Joana Filipa Leandro Horta

Pochonia chlamydosporia - Biological control agent of root-knot and potato cyst nematodes

Master thesis project in Biodiversity and Vegetable Biotechnology, guided by professor Dr.ª Isabel Luci Pisa Mata da Conceição and Doctor Maria Clara de Almeida Vieira dos Santos, presented at the Department of Life Sciences of Faculty of science and technology of the University of Coimbra.

Junho de 2017

Universidade de Coimbra

Front cover from left to right: *Pochonia chlamydosporia* spores (light microscope photograph); Corn meal agar plate inoculated with the fungus; Eric-PCR profiles; β-tubulin- and ITS-PCR products; *In vitro* bioassays (chlamydospores production, rhizosphere colonisation and nematode egg parasitism. All photographs by M.C. Vieira dos Santos and J. Horta.

Pochonia chlamydosporia - Biological control agent of root-knot and potato cyst nematodes

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica da Prof. Doutora Isabel Luci Pisa Mata da Conceição (Universidade de Coimbra) e da Doutora Maria Clara de Almeida Vieira dos Santos (Universidade de Coimbra).

Joana Filipa Leandro Horta

Departamento de Ciências da Vida

Universidade de Coimbra

2017

Table of contents

Table of figures iv

Abbreviations vi

Agradecimentos vii

Abstract ix

Resumo xi

1. Introduction 14

1.1. Tomato culture 14

1.2. Plant-parasitic nematodes 15

1.2.1. Root-knot nematodes, *Meloidogyne* spp. 16

1.2.2. Potato cyst nematodes, *Globodera* spp. 17

1.3. Management strategies 18

1.3.1. Biological control 20

1.4. *Pochonia chlamydosporia*, a potential biological control agent 23

1.4.1. *Pochonia chlamydosporia* isolates screening 27

2. Materials and Methods 30

2.1. *Pochonia chlamydosporia* isolates 30

2.2. Molecular identification and characterization of *Pochonia chlamydosporia* isolates 31

2.2.1. DNA extraction 31

2.2.2. *Pochonia chlamydosporia* identification 31

2.2.3. Enterobacterial repetitive intragenic consensus (ERIC)-PCR 32

2.2.4. Internally trancribed spacer-restriction fragment length polymorphism (ITS-RFLP) 32

2.3. Biological characterization of *Pochonia chlamydosporia* isolates 33

2.3.1. Chlamydospore production 33

2.3.2. Rhizosphere colonisation 33

2.3.3. Nematode egg parasitism 34

2.4. Data analysis 35

3. Results 37

3.1. Molecular identification and characterization of *Pochonia chlamydosporia* isolates 37

3.1.1. *Pochonia chlamydosporia* identification 37

3.1.2. Enterobacterial repetitive intragenic consensus (ERIC)-PCR 38
3.1.3. Internally transcribed spacer-restriction fragment length polymorphism (ITS-RFLP).. 39
3.2. Biological characterization of *Pochonia chlamydosporia* isolates.................. 43
3.2.1. Chlamydospore production.. 43
3.2.2. Rhizosphere colonisation 46
3.2.3. Nematode egg parasitism.. 46
4. Discussion 51
5. Conclusion 58
6. Future perspectives... 58
7. Bibliography 61

Table of figures

Figure 1 - Molecular characterization of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10, 392). PCR product of DNA amplified using primers for the specific detection of: (a) *Pochonia chlamydosporia* var. *chlamydosporia* (Hirsch et al., 2000) and (b) *Pochonia chlamydosporia* var. *catenulata* (Atkins et al., 2003a). nc: negative control without template DNA; M: DNA size marker Hyperladder^{TM} IV, 100 bp ladder (Bioline, UK).. 37

Figure 2 – ERIC-PCR profiles Molecular characterization of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). PCR product of DNA amplified with ERIC-PCR profiles. nc: negative control without template DNA; M: DNA size marker HyperladderTM IV, 100 bp ladder (Bioline, UK). 38

Figure 3 - Dendrogram showing linkage distances among groups based on the ERIC-PCR profiles of of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). The dendogram was constructed by Unweighted Pair Group Method with Arithmetic Mean in the hierarchial clustering based on Euclidean distances using Statistica version 10 (StatSoft Inc., US). 38

Figure 4 - PCR product of DNA from 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392) amplified with a) ITS4/ITS1 primers and b) ITS4/ITS5 primers (White et al.,1990). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK).. 39

Figure 5 - RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Eco*RI of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK)... 40

Figure 6 – RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Hae*III of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK)... 41

Figure 7 - RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Hinf*I of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK)... 42

Figure 8 – Chlamydospore production (1st assay): number of chlamydospores (a), and chlamydospore viability (b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05). 43

Figure 9 – Chlamydospores production (2nd assay): number of chlamydospores (a) and chlamydospore viability(b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05). 44

Figure 10 – Chlamydospores production (3rd assay): number of chlamydospores (a) and chlamydospore viability (b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05). 45
Figure 11 – Percentage of colonised roots of tomato cv. Tiny Tim by 11 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10). Bars represent standard error of means and columns with the same letter are not significantly different according to LSD test (p>0.05). 46
Figure 12 – Percentage of parasitised *Meloidogyne incognita* eggs by 10 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10) grown for 10 days on 1.7% corn meal agar (CMA). (a) First assay; (b) second assay; (c) third assay. Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05). 48
Figure 13 - Percentage of parasitised *Globodera pallida* eggs by 10 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10) grown for 10 days on 1.7% corn meal agar (CMA). (a) First assay; (b) second assay; (c) third assay. Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05). 49

Abbreviations

BTH - Benzothiadiazole

CFE - Centre for Functional Ecology

CMA - Corn meal agar

DNA - Deoxyribonucleic acid

EPPO - European and Mediterranean Plant Protection Organization

ERIC - Enterobacterial repetitive intergenic consensus

FAO - Food and Agriculture Organization of the United Nations

IGS - Intergenic spacers

ITS - Internally transcribed space

JA - Jasmonic acid

J2 - Second-stage juvenile

J3 - Third-stage juvenile

J4 - Fourth-stage juvenile

OMAIAA - Observatório dos mercados agrícolas e das importações agroalimentares

PCN - Potato cyst nematode

PCR - Polymerase chain reaction

PDA - Potato dextrose agar

PPN - Plant Parasitic Nematodes

RFLP - Restriction fragment length polymorphism

RKN - Root-knot nematode

SA - Salicylic acid

UC - University of Coimbra

WA - Water agar

Agradecimentos

Este trabalho foi parcialmente financiado pelo FEDER - Fundo Europeu de Desenvolvimento Regional através do COMPETE 2020 – Programa Operacional Competitividade e Internacionalização (POCI), e pelo orçamento da Fundação para a Ciência e a Tecnologia na sua componente OE no âmbito do projeto POCI-01-0145-FEDER-016611 (PTDC/AGR-PRO/3438/2014).

A realização desta dissertação de mestrado contou com importantes apoios e incentivos, sem os quais não se teria tornado uma realidade e aos quais estou bastante grata.

Em primeiro lugar gostaria de agradecer à Professora Doutora Isabel Luci Conceição por me ter aceite como orientanda, e por me ter guiado para o projeto no qual me encontro integrada. Quero ainda agradecer todo o apoio prestado ao longo do meu projeto de tese, e agradecer todas as oportunidades que me proporcionou durante o meu percurso científico, inclusive a oportunidade de participar em dois cursos relacionados com o meu tema do projeto de tese, que permitiram fundamentar os meus conhecimentos e evoluir a nível curricular.

Quero agradecer à Doutora Maria Clara Vieira dos Santos, em primeiro lugar por me ter aceite como orientanda, e em segundo lugar pela grande prova de confiança ao criar-me oportunidades que me ajudaram a evoluir na minha carreira profissional. Agradeço também a total disponibilidade para me ensinar a ser mais autónoma a nível prático, pelo grande contributo no trabalho prático e escrito, por me ter ensinado a aprender com os meus erros e por me mostrar que as barreiras da ciência se ultrapassam com esforço e dedicação. Agradeço ainda a amizade e paciência durante este último ano.

À Professora Doutora Isabel Abrantes pela oportunidade de trabalhar no laboratório de Nematologia, pela sua disponibilidade para solucionar dúvidas e problemas que pudessem surgir ao longo do projeto de tese e por me dar a oportunidade de participar em eventos que contribuíram para o meu progresso como profissional.

Quero também agradecer ao Professor Doutor Jorge Canhoto por me ter aceite neste mestrado e por todo o conhecimento adquirido ao longo destes dois anos. Quero ainda agradecer a sua disponibilidade e apoio sempre que necessitei.

Ao Professor Doutor António Portugal por me ter guiado para o meu projeto de dissertação, quando recorri à sua ajuda para encontrar um projeto que se enquadrasse na minha área de interesse.

Aos meus colegas de laboratório à Ivânia, à Carla, ao Luís, à Joana Sá, à Joana Duarte e à Joana Figueiredo por terem contribuído para a evolução e realização do meu

projeto de tese. E um especial obrigada à Soraia, por ter estado sempre disposta a ajudar-me durante o meu trabalho prático, pela sua colaboração ao solucionar-me dúvidas que foram surgindo ao longo do trabalho prático e escrito, e por me permitir trabalhar ao som da melhor rádio portuguesa, a m80.

Um especial obrigada a todos os meus amigos que contribuíram de alguma forma para o sucesso do meu trabalho. Quero então agradecer aos meus colegas de casa, à Daniela, à Cátia Gomes, ao Bruno e à Cátia Martins e ao pseudo-colega e amigo Filipe, por todo o apoio ao longo do meu trabalho, pelas músicas que me proporcionaram à hora de jantar, por estarmos todos na mesma situação e ainda assim mantermos a calma e pela compreensão, por todas as vezes que estive mais ausente. Às restantes colegas de mestrado, Bruna, Daniela Cordeiro, Matilde e Joana Pereira, por me prestarem auxílio sempre que precisei, durante estes dois anos. Às minhas amigas de infância, Vanessa, Inês e Mafalda, que participaram e me apoiaram em todos os momentos da minha vida, por serem um dos meus pilares, por me fazerem acreditar que eu posso sempre chegar mais longe e por de alguma forma contribuírem para o meu sucesso ao longo de cada etapa. À Carina, à Lili, à minha afilhada e amiga Daniela Pedrosa e ao Zé Tó, por todo o apoio e amizade, e por continuarem a fazer parte da minha vida apesar da distância e das atuais responsabilidades que nos limitam o tempo.

Ao Rodrigo, uma das pessoas mais importantes, pelo companheirismo, atenção, compreensão, por todo o apoio prestado especialmente ao longo deste último ano, por me motivar, por sempre acreditar nas minhas capacidades e por ter estado sempre presente. Quero também agradecer à sua família por me auxiliarem sempre que precisei.

À minha família, mãe, pai, Tiago, Natália e à minha sobrinha Madalena, agradeço não só por estes dois anos, mas por tudo o que proporcionaram ao longo da minha vida. Posso afirmar que sem a vossa ajuda eu não teria chegado até aqui e que o vosso apoio foi crucial até ao final desta etapa. Pelo amor, união e cumplicidade, um grande obrigada.

Abstract

Plant-parasitic nematodes (PPN) are highly specialised parasites recognized as one of the greatest threat of agricultural crops. The most economically important PPN which have a great impact on crops worldwide are the sedentary root-knot nematode (RKN), *Meloidogyne* spp. and potato cyst nematodes (PCN), *Globodera* spp. Root-knot nematodes are among the most economically damaging agricultural pests attacking a wide range of horticultural and field crops. *Meloidogyne* species, such as *M. incognita*, are serious ubiquitous pests being considered as "major" species and infect many vegetable crops, such as tomato. Potato cyst nematodes (PCN), *G. pallida* and *G. rostochiensis*, are one of the most specialized and successful PPN pests in agriculture around the world causing major losses in potato crops.

The control of PPN involves the manipulation of nematode densities to non-injurious levels using several strategies to reduce or eliminate nematodes. Biological control through the action of living organisms may provide an additional method for the management of these pests. This management strategy is set to promote environmental and economically efficient methods to eliminate diseases and pests. However biological control needs to be integrated with other control methods, as it is less effective and slower acting than chemical control. The facultative nematophagous fungus *Pochonia chlamydosporia* parasitises sedentary females and eggs of RKN and PCN. The biological control potential of *P. chlamydosporia* isolates has been widely studied in pots and fields experiments. However, the reduced efficacy of selected isolates in controlling nematode populations has been demonstrated in some studies. The intrinsic variation between different isolates of *P. chlamydosporia* requires prior selection. The selection process is based on rapid *in vitro* tests to evaluate three important criteria, the ability: to produce chlamydospores; to colonise the rhizosphere; and to parasitise egg nematodes.

