaging nematode. The effect of the plant activators BABA, BTH (Bion®), jasmonates (*cis*-jasmone and methyl-jasmonate) and SA on the development and reproduction of *M. chitwoodi* was determined on tomato and the effects of the commercial plant defence activator (Bion®) and jasmonates were further investigated in potato.

4.2. Material and Methods

4.2.1. *Meloidogyne chitwoodi* isolate

An isolate of the root-knot nematode *M. chitwoodi*, obtained from infected potato tubers collected in Porto, Portugal (Conceição et al., 2009), was maintained on susceptible host tomato plants cv. Easypeel, in pots with sterilised sandy loam soil and sand (1:1) in the Nematology laboratory at the University of Coimbra.

4.2.2. Plant material

Tomato cv. Tiny Tim plants grown in plastic pots, with 250 cm^3 and 400 cm^3 of peat compost mixed with coarse sandy soil, were used to perform the nematode development and reproduction studies, respectively. These experiments were conducted at Rothamsted Research, UK, under temperature controlled glass house (at 25 °C with 12 hours of artificial light: 400 W SON-T agro bulbs, placed 1 m above pots).

Nematode development and reproduction assays were also conducted in plants grown from a single potato sprout from cv. Désirée in plastic pots with 300 cm^3 and 800 cm^3, respectively, of coarse sandy soil, commonly used for growing potato plants in pots. These experiments were conducted at the Nematology laboratory, University of Coimbra, Portugal, in a growth chamber at 23 °C and a 16h photoperiod, watered every two days and fertilised weekly with Hyponex® (N:P:K 7:6:19).

4.2.3. Foliar sprays

Three week-old tomato plants were sprayed twice using an automatic hydraulic sprayer (Sprayer 015 F110 brown) purposely built to deliver compounds quantities in a similar way used for foliar spray in field conditions and it was set for the following conditions: 1.0 m/s at 3.0 bar; 35 cm above crop; 0.5 m swath width; and application rate 200l/h.

The plants were sprayed with a seven days interval, with standard solutions 20 mM (412 g/ha) BABA (Sigma, USA), 2.5 mM (210 g/ha) BTH (Bion® Syngenta, USA), 1.5 mM (50 g/ha) *cis*-jasmone, 1.1 mM (50 g/ha) methyl-jasmonate (Sigma, USA) or 10 mM (276 g/ha) SA (Sigma, USA) in 1% ethanol (EtOH). These correspond to ca.: 0.202 mg/plant (BABA), 0.103 mg/plant (BTH), 0.025 mg/plant (*cis*-jasmone and methyl-jasmone) and 0.135 mg/plant (SA). Before foliar spray applications, all plant activators were mixed with 0.1% of the non-ionic surfactant ethylan BV (EBV) (Tennants Distribution Ltd, UK). Plants sprayed with water or 0.1% EBV were considered as untreated and carrier solution control, respectively, for all treatments. An additional control for SA treatment was included by spraying plants with 1% EtOH+0.1% EBV. Special care was taken to avoid contact of the plant activators with the soil and roots by covering the soil surface and the pots with aluminium foil. Five replicates per treatment and per experiment were prepared and the experiments were conducted twice as independent biological replicates. Following each spray, the pots were kept separate for two days to avoid cross-contamination from volatiles. The pots were then transferred to the same compartment in the glasshouse and the plants were watered daily.

Three week old potato plants were sprayed twice with a fine mist sprayer, 35 cm above plant; at a rate of 1ml/plant, with a seven days interval, with 2.5 mM (ca. 1338 g/ha) BTH, 1 mM (ca. 270 g/ha) *cis*-jasmone (Sigma, USA) and 1 mM (ca. 270 g/ha) methyl-jasmonate (Sigma, USA). These correspond to ca.: 1.050 mg/plant (BTH), 0.213 mg/plant (*cis*-jasmone) and 0.238 mg/plant (methyl-jasmone). All plant activators were mixed with 0.1% EBV prior to application. Five plants per treatment per experiment were prepared and controls were made with water or 0.1% EBV. The pots were also covered with aluminium foil during spray application and kept in separate rooms for 48 h following spray application.

4.2.4. Nematode inoculation

Second-stage juveniles were obtained from egg masses, hand-picked from tomato cv. Easypeel infected roots, and placed on a plastic sieve (10 µm) with tap water, in the dark, at room temperature. The plants were inoculated with 300 second-stage juveniles (J2)/plant two days after the first spray.

4.2.5. Nematode development assessment

Twenty-one days after inoculation (DAI), five plants per treatment were uprooted. The roots were washed free of soil and shoot and root length and fresh weight were recorded. The acid fucsin technique (Byrd et al., 1983) was used to stain nematodes within root tissues for nematode development analysis. The nematode developmental stage was identified on the basis of body shape: swollen sexually undifferentiated J2, fourth stage juveniles and adult females (Eisenback and Hirschmann Triantaphyllou, 1991) and the numbers of nematodes for each developmental stage were counted under a stereomicroscope.

4.2.6. Nematode reproduction assessment

Forty-five DAI, five plants per treatment were uprooted. The roots were washed free of soil and length and fresh weight of shoot and root were recorded. Nematode eggs were extracted from each root system using 0.52% sodium hypochlorite (Hussey and Barker, 1973). The number of galls, egg masses and eggs per root system were counted to estimate the final population density (Pf). The reproduction factor (Rf=Pf/Pi where Pi=300 J2), the numbers of eggs/egg mass and eggs/g of root were assessed for each plant.

In the experiments with potato plants, the new potato tubers produced were weighted and sliced under a stereomicroscope to check for nematode infection.

4.2.7. Data analysis

Data were compared by ANOVA using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA). A square root transformation was used to ensure a Normal distribution and constant variance of the data that otherwise would not meet the ANOVA assumptions (percentage of swollen undifferentiated J2 in both plants, Rf, eggs/egg mass in tomato plants and galls/g of root in potato plants). Statistically significant differences between treatments were computed using the LSD test ($P<0.05$).

4.3. Results

4.3.1. The effect of plant activators against *M. chitwoodi* on tomato cv. Tiny Tim

Differences were detected in root length among treatments 21 DAI with *M. chitwoodi*. Plant length was smaller in plants sprayed with EtOH (control) but larger in the water control and in plants sprayed with *cis*-jasmone. No significant differences were observed in plant weight, but an increase in root weight was detected in plants treated with SA. These plants also had the shortest roots (Table 4.1). Forty-five DAI plants sprayed with BTH and EtOH had the largest shoot length; on the other hand root length was largest in plants treated with methyl-jasmonate and in the water control. Shoot weight was lower in plants treated with cis-jasmone and root weight was higher in plants sprayed with SA (Table 4.1). Phytotoxicity symptoms were not observed in any of the plants treated.

The number of J2 per g/root was higher in plants treated with BTH, *cis*-jasmone and SA foliar sprays, after 21 DAI (Fig. 4.1a). No significant differences were detected among treatments in the number of fourth stage juveniles (Fig. 4.1b). However, plants treated with *cis*-jasmone had a significantly lower number of adult females per g/root. In plants treated with SA a lower number of adult females was also observed. However, no significant differences were detected when compared to the control EtOH (Fig. 4.1c). Foliar spray with BTH, *cis*-jasmone or methyl-jasmonate significantly reduced the numbers of nematodes inside roots 21 DAI (Fig. 4.1d).

BABA, *cis*-jasmone and methyl-jasmonate were the most effective plant activators in reducing nematode penetration, as plants treated with these activators showed a smaller number of galls and egg masses, although significant differences were detected only in plants treated with methyl-jasmonate compared to the control EBV (Fig. 4.2a and b). The numbers of eggs/egg mass were small in all treatments and controls, except in the untreated plants (water control) (Fig. 4.2c). Also, the Rf was lower in all treatments compared to the water control (Fig. 4.2d). The numbers of eggs/egg mass and Rf were small in EtOH control, although not significantly smaller than values observed in plants treated with SA (Fig. 4.2c and d).

Table 4.1 – Plant, shoot and root length and weight of tomato cv. Tiny Tim, 21 and 45 days after inoculation with 300 *Meloidogyne chitwoodi* second-stage juveniles, treated with foliar sprays of: 20 mM β-aminobutyric acid (BABA); 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 10 mM salicylic acid (SA); 1% ethanol (EtOH); 0.1 % ethylan BV (EBV); and water (H2O).[a)]

Days after inoculation	Treatment	Length (cm)			Weight (g)		
		Plant	Shoot	Root	Plant	Shoot	Root
21	BABA	37.6±1.9 abc	19.2±1.2 bc	18.4±1.1 ab	19.9±0.7 a	12.6±0.6 ab	7.3±0.3 ab
	BTH	38.1±3.0 abc	20.0±1.4 c	18.1±2.0 ab	21.4±4.2 a	12.5±2.3 ab	8.9±2.0 ab
	Cis-J	38.3±1.5 bc	18.6±1.1 abc	19.7±1.9 b	20.1±2.6 a	12.5±1.6 ab	7.6±1.0 ab
	Me-J	34.5±1.9 abc	17.1±1.4 abc	17.4±0.7 ab	16.7±1.7 a	10.4±1.0 a	6.3±0.9 a
	SA	33.5±1.1 ab	17.6±1.2 abc	15.9±0.7 a	23.6±2.2 a	12.8±1.1 ab	10.8±1.1 b
	EtOH	33.2±1.3 a	15.4±0.8 a	17.8±1.0 ab	19.4±2.3 a	11.8±1.2 ab	7.6±1.2 ab
	EBV	35.3±0.7 abc	16.6±0.7 ab	18.5±0.6 ab	18.9±0.8 a	11.9±1.0 ab	7.0±0.7 ab
	H2O	39.4±1.7 c	19.7±1.3 bc	19.7±0.6 b	21.9±3.5 a	15.5±2.7 b	6.4±0.8 a
45	BABA	52.4±3.2 ab	34.6±2.9 abc	17.8±0.7 a	50.2±2.6 bc	22.6±1.9 abc	27.5±1.9 bc
	BTH	66.5±0.9 c	45.0±0.0 d	21.5±1.0 bcd	45.4±2.6 ab	26.9±1.1 cde	18.5±1.6 a
	Cis-J	47.4±6.0 a	27.0±4.4 a	20.4±2.0 abc	39.4±1.6 a	18.7±3.1 a	20.7±2.5 ab
	Me-J	57.2±1.7 abc	31.7±1.7 abc	23.3±2.3 d	52.0±1.7 bcd	19.4±1.0 ab	32.8±1.5 c
	SA	59.7±1.3 bc	40.0±0.0 cd	19.7±1.3 abc	61.0±3.4 d	29.6±0.7 de	31.5±3.5 c
	EtOH	64.5±1.1 c	45.0±0.0 d	19.5±1.1 ab	55.5±2.1 cd	32.2±1.5 e	23.3±1.9 ab
	EBV	54.2±0.9 ab	35.0±0.0 bc	19.2±0.9 ab	48.6±2.3 bc	24.8±1.2 bcd	23.8±2.7 ab
	H2O	53.3±4.8 ab	30.0±5.0 ab	23.3±0.9 cd	47.8±6.2 abc	23.5±3.9 abcd	24.3±2.7 ab

[a)] values are means of five replicates±standard error. Values followed by the same letter within a column are not significantly different according to LSD test ($p<0.05$).