The main goal of the present study was to evaluate the potential of Portuguese *P. chlamydosporia* isolates associated with *Meloidogyne* spp. Specifically isolates were identified and characterized at the molecular level and their ability to produce chlamydospores, colonise the rhizosphere and parasitise PPN eggs was evaluated.

Thirteen *P. chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX and reference isolates Pc2, Pc10 and 392) were evaluated. Genetic variation between isolates was examined using β-tubulin-PCR, ERIC-PCR and ITS-RFLP and isolates were screened by *in vitro* bioassays for their ability to produce chlamydospores, colonise the rhizosphere of tomato and parasitise eggs of *M. incognita* and *G. pallida.*

Molecular characterization of *P. chlamydosporia* revealed differences between *P. chlamydosporia*: var. *chlamydosporia* and var. *catenulata*. For some isolates, very similar ERIC-PCR patterns were obtained. ERIC-PCR can be used to identify intra-specific variation within the species.

The best producers of chlamydospores in the three biological replicates were isolates II, III, IV and VI. The viability was higher than 80% for most isolates. Most of isolates extensively colonised the rhizosphere of tomato (more than 80% root fragments) except isolates Ib, IX, IV and V. The proportion of parasitised eggs, detected on agar plates, was low for both nematode species (less than or equal to 60% for RKN and less than 55% for PCN). Isolates Ia and VIII were best parasites of RKN, and isolates V and Pc2 were the best parasites of PCN. Isolates Ib and IX were the worst isolates in the three *in vitro* bioassays. Isolates Ia and VIII should be considered as good candidates to be developed as potential biological control agents. Although, they were not the best chlamydospore producers, the results were consistent between biological replicates. In addition, they presented a high rate of germinated chlamydospores, were considered good colonisers of tomato roots and revealed to be good parasites of eggs of both species of nematodes. Molecular, biochemical and biological analyses are determinant in the screening of potential biological control agents, particularly in the case of *P. chlamydosporia*, due to the high variability among isolates.

Keywords – Biological control, *Globodera* spp., isolate variability, *Meloidogyne* spp. *Pochonia chlamydosporia*.

Resumo

Os nemátodes parasitas de plantas (NPP) são altamente especializados, sendo considerados como uma das maiores ameaças às culturas agrícolas. Os NPP economicamente mais importantes, com grande impacto nas culturas a nível mundial, são os nemátodes-das-galhas-radiculares (NGR), *Meloidogyne* spp., e os nemátodes de quisto da batateira (NQB), *Globodera* spp. Os NGR estão entre as pragas agrícolas economicamente mais prejudiciais que atacam uma grande variedade de culturas. Espécies de *Meloidogyne*, como por exemplo *M. incognita*, são pragas graves presentes em todos os locais, que têm vindo a ser consideradas como as "principais" espécies pois infetam diversas culturas hortícolas importantes, como, por exemplo, o tomate. Os NQB, *G. pallida* e *G. rostochiensis*, são uma das pragas mais especializadas e bem-sucedidas na agricultura em todo o mundo, causando grandes perdas nas culturas de batata.

O controlo dos NPP envolve a manipulação das densidades de nemátodes para níveis não prejudiciais utilizando diversas estratégias para os reduzir ou eliminar. O controlo biológico através da ação de organismos vivos pode fornecer um método suplementar para a gestão dos NPP. Esta estratégia de gestão baseia-se em métodos ambientais e economicamente eficientes para eliminar pragas e doenças. No entanto, o controlo biológico precisa de ser integrado com outros métodos de controlo, uma vez que é menos eficaz e atua mais lentamente que o controlo químico. O fungo nematófago *Pochonia chlamydosporia* parasita fêmeas sedentárias e ovos de NGR e NQB. O potencial doa isolados de *P. chlamydosporia* como agentes de controlo biológico tem sido bastante estudado em ensaios de vasos e campo. No entanto, a eficácia reduzida de alguns isolados no controlo de populações de nemátodes tem sido demonstrada em alguns estudos. A variação intrínseca entre diferentes isolados de *P. chlamydosporia* requer uma seleção prévia. O processo de seleção é baseado em três critérios-chave, baseados na capacidade de: produzir clamidósporos; colonizar a rizosfera; e parasitar ovos de nemátodes.

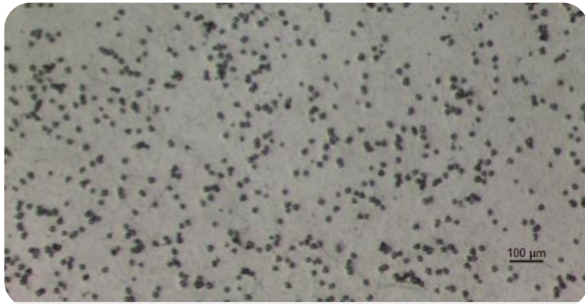
O objetivo principal do presente estudo foi avaliar o potencial de isolados portugueses de *P. chlamydosporia* associados a *Meloidogyne* spp. Especificamente, os isolados foram identificados e caraterizados a nível molecular e foi avaliada a sua capacidade de produzir clamidósporos, de colonizar a rizosfera e de parasitar ovos de NPP.

Foram utilizados treze isolados de *P. chlamydosporia* (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX e os isolados de referência Pc2, Pc10 e 392). A variação genética entre isolados foi avaliada através da técnica de PCR com primers de β-tubulina, ERIC e ITS. Os isolados foram selecionados através de bioensaios *in vitro* no que diz respeito à sua capacidade de produzir clamidósporos, de colonizar a rizosfera do tomate e parasitar os ovos de *M. incognit*a e de *G. pallida*.

A caraterização molecular de *P. chlamydosporia* revelou diferenças entre *P. chlamydosporia* var. *chlamydosporia* e var. *catenulata*. Para alguns isolados foram obtidos padrões ERIC-PCR muito semelhantes. A técnica de ERIC-PCR pode ser usada para identificar variações intraespecíficas dentro da espécie.

Os melhores produtores de clamidósporos nas três réplicas biológicas foram os isolados II, III, IV e VI. A viabilidade foi superior a 80% para a maior parte dos isolados. A maioria dos isolados colonizou extensivamente a rizosfera do tomateiro (mais de 80% dos fragmentos da raiz), exceto os isolados Ib, IX, IV e V. A proporção de ovos parasitados, detetados em placas de agar, foi baixa para ambas as espécies de nemátodes (inferior ou igual a 60% para NGR e inferior a 55% para NQB). Os isolados Ia e VIII foram os melhores parasitas de NGR, e os isolados V e Pc2 foram os melhores parasitas de NQB. Os isolados Ib e IX foram os piores isolados nos três bioensaios *in vitro*. Os isolados Ia e VIII poderão ser considerados como potenciais agentes de controlo biológico. Embora, estes isolados não tenham sido os melhores produtores de clamidósporos, os resultados foram consistentes entre replicações biológicas. Além disso, os clamidósporos produzidos por estes dois isolados possuíam elevada viabilidade. São também bons colonizadores das raízes do tomateiro e revelaram serem bons parasitas de ovos em ambas as espécies de nemátodes estudadas. As análises moleculares, bioquímicas e biológicas são decisivas na seleção de potenciais agentes de controlo biológico, particularmente no caso de *P. chlamydosporia*, devido à elevada variabilidade entre isolados.

Palavras-chave – Controlo biológico, *Globodera* spp., *Meloidogyne* spp., *Pochonia chlamydosporia*, variabilidade dos isolados.



1. Introduction

1. Introduction

One of the major global challenges in the future will be to ensure security and food quality due to a projected increase of 35% of the world population by 2050. It is expected an increase in food demand near to 75%, which means more food will have to be produced, using resources more efficiently and with less waste (Keating et al., 2010; Fitzpatrick, 2013). However, food production will be constrained by the finite resources provided by Earth's lands, oceans, and atmosphere (Godfray et al., 2010). Food security is the sustainable production of sufficient amounts of high quality, safe food required to increase health and human well-being. Regarding food security, the emergence of infectious diseases has to be considered. The safety of food supplies is threatened by existing and emerging pathogens, which have widely differing effects on food production and are altogether responsible for great losses in global agricultural productivity. Parasites, such as plant-parasitic nematodes (PPN), are recognized as responsible for the greatest overall risk to crop production (Savary et al., 2012; Fitzpatrick, 2013). Plant-parasitic nematodes cause losses equal to or great than 10 % in world crop production (Nicol, 2002). Crop protection against these parasites is a key factor to be considered in the demand for food security (Savary et al., 2012).

1.1. Tomato culture

The tomato, *Solanum lycopersicon*, belongs to the genus *Solanum*, a small genus within the diverse family of *Solanaceae,* the same family as potato (*S. tuberosum*), which includes more than 3000 species (Costa and Heuvelink, 2005; Raiola et al., 2014). The genus *Solanum* is believed to have originated in South America (Costa and Heuvelink, 2005). In the *Solanum* (section *Lycopersicon*) 13 species are recognized, but only *S. lycopersicum* is a domesticated species (Raiola et al., 2014). Tomato was introduced in Europe in the middle of the 16th century. Generally, it grows in temperate regions and does not withstand low temperatures (Jones, 2007).

Domesticated tomato is the most important horticultural crop worldwide. Besides, tomato is one of the most studied fruits and it is used as a model plant due to its nutritional properties (Schwarz et al., 2014). Tomato is an important plant for human nutrition and has the advantage of being a healthy food available and can be included in various gastronomic cultures (Burton-Freeman and Reimers, 2011; Zavala-González et al., 2015). The tomato fruits are an excellent source of large amount of substances with all kinds of health benefits for the body, which give the tomato a unique nutritional and chemical profile. Among the main substances are vitamins, minerals and antioxidants, such as Vitamin C, Vitamin A, fiber, potassium and the antioxidant lycopene. Several studies have shown that a dietary intake of tomatoes is associated with a reduced risk of inflammatory processes, cancer, cardiovascular diseases, diabetes, obesity and skin protection (Burton-Freeman and Reimers, 2011; Raiola et al.,

2014). Furthermore, it is an excellent moisturizer due to a high content in water and potassium (OMAIAA, 2011).

There is a large variety of tomatoes, the round red-fleshed tomato which is the most popular, yellow and orange round types, cluster, plum, and small-sized red-types such as cherry (Nunes, 2008). Tomato fruit is one of the horticultural crops most consumed worldwide and one of the most important vegetables in the European Union in 2015 (Nunes, 2008; Eurostat, 2017). Asia is currently the major producer of tomato and Europe is the third (FAO, 2017). The EU-28 produced approximately 17.6 million tonnes of tomatoes in 2015 and Portugal was the third largest producer (more than 1 million tonnes), after Italy and Spain (Eurostat, 2017; FAO, 2017). In Portugal, the larger areas of tomato production are the Ribatejo-Oeste, Algarve and Entre-Douro e Minho (OMAIAA, 2011).

Tomato crops are hosts of several pathogens including viruses, bacteria, fungi and nematodes and are easily affected by abiotic factors like inadequate rainfall (Moshe et al., 2012; Onuorah and Orji, 2015). The main enemies of tomato crops are whiteflies, leaf-mining larvae, caterpillars and nematodes. In addition, some of these pests are vectors of other diseases that can cause large losses in production (Abrantes et al., 2007).

1.2. Plant-parasitic nematodes

Plant-parasitic nematodes are highly specialised parasites recognized as one of the greatest threat of agricultural crops and are frequently one of the most insidious and costly (Handoo, 1998; Nicol et al., 2011; Haegeman et al. 2012). Plant-parasitic nematodes are considered as one of the limiting factors for agricultural production (Prakash et al., 2014). They are called plant-parasitic because they feed on plant nutrients (Handoo, 1998). The majority of PPN are parasites of plant roots and a minority prefers other plant organs (stems, leaves, flowers and seeds) (Lambert and Bekal, 2002; Haegeman et al. 2012). This group of nematodes is usually subdivided into groups according to their infection style (Kyndt et al., 2013). They can be ectoparasitic nematodes that do not enter the plant tissue, but feed on the roots in the soil, or they can be endoparasitic nematodes, spending at least part of their lifecycle within the host (Haegeman et al. 2012).

The most economically important PPN which have a great impact on crops worldwide are the sedentary root-knot nematode (RKN), *Meloidogyne* spp. and potato cyst nematodes (PCN), *Globodera* spp. (Haegeman et al. 2012, Jones et al., 2013).