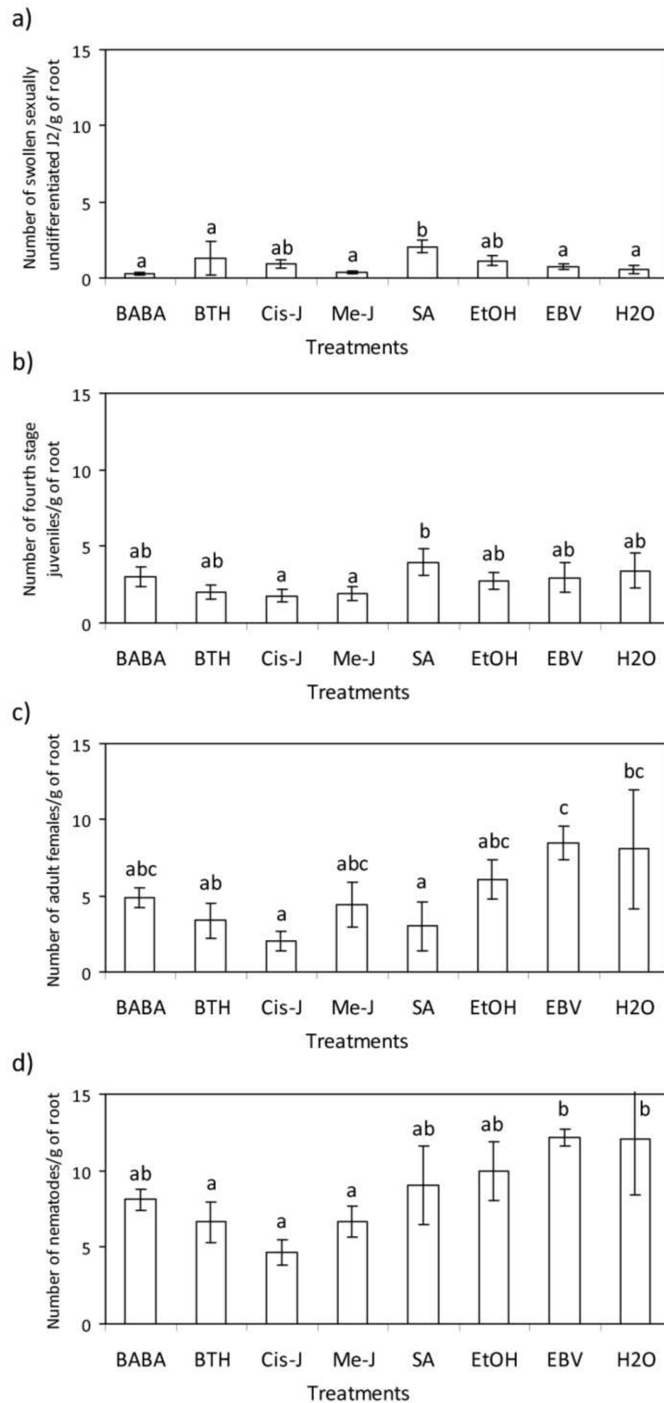

Figure 4.1 - Numbers of swollen sexually undifferentiated second-stage juveniles (J2) (a), fourth-stage juveniles (b), adult females (c), and of nematodes (d) per gram of root in tomato cv. Tiny Tim 21 days after inoculation with 300 *Meloidogyne chitwoodi* J2 and treated with foliar sprays of: 20 mM β-aminobutyric acid (BABA); 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 10 mM salicylic acid (SA); 1% ethanol (EtOH); 0.1 % ethylan BV (EBV); and water (H2O). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).

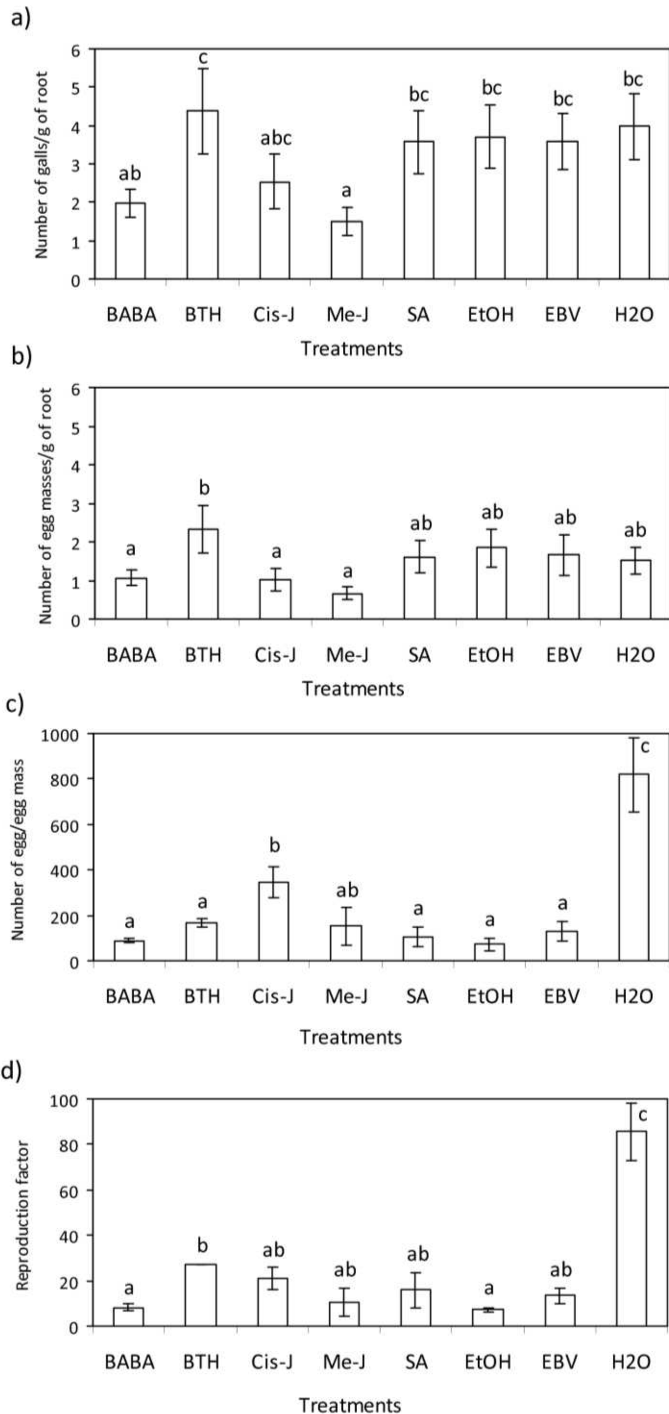

Figure 4.2 – Numbers of galls (a) and egg masses (b) per g of root, eggs/egg mass (c) and reproduction factor (Rf) (d) in tomato cv. Tiny Tim 45 days after inoculation with 300 *Meloidogyne chitwoodi* second stage juveniles (J2) and treated with foliar sprays of: 20 mM β-aminobutyric acid (BABA); 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 10 mM salicylic acid (SA); 1% ethanol (EtOH); 0.1 % ethylan BV (EBV); and water (H2O). Rf=final population (Pf)/300 J2 (Pi). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).

4.3.2. The effects of plant activators against *M. chitwoodi* on potato cv. Désirée

Twenty-one DAI with the nematode, a decrease in shoot and root length was observed in the plants sprayed with the plant activators, compared to the water control, but differences were not observed in shoot and root weight. In plants treated with *cis*-jasmone, root and shoot weight was larger than all the other treatments (Table 4.2). Some of the plants sprayed with this plant activator showed yellow spots on leaves, indicative of phytotoxicity.

No differences in shoot length were detected in the plants treated with the plant activators 45 DAI. However, root length was smaller in plants sprayed with methyl-jasmonate. Shoot weight was larger in plants treated with both jasmonates and in the water control, whereas root weight was significantly larger in plants treated with BTH and *cis*-jasmone (Table 4.2). Plants treated with *cis*-jasmone did not produce tubers and phytotoxicity symptoms were observed on the leaves of these plants. Weight of tubers produced in plants treated with methyl-jasmonate was very small when compared to plants sprayed with BTH or the water control (Table 4.2). In two tubers from plants treated with BTH and EBV, adult females with egg masses were detected: one female, in a tuber from a plant sprayed with BTH produced 127 eggs and two females in a tuber of a plant treated with EBV produced 101 and 73 eggs.

Nematode development was delayed in plants treated with BTH, *cis*-jasmone and methyl-jasmonate as shown by the higher percentage of swollen sexually undifferentiated J2 (Fig. 4.3a) and fourth stage juveniles (Fig. 4.3b) and a low percentage of adult females (Fig. 4.3c). Only treatments BTH and *cis*-jasmone had an effect on the total numbers of nematodes inside the roots 21 DAI (Fig. 4.3d).

In potato plants sprayed with the plant activators, the numbers of galls and egg masses/g of root were smaller than in the controls (Fig. 4.4a and b). Although the numbers of eggs/egg mass were higher, they were not different from the controls, with the exception of plants sprayed with methyl-jasmonate (Fig. 4.4c). The Rf values were lower in plants treated with the plant activators but only foliar sprays of BTH and methyl-jasmonate were effective in reducing nematode reproduction (Fig. 4.4d).

Table 4.2 – Plant, shoot and root length and weight of potato cv. Désirée 21 and 45 days after inoculation with 300 *Meloidogyne chitwoodi* second-stage juveniles and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 0.1 % ethylan BV (EBV); and water (H2O).[a)]

Days after inoculation	Treatment	Length (cm)			Weight (g)			
		Plant	Shoot	Root	Plant	Shoot	Root	Tuber
21	BTH	50.7±2.6 a	30.1±2.3 a	20.6±1.2 a	9.1±1.3 a	5.6±0.8 a	3.5±0.6 ab	-
	Cis-J	58.8±2.0 b	38.3±2.0 bc	20.5±1.5 a	13.2±1.0 b	8.0±0.4 b	5.2±0.7 b	-
	Me-J	50.8±1.5 a	27.8±0.9 a	23.0±1.9 ab	8.0±1.2 a	4.8±0.5 a	3.2±0.8 a	-
	EBV	59.9±4.4 b	37.5±3.6 b	22.4±1.1 ab	8.4±0.9 a	5.3±1.1 a	3.1±0.5 a	-
	H2O	71.8±3.3 c	44.5±1.7 c	27.3±2.4 b	8.9±0.6 a	6.0±0.3 a	2.9±0.4 a	-
45	BTH	59.2±7.9 a	30.8±8.3 b	28.3±0.8 ab	10.1±0.3 b	2.4±0.5 ab	7.7±0.6 b	8.7±0.9 c
	Cis-J	46.7±2.1 a	18.0±1.0 a	28.7±1.7 ab	10.2±1.3 b	3.6±0.4 b	6.7±1.0 b	-
	Me-J	52.5±4.3 a	29.0±4.5 b	23.5±1.3 a	6.0±0.5 a	3.3±0.6 b	2.6±0.3 a	1.6±0.3 a
	EBV	48.0±1.4 a	22.3±0.9 ab	25.7±1.8 ab	5.9±1.8 a	1.5±0.3 a	2.3±0.5 a	3.3±1.5 ab
	H2O	59.0±5.4 a	28.8±2.6 b	30.2±3.2 b	3.8±0.7 a	3.1±0.8 ab	2.8±1.2 a	5.5±0.8 b

[a)] values are means of five replicates±standard error. Values followed by the same letter within a column are not significantly different according to LSD test ($p<0.05$).

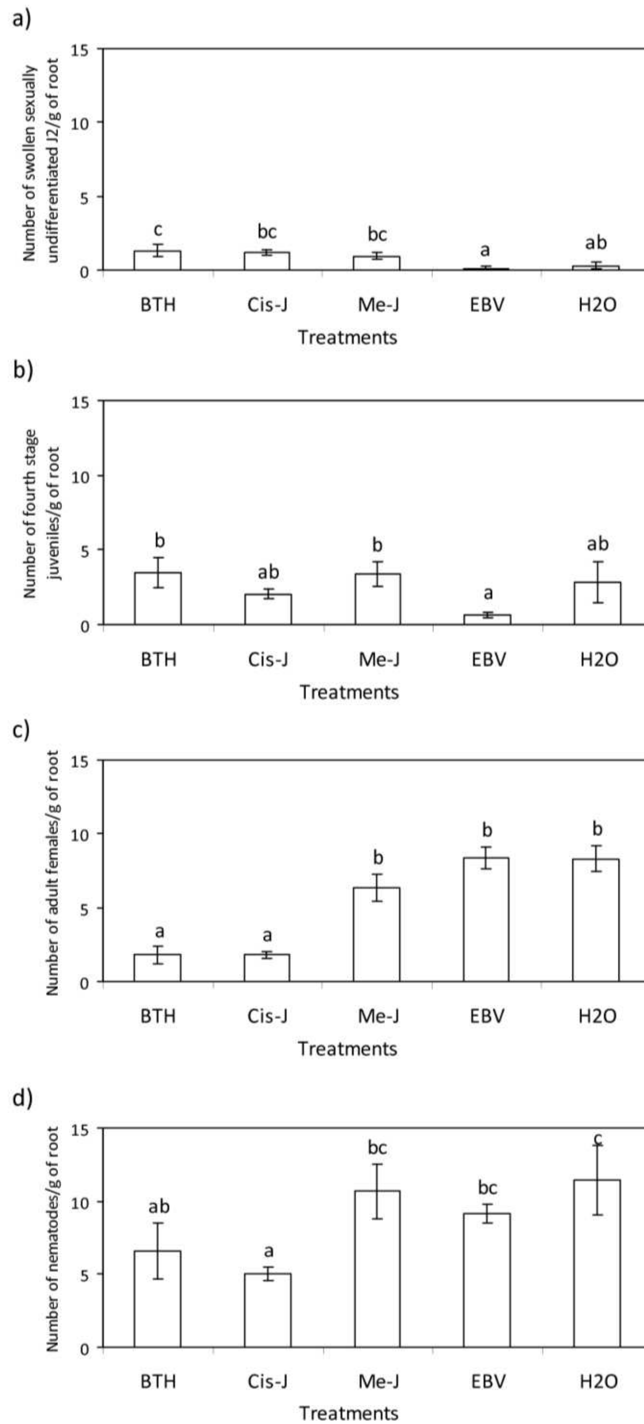

Figure 4.3 – Numbers of swollen sexually undifferentiated second-stage juveniles (J2) (a), fourth-stage juveniles (b), adult females (c), and of nematodes (d) per gram of root in roots of potato cv. Désirée 21 days after inoculation with 300 *Meloidogyne chitwoodi* J2 and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 0.1 % ethylan BV (EBV); and water (H2O). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).

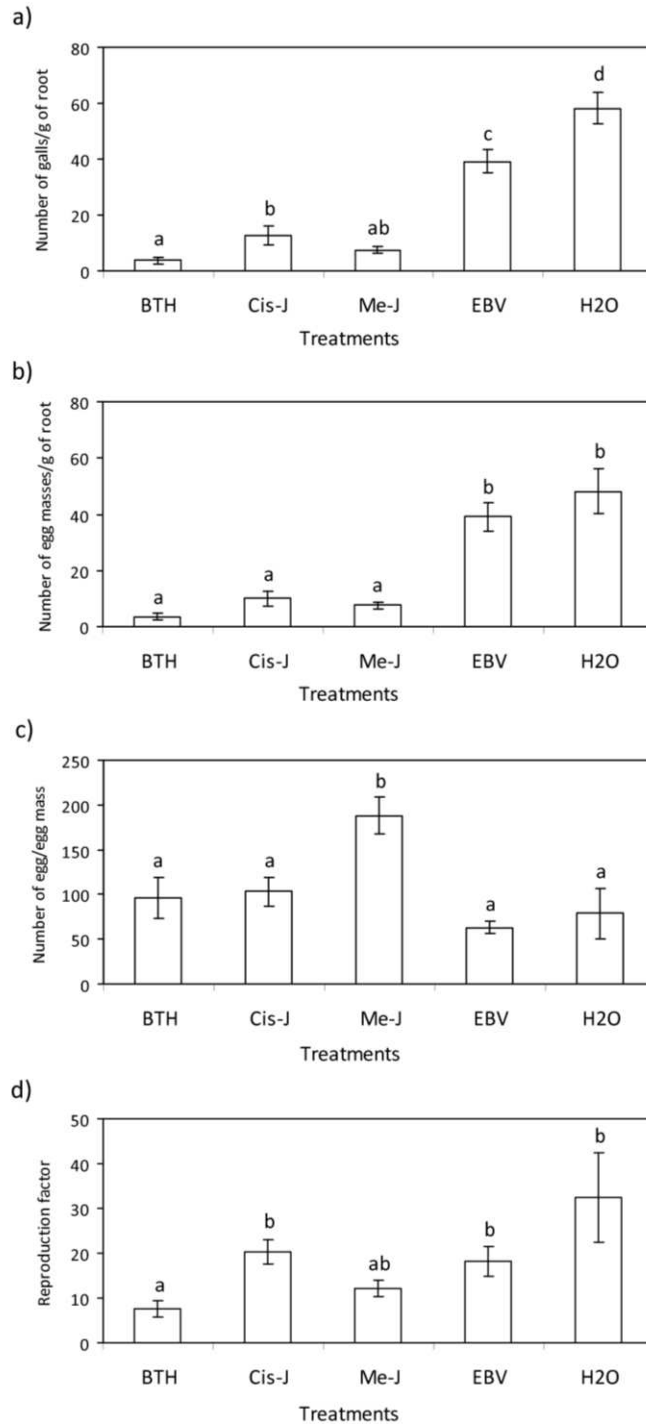

Figure 4.4 - Numbers of galls (a) and egg masses (b) per g of root, eggs/egg mass (c) and reproduction factor (Rf) (d)in potato cv. Désirée 45 days after inoculation with 300 *Meloidogyne chitwoodi* second stage juveniles (J2) and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 1 mM methyl-jasmonate (Me-J); 0.1 % ethylan BV (EBV); and water (H2O). Rf=final population (Pf)/300J2 (Pi). Bars represent standard error of means. Columns with the same letter are not significantly different according to LSD test ($p<0.05$).

4.4. Discussion

In this study, foliar application of SA or *cis*-jasmone were successful in reducing nematode development in tomato plants (Fig. 4.1). In preliminary *in vitro* attraction assays, using tomato cv. Tiny Tim plantlets sprayed with these plant activators, and placed in 23% pluronic gel with 250 *M. chitwoodi* J2, a delay in root penetration was observed when compared to non-treated plants. A strong reduction (>70%) on the numbers of J2 inside the roots after 24 h was detected for treatments with the jasmonates, although no reduction on the numbers of J2 inside the roots was observed for SA treatment (Vieira dos Santos, unp. results).

The plant activators BABA and methyl-jasmonate were the most effective in reducing nematode penetration (45 and 58 % respectively), but only BABA was effective in reducing nematode reproduction in tomato plants (Fig. 4.2). Both *cis*-jasmone and methyl-jasmonate had a strong effect in nematode penetration, but egg production was increased. Thus, the effects on nematode development did not influence nematode reproduction.

Previous research showed that foliar sprays of JA or methyl-jasmonate induced a systemic defence response by reducing nematode reproduction on tomato plants without visible phytotoxic effects (Cooper et al., 2005; Fujimoto et al., 2011). Moreover, foliar sprays of *cis*-jasmone and methyl-jasmonate caused a considerable reduction of potato cyst nematodes, *Globodera pallida* infection of tomato plants. Treatment with methyl-jasmonate also significantly reduced infection by *M. incognita* (Curtis et al., 2009). In contrast, a number of chemical elicitors including JA and SA were considered phytotoxic to tomato plants and did not improve control of RKN, neither in cereals nor in tomato plants (Oka et al., 1999; Oka and Cohen, 2001). Variation in the efficacy of SA as a plant defence inducer has also been reported. Foliar sprays of SA to okra and cowpea reduced RKN infestation and had no effect on plant growth (Nandi et al. 2003), but, on pineapple, they did not had an effect on nematode reproduction though they reduced plant growth (Chinnasri et al., 2006). In the present study, phytotoxicity symptoms were not detected in any of the tomato plants treated with plant activators. It is possible that these contrasting data is due to differences in treatment applied (foliar spray, drench application) and concentrations used.