1.2.1. Root-knot nematodes, *Meloidogyne* spp.

Root-knot nematodes are among the most economically damaging agricultural pests attacking a wide range of horticultural and field crops. They are distributed worldwide and are sedentary and obligate parasites that infect the roots of hundreds of plant species (Radwan et al., 2012). The first report of root-knot disease was in cucumber roots in the year 1855. Root-knot nematodes belong to the order *Tylenchida* and the genus *Meloidogyne* that comprises more than 90 species (Moens et al., 2009).

Large annual yield losses of about 10%, and even 30% in the case of susceptible vegetable crops have been attributed to RKN (Radwan et al., 2012). The impact of RKN on roots of host plants is similar to symptoms in plants with a malfunctioning root system. Symptoms include: supressed plant yields and product quality, nutritional deficiencies, supressed shoot growth and temporary wilting even when soil moisture is adequate (Karssen et al., 2006). These nematodes developed very specific interactions with their host. They use the stylet to inject secretions into plant cells and withdraw nutrients from the infected root cells (Castagnone-Sereno, 2006). The feeding cells are an exclusive source of nutrients for the developing nematode and they are called giant cells leading to the formation of the typical galls in the root system, the primary visible symptom (Caillaud et al., 2008). After 24-48h of penetration, the cells surrounding the developing juvenile begin to proliferate and enlarge to form galls (Dropkin, 1972; Trudgill and Blok, 2001). Reproduction in *Meloidogyne* varies from obligatory sexual reproduction (amphimixis), in a small number of species, to obligatory parthenogenesis (apomixis), with a large proportion of facultative parthenogenetic species (Trudgill and Blok, 2001; Moens et al., 2009). The parthenogenesis is generally associated with shorter life cycles and higher reproductive rates. The apomictic species are related to greater agronomic impacts (Moens et al., 2009). Root-knot nematode species further differ in their male-to-female ratio, as males are largely suppressed in parthenogenetic species (Trudgill and Blok, 2001; Moens et al., 2009). The males are found when conditions are unfavourable for female development, for example when there is a limitation of food supply (Moens et al., 2009). Males do not feed and are vermiform, rounded to transversely elongate and have a long stylet (23-26µm) (Moens et al., 2009; Hunt and Handoo, 2009). Mature females have a globose structure with a short neck (Mitkowski and Abawi, 2003; Hunt and Handoo, 2009). The pear-shaped female produces eggs that are released on the surface of the galled root. Eggs are encapsulated in egg masses, composed of a glycoprotein matrix (Hashem and Abo-Elyousr, 2011). The egg mass is initial soft and sticky but becomes firmer and dark brown according to age (Moens et al., 2009). The number of eggs produced by a single female depends on environmental conditions. If conditions are favourable, the female can produce between 500–2000 eggs (Calderón-Urrea et al., 2016). Within the egg, embryogenesis is followed by the first moult to the infective second-stage juvenile (J2). In the soil, J2 penetrate the root of the host and migrate between cells and starts feeding (Abad et al.,

2008; Moens et al., 2009). Infective J2 moved through the root and develop a permanent feeding site (Moens et al., 2009). Second-stage juveniles use the stylet to induce the dedifferentiation of root cells in specialized giant cells, which supply nutrients to the nematode (Abad et al., 2008; Moens et al., 2009). At this stage, the nematode becomes sedentary and moults to the third-stage juvenile (J3), then to the fourth-stage juvenile (J4) and finally to the adult stage (Moens et al., 2009).

Among the most important *Meloidogyne* species, there are three polyphagous and apomictic species, *M. arenaria, M. javanica, and M. incognita*, and one facultative parthenogenetic, *M. hapla*, which are serious ubiquitous pests being considered as "major" species (Trudgill and Blok, 2001; Karuri et al., 2017). These four species are morphologically similar and infect many common vegetable crops, such as tomato, considered a universal host for *Meloidogyne* spp*. Meloidogyne incognita*, *M. javanica* and *M. arenaria* are distributed mainly in cultivated soils in tropical regions, while *M. hapla* occurs in regions with cooler, temperate climates (Moens et al., 2009).

Meloidogyne incognita has been considered as one of the most prevalent species in warmer conditions of southern Europe, and also in glasshouses in northern Europe and is able to infect almost all cultivated plants, including tomato, soybean and cotton, and they can develop quickly under appropriate conditions (Dropkin, 1972; Calderón-Urrea et al., 2016). Studies with mitochondrial DNA indicate that *M. incognita*, *M. arenaria* and *M. javanica* derive from closely related sexual species (Trudgill and Blok, 2001). *Meloidogyne incognita* reproduces by mitotic parthenogenesis but males can be found in this nematode species when the conditions are unfavourable (limitation of food supply) (Ehwaeti et al., 1999; Abad et al., 2008).

Management of RKN is difficult due to short generation times, high reproduction rates and wide host range (Jang et al., 2016).

1.2.2. Potato cyst nematodes, *Globodera* spp.

Potato cyst nematodes (PCN), *Globodera pallida* and *G. rostochiensis*, are obligate biotrophs and one of the most specialized and successful PPN pests of agriculture around the world (Nicol et al., 2011; Jones et al., 2013). *Globodera pallida* and *G. rostochiensis* are listed in the European and Mediterranean Plant Protection Organization (EPPO) A2 List (EPPO, 2013). Potato cyst nematodes belong to the family *Heteroderidae* and they are a native species of the Andean Cordillera in South America, the same origin of his unique host genus *Solanum*. These nematodes were probably introduced in Europe through potato breeding material in 1850 (Brodie and Mai, 1989; Nicol et al., 2011). Furthermore, tomatoes and aubergines are also good hosts (Nicol et al., 2011). Symptoms in infested potato plants are the reduction of root system, because of the decrease water uptake, yellow or death foliage, reduction of tube size, and the plant may eventually die (EPPO, 2013). Control strategies of these nematodes are based

on crop rotation and the use of resistant varieties and chemicals (Turner et al., 2006). Because of intensive potato production with reduced rotation periods, PCN levels have continue increased (Kyndt et al., 2014).

Infestation occurs when the J2 hatches from the egg and his stylet enters the root near the growing tip by piercing the epidermal cell walls, and then internal cell walls. The infestation can be propagated through infested seed potatoes and movement of soil (e.g. in farm machinery) (EPPO, 2013). Eggs are produced inside the female body that becomes a cyst with a very resistant protective wall, after the death of the female (Riggs and Schuster, 1998). The cyst wall prevents the invasion by potential parasites and protect the eggs on the inside, and they can remain dormant within the cyst for many years in the absence of a host (Riggs and Schuster, 1998; Jones et al., 2013). This ability to survive for prolonged periods in the soil makes control by rotation difficult (Jones et al., 2013). The cyst of the PCN is globose, spheroid and contains the eggs that hatch in the presence of host root diffusate (Nicol et al., 2011). The J2 then locates its host, invades and migrates intracellularly thought the root using the stylet (Lilley et al., 2005). Once in the vascular cylinder, they establish a feeding site, injecting stylet secretions. Formation of the feeding site is characterized by the destruction of the cell walls between the initial site of infection and its vicinity, thus forming a multinuclear syncytium which guarantees nourishing supplement for the nematodes (Lilley et al., 2005; Nicol et al., 2011; Jones et al., 2013). The J2 become sedentary, until development is complete, and undergo three moults (J3, J4 and adult stage) (Lilley et al., 2005; Nicol et al., 2011). Cyst nematodes are sexually dimorphic (Lilley et al., 2005). Female is round, white and swollen and break through the root surface (Nicol et al., 2011; EPPO, 2013). Males are slender, vermiform and leave the roots (Lilley et al., 2005; Nicol et al., 2011). Egg production starts between 3 and 6 weeks after infection, depending on species and environmental conditions. Potato cyst nematodes reproduce sexually. Receptive females release sex pheromones that attract males nearby in the soil. After mating, the males die and the female forms eggs and remain on the roots until the eggs are completely formed (Brodie and Mai, 1989). The females die when they become fully mature, their cuticle turns brown and form a protective cyst with 200-500 eggs within (Nicol et al., 2011; EPPO, 2013). The cyst drops from the surface of the root and the J2 wait for the next suitable host plant (Nicol et al., 2011). The eggs can hatch immediately or remain dormant in the absence of solanaceous hosts (Brodie and Mai, 1989). Sex is determined epigenetically, that is, it depends on environmental conditions. When there is nutritional scarcity there is a greater abundance of males (Lilley et al., 2005).

1.3. Management strategies

The control of nematodes involves the manipulation of nematode densities to non-injurious levels using several strategies to reduce or eliminate nematodes (Moens et al., 2009).

Plant-parasitic nematodes are generally controlled by conventional methods, cultural practices and the use of resistant cultivars (Oka et al., 2000). There is no single rotation, rootstock, or nematicide that can control all nematode pest problems, therefore there must be flexibility in the control strategies (Chen and Tsay, 2006).

Conventional methods to manage PPN, include the use of chemicals, such as soil fumigant and non-fumigant nematicides (Khalil, 2013). Soil fumigant nematicides may help prevent serious losses in agricultural crops when combined with good management practices (Hill, 1988). Soil fumigants, for example, have been used to control *M. incognita* for over 20 years (Egel et al., 2014). However, nematicides are very toxic to humans and animals, causing both soil and water pollution, and have a long persistence in the ecosystem (Lopes et al., 2011; Seo and Kim, 2014). They are not environmental friendly and they lead to other problems, such as high application costs (Anastasiadis et al., 2008; Egel et al., 2014). Additionally, many nematicides will be withdrawn further limiting control options, emphasizing the need for alternative control strategies (Nicol et al., 2011).

Other methods of control used are cultural practices, such as crop rotation, one of the oldest and most important methods for managing nematodes in annual crops (Hill, 1988). However, development of crop rotation programs is generally constrained by specialized cropping practices, equipment requirements, local climate, and the market value of crops (Chen and Tsay, 2006). This technique is based on the reduction of harmful levels of nematodes by rotating a susceptible host crop with a nonhost crop to facilitate the subsequent crop to produce an acceptable yield (Hill, 1988). In an ideal crop rotation system, the previous crop prevents damage to the next crop by suppressing the target nematode population (Chen and Tsay, 2006). A large number of crops used in rotations can increase nutrient cycling in soil leading to a higher yield for the main economic crop (Widmer et al., 2002). Nonetheless, an efficient crop rotation scheme must be based on knowledge of the nematode species present and on the host range of the nematode (Briar et al., 2016). This strategy does not allow intensive agricultural practices and resistant or tolerant crops may not be an economically attractive option. Additionally, crop rotation may further increase selection pressure for virulent populations (Turner and Rowe, 2006). Furthermore, the success of these methods is limited because of the wide host ranges of most RKN species and the frequent occurrence of infestations induced by more than one species (Kerry, 2001).

However, unconventional methods have been used as alternatives to nematicide chemicals, and represent sustainable and environmentally friendly practices (Khalil, 2013; Jang et al., 2016). Since some nematicides have been withdrawn from the market, there is an intensive search for alternative control measures to manage PPN, like those based on soil amendments with inorganic and organic matter and the use of biological control agents (Lopes et al., 2011).

The use of organic amendments have many advantages, such as the changes in properties of soil that improve plant growth, tolerance to pathogens and improve soil structure. Also, enhancement of natural enemie's densities, nutrient supplement and increased organic matter levels are associated with the use of organic amendments (Dias-Arieira et al., 2015; Briar et al., 2016). This is a non-traditional method, which include organic materials, oil cakes and chitinaceous and proteinaceous compounds (Oka et al., 2000; Khalil, 2013). However, the results of the application of organic amendments for reducing PPN populations varied between success and failure (Zakaria et al., 2013). The effects are only visible in the long term and the use of large amounts to obtain control is necessary (McSorley, 2011; Wani and Bhat, 2012).

Alternative control strategies, such as biological control, need to be integrated with other control strategies, as they are less effective and slower acting than the chemical control. However, a better understanding of the ecological factors that allow these organisms to persist, compete, and function will support the practical implementation of biological control in the future. Through this knowledge, it may be possible to develop effective and sustainable biological control strategies for the management of PPN (Bent et al., 2008).

1.3.1. Biological control

Biological control is the management of plant diseases and pests through the action of living organisms, such as parasites of organisms that kill or damage their hosts and also microbes that have an indirect action on the survival and function of pathogens and pests (Moens et al., 2009). This management strategy is set to promote environmental and economically efficient methods to eliminate diseases and pests (Trainer et al., 2014).