The Rf was significantly lower (Rf = 8.3) in tomato plants sprayed with BABA, when compared to the control (Rf = 13.5) (Fig. 4.2). β-aminobutyric acid is an effective plant

defence inducer not only against nematodes but also against a large number of plant parasites. Although its efficacy, under controlled conditions, is dependent on host, pathogen and mode of application, it exhibits post-infectional activity, unlike other plant activators (Cohen, 2002). Foliar sprays of BABA were effective in reducing *M. javanica* reproduction in pineapple (Chinnasri et al., 2006) and *M. incognita* infection in sugarcane (Berry et al., 2011). In addition, protection against cyst nematodes, *Heterodera* spp., and RKN was obtained not only by soil drenches but also by foliar application. On wheat and barley, only the application as soil drench was effective in reducing RKN populations, whilst in tomato, foliar sprays were effective in decreasing infection and reproduction (Oka et al., 1999; Oka and Cohen, 2001).

When applied to potato plants, BTH and *cis*-jasmone significantly affected nematode development (Fig. 4.3). Some of the potato plants sprayed with *cis*-jasmone showed yellow spots on leaves indicating some phytotoxic effect of direct application. No significant differences in plant growth were detected when compared to other treatments or controls but none of the plants treated with this plant activator produced tubers; in all the other treatments tuber production occurred in almost all replicates (Table 4.2). Nonetheless, there was no evidence of phytotoxic effects associated with *cis*-jasmone application on tomato plants. The observed phytotoxicity symptoms on potato leaves could be due to the different dosage application of the activator. Many of the side effects in studies based on exogenous application of plant defence inducers are strongly dosage dependent and can not be directly linked with fitness costs (Heil and Baldwin, 2002).

Foliars sprays of BTH, *cis*-jasmone and methyl-jasmonate effectively reduced nematode penetration after 21 days by 90, 67 and 81%, respectively. On the other hand, nematode reproduction was only affected by BTH treatment (Rf=7.6) when compared with EBV treatment (Rf=18.1). The effect of foliar sprays of BTH on potato against *M. chitwoodi* has been reported in field conditions and it has been suggested that the efficacy of the plant elicitors in reducing plant-parasitic nematode densities and protecting potato tubers from nematode infection could be improved if nematode densities are low to moderately high (Collins et al., 2006). The use of the commercial formulations of BTH (Bion®) on glasshouse-grown Cabernet Sauvignon grapevines had a significant effect on *Meloidogyne* spp. in the roots, slowing nematode development and decreasing egg deposition (Owen et al., 2002) and reducing nematode infection when applied to sugarcane (Berry et al., 2011).

Foliar application of the commercial formulation Actigard® to cowpea or soybean plants reduced reproduction and delayed the development of the RKN, *M. javanica* (Chinnasri et al., 2003). Differences detected in the effects of foliar sprays of BTH, *cis*-jasmone and methyl-jasmonate between the two plant species tested may be related with the different dosage application. A stronger reduction in nematode infection was observed in potato plants where a ca. 10-fold concentration of the plant activators was applied when compared to the dosage applied in tomato plants. Dosage application of methyl-jasmonate has been directly related with the decrease in the number of RKN and egg masses in roots of tomato plants (Fujimoto et al., 2011). A linear relationship between dosage application and nematode reproduction was observed in pineapple plants treated with BTH (Chinnasri et al., 2006). But, the observed effects might not be explained by the foliar spray concentrations alone: differences in susceptibility of the host plants to nematode attack were also detected. The reproduction factor in untreated plants (water control) was higher in tomato plants (Rf=85.5) than in potato plants (Rf=32.5). It is unclear whether this is due to differences in nematode attraction and penetration of the two plant hosts or to differing natural active defence responses to nematode attack between the plant species. Our studies cannot discriminate the potentially variable effects of a natural vs the artificial elicitation pathways of plant defence, which possibly differ between plant species. However, it has been suggested that the natural resistance response is down-regulated and surpassed when exogenous chemical elicitors are applied (Heil and Bostock, 2002).

Different activation points along the signal transduction pathways mediated by JA and SA can influence the performance of plant activators (Heil and Bostock, 2002). Furthermore, when insects and pathogens concurrently occur in the field, negative interactions may arise between the two defence pathways of induced resistance. These conflicts potentially increase vulnerability to insects when plants are induced to express SAR to pathogens (Gozzo, 2003).

Our results suggest that activation of the SA pathway by exogenous applications of BABA and BTH has a larger effect on nematode development and reproduction for both tomato and potato plants. The underlying mechanisms responsible for the observed effects have not been determined, and future research should address gene/protein expression to provide a finer understanding of the specific plant-nematode interactions, that could be exploited in

nematode control strategies. The effects of inducing plant defence pathways on the complex and diverse naturally occurring interactions between plants and their associated soil microbial diversity should also be considered.

The success of management strategies based solely on chemical plant defence activators may be compromised by the complex and diverse range of pathogens potentially present in the rhizosphere that can be either positively or negatively affected by the immunity response induced by a given plant elicitor (Vallad and Goodman, 2004; Lucas, 2010). Integrated control regimes that combine the use of plant activators to strengthen the endogenous defence of plants with resistant crop genotypes and management of natural suppressive processes may be a practical alternative to current methods of root-knot nematode control.

4.5. Acknowledgements

The first author was supported by ESF funds through the "Programa Operacional Potencial Humano - POPH" of "The National Strategic Reference Framework" (SFRH/BD/27317/2006). Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

We thank Barry Pye for spraying the tomato plants using the Rothamsted Research Experimental Station automatic hydraulic sprayer.

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Chapter 5

Pochonia chlamydosporia combined with plant activators – a potential sustainable strategy for M*eloidogyne chitwoodi* control

Written as a journal article:
Vieira dos Santos, M.C., Curtis, R.H.C., and Abrantes, I. (2012). *Pochonia chlamydosporia* combined with plant activators - a potential sustainable strategy for M*eloidogyne chitwoodi* control. Biocontrol Science and Technology (*submitted*).

Abstract - Sustainable strategies for the control of the root-knot nematode *Meloidogyne chitwoodi* are required to reduce dependence on highly toxic chemical pesticides. The biocontrol efficacy of *Pochonia chlamydosporia* could be enhanced through integration with control measures that reduce initial nematode infestations. Foliar sprays with plant defence activators reduce the susceptibility of certain crop plants for root feeding nematodes and, in combination with the application of *P. chlamydosporia* to the soil, could boost the biocontrol potential of this fungus. The aim of this study was to assess the effect of combined soil application of *P. chlamydosporia* with foliar spray of the activators of plant defence, benzothiadiazole (BTH) or *cis*-jasmone, on *M. chitwoodi* infection of potatoes. Three-week-old potato, *Solanum tuberosum*, cv. Désirée plants grown in soil, previously mixed with 5000 chlamydospores/g, were sprayed twice with BTH or *cis*-jasmone and inoculated with 300 *M. chitwoodi* second-stage juveniles. Forty-five days after inoculation, nematode reproduction, numbers of colony-forming units (CFU)/g of soil and root and egg parasitism were assessed by standard techniques. Shoot and root length and weight were variable among treatments and root weight was higher in infected plants. Foliar sprays of BTH or *cis*-jasmone significantly affected nematode penetration although only BTH treatments or *cis*-jasmone combined with the fungus were significantly effective in reducing nematode reproduction (RF=8.4, 7.6 and 12.5, respectively). The presence of the fungus slightly increased the efficacy of the plant activators. The fungus was able to proliferate above the initial inoculation rate, in soil and roots, in the presence of the nematode. The activation of induced plant defences may have influenced rhizosphere colonization by the fungus as the number of CFU/g of root was lower in plants treated with the foliar sprays. Egg parasitism was higher in the *cis*-jasmone treatment where rhizosphere colonisation was lower, suggesting that *P. chlamydosporia* became a poorer rhizosphere coloniser but a more efficient nematode parasite. The effects of plant activators in the saprophytic-parasitic switch of the fungus are not known, but results suggest that the JA defence pathway might affect *P. chlamydosporia* biology and its biocontrol potential. Egg deposition was lower in plants treated with BTH and *cis*-jasmone in the presence of the fungus, which may be indicative of an additive effect of both treatments. An integrated pest management strategy based on the use of *P. chlamydosporia* associated with inducers of plant defence could thus be a potential effective alternative control strategy for *M. chitwoodi* in potatoes.

Key words: benzothiadiazole (BTH); *cis*-jasmone; root-knot nematodes; systemic acquired resistance, biological control.

5.1. Introduction

The root-knot nematode (RKN), *Meloidogyne chitwoodi*, is an economically important damaging species of plant-parasitic nematodes recognised by its effects on potato production. This nematode has been reported in Argentina, Belgium, Germany, the Netherlands, Mexico, Portugal and South Africa and has been listed as a quarantine organism in Europe since 1998 (Conceição et al., 2009; OEPP/EPPO 2009).

Along with a wide host range, including several economically important crops such as potato, *Solanum tuberosum*, and tomato, *S. lycopersicum*, this species is also tolerant to low soil temperatures (Santo et al., 1980; Santo and O'Bannon, 1982; O'Bannon et al., 1982; Ferris et al., 1993). Current control measures for the management of RKN as, for example, the use of nematicides, proved to be less effective against this nematode, so that control measures have been relying on multiple highly toxic nematicidal treatments (Pinkerton et al., 1986; Santo and Wilson, 1990). The potential negative impact of most chemical nematicides on non-target organisms and the environment led to a total ban or restricted use of these chemicals (Chitwood, 2003). Therefore, the development of new control approaches that can be integrated in environmentally friendly pest management strategies has become an important research subject (Oka et al., 2000).

The exploitation of natural enemies of nematodes and natural defence mechanisms in plants could lead to non-chemical, sustainable and environmentally friendly management control strategy to reduce RKN population densities.

Fungal antagonists are among the most promising nematode biological control agents (Stirling, 1991) and *Pochonia chlamydosporia* (Goddard) Gams and Zare is an ubiquitous facultative nematophagous fungus with parasitic activity against eggs and sedentary females of economically important plant-parasitic nematodes. It has also been associated with soils that suppress the multiplication of cyst nematode populations (Kerry and Crump, 1977; Kerry et al., 1982). The biocontrol potential of *P. chlamydosporia* has been widely studied in pots and field experiments (De Leij et al., 1993; Bourne and Kerry, 1999; Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003; Atkins et al., 2003; Tobin et al., 2008; Tzortzakakis, 2000, 2007, 2009; Puertas and Hidalgo-Díaz, 2009).

Pochonia chlamydosporia is a relatively poor competitor in soil, but it seems to be able to proliferate in the rhizosphere by using nutrients released in root exudates (De Leij et al.,

1993; Kerry et al., 1993; Bourne and Kerry, 2000). Limited endophytic growth (within the roots) has been detected, but the fungus is predominantly confined to the rhizosphere (De Leij and Kerry, 1991; Lopez-Llorca et al., 2002). *Pochonia chlamydosporia* is thought to promote plant growth in the initial stages of root colonization and elicits plant defence against endophytic colonization (Bordallo et al., 2002; Mácia-Vicente et al., 2009a, 2009b). Rhizosphere colonization by the fungus is essential for nematode control and is mediated by the plant species since they differ in their ability to support the fungus (Bourne et al., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999). Fungal abundance in the rhizosphere is not always related to the extent of parasitism of nematode eggs, although isolates of *P. chlamydosporia* were more abundant on nematode infected than healthy roots. This may be related to changes in root exudates due to egg mass protrusion (Bourne et al., 1996; Bourne and Kerry, 1999; Atkins et al., 2003). However, in the presence of very susceptible hosts or large nematode population densities, galls are predisposed to be larger, containing several females leading to egg deposition in egg masses inside the roots. So, highly susceptible hosts have a negative effect on the efficacy of *P. chlamydosporia* control as the egg masses inside the roots become protected from fungal attack and, in addition, the fungus does not prevent the initial infestation of roots by nematode second-stage juveniles (J2). Therefore, biocontrol efficacy could be enhanced by combining its use with control measures that prevent or reduce initial infestations, such as crop rotation with poor hosts for the nematode (Bourne et al., 1996; Kerry and Bourne, 1996). Nevertheless, crop rotation has been the basis of control programmes worldwide, but it is not an economically attractive strategy, especially against *M. chitwoodi*. Cereal crops favoured for rotation with potato (wheat, oats, barley and maize) are considered good hosts of this nematode (OEPP/EPPO 2009).

Plant defences are triggered by pathogen attack, but can also be activated by exogenous application of compounds, such as salicylic acid (SA) and its synthetic mimics like benzothiadiazole (BTH), or jasmonic acid (JA) and its derivatives, and by the non-protein amino acid β-aminobutyric acid (BABA) (Cohen, 2002; Gozzo, 2003; Lucas, 2010). Induced resistance is regulated by two essential mechanisms: systemic acquired resistance mediated by accumulation of endogenous SA, and induced systemic resistance related with JA and ethylene pathways that trigger an array of biochemical reactions targeting the pathogen in

the site of the attempted invasion (Pieterse and Van Loon, 1999; Kuć, 2001; Gozzo, 2003; Lucas, 2010).

It has been demonstrated that the use of foliar sprays of BABA, BTH, JA and derivatives and SA can reduce plant-parasitic nematode invasion, development and reproduction (Oka et al., 1999; Oka and Cohen, 2001; Owen et al., 2002; Nandi et al., 2003; Chinnasri et al., 2003, 2006; Cooper et al., 2005; Collins et al., 2006; Curtis et al., 2009; Berry et al., 2011; Fujimoto et al., 2011; Nahar et al., 2011; Vieira dos Santos et al., submitted).

The application of plant defence activators that reduce the overall susceptibility of crop plants for root feeding nematodes could be a key strategy for boosting the control potential of a biological control agent shown to be more efficient when associated with poor plant hosts. The aim of this study was to assess the effect of combining soil application of *P. chlamydosporia* with foliar sprays of the activators of plant defence BTH (Bion®) or *cis*-jasmone, on *M. chitwoodi* infection of potato.

5.2. Material and Methods

5.2.1. *Meloidogyne chitwoodi* isolate

An isolate of the RKN *M. chitwoodi*, obtained from infected potato tubers collected in Porto, Portugal (Conceição et al., 2009), was maintained on susceptible host tomato plants cv. Easypeel, in pots with sterilised sandy loam soil and sand (1:1) in the Nematology laboratory at the University of Coimbra.

5.2.2. *Pochonia chlamydosporia* isolate

Pochonia chlamydosporia isolate Pc2, obtained from *Globodera rostochiensis* eggs extracted from cysts from soil samples collected in a Portuguese potato field, was maintained in 1.7 % corn meal agar (CMA, Oxoid, UK), at 25 °C (Vieira dos Santos et al., submitted). Production, using a barley:sand substrate (1:1), and extraction of chlamydospores was performed following the methodology described by De Leij and Kerry (1991). Chlamydospore viability and germination were assessed on sorbose agar with antibiotics (Abrantes et al., 2002).

5.2.3. Plant material and *Pochonia chlamydosporia* inoculation

Potato cv. Désirée plants were grown from a single sprout in plastic containers with 800 cm^3 of coarse sandy soil previously mixed thoroughly with 5000 chlamydospores/g of soil. Controls were made with non-inoculated soil. The pots were placed in a growth chamber at 23 °C and a 16 h photoperiod and watered every two days.

5.2.4. Foliar sprays

All plant activators, before foliar spray applications, were mixed with 0.1% non-ionic surfactant ethylan BV (EBV) (Tennants Distribution Ltd, UK). Three weeks after the sprout plantation, the potato plants were sprayed twice with a seven days interval, with a fine mist sprayer, 35 cm above the plant at a rate of 1ml/plant, with 2.5 mM BTH (Bion®, Syngenta, USA) or 1 mM *cis*-jasmone (Sigma, USA). These concentrations correspond to ca.: 1.050 mg/plant (BTH) and 0.213 mg/plant (*cis*-jasmone). Ten pots with soil inoculated with *P. chlamydosporia* and five without fungal inoculum per treatment were sprayed and controls were made with water or 0.1 % EBV. Special care was taken to avoid contact of the plant activators with the soil and roots by covering the soil surface and the pots with aluminium foil. Following each spray, the pots were kept separate for two days to avoid cross-contamination from volatiles. The pots were then transferred back to the growth chamber and the plants were watered every two days.

5.2.5. Nematode inoculation

Two days after the first spray, five plants/treatment with *P. chlamydosporia* or without fungal inoculum were inoculated with 300 J2/plant obtained from egg masses, hand-picked from tomato cv. Easypeel infected roots, and placed on a plastic sieve (10 µm) with tap water, in the dark, at room temperature.

5.2.6. *Meloidogyne chitwoodi* reproduction

Forty-five days after inoculation, the plants were uprooted, the roots were washed free of soil and the length and the fresh weight of shoot and root were recorded. The numbers of galls and egg masses per root system were determined and the number of eggs, extracted from each root system using 0.52% sodium hypochlorite (Hussey and Barker, 1973), counted

to estimate the final population density (Pf). The reproduction factor (Rf=Pf/Pi where Pi=300 J2), the numbers of eggs/egg mass and eggs/g of root were assessed for each plant.

5.2.7. *Pochonia chlamydosporia* proliferation and parasitism

The numbers of colony-forming units (CFU)/g of soil and CFU/g of root were evaluated by dilution plating (De Leij and Kerry, 1991). Egg parasitism was assessed in eggs, mechanically extracted from 10 egg masses collected randomly in each root system, plated in 0.8% technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg/l of each) for three days, at 25 °C. The number of parasitized eggs was quantified using standard methods (Kerry and Crump, 1977).

5.2.8. Data analysis

Data were compared by ANOVA using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA). A square root transformation was used to ensure a Normal distribution and homogeneity of variance of the data that otherwise would not meet the ANOVA assumptions. Statistically significant differences between treatments were computed using the LSD test ($P<0.05$).

5.3. Results

5.3.1. Effects of *Pochonia chlamydosporia* and plant activators against *M. chitwoodi* on potato cv. Désirée

Although variable, plant shoot and root length, in the presence of both the nematode and the fungus, were overall lower than the water control (Table 5.1). Shoot and root weight were consistently higher in plants treated with the activators of plant defence. In these treatments, root weight was significantly higher in the presence of the nematode (Table 5.1). No evidence of phytotoxic effects associated with BTH and *cis*-jasmone application was detected.

Table 5.1 – Shoot and root length and weight of potato cv. Désirée, grown in soil with *Pochonia chlamydosporia* (Pc), 45 days after inoculation with 300 *Meloidogyne chitwoodi* (Mc) second-stage juveniles and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 0.1 % ethylan BV (EBV); and water (H2O).[a)]

Treatment	Shoot		Root	
	Length (cm)	Weight (g)	Length (cm)	Weight (g)
BTH+Pc[b)]+Mc	18.2 ± 1.7 ab	3.2 ± 0.7 cd	27.8 ± 1.8 ab	6.1 ± 1.1 b
BTH+Pc[b)]	23.7 ± 2.9 bcd	4.2 ± 0.5 d	27.8 ± 4.0 abc	2.8 ± 0.7 a
BTH+Mc	30.8 ± 8.3 d	2.4 ± 0.5 abc	28.3 ± 0.8 abc	7.7 ± 0.6 b
Cis-J+Pc[b)]+Mc	23.6 ± 0.6 bcd	4.2 ± 0.2 d	33.2 ± 3.4 bc	6.6 ± 0.3 b
Cis-J+Pc[b)]	26.7 ± 2.7 cd	1.8 ± 0.1 ab	30.2 ± 1.1 abc	3.5 ± 0.7 a
Cis-J+Mc	18.0 ± 1.0 ab	3.6 ± 0.4 cd	28.7 ± 1.7 abc	6.7 ± 1.0 b
EBV+Pc[b)]+Mc	20.8 ± 0.5 abc	4.4 ± 0.3 d	34.8 ± 3.0 c	2.1 ± 0.3 a
EBV+Pc[b)]	24.8 ± 0.4 bcd	1.8 ± 0.3 ab	31.5 ± 1.5 abc	3.0 ± 0.7 a
EBV+Mc	22.3 ± 0.9 bcd	1.5 ± 0.3 a	25.7 ± 1.8 a	2.3 ± 0.5 a
H2O+Pc[b)]+Mc	15.5 ± 1.0 a	3.4 ± 0.5 cd	27.0 ± 0.2 a	1.9 ± 0.3 a
H2O+Pc[b)]	18.3 ± 2.0 ab	1.6 ± 0.3 a	29.7 ± 1.5 abc	2.8 ± 0.2 a
H2O+Mc	28.8 ± 2.6 d	3.1 ± 0.8 bcd	30.2 ± 3.2 abc	2.8 ± 1.1 a

[a)] values are means of five replicates±standard error. Values followed by the same letter within a column are not significantly different according to LSD test ($p<0.05$).
[b)] 5000 chlamydospores/g of soil.