Four main approaches can be considered based on biological control: the exploitation of naturally suppressive soils; soil amendments to enhance the activity of indigenous soil microbes; application of selected strains of bacteria or fungi; and microbial enzymes and toxic metabolites (Tian et al., 2007). The first step is to identify organisms that can be applied to the seed or transplant medium to reduce nematode population (Timper, 2014). Some bacteria, fungi, protozoa, predatory nematodes are enemies of nematodes of the soil and this creates the possibility of using soil microorganisms to act as biopesticides to control PPN (Oka et al., 2000; Tian et al., 2007). These natural enemies have been found in nematode suppressive soils, where, even in the presence of nematodes, the plant damage less significant (Oka et al., 2000). Biological agents can provide long-term effective control of cyst and RKN in the field (Kerry, 1990). The establishment and activity of biological control agents depend on factors such as soil condition, nematode species, rate of development and density and host plant (Kerry, 1997).

Bacteria are the most abundant organisms in soil, and some species of bacteria, such as the species of the genera *Pasteuria*, *Pseudomonas* and *Bacillus* have shown great potential for the biological control of nematodes pest populations. A wide variety of nematophagous bacterial groups have been found in soil, host-plant tissues and nematodes, and their eggs and cysts. Bacteria infect nematodes in different ways: parasitising them, producing metabolites (toxins, antibiotics or enzymes), interfering in recognition between nematode and plant-host and promoting plant health (Tian et al., 2007). As biological control agents they can be applied to seed and can greatly reduce nematode invasion of roots (Kerry, 1990). Some results *in vitro* have shown that bacteria can affect nematode activity possibly through the modification of root exudates, which affect, for example, nematode hatch and host recognition processes (Kerry, 1990). However, only a few commercial biological control products with nematicidal potential from bacteria have been developed (Tian et al., 2007). The *Bacillus* genus are currently registered in European regulation no. 1107/2009 (Gerbore et al., 2014). It is present in several environments, and could be one of the major sources of potential microbial biopesticides. Being relatively easy to produce industrially as no specific nutritional source is required bacteria belonging to this genus produce spores that are extremely resistant to harsh environmental conditions. However, *Bacillus* products often offer only partial protection against nematodes attacks. Another limitation is the lack of information on the deficiency often observed in the connections between field trials and controlled laboratory experiments (Cawoy et al., 2011). Another bacteria group, the *Pasteuria* group, has great potential to be developed as biological control agent. *Pasteuria penetrants* is the most studied species of the genus and parasitises *Meloidogyne* species, whereas *P. nishizawae* can parasitise cyst nematodes. These species produce adhesive spores that are very resistant to drying and significantly reduce nematode densities by interfering with their reproduction (Ravichandra, 2008). However, lack of efficient technology for large-scale production and host specificity are important constrains for the development of efficient control strategies (Chen and Dickson, 1998; Ravichandra, 2008; Davies, 2009).

Nematophagous fungi are natural enemies of nematodes and they are among the most promising biological control agents of nematodes (Nordbring-Hertz et al., 2006; Vieira dos Santos et al., 2012). Fungal antagonists of nematodes play a major role in recycling nitrogen, carbon and other elements (Mankau, 1980). Fungi have great potential as biological control agents, in addition, they are easy to grow and maintain in the laboratory, rhizosphere competent, and providing an excellent model system for interaction studies (Kerry, 1997; Nordbring-Hertz et al., 2006). More than 200 species of nematophagous fungi have been described and they are subdivided into three main groups, according to the infection mechanism: nematode trapping fungi, with specialized morphological structures to capture free-living nematodes; endoparasitic fungi that infect nematodes through spores; and egg and cyst parasitic fungi. Fungi vary in their parasitic and saprophytic ability. The endoparasites are mostly obligate

parasites, since they depend on nematodes as nutrient, and most of the trap-forming and egg-parasitic fungi can survive saprophytically in soil. The ability to parasite nematodes is related to different phases of mycelial development, for example trapping fungi attack nematodes with hyphal structures, endoparasitic fungi attack nematodes with their spores and the egg-parasitic fungi use apressoria to parasitise the eggs of nematodes (Nordbring-Hertz et al., 2006).

Nematode trapping fungi are present in soil and attack various stages of nematodes using trapping structures of different shapes and sizes. The structures produced by these fungi vary by species and can range from simple fungal hyphae covered with sticky secretions to much more complex structures. *Arthrobotrys oligospora* is the most commonly nematode-trapping fungus in the environment. *In vitro* tests revealed that its efficacy depends very heavily on environmental factors (Duponnois et al., 1995). Additionally, complexity in soil establishment and their limited capturing activity reduce its potential as biological control agents (Moosavi and Zare, 2012).

Endoparasitic fungi infect nematodes with spores (conidia or zoospores), that can be ingested by the nematode or adhere on the nematode cuticle (Nordbring-Hertz et al., 2006; Moosavi and Zare, 2012). Most of these fungi are obligate parasites with a broad nematode host range. However, they are poor saprotrophic competitors in soil (Moosavi and Zare, 2012). Among the endoparasites, *Hirsutella rhossoliensis* has a broad host range, which include species of *Meloidogyne*, *Heterodera*, *Pratylenchus* and *Criconemella* (Jaffee et al., 1994). Some endoparasitic fungi, as *Catenaria anguillulae,* infect live vermiform nematodes and produce spores within the nematode body, and others, as *Nematophthora gynophila* and *C. auxiliaris,* are obligate parasites that infect sedentary young female cyst nematodes (Viaene et al., 2006). Compared with nematode trapping fungi, endoparasitic fungi are more adaptable to practical application (Moosavi and Zare, 2012). However, endoparasitic fungi are considered unsuitable for a large scale application as they are difficult to culture *in vitro* and establish in soil (Viaene et al., 2006).

Egg-parasitic fungi are facultative fungal parasites of sedentary stages (Viaene et al., 2006). Nematode eggs are the most vulnerable stage of the nematode life cycle to nematophagous fungi (Verdejo-Lucas et al., 2002). These parasites attack eggs and females and they cannot prevent plant damage but they can reduce significantly the multiplication of nematodes (Kerry, 1997). They produce internal hyphae that grow toward the eggs and then form apressoria which penetrate the eggshell (Nordbring-Hertz et al., 2006). Few egg-parasitic fungi have been considered as promising biological control agents, and the most frequently isolated fungi are *Paecilomyces lilacinus* and *Pochonia chlamydosporia* (Moosavi and Zare, 2012). Some features makes them promising biological control agents, such as the ability to produce spores in *in vitro* conditions, rhizosphere competence and more effectiveness in infecting (because their

target pest is immobile and more easier to infect) (Kerry, 1997; Moosavi and Zare, 2012). They also attracted more attention because of their high potential as biological control agents of economically important nematodes, such as *Meloidogyne*, *Globodera* and *Heterodera* species (Moosavi and Zare, 2012).

Paecilomyces lilacinus is a known parasite with a wide host range, including *Radopholus similis* and *Tylenchulus semipenetrans*, but the greater interest in this species is due to the fact that *P. lilacinus* parasitises *Meloidogyne* spp. and *G. rostochiensis* eggs. The presence of the fungus is common in subtropical and tropical regions. Several companies around the world are evaluating and developing the efficiency of the fungus, since it is able to reduce nematode damage to a range of crops in field trials (Viaene et al., 2006). The isolate 251 of *P. lilacinus* is being marketed as a biological control agent in Germany (BioAct®WP) and in South Africa (PL Plus®) for cysts and RKN management (Viaene et al., 2006). The fungus is rhizosphere competent and attacks the eggs of several nematode species (Kerry, 1997). However, this fungus requires high soil temperatures, has given variable control in a range of conditions and requires a large number of propagules (10^6/g soil) for nematode control (Kerry, 1997). Furthermore, the efficacy of biological control under glass house and field conditions was often inconsistent and some isolates are pathogenic to humans (Kerry, 1997; Kiewnick and Sikora, 2006).

The facultative nematophagous fungus *Pochonia chlamydosporia* parasitises sedentary females and eggs of economically important PPN, such as RKN and PCN (Kerry and Hirsch, 2011). *Pochonia chlamydosporia* var. *catenulata* has been developed as a biological control agent for use in Cuba, with the name KlamiC®. This commercial biological control agent is being evaluated for nematode management because it has potential effect against *Meloidogyne* spp. and has no harmful action in human health or risk of contaminating the environment and groundwater (Hernandez and Hidalgo-Diaz, 2008; Kerry and Hirsch, 2011; Manzanilla-López at al., 2013). *Pochonia chlamydosporia* has also been developed as a commercial product in Italy, with the name Pochar™, and in Brasil, with the name Rizotec® (Sellito et al., 2016; Bontempo et al., 2017).

1.4. *Pochonia chlamydosporia*, a potential biological control agent

Pochonia chlamydosporia is a facultative nematophagous fungus with parasitic activity against eggs and females of economically important species of the genera *Heterodera*, *Globodera*, *Meloidogyne*, and more recently *Nacobbus* and *Rotylenchus* spp. (Flores-Camacho et al., 2008; Manzanilla-López at al., 2013). This nematophagous fungus has an ubiquitous distribution and has been found to parasitise nematode eggs in nematode suppressive soils (Kerry and Crump, 1977; Kerry et al., 1982; Manzanilla-López at al., 2013).

Pochonia chlamydosporia was first reported as a parasite of nematode eggs in the United Kingdom by Wilkox and Tribe, in 1974 associated with cyst nematodes, *H. schachtii* and *H. avenae* (Leij and Kerry, 1991; Manzanilla-López at al., 2013). It has also been associated with a marked decline in populations of cereal-cyst nematode, in monocultures of susceptible crops (Leij and Kerry, 1991). The eggs of nematodes are not the sole nutrition source, as the fungus is able to colonise other organisms, including other fungi, and mollusc eggs (for example, snail eggs) and the rhizospheres of healthy plants (Kerry and Bourne, 1996; Manzanilla-López at al., 2013). The fungus is a poor competitor in soil, but is able to colonise the rhizosphere of plants by using the nutrients present in root exudates (Bourne and Kerry, 2000; Manzanilla-López at al., 2013).

All stages of the fungus occur in soil (Kerry and Bourne, 1996). This nematophagous fungus produces two types of spores, conidia and chlamydospores (Mo et al., 2005; Kerry and Hirsch, 2011). Conidia production can be induced in large amounts. However, they have low rates of survival in the soil and need a supplementary energy source. Although, chlamydospores have sufficient food reserves to enable the fungus to establish in the soil and initiate fungal growth in favourable conditions. The propagules applied to soil survive in sufficient numbers to infect nematode eggs produced 1-3 months later (Kerry and Hirsch, 2011).

In the rhizosphere, the fungus has the ability to colonise the roots of host plants, such as some Gramineae and Solanaceae species (Manzanilla-López at al., 2013). Plants species differ in their ability to support the fungus in their rhizosphere (Abrantes et al., 2002). In several studies of a range of crops (e.g. tomato, potato, beans, cabbage and pumpkin), *P. chlamydosporia* has been reported to show endophytic behaviour in the roots and no damage to crops has been observed (Kerry and Bourne, 1996; Manzanilla-López at al., 2013). In addition, some studies revealed that the fungus can promote the growth of several crops, such as tomato, potato, cotton, lettuce and eggplant (Zavala-González et al., 2015).

Pochonia chlamydosporia proliferates in the rhizosphere of infected plants and colonises egg masses of RKN or cyst nematodes. When *P. chlamydosporia* contacts a nematode egg in the rhizosphere its behaviour switch from saprophyte to nematode parasite (Kerry and Bourne, 1996). The conditions required to induce the switch are not well understood, but probably nutrition and nematode host preference at the fungal infra-specific level are involved (Kerry, 2001; Mauchline et al., 2004). The infection process does not involve specialised structures to capture nematodes (Kerry and Bourne, 1996). The fungus infects nematode eggs through the development of apressoria produced terminally or laterally on a hypha. The apressoria attacks tightly the eggshell surface, and gives rise to an infection peg which grows through the eggshell. A post-infection bulb leads to the development of a mycelium within the egg that will destroy the egg contents (Kerry and Bourne, 1996; Kerry and Hirsch, 2011). The fungus produces a range of enzymes, among them an alkaline serine protease enzyme, VCP1,

which is responsible for the degradation of the outer vitelline membrane of the egg (Kerry and Hirsch, 2011). The fungus can attack all stages of embryonic development, but immature eggs are more susceptible (Kerry and Bourne, 1996). The colonised eggs are destroyed within a few days of infection (Kerry and Hirsch, 2011).

The fungus is a promising biological control agent for RKN and cyst nematodes as it has several advantages, including easy laboratory culturing in artificial liquid or solid media, worldwide distribution, its easy dispersion and colonisation in the rhizosphere as well as its host specificity and effectiveness in nematode control (Flores-Camacho et al., 2008; Manzanilla-López at al., 2013). Another advantage of the fungus is the ability to survive in the absence of the nematode (Flores-Camacho et al., 2008).