In plants sprayed with BTH or *cis*-jasmone, the numbers of galls, egg masses and eggs/g of root were significantly smaller than in the controls and were not affected by the fungus (Table 5.2). The number of eggs/egg mass was lower in the absence of the fungus in the controls (water and EBV) whereas the opposite was observed in BTH and *cis*-jasmone treatments (Table 5.2). The Rf values were lower in plants treated with the activators of plant defence and were not significantly affected by the presence of the fungus. Nematode reproduction was significantly affected by the foliar spray of BTH in the presence or absence of the fungus (RF=8.4 and 7.6, respectively) when compared to the control water (Rf=35.9 and 32.5, respectively) and to the EBV control (Rf=21.7 and 18.1, respectively). For the *cis*-jasmone treatment, this effect was only statistically significant when foliar sprays were combined with the fungus (Rf=12.5) (Table 5.2).

Table 5.2 – Numbers of galls, egg masses and eggs/g of root, eggs/egg mass and reproduction factor (Rf) in potato cv. Désirée, grown in soil with or without *Pochonia chlamydosporia* (Pc) and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 0.1 % ethylan BV (EBV); and water (H2O), 45 days after inoculation with 300 *Meloidogyne chitwoodi* second stage juveniles.[a)]

Treatment	Galls/g of root (Nº)	Egg masses/g of root (Nº)	Eggs /g of root (Nº)	Eggs/egg mass (Nº)	Rf[c]
BTH+Pc[b)]	5.9±0.9 a	6.0±1.0 a	416.4±40.7 a	74.6±10.0 ab	8.4±0.8 a
BTH	3.9±1.2 a	3.6±1.2 a	307.0±90.9 a	95.9±22.3 abc	7.6±1.7 a
Cis-J+Pc[b)]	10.2±1.3 a	9.9±1.1 a	543.7±76.8 a	50.6±7.3 a	12.5±1.2 ab
Cis-J	12.7±3.3 a	10.1±2.7 a	952.4±171.8 ab	103.1±16.4 bc	20.3±2.7 bc
EBV+Pc[b)]	38.4±10.5 b	38.3±5.0 b	3564.6±1102.0 cd	94.7±12.0 bc	21.7±4.6 c
EBV	39.2±4.2 b	39.1±10.5 b	2398.9±114.6 bc	63.1±7.4 ab	18.1±3.3 abc
H2O+Pc[b)]	43.1±9.3 b	43.1±9.2 b	6007.1±1535.3 d	145.0±30.5 c	35.9±8.5 c
H2O	58.1±5.6 b	48.2±8.2 b	3374.4±815.1 cd	78.9±28.4 ab	32.5±17.8 c

[a)] values are means of five replicates±standard error. Values followed by the same letter within a column are not significantly different according to LSD test ($p<0.05$).
[b)] 5000 chlamydospores/g of soil.
[c)] Rf= final population (Pf)/300 J2 (Pi).

The numbers of CFU/g of soil were significantly higher in the presence of the nematode ($p<0.001$) and no differences were detected between treatments (Fig. 5.1a). Similar results were also observed in the numbers of CFU/g of root (Fig. 5.1b). Egg parasitism was high in all treatments (> 32%), being significantly higher in plants treated with *cis*-jasmone (> 52%) (Fig. 5.1c).

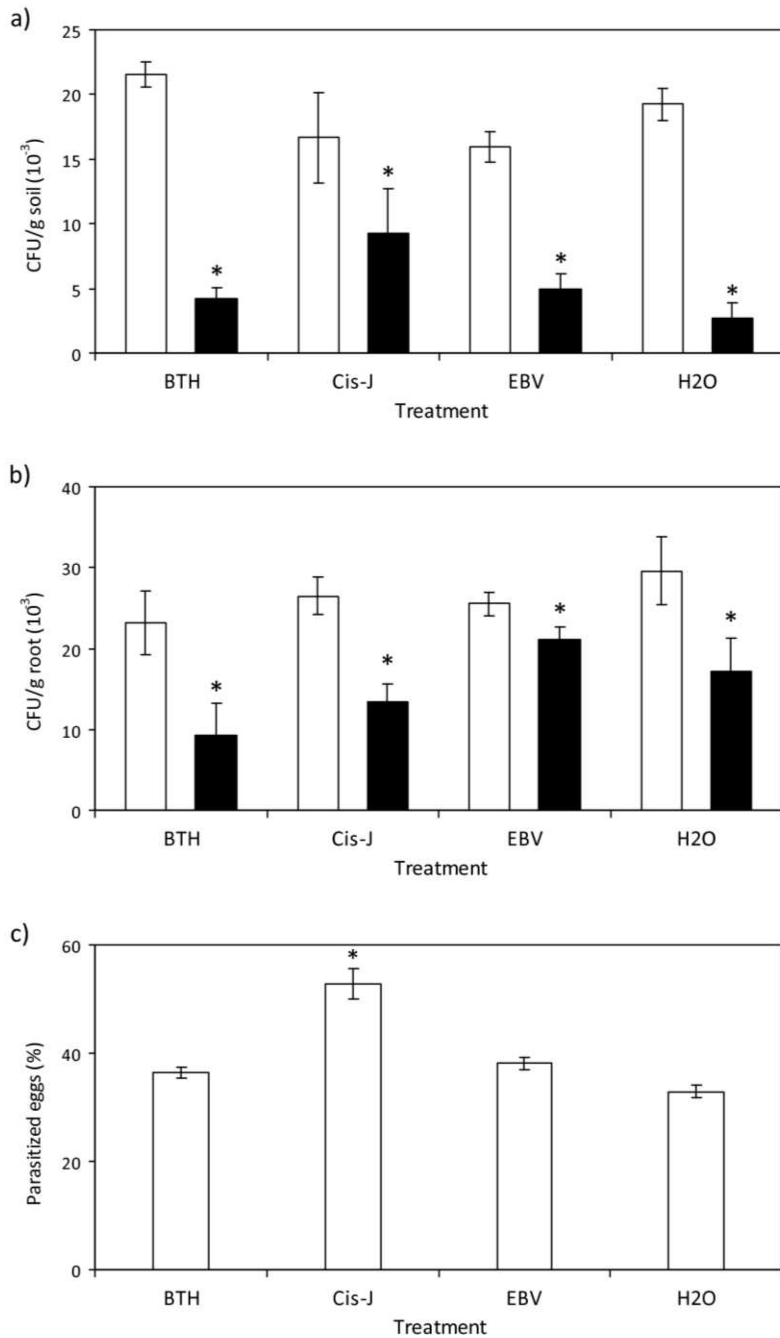

Figure 5.1 - Numbers of colony forming units (CFUs) of *Pochonia chlamydosporia* in soil (a) and roots (b) of potato cv. Désirée and (c) egg parasitism. Treatments: foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 0.1 % ethylan BV (EBV); and water (H2O). (□) with 300 *Meloidogyne chitwoodi* second-stage juveniles and (■) without the nematode. Bars represent standard error of means. * significantly different according to Fisher LSD test (P<0.001).

5.4. Discussion

This study investigates for the first time the potential of *P. chlamydosporia* as a biological control agent combined with the use of plant defence activators against the RKN *M. chitwoodi*. The spray of potato leaves with BTH or *cis*-jasmone significantly affected nematode penetration of the plants, but only BTH treatment was effective in reducing nematode reproduction, whereas *cis*-jasmone treatment was only significantly effective in reducing nematode reproduction when combined with the fungus (Table 5.2).

The efficacy of foliar sprays with BTH in reducing plant-parasitic nematode densities, including *M. chitwoodi*, on potato under field conditions, has been reported before (Collins et al., 2006). Moreover, it has been shown that commercial formulations of BTH (Bion® and Actigard®) on glasshouse-grown Cabernet Sauvignon grapevines, sugarcane, cowpea and soybean plants had a significant effect on *Meloidogyne* spp. by reducing nematode infection and reproduction, slowing nematode development and decreasing egg deposition (Chinnasri et al., 2003; Owen et al., 2002; Berry et al, 2011).

The ability of *Pochonia chlamydosporia* to grow in soil depends, to a certain extent, on available nutrients, as isolates of the fungus have a tendency to be more abundant in organic than in mineral soils (De Leij et al., 1992, 1993; Kerry et al., 1993; Bourne and Kerry, 2000). *Pochonia chlamydosporia* densities detected in soil were low in the absence of the nematode. However, once in the presence of the nematode, the fungus was able to proliferate above the initial inoculation rate (>15000 chlamydospores/g of soil). Fungal growth and proliferation in soil is not related with the presence of RKN (Mauchline et al., 2002). But the germination of chlamydospores inoculated in soil could be triggered by nutrients leaking from the roots (De Leij et al., 1993, Mauchline et al., 2002). It is very likely that the soil samples were in fact under a strong influence of root exudates, because the whole system was confined by the pots. Changes in root exudates due to egg masses protrusion (and possibly higher nutrient leakage) may have been responsible for the proliferation of the fungus in rhizosphere soil, as previously suggested (Bourne et al., 1996). Similarly, rhizosphere colonization was higher in the presence of the nematode. Although fungal abundance is not always related to the presence of nematode eggs, rhizosphere colonization is strongly related to egg production and changes in the root exudates (Bourne et al., 1996).

These are important aspects of the biology of *Pochonia chlamydosporia* which are essential for efficient nematode control. Isolates of *P. chlamydosporia* tend to be more abundant on nematode infected rather than healthy roots (Bourne et al., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999; Atkins et al., 2003). Interestingly, the number of CFU/g of root was lower in plants treated with the foliar sprays when compared to the controls in the presence and absence of the nematodes, thus implying that activation of induced plant defences may have influenced rhizosphere colonization by the fungus. Fungal growth within root tissues induces cell wall modifications including papillae and appositions along with the presence of compounds associated with plant resistance. However, this elicitation of plant defence mechanisms does not seem to affect fungal growth (Bordallo et al., 2002; Mácia-Vicente et al., 2009a).

More importantly, egg parasitism was higher in the *cis-jasmone* treatment where rhizosphere colonisation was lower when compared to untreated plants. This would suggest that, under these conditions, *P. chlamydosporia* revealed to be a poorer rhizosphere coloniser but a more efficient nematode parasite. When the colonizing *P. chlamydosporia* contacts a nematode egg mass in the rhizosphere, the fungus ceases its saprophytic stage and switches to a parasitic stage. The factors that affect this switch are not well understood and, although nematode host preference at the infra-specific level may be involved, nutrients either released by plants or available in nematode eggs may also play an important role (Kerry, 2000; Morton et al., 2004; Esteves et al., 2009). The effects of plant activators in this saprophytic-parasitic switch are not known, but results of this study would suggest the involvement of the JA defence pathway might play a key role in the *P. chlamydosporia* biology and its use as biological control agent.

Egg deposition was lower in plants treated with BTH or *cis*-jasmone in the presence of the fungus, which may be indicative that the two treatments combined have an additive effect on the control of the RKN *M. chitwoodi*.