The efficacy of *P. chlamydosporia* as a biological control agent of several nematodes (*Meloidogyne* spp. and *Globodera* spp.) in tomato plants, has been showed in *in vitro* assays and under glasshouse conditions. Additionally, studies on toxic, ovicidal, and larvicidal effects revealed that this fungus can be a potential biological control agent against animal parasitic nematodes (Manzanilla-López at al., 2013). However, the efficacy of the fungus depends on nematode species, nematode density and plant host (Kerry, 1997). The fungus is mostly confined to the surface of roots, so RKN eggs within large galls are physically isolated from fungal infection. Gall size depends on two factors, host plant susceptibility and nematode density (Bourne et al., 1994). The presence of a susceptible host plant or the presence of a high nematode population density in abundance originate large galls, with several females, and the egg masses can be deposited inside the roots and protected from fungal attack. The efficacy of the fungus is negatively affected by highly susceptible hosts. Furthermore *P. chlamydosporia* does not prevent initial infestation of roots by J2. The fungus causes a decrease of the nematode population in soil due to its ability to affect nematode reproduction (Kerry and Bourne, 1996; Bailey et al., 2008). To enhance the performance of *P. chlamydosporia* as a biological control agent a better understanding of the tri-trophic interactions plant-nematode-fungus is extremely important (Manzanilla-López at al., 2013).

The biological control potential of *P. chlamydosporia* isolates has been widely studied in pots and fields experiments (Leij and Kerry, 1991; Leij et al., 1993; Sorribas et al., 2003; Atkins et al., 2003b; Vieira dos Santos et al., 2014a). However, the reduced efficacy of this fungus in controlling nematode populations has been demonstrated in other studies (Tzortzakakis and Petsas, 2003; Verdejo-Lucas et al., 2003; Tzortzakakis, 2009; Vieira dos Santos et al., 2014b). The efficacy of selected isolates against egg nematodes has been demonstrate but the establishment in the soil has inconsistent results (Vieira dos Santos et al., 2014b). The variability of *P. chlamydosporia* isolates has been related to the host nematode and the soil environmental where the fungus was isolated (Zavala-González et al., 2015). Field efficacy of the isolates depends not only one the plants and their roots, but also on the nematode host, soil biotic and abiotic

factors and inherent variability in geographical isolates (Nagesh et al., 2007). For example, the use of native isolates have more advantages over introduced exotic isolates as they probably show higher levels of virulence against local populations of nematodes, compete better with indigenous microflora, and can adapt better to environmental conditions than introduced exotic isolates. Potential adverse effects and environmental risks may come from the introduction of exotic isolates in the soil (Sorribas et al., 2003).

Pathogenicity and toxicity of *P. chlamydosporia* have been studied in non-target organisms. No adverse effects were observed in vertebrate tests with rats and rabbits, invertebrates and beneficial plants and its application did not affect plant germination and growth. Studies have not reported harmful effects on human health and risks for animals and plants (García et al., 2004; García et al., 2008a; García et al., 2008b).

The fungus can be isolated from roots, soil and eggs and can be cultivated in solid and liquid media (Manzanilla-López at al., 2013). Molecular techniques are useful tools to identify and screening potential biological control agents. These studies at a genomic level allow the evaluation of a large numbers of isolates and are rapid and economically feasible to perform (Yang et al., 2012). When through morphology it is not possible to differentiate isolates which are closely related, they can be discriminated using a variety of molecular methods based on PCR, such as β-tubulin-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and ribosomal internal transcribed spacers (ITS)-PCR (Kerry and Hirsch, 2011). The β-tubulin gene of *P. chlamydosporia* var. *chlamydosporia* was found to have an intron not present in other fungi, and this provides considerable utility for *Pochonia*-selective assays. There are two infraspecific groups in *P. chlamydosporia*, var. *chlamydosporia* and *var. catenulata*, the specific β-tubulin-PCR only amplifies the var. *chlamydosporia*. ERIC-PCR profiling showed that isolates can be grouped according both their geographical and host nematode origin. The primers act arbitrarily in fungi by amplifying repetitive sequences in the genome and produce a multiple band profile (Morton et al., 2003a). The sequence of the internally transcribed spacer (ITS) regions of the ribosomal RNA gene cluster is an important feature of fungal studies, since conservation and smooth sequence variation in these spacers can be used to determine phylogenetic relationships, define species and generate species specific-primers. Sequence variation in certain species vary according to the species considered and the spacer studied (Zare et al., 1999). ITS region is used to determine phylogenetic relationships between *Pochonia* species. Within *P. chlamydosporia* there is a considerable intraspecific variation as determined by phenotypic variations (Morton et al., 2003a). The restriction fragment length polymorphism (RFLP) technique uses restriction enzyme digestion of the DNA to detect differences in the size of DNA fragments. Fungal species can be distinguish through the polymorphisms in the restriction enzyme cleavage sites (Capote et al., 2012). PCR-RFLP with ITS sequences is an easy and rapid technique which separates *P. chlamydosporia* var. *chlamydosporia*

from var. *catenulata* isolates by distinctive band fragments. RFLP analysis of ITS sequences from *P. chlamydosporia* may provide an extra level of discrimination between isolates, however their results may failed in differentiating closely related species (Atkins et al., 2003a; Capote et al., 2012). The importance of this intraspecific variation suggests that potential biological control agents need careful selection (Abrantes et al., 2002).

1.4.1. *Pochonia chlamydosporia* isolates screening

The intrinsic variation between different isolates of *P. chlamydosporia* requires prior selection. The selection process is based on rapid *in vitro* tests. These bioassays enable many isolates to be eliminated before being tested in time consuming assays such as pot trials in the glasshouse (Abrantes et al., 2002).

Fungus isolates vary in their ability to colonise the rhizosphere, to proliferate in soils (tend to be more abundant in organic soils than mineral soils) and its pathogenicity against nematode eggs (Kerry and Hirsch, 2011; Moosavi and Zare, 2012). Also, different *P. chlamydosporia* isolates have been detected in the same niche in soil and rhizosphere (Manzanilla-López et al., 2013).

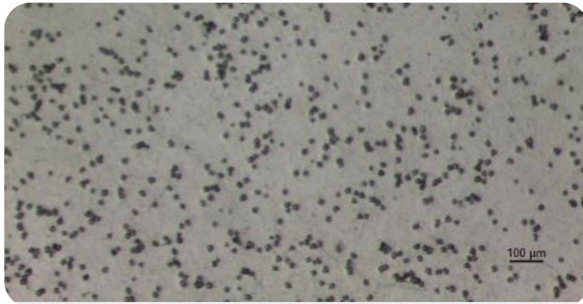
After identification, isolates should be submitted to *in vitro* bioassays based on three important criteria that lead to a first selection of isolates: the ability to produce chlamydospores; the ability to colonise the rhizosphere; and the ability to parasitise egg nematodes (Abrantes et al., 2002).

Among the three types of inoculum, mycelium, conidia and chlamydospores, chlamydospores are the preferred source of inoculum, since they contain sufficient internal resources for fungal growth (Kerry and Hirsch, 2011; Manzanilla-López at al., 2013). They are effective in establishing the fungus in the soil and rhizosphere. For production of chlamydospores, a culture media with milled, maize, barley, wheat or rice is used. The amount of substratum in culture media will affect the production of chlamydospores, the percentage used is 50%. If the amount used is 25% or 100% chlamydospores production decrease and for 75% increases the number of chlamydospores (Abrantes et al., 2002).

The efficacy of fungus as a biological control agent depends on fungal rhizosphere colonisation to facilitate colonisation of nematode eggs (Manzanilla-López at al., 2013). Studies have demonstrated that the interactions in the rhizosphere have a great effect on the number of nematodes parasitised (Kerry and Bourne, 1996). Rhizosphere colonisation is mediated by the plant species and is fundamental for nematode control (Leij and Kerry, 1991; Bourne et al., 1994; Kerry and Bourne, 1996). Isolates that colonise more than 80% of the root segments in semi-sterile conditions are selected (Abrantes et al., 2002).

The third criteria is the ability to parasitise nematode eggs. The fungus can exhibit different grades of virulence that depend on the isolates and nematode host (Abrantes et al., 2002; Morton et al., 2003b). Reported studies have shown that *P. chlamydosporia* infects eggs of *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. chitwoodi,* but usually *M. incognita* is used as standard in experimental studies (Abrantes et al., 2002).

The main goal of the present study was to evaluate the potential of Portuguese *P. chlamydosporia* isolates associated with *Meloidogyne* spp. as biological control agents. Specific objectives were to characterise and compare 10 isolates associated with RKN and three reference isolates, using molecular techniques and standard *in vitro* bioassays to assess their ability to produce chlamydospores, colonise the rhizosphere and parasitise nematode eggs.



2. Materials and Methods

2. Materials and Methods

2.1. *Pochonia chlamydosporia* isolates

Thirteen *P. chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX and reference isolates Pc2, Pc10 and 392) used in this study belong to the collection of the Nematology Laboratory at Centre for Functional Ecology (CFE), University of Coimbra (UC) (Table I). Isolates Ia, Ib, II, III, IV, V, VI, VII, VIII and IX were obtained from root samples infected with *Meloidogyne hispanica*, *M. incognita* and *M. javanica*, originating from three different plots of a greenhouse in Alcochete, Setúbal. Isolate Pc2 was obtained from *Globodera rostochiensis* eggs extracted from cysts in soil samples collected in a potato field in Guarda (Vieira dos Santos et al., 2013). Isolate Pc10 (IMI 331547) was obtained from *M. incognita* eggs from Brasil, and isolate 392 (IMI SD187) was extracted from *M. incognita* from Cuba. Both were kindly supplied by Professor B. Kerry, Rothamsted Research, UK. Isolate Pc10 was used as a reference isolate of *P. chlamydosporia* var. *chlamydosporia* and 392 was used as a reference isolate of *P. chlamydosporia* var. *catenulata*.

Table I – Origin of *Pochonia chlamydosporia* isolates used in this study

Isolate reference	Substrate	Nematode species	Geographic origin
Ia	eggs	*Meloidogyne hispanica*	Setúbal, Portugal
Ib	eggs	*M. hispanica*	Setúbal,Portugal
II	eggs	*M. javanica*	Setúbal, Portugal
III	eggs	*M. javanica*	Setúbal, Portugal
IV	eggs	*M. incognita*	Setúbal, Portugal
V	eggs	*M. incognita*	Setúbal, Portugal
VI	roots	*M. hispanica*	Setúbal, Portugal
VII	roots	*M. hispanica*	Setúbal, Portugal
VIII	roots	*M. javanica*	Setúbal, Portugal
IX	roots	*M. javanica*	Setúbal, Portugal
Pc2	eggs	*Globodera rostochiensis*	Guarda, Portugal
Pc10	eggs	*M. incognita*	Brasil
392	eggs	*M. incognita*	Cuba

Isolates are being maintained in the Nematology Laboratory, at CFE, UC, in 1.7% corn meal agar (CMA) (Oxoid, UK) at 25ºC. The maintenance is performed through agar plugs colonised with the fungus. Colonies of each isolate were transferred to new plates with 1.7% CMA, every two months. During this procedure, a huge decrease in viability was observed for isolate VII. Thus, this isolate was only used in the molecular characterization and in the 1st biological replicate of the chlamydospore production assays. Hence, it was decided to preserve the isolates in 20 % sterilised glycerine in criotubes, stored at -80ºC (Franco-Navarro et al., 2009).

2.2. Molecular identification and characterization of *Pochonia chlamydosporia* isolates

2.2.1. DNA extraction

DNA of the *P. chlamydosporia* isolates was extracted from mycellium grown for 10 days in 1.7% potato dextrose agar (PDA) (Difco^{TM}, France) using the E.Z.N.A. Fungal DNA miniKit (OMEGA bio-tek, US) according to the manufacter instructions with some modifications regarding sample preparation. The mycellium was transferred to 1.5 ml Eppendorf tubes and frozen at -80ºC overnight. After addition of 600 µl of FG1 Buffer, the samples were placed in a tissue lyser (Qiagen, Germany) for 5 min. In the resuspension step, the samples were incubated 5 min at 65 ºC in a thermomixer (Eppendorf AG., Germany). DNA was kept at -20 ºC until needed.