The potential of *P. chlamydosporia* isolates as biological control agents against RKN is greatly influenced by a number of traits, including their establishment in soil, establishment in the rhizosphere and their ability to parasitize nematode eggs (Abrantes et al., 2002). Such traits did not seem to be adversely affected by the application of plant defence activators. Instead, the application of the plant activator *cis*-jasmone may have created the conditions

for a fungal switch from saprophytic to parasitic, thus increasing nematode parasitism. Furthermore, the effects of these activators of plant defence on nematode reproduction were also enhanced by the presence of the fungus. Therefore, an integrated pest management strategy based on inducing plant defence mechanisms and the use of the nematophagous fungus *P. chlamydosporia* could be a potential alternative control strategy for the management of *M. chitwoodi*. These experiments were conducted in controlled conditions and sterilized soil, but to better understand the intricate interactions between the plant, the nematode and the fungus under the effect of these plant defence activators further *in vitro* experiments should be conducted. These will be important for testing the putative involvement of the JA defence pathway in the saprophytic/parasitic switch of *P. chlamydosporia.* Both abiotic and biotic factors, which influence the performance and efficacy of *P. chlamydosporia*, and the effects of inducing plant defence pathways on the complex and diverse naturally occurring interactions between plants and their associated soil microbial diversity should also be evaluated in field studies.

5.5. Acknowledgements

The first author was supported by ESF funds through the "Programa Operacional Potencial Humano - POPH" of "The National Strategic Reference Framework" (SFRH/BD/27317/2006). Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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General Discussion

The sustainable management of economically important soil-dwelling pests such as potato cyst and root-knot nematodes calls for the integration of novel control strategies. The development and application of successful biocontrol measures may rely on studies on native enemies of nematodes in agricultural systems and natural plant defence mechanisms.

1. *Pochonia chlamydosporia*: factors that may determine its efficacy as a biological control agent

Pochonia chlamydosporia Portuguese isolates Pc1, Pc2 and Pc3, associated with potato cyst nematode (PCN) eggs, and 2 non-native isolates: Vc10, originally obtained from *Meloidogyne incognita* eggs, Brazil; and Pc280 isolated from *Globodera pallida* eggs, UK, were identified using specific primers based on the *β*-tubulin primers and ERIC-PCR (Chapter 1). ERIC-PCR fingerprinting allow rapid identification and has been used to identify specific isolates, in mixed infection studies, re-isolated from soil and roots and to assess intraspecific variation among isolates of *P. chlamydosporia* (Arora, 1996; Hirsch et al., 2000; Morton et al., 2003; Mauchline et al., 2004). Genetic variability among isolates showed that isolates can be grouped according to both their geographical and the host nematode species; however, the importance of this genetic variation in the regulation of a host population is unknown. Isolates with different geographical origins may share identical ERIC-PCR patterns, indicating that this fungus is less ubiquitous and diverse in different agricultural systems than many other micro-organisms (Morton et al., 2003; Flores-Camacho et al., 2008; Manzanilla-López et al., 2009). The isolates Pc1 and Pc3, originating from cysts of two different regions, shared an identical profile, whereas Pc2, Vc10 and Pc280 exhibited different profiles.

Fungal growth and proliferation in the rhizosphere, the ability to parasitise nematode eggs, low host specificity and the potential for mass production are key features that are tested in standard bioassays for the selection of *P. chlamydosporia* isolates (Abrantes, 2002). All the isolates revealed marked differences in their performance in the *in vitro* bioassays (chlamydospores production, rhizosphere of barley colonization and *G. rostochiensis* and *M. chitwoodi* eggs parasitism (Chapter 1).

Isolate Pc1 was a poorer rhizosphere colonizer but a better egg parasite and produced ca. 20 fold more chlamydospores than Pc3. These differences suggest that genetic variability

accessed by ERIC-PCR profiling is not related with *in vitro* bioassays performance. Both isolates failed in the rhizosphere competence bioassay (Pc1) or produced few chlamydospores (Pc3) and therefore were considered to have a limited potential as a biological control.

For isolate Pc2, egg parasitism was moderate (< 43 %) against both nematode species, but this isolate revealed desirable traits such as high rhizosphere competence and chlamydospore production, thus having a greater potential to be exploited as a biological control agent. The two non-native isolates tested, Vc10 and Pc280, were poor rhizosphere colonizers and chlamydospore producers when compared to Pc2, but Vc10 was the best parasite of PCN eggs (ca. 55 %).

Enzymatic activity of the isolates, grown under different nutrient media, and their response to abiotic factors such as temperature and water availability may influence their biological control potential. Therefore, information about the enzymatic activity of the isolates was collected and compared (Chapters 1 and 2). The highest enzymatic activity was detected in Pc2 in low nutrient media. All isolates produced esterases in liquid media, but protease activity was only observed in Pc2 and Vc10. Proteases are thought to be involved in the first steps of nematode infection (St. Leger et al., 1986). Interestingly, isolates Pc2 and Vc10 also performed best in the standard bioassays and their enzymatic activity was further assessed in the presence and absence of *G. rostochiensis* and *M. chitwoodi* eggs. The enzymatic activity decreased in the presence of nematode eggs for both Pc2 and Vc10, and this phenomenon, although not fully understood, may be related to a metabolic switch between saprophytic and parasitic activity. The egg infection process depends on the activity of specific enzymes that are locally expressed in dedicated fungal structures (appressoria) that degrade the nematode egg shell (Segers et al., 1994; Tikhonov et al., 2002), and may have evaded the standard enzymatic tests performed.

The influence of abiotic factors such as temperature and water availability is essential in the success of *P. chlamydosporia* isolates as potential biological control agents (Bourne and Kerry, 2000; Magan, 2001). Different isolates can differ in their optimal temperature for growth and isolates obtained from cyst nematodes have lower optimal temperatures than isolates associated with root-knot nematode (RKN) eggs (Kerry et al., 1986; Bourne and Kerry, 2000; Olivares-Bernabeu and Lopez-Llorca, 2002). Differences in growth and tolerance

to osmotic and matric water stress have been detected, although the optimal water potential for growth is likely to occur between -1.0 and -2.0 MPa at 25 to 30 °C (Bourne and Kerry, 2000; Esteves et al., 2009). The impact of temperature and water stress on sporulation, germination and egg parasitism of plant-parasitic nematodes was evaluated for the first time in isolates of *P. chlamydosporia*.

The optimum temperature for growth of all isolates was 25 °C, whereas high temperatures (35 °C) were lethal to the mycelium of some isolates (Pc1, Pc2 and Pc280). Although growth was inhibited at 33 and 10 °C, spores/hyphal fragments can remain viable for almost one month at these temperatures and the isolates can recover their growth when temperatures are set back to 25 °C. Nevertheless, growth, spore production and viability, and egg parasitism were affected by exposure to limiting growth temperatures (Chapter 1). Isolate Pc3 is fitter in withstanding lower temperatures as its growth rate was higher in colonies growing out of colonised plugs submitted to 10 °C for 25 days and subsequently revealed a significant increase in chlamydospore production and PCN egg parasitism. On the other hand, for isolate Pc1, an increase in growth rate and chlamydospore production was observed in colonies growing out of colonised plugs submitted to 33 °C for 25 days. However, no differences in egg parasitism were detected. For the other isolates, the variability in growth rate, chlamydospore production and egg parasitism could not be related to exposure to limiting for growth temperatures. Whether temperature tolerance of each isolate is related to the climatic conditions of its geographical origin remains unknown, but the observed variability emphasizes the need to understand the influence of abiotic conditions on the performance of the isolates.

All isolates were able to grow under a range of water potentials, and behaved similarly when exposed to various degrees of osmotic and matric stress (Chapter 2). Isolates were more tolerant to osmotic stress than to matric stress: water potentials limiting for growth were -10 and -7.1 MPa, respectively. Radial growth rates decreased with the increase of osmotic and matric stress, which is in agreement with other studies (Kerry et al., 1986; Esteves et al., 2009). Furthermore, the production of chlamydospores, one of the key factors to be assessed in the development of isolates as biological control agents, was severely repressed in both osmotic and matric modified medium, and a negative effect of osmotic stress on egg parasitism was also observed. Previous studies on the tolerance levels of

P. chlamydosporia to environmental stress did not investigate the ability of the fungi to resume growth when conditions became favourable again, as would be expected in the often variable field conditions (Kerry et al., 1986; Bourne and Kerry, 2000; Olivares-Bernabeu and Lopez-Llorca, 2002; Esteves et al., 2009). When transferred to non-restricting conditions all the tested isolates were able to resume growth after exposure to water potentials limiting for growth. With the exception of Pc2 and Pc3 under osmotic stress, chlamydospore production increased and the percentage of parasitism was higher when fungi were removed from water stress than in the control conditions.

In vitro screening bioassays can speedy the selection of isolates with biological control potential using time and resources efficiently. However, isolates that perform well in laboratory tests may not always be effective in soil (Abrantes et al., 2002), and the opposite also occurs (Hidalgo-Diaz et al., 2000). The native isolate Pc2, that produced the largest numbers of chlamydospores, and the reference non-native Vc10 were selected for further studies. Their survival and proliferation in soil and rhizosphere and their efficacy against *M. chitwoodi* during a potato-maize-potato-potato crop rotation in a pot trial was evaluated (Chapter 3). Isolate Vc10 has been widely studied and is possibly the best characterized isolate with proven efficiency against *Meloidogyne* spp. (De Leij and Kerry, 1991; De Leij et al., 1993). Subtle differences in host preference, plant compatibility or tolerance to abiotic conditions were detected between the isolates. Colonisation of the rhizosphere of crop plants was low, which may explain the generally low levels of egg parasitism. Growth and proliferation in soil were slow and were only achieved, for both isolates, after multiple inoculations. Nutrient availability and temperature had a strong effect on the efficacy of both isolates, as expected from the above bioassays and previous studies (De Leij et al., 1992; Magan, 2001). When applied to non-sterile soil, the fungal isolates were not only submitted to varying and sometimes harsh abiotic conditions, but were also exposed to the native microbiota, with which they may have interacted differently (Monfort et al., 2006). However, soil microorganisms are themselves subjected to environmental stress and their populations have collapsed after the summer 2009, allowing the establishment of *P. chlamydosporia*. These results suggest that this fungus is overall a poor competitor in soil, and its application can reach higher biocontrol efficiency when applied after the summer, even if native isolates, putatively better adapted to local conditions, are used, which is in

accordance with the results obtained in studies in Mediterranean conditions (Verdejo-Lucas et al., 2003; Tzortzakakis, 2009).