2.2.2. *Pochonia chlamydosporia* identification

DNA was amplified with the β-tubulin primers specific for *P. chlamydosporia* var. *chlamydosporia*: tub1f, 5’-TTT GCA GTA TCT CAG TGT TC-3’; and tub1r, 5’-ATG CAA GAA AGC CTT GCG AC-3’ (Hirsch et al., 2000). The reaction mixture in a final volume of 25 µl with sdH2O was prepared with 1 mM of MgCl2, 0.4mM of dNTPs, 1 µM of each primer, 1 U of BioTaqTM polymerase (Bioline, UK) and 2 µL of genomic DNA. PCR was performed in a thermocycler (Applied Biosystems, US) using the following reaction conditions: 1 min at 95ºC, followed by 30 cycles at 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 2 min and a final extension at 72ºC for 1 min.

DNA of all isolates was also amplified using primers specific for *P. chlamydosporia* var. *catenulata*: PcatF, 5’-GTG AAC TTA TAC CAT TTT TTG-3’ and PcatR, 5’-CAC AAG TCC CCA TCC GC-3’ (Atkins et al., 2003a). The reaction mixture in a final volume of 20 µl with sdH2O was prepared with 1.5 mM of MgCl2, 0.4mM of dNTPs, 0.1 µM of each primer, 1 U of BioTaqTM polymerase and 3 µL of genomic DNA. Amplification was performed in a thermocycler using the following reaction conditions: 3 min at 95ºC, followed by 35 cycles at 95ºC for 1.30 min, 50ºC for 1 min, and 72ºC for 2 min and a final extension at 72ºC for 5 min.

PCR products were separated in 1% agarose gels stained with GreenSafe Direct Load (Nzytech genes & enzymes, Portugal) and visualised in a transilluminator (Vilbert Lourmat, France)

The relative mobilities of the PCR bands were calculated with the Bio-print Mega software version 12.15 for Windows (Vilbert Loumart, France).

2.2.3. Enterobacterial repetitive intragenic consensus (ERIC)-PCR

DNA extracted was fingerprinted using ERIC-PCR following the protocol described by Arora et al. (1996) with some modifications. Primers used were R1CIRE, 5'-CAC TTA GGG GTC CTC GAA TGT A-3', and ERIC2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3'. Reactions were prepared to a final volume of 25 µl with sdH_2O contained 2.5 mM of MgCl2, 0.5 mM of dNTPs, 1.25 µM of each primer, 1 U of RedTaq polymerase (Sigma-Aldrich, US) and 5µL DNA. The PCR thermocycling conditions were set at 95ºC for 7 min, 30 cycles of 94ºC for 1 min, 46ºC for 1 min and 72º C for 8 min and a final extension at 72ºC for 16 min. PCR products were separated in a 1% agarose gel stained with GreenSafe Direct Load and visualised in a transilluminator.

Two replicates of each amplification were performed independently to check the reproducibility of the reactions.

2.2.4. Internally trancribed spacer-restriction fragment length polymorphism (ITS-RFLP)

ITS regions were amplified by PCR using specific ITS primers according to White et al.,1990: ITS1 (TCCGTAGGTGAACCTGCGG), ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGC). Reactions were prepared to a final volume of 25 µl with sdH_2O contained: 1X Buffer, 1.7 mM of MgCl2, 1 mM of dNTPs, 1 µM of each primer, 1 U of Biotaq^{TM} polymerase and 5 µL DNA. The PCR thermocycling conditions were set at 95ºC for 3 min, 30 cycles of 95ºC for 1.30 min, 50ºC for 1 min, 72ºC for 2 min and a final extension at 72ºC for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel stained with GreenSafe Direct Load and visualised in a transilluminator.

PCR products were kept at -20 ºC until needed. Four restriction enzymes: *Eco*RI (Bioron, Germany), *Hae*III (United States Biological Life Sciences, US), *Hind*III (Invitrogen, Thermo Fisher Scientific, US) and *Hinf*I (Bioron, Germany) were tested. The digestion of ITS-PCR products was made in a final volume of 25µl with sdH_2O with: 2 µl Buffer 10 X, 20 U of restriction enzyme and 3 µl of PCR products. Samples were incubated at 37 ºC for 1 h followed by an incubation at 65 ºC for 20 min to inactivate the enzymes. The digestion of the PCR products was analysed by electrophoresis on 2% agarose gels stained with GreenSafe Direct Load and run at 100V for 2 hr. Relative mobilities of the

generated bands were calculated with the Bio-Print print Mega software version 12.15 for Windows (Vilbert Loumart, France).

2.3. Biological characterization of *Pochonia chlamydosporia* isolates

The isolates were evaluated to assess their ability to produce chlamydospores, to colonise the rhizosphere of tomato plants and to parasitise RKN and PCN nematodes.

Standards bioassays were performed using the methodologies described in Abrantes et al. (2002) with some modifications regarding the rhizosphere colonisation assays. Isolates Ib and 392 were not evaluated regarding egg parasitism as they did not produce enough spores in 1.7% CMA to perform the assay.

2.3.1. Chlamydospore production

Chlamydospore production was evaluated following the method described by Leij and Kerry (1991). Milled barley was washed through a sieve (53 µm) and mixed with sterilized coarse sand (1:1). The mixture was left to dry at room temperature for ca. 4 h. Sixty g of substrate medium were transferred into a conical flask (250 ml) and autoclaved. Then, 3 flasks per isolate were inoculated with 3 agar plugs (0.7 cm) colonised with the fungus transferred from 10 days-old colonies on 1.7% CMA. The flasks were incubated for 21 days at 25ºC in the dark. After, 1 g of the colonised medium was suspended in 9 ml of 0.05% water agar solution and the number of chlamydospores was estimated under a stereomicroscope using a Neubauer chamber. Three replicates per flask were prepared. Plates containing sorbose agar with antibiotics (12 g/l technical agar (DifcoTM, France), 2 g/l of sorbose (Sigma, US) and 50 mg/l of each antibiotic: streptomycin sulfate (Sigma, US); chloramphenicol (Sigma, US); and chrortetracycline (Sigma, US) were inoculated with 0.2 ml of the chlamydospore suspension to evaluate chlamydospore viability. Three plates per flask were incubated for 2 days at 25ºC. Then, the number of germinated chlamydospores was counted under a stereomicroscope and the percentage of germinated chlamydospores was estimated. The experiment was repeated three times.

2.3.2. Rhizosphere colonisation

Rhizosphere colonisation was assessed using tomato plants cv. Tiny Tim. Tomato seeds were sterilized with 1.6% sodium hypochlorite (Panreac AppliChem, Spain), for 10 min with agitation. The sterilised solution was discarded and seeds were washed with sterile distilled water for 1 min. This procedure was repeated 3 times. Tomato seeds were then transferred to Petri dishes containing 1X Gamborg's B5 medium (Gamborg's B5 (Sigma, US), amended with 15 g/l sucrose (Sigma-Aldrich, US) and 8 g/l purified Agar (Sigma-Aldrich, US)); pH adjusted to 6.4. To increase the proportion of germinated seeds, plates were incubated at 4ºC for 24h and then transferred to 25ºC for 3 days.

Plates with 10 days-old colonies grown on 1.7% CMA were washed with 8 ml of sterile distilled water and scraped with an inoculating loop to suspend fungal material. The suspension was transferred to a flask and the number of spores was counted under a stereomicroscope using a Neubauer chamber. Five flasks/isolate filled with autoclaved vermiculite were inoculated with 10^5 spores and a 3 days-old single germinated tomato seed was planted. Flasks without fungal inoculum were used as controls.

Flasks were incubated for 21 days at 25ºC, 12h photoperiod. Then, the roots were cleaned of vermiculite and placed onto 0.8% water agar. After incubation for 3 days at 25ºC, root fragments with evident hyphal material were considered colonised by the fungus and the percentage of colonised root fragments was estimated. Two biological replicates were performed.

2.3.3. Nematode egg parasitism

Two PPN isolates, *M. incognita* and *G. pallida* were selected from the collection of the Nematology Laboratory at CFE, UC. The *M. incognita* isolate originating from infected potatoes cv. Asterix in Sobreda, Vale da Caparica, Setúbal, was propagated on tomato cv. Tiny Tim grown in 800 cm^3 pots with sterilized sandy loam soil and sand (1:1). Pots were inoculated with 10 egg masses/plant and placed in a glasshouse at 25ºC, 12h photoperiod, 70% humidity and watered regularly. After 2 months, the plants were uprooted and 50 egg masses were manually removed and disrupted in sterilised distilled water by agitation.

The *G. pallida* isolate was originally obtained from infected potato fields in Bolho, Cantanhede, Coimbra. *Globodera pallida* was propagated on susceptible potato cv. Désirée, in plastic containers filled with 800 g of sterile sandy soil. Pots were inoculated with a polyester bag containing cysts, to give approximately 5 eggs/g of soil. The number of eggs/cyst was calculated by counting 5 replicates of a suspension of eggs obtained by crushing 50 cysts in water. Pots were placed in a glasshouse at 20º-22ºC, 70% humidity and 16 h of photoperiod and watered regularly. After 3 months, new cysts were extracted from the soil using a modified Fenwick can (Shepherd, 1986). Fifty cysts crushed with a forceps were suspended in water by agitation to obtain eggs.

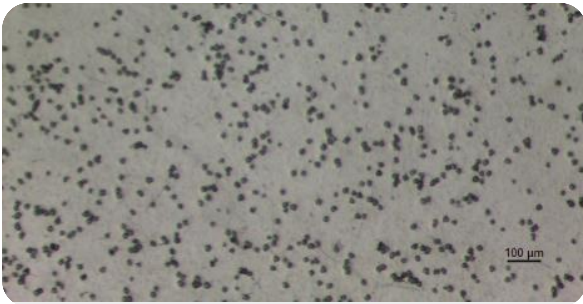
Virulence of the isolates was evaluated by assessing the ability to parasitise RKN and PCN eggs. Plates of the fungus growing on 1.7% CMA were washed with 8 ml of sterile distilled water and scraped with an inoculating loop to suspend fungal material. The suspension was transferred to a flask and the number of spores was counted under a stereomicroscope using a Neubauer chamber. Petri dishes with 0.8% technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg/l of each) were inoculated with a $2x10^4$ spores/ml suspension of *P. chlamydosporia* and incubated for two days at 25ºC. After this time, 250 eggs of the two nematodes species were spread on each plate colonised by the fungus and the plates were incubated again

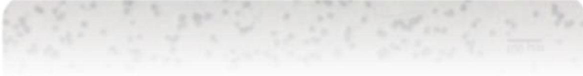
at 25ºC, during 3 days. Controls were inoculated with eggs of each nematodes species in the absence of fungus. After incubation, the number of parasitised eggs by the fungus was counted and the percentage of parasitised eggs estimated. Only eggs colonised by fungal mycelium with characteristic conidia were considered parasitised by *P. chlamydosporia*. Three biological replicates were performed.

2.4. Data analysis

ERIC-PCR fingerprints produced distinct bands that were visually scored for presence (1) or absence (0) from gel photographs. The results obtained were compared and a dendogram was constructed by Unweighted Pair Group Method with Arithmetic Mean in the hierarchical clustering based on Euclidean distances using Statistica version 10 (StatSoft Inc., US).

Data on chlamydospore production, rhizosphere colonisation and nematode egg parasitism were compared by one-way ANOVA, using the General Linear Model command in Statistica version 10 (StatSoft Inc., US). A square root transformation was used when needed to ensure a normal distribution and constant variance of data, that otherwise, would not meet the ANOVA assumptions. Statistically significant differences among treatments were computed using LSD test ($P>0.05$).





3. Results

3. Results

3.1. Molecular identification and characterization of *Pochonia chlamydosporia* isolates

3.1.1. *Pochonia chlamydosporia* identification

The identity of the isolates was confirmed using β-tubulin primers specific for *P. chlamydosporia* var. *chlamydosporia* (Hirsch et al., 2000) and *P. chlamydosporia* var. *catenulata* (Atkins et al., 2003a). All isolates presented a specific band for *P. chlamydosporia* var. *chlamydosporia* (ca. 270 bp), except isolate 392 (Fig. 1a). Only isolate 392 presented a specific band for *P. chlamydosporia* var. *catenulata* (ca. 370 bp) (Fig. 1b).

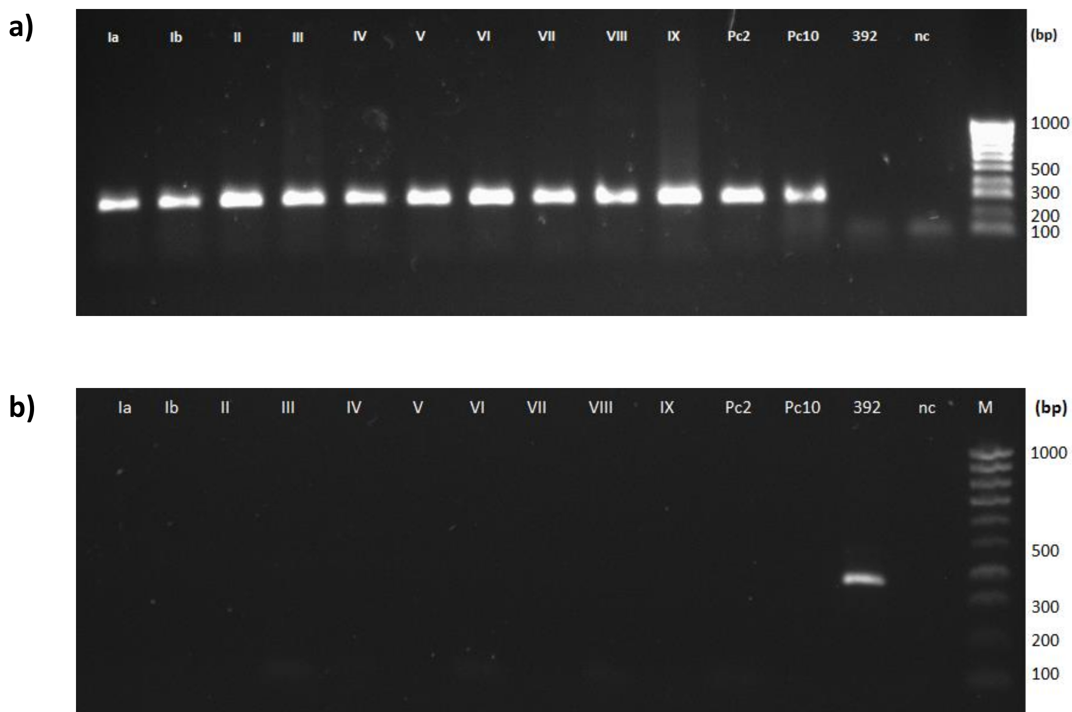


Figure 1 - Molecular characterization of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10, 392). PCR product of DNA amplified using primers for the specific detection of: (a) *Pochonia chlamydosporia* var. *chlamydosporia* (Hirsch et al., 2000) and (b) *Pochonia chlamydosporia* var. *catenulata* (Atkins et al., 2003a). nc: negative control without template DNA; M: DNA size marker Hyperladder^{TM} IV, 100 bp ladder (Bioline, UK).

3.1.2. Enterobacterial repetitive intragenic consensus (ERIC)-PCR

Molecular characterization of the isolates using ERIC-PCR showed that isolates associated with *Meloidogyne* spp. have very similar patterns. A different band pattern was observed for isolate Pc2 associated with *G. rostochiensis* eggs (Fig. 2).

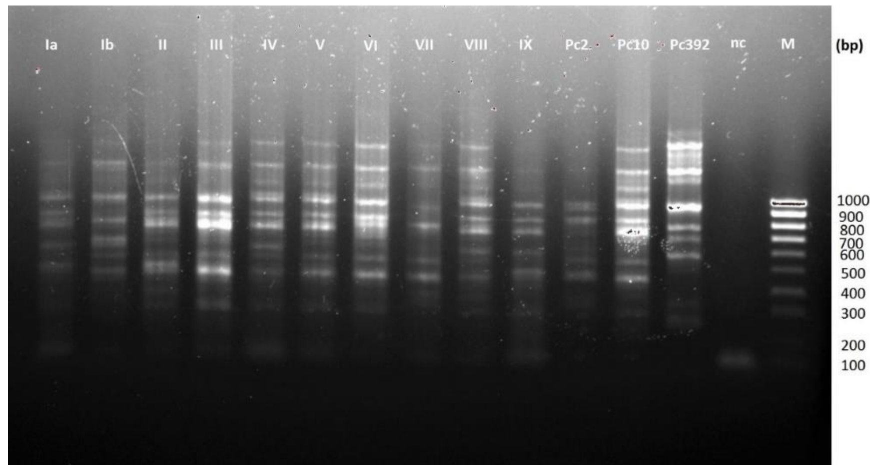


Figure 2 – ERIC-PCR profiles Molecular characterization of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). PCR product of DNA amplified with ERIC-PCR profiles. nc: negative control without template DNA; M: DNA size marker HyperladderTM IV, 100 bp ladder (Bioline, UK).

Cluster analysis of the ERIC-PCR profiles revealed that the isolates were grouped according to their geographic origin (Fig. 3) and to their host population except isolate PcVI that clustered apart from the other isolates associated with *M. hispanica* (PcIa, PcIb and PcVII).

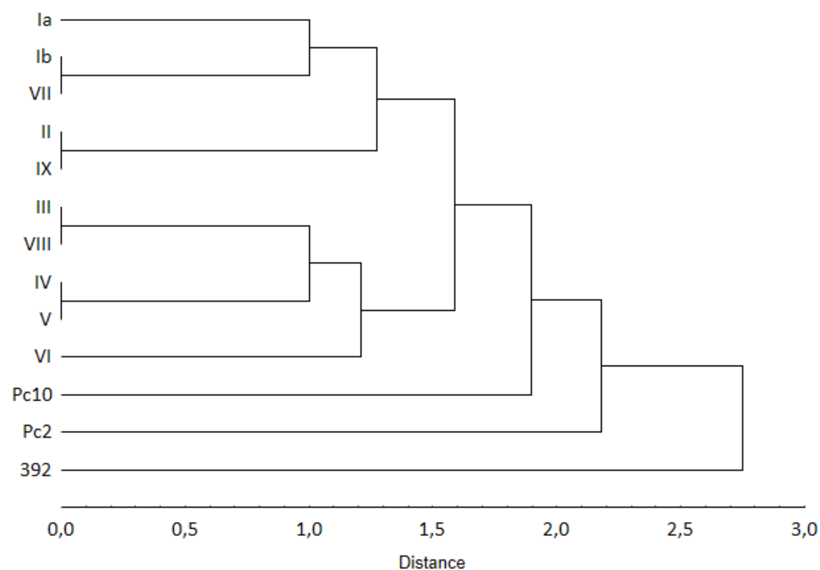


Figure 3 - Dendrogram showing linkage distances among groups based on the ERIC-PCR profiles of of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). The dendogram was constructed by Unweighted Pair Group Method with Arithmetic Mean in the hierarchial clustering based on Euclidean distances using Statistica version 10 (StatSoft Inc., US).

3.1.3. Internally transcribed spacer-restriction fragment length polymorphism (ITS-RFLP)

PCR using ITS primers generated a single PCR product of ca. 614 bp for primers ITS1-ITS4 (Fig. 4a) and a single band with ca. 655 bp for primers ITS4-ITS5 (Fig. 4b) for all isolates.

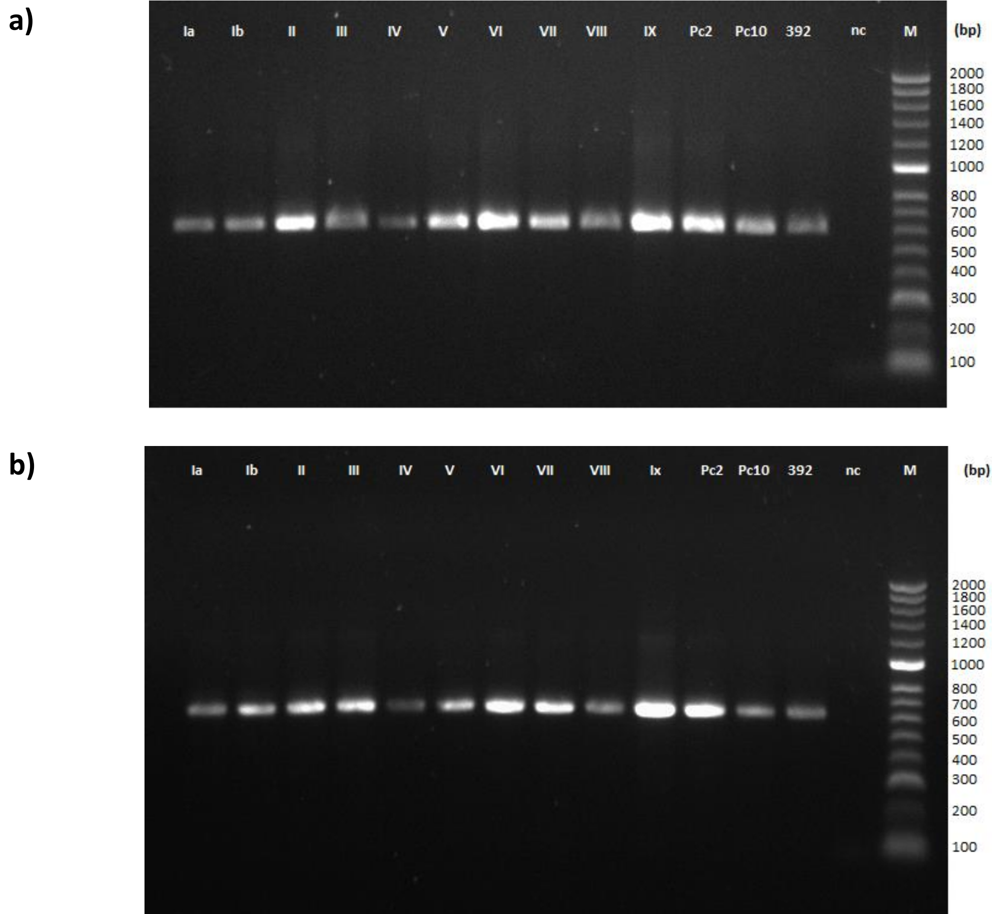


Figure 4 - PCR product of DNA from 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392) amplified with a) ITS4/ITS1 primers and b) ITS4/ITS5 primers (White et al.,1990). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK).

The restriction pattern produced using the *Eco*RI enzyme on products from the ITS region revealed two distinct bands for all isolates. Differences in band size were detected for the two sets of primers used: ITS1/ITS4 the band sizes were ca. 350, 290 bp (Fig. 5a) and for ITS4/ITS5, band sizes were 350, 305 bp (Fig. 5b).

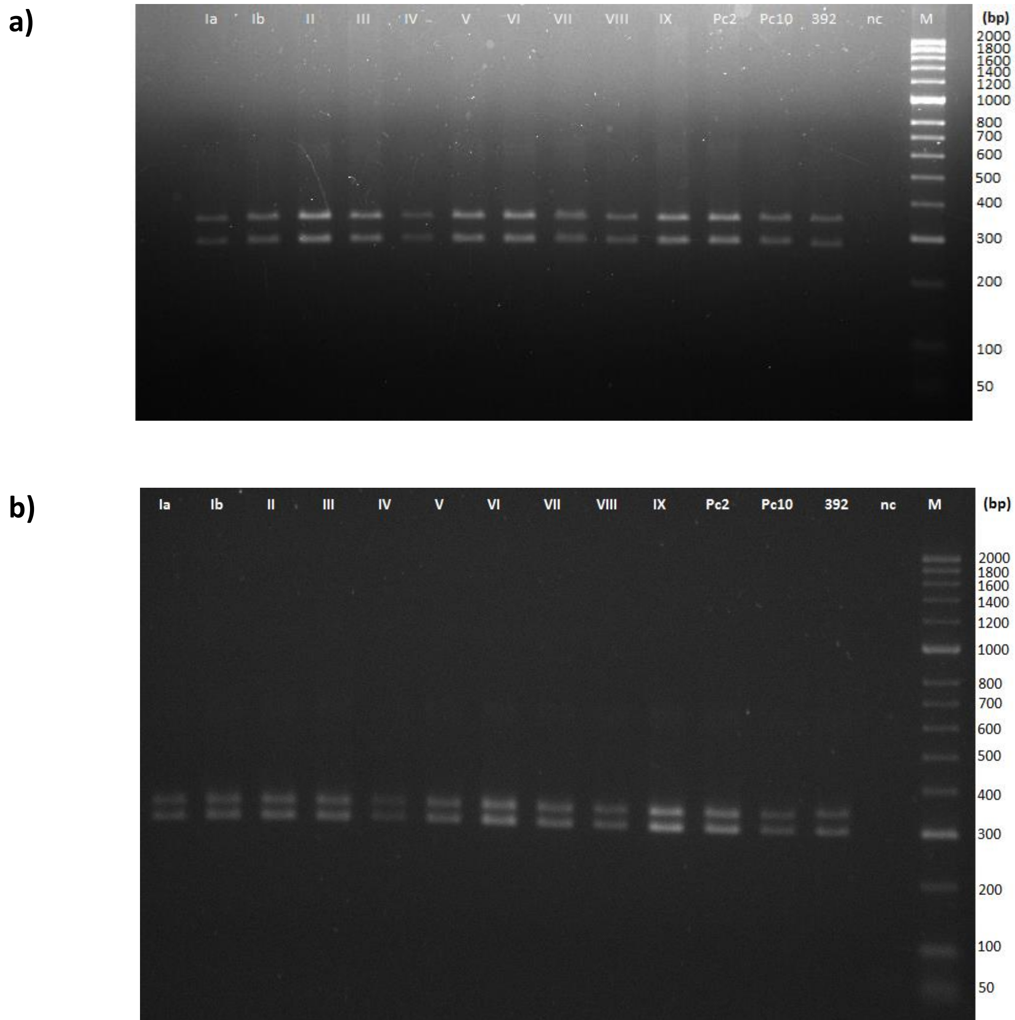


Figure 5 - RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Eco*RI of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK).