Nematode control strategies involving the application of parasites of sedentary nematode stages such as *P. chlamydosporia* cannot prevent the initial infestation of roots by the mobile nematode juveniles and should be integrated with other control methods such as, for example, crop rotation with poor hosts for the nematode, taking into account that plant species differ in their ability to support *P. chlamydosporia* growth in their rhizosphere (Bourne et al., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999). Thus, crop rotation requires a careful selection of cultivars that should not only be less susceptible to the nematode but also that support extensive growth of the fungus in their rhizosphere (Manzanilla-López et al., 2011). The application of plant defence activators is an emerging nematode control strategy that could possibly be integrated with biological control agents to promote plant defence against RKN.

2. The use of plant elicitors to strengthen the endogenous defence of plants

Two essential mechanisms regulate induced resistance in plants: systemic acquired resistance mediated by accumulation of endogenous salicylic acid (SA) and induced systemic resistance related with jasmonic acid (JA) and ethylene pathways (Kuć, 2001). Plant defence mechanisms naturally triggered by pathogen attack can also be activated by the exogenous application of compounds, such as SA and benzothiadiazole (BTH), JA and its derivatives, and by the non-protein amino acid *β*-aminobutyric acid (BABA) (Cohen, 2002; Gozzo, 2003; Lucas, 2010). Pot trials were conducted to analyse the enhancement of the plant defence activators JA pathway (*cis*-jasmone and methyl-jasmonate) and SA pathway (BABA, BTH, and SA) against *M. chitwoodi* in the host tomato plant and of the activators of both pathways (*cis*-jasmone, BTH and methyl jasmonate) against this RKN in the important host potato plant (Chapter 4). These plant defence activators had already shown promising levels of plant parasitic nematode control on different plant hosts, either through a local or a systemic effect (reviewed in Chapter 4).

Foliar applications of BABA, *cis*-jasmone or methyl-jasmonate effectively reduced nematode penetration in tomato roots, but for those nematodes that were able to invade

the roots, reproduction was only reduced in BABA treatment. Even though both SA and *cis*-jasmone effectively reduced nematode development, egg production increased.

Although stronger effects were generally obtained in potato than in tomato plants, a similar trend was observed in the outcomes of the application of BTH, *cis*-jasmone or methyl-jasmonate against *M. chitwoodi*. Nematode reproduction was more markedly affected by BTH, and only BTH or *cis*-jasmone affected nematode development significantly. However, all activators produced comparable results in reducing nematode penetration. While differences in susceptibility of the different host plants to nematode attack were detected, the stronger effects of foliar sprays of BTH, *cis*-jasmone or methyl-jasmonate on *M. chitwoodi* in potato may be related to the different dosage application. Linear relationships between dosage application of plant activators and the effects on RKN have been reported (Chinnasri et al. 2006; Fujimoto et al. 2011). Overall, plant defence activators of the SA pathway were more effective in reducing nematode reproduction than the JA pathway. The mechanisms responsible for the observed effects have not been assayed, thus the potential effects of the nematode modulation of these biochemical pathways in its interaction with the plant are not fully understood. Until such mechanisms and their outcomes are further investigated, under different conditions, all the plant defence activators should be considered valid options for nematode control. On the other hand, the intricate and diverse naturally occurring trophic interactions among plant, pathogens and environmental conditions are a major constraint of a successful management strategy based only on chemical plant defence activators (Vallad and Goodman, 2004; Lucas, 2010). However, reduction of nematode penetration of roots is a highly desirable outcome for a nematode control strategy and it can also be used in combination with the application of *P. chlamydosporia* in the soil to enhance the performance of the biological control agent. This practice can be integrated as a nematode management control strategy.

3. Evaluation of a sustainable integrated management strategy that exploits the combined use of a biological control agent with the use of plant activators

In order to assess the efficacy of the combined application of *P. chlamydosporia* and of plant defence activators against *M. chitwoodi* in potato in controlled conditions (Chapter 5), the native isolate Pc2 was selected for this study due to its favourable traits inferred from the bioassays and pot trials (Chapters 1, 2 and 3). Plant activators BTH and *cis*-jasmone were taken as representative of each of the SA or JA pathways, and selected for their effects in the pot trials (Chapter 4).

Foliar treatments with BTH or *cis*-jasmone significantly affected nematode penetration in potato plant roots, confirming the results obtained in the pot trials. The plant defence activator BTH significantly reduced nematode reproduction when compared to untreated plants. The same effect was obtained with the combined application of *cis*-jasmone and the fungus.

The performance of the fungal isolate Pc2 under the controlled conditions of this assay, where temperature and humidity were maintained without fluctuations, was markedly superior to that observed in the outdoor pot trials with the fungi alone. Rhizosphere proliferation by Pc2 was lower in treatments where the plant defence activators were applied, which suggests that the plant actively responds to the endophytic colonisation of its roots by the fungus. As mentioned in Chapter 5, during rhizosphere colonisation, *P. chlamydosporia* can also elicit plant defence and can be regarded as a plant growth promoter (Bordallo et al., 2002; Mácia-Vicente et al., 2009a, 2009b). It should be pointed out that the biocontrol efficacy of Pc2 was enhanced by *cis*-jasmone application, as egg parasitism by the fungus was significantly increased in this treatment, while the application of BTH did not seem to affect fungal parasitism. This may reveal that activation of the JA pathway might produce changes in the plant and/or the rhizosphere that prompt the fungal transition from the saprophytic to the parasitic trophic phase.

Pending further research to clarify the mechanisms and interactions involved in the above results, it seems likely that activation of the JA pathway by defence activators that reduce RKN penetration, whilst promoting egg parasitism by a selected *P. chlamydosporia* isolate, can result in a sustainable, natural and effective strategy of nematode control.

4. Future work

The work described in this Thesis seeks not only to contribute for the development of potential non-chemical, sustainable and environmentally friendly management strategies of reducing plant-parasitic nematode population densities, but also to reveal and propose new research paths and ideas.

The fungus seemed to be less tolerant to water stress at higher temperatures (Bourne and Kerry, 2000). In Mediterranean climates, water stress is frequently associated with high temperatures in long, dry summers. Tolerance of such harsh and potentially additive abiotic factors, as well as the capacity to resume growth and sporulate once conditions are favourable, needs to be addressed during screening for novel biological control agents. To avoid having to perform large pot trials in field conditions, a miniaturised system similar to baiting techniques (Kerry, 1991) could be devised and optimised for high-throughput screening of the influence of the interaction between water stress and temperature on the survival of *P. chlamydosporia*.

Future work should also be focused on the research of the mechanisms responsible for the switch between the two trophic phases of *P. chlamydosporia*. Sequence analysis of the upstream regulatory region of the serine protease gene *vcp1* revealed that gene expression was influenced by readily metabolised sources of carbon and nitrogen and pH (Ward et al., 2012). The results obtained in the pot trials (Chapter 5) confirmed that nutrient quality and availability seem to play an important role in this transition, but the effect of the key sources of nutrients in the rhizosphere has not been investigated. Therefore, further research needs to be conducted on the role of root exudation composition and exudation patterns as affected by plant species and cultivars, as well as on natural and artificially activated plant defence in promoting fungal rhizosphere colonisation and the switch to parasitism.

In order to parasitize RKN eggs, the fungus must colonise and overcome the gelatinous matrix and the mucilage enveloping the eggs. The gelatinous matrix can be regarded as a source of nutrients for the saprophytic growth of the fungus and the mucilage embedding the eggs has been considered to stimulate egg parasitism (Irving and Kerry, 1986). On the other hand, densely populated microbial niche and microflora content of the rhizosphere can also influence fungal parasitism (Kok et al., 2001). The influence of these biotic factors and

their interactions in the saprophytic-parasitic trophic phases of the fungus can be elucidated in straightforward bioassays. To unveil metabolic pathways involved in the saprophytic-parasitic switch and other desirable traits of candidate biocontrol agents, studies of gene expression in selected specific conditions could be performed.

Transcriptome analyses of *P. chlamydosporia* revealed the up-regulation of specific genes that would otherwise be considered housekeeping genes, detected only in the presence of nematode eggs, and that include genes involved in metabolic functions, cellular signal regulation, cellular transport, regulation of gene expression and DNA repair (Rosso et al., 2011). These findings imply that gene involvement in parasitism and their regulation cannot necessarily be anticipated and general techniques should be integrated (i.e. transcriptomics and proteomics).

To fully access the enormous potential of chemically-induced resistance to plant-parasitic nematodes, further studies should take into account a number of variables such as the effects of different chemical concentrations and application methods, along with the effects of inducing plant defence pathways on the complex and diverse naturally occurring interactions between plants and their associated soil microbial diversity (Molinari, 2011). It has also been suggested that induced plant defence mechanisms would incur fitness costs for the plant (Heil and Baldwin, 2002). Research is needed on the evaluation of the potential trade-offs in investment in plant defence and susceptibility to nematode attack to determine the most successful combinations of different forms of induced defence in different plant species/cultivars under different growing conditions. This can be experimentally tested in studies that integrate various aspects of biology: molecular analyses, plant physiology and phenotyping and ecological studies of direct and indirect effects of plant defence activators.

The putative modulation of the interactions among the host plant, nematode and fungal isolate by the activation of the JA pathway represents not only a potential application as a new control strategy but also poses new questions on how the organisms interact and how these interactions can be further exploited to promote nematode control. Therefore, trials should be conducted in conditions closer to those found in agricultural systems to assess how these interactions can be affected by both biotic (competition by native microbiota, effects of other herbivores and pathogens in the activation of plant defence mechanisms) and abiotic factors. Also, *in vitro* assays under controlled conditions, which focus on selected

key components of all these interactions, need to be performed, in order to better understand the biochemical and molecular mechanisms involved.

The involvement and regulation of various pathogenesis related (PR) proteins of the defence pathways can be assessed at different stages and time points of the previous suggested assays through current proteomic tools. While gel-based protein separation techniques have led to the identification of many pathogenicity and defence-related genes and proteins expressed during plant-pathogen interactions, key proteins are often expressed in low abundance, and can either be overlooked by the analyses or can be difficult to extract and identify. Several emerging techniques such as gel-free high-throughput screening technologies could overcome the setbacks of gel-based protein separation techniques (Afroz et al., 2011). Nonetheless, initial screening using current 2-D DIGE and e.g. mass spectrometry coupled with the various techniques for functional analysis of proteome and protein-protein interactions, such as protein microarrays, would provide important information.

Several aspects of the factors affecting the performance of *P. chlamydosporia*, the use of plant elicitors to strengthen the endogenous defence of plants and the development and application of alternative integrated biocontrol strategies against plant-parasitic nematodes must continue to be addressed. Nevertheless, the results obtained and the conclusions discussed help to further the knowledge on biocontrol strategies against economically important plant-parasitic nematodes.

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