A three band pattern was observed in the restriction pattern obtained with *Hae*III. Again no differences were detected among isolates but different sizes were observed for ITS1/ITS4 products (350, 142, 100 bp) (Fig. 6a) and ITS4/ITS5 products (360, 165, 105 bp) (Fig. 6b).

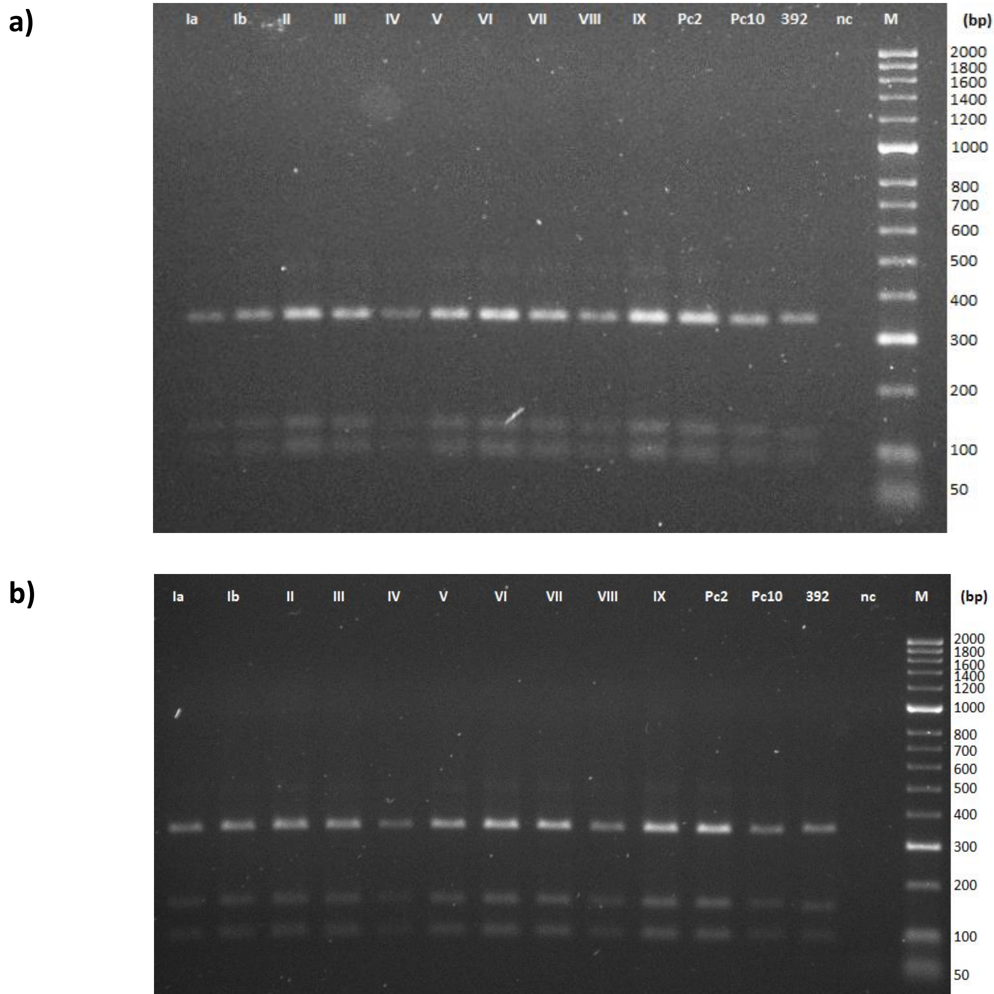


Figure 6 – RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Hae*III of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK).

ITS-PCR products were not cleaved by the restriction enzyme *Hind*III (data not shown).

The three band restriction patterns produced by the cleavage of products from the ITS regions by *Hinf*I enzyme revealed differences between isolate 392 and all the other isolates. Regarding ITS1/ITS4 products (Fig. 7a), for isolate 392, band sizes were 297, 167, 116 bp, whereas for all the other isolates the band sizes were 297, 199, 116 bp. For the ITS4/ITS5 products (Fig. 7b), isolate 392 presented a three band pattern with 295, 180, 115 bp and all the other isolates presented a band pattern with 295, 230, 115 bp.

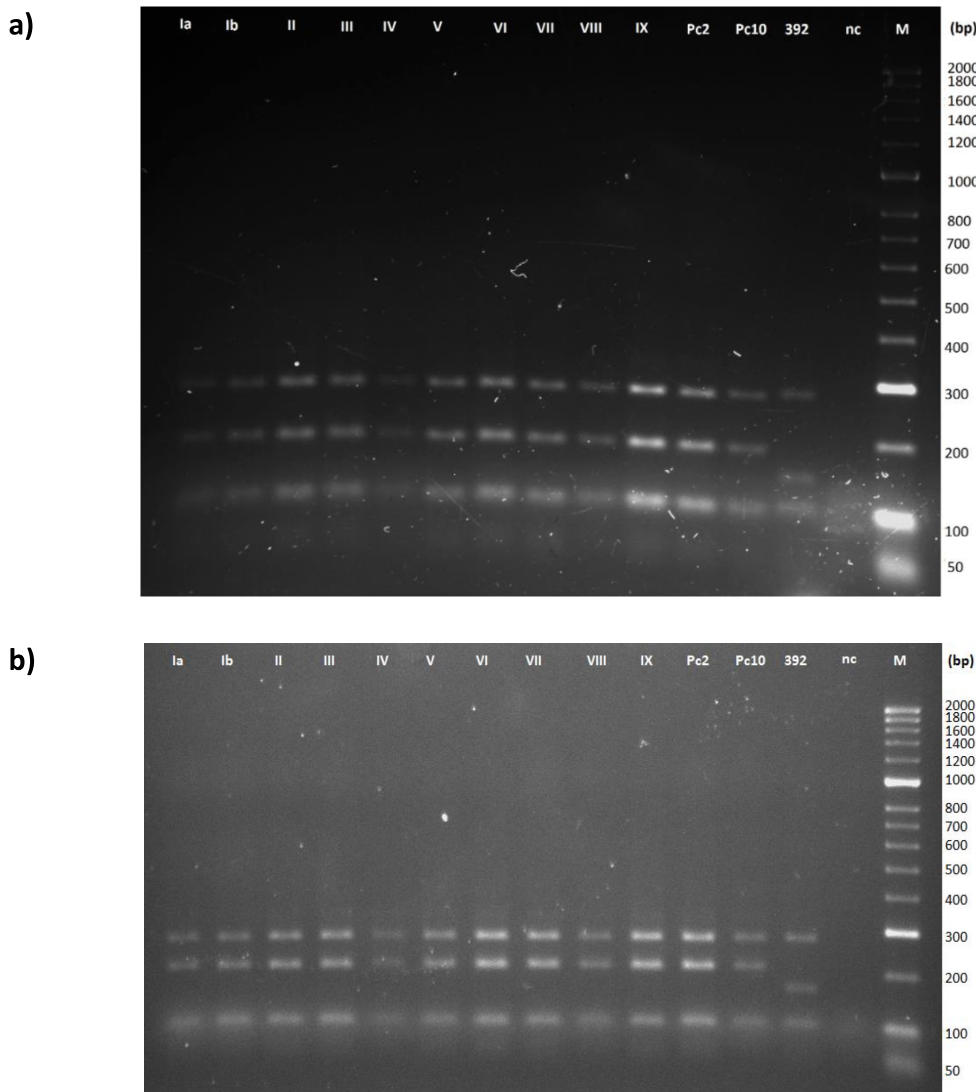


Figure 7 - RFLP of (a) ITS 4/ITS1 and (b) ITS4/ITS5 sequences digested with *Hinf*I of 13 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc2, Pc10 and 392). nc: negative control without template DNA; M: DNA size marker HyperladderTM II, 100 bp ladder (Bioline, UK).

3.2. Biological characterization of *Pochonia chlamydosporia* isolates

3.2.1. Chlamydospore production

Results between the three biological replicates were not consistent and differences were detected among isolates in the ability to produce chlamydospores.

3.2.1.1. First assay

Differences were detected among isolates in their ability to produce chlamydospores. Isolates III and 392 produced the most spores (108.4×10^5/g and 106×10^5/g, respectively) and the isolate IX (1.1×10^5/g) was the least producer (Fig. 8a). The viability of the chlamydospores was greater than 80% for all isolates, except for isolate 392 (77.4%) (Fig. 8b). The isolate with the highest percentage of germination of chlamydospores was Pc10 (92.3%).

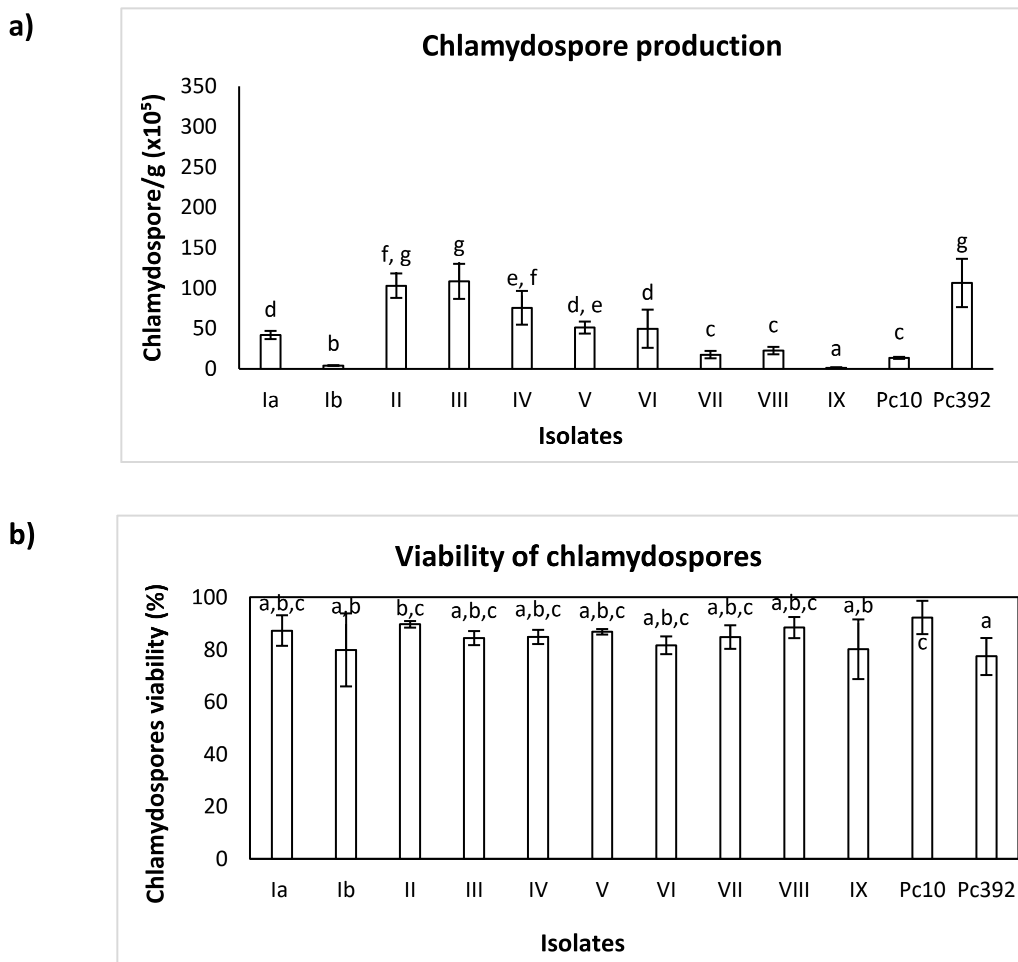


Figure 8 – Chlamydospore production (1st assay): number of chlamydospores (a), and chlamydospore viability (b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test ($p>0.05$).

3.2.1.2. Second assay

Differences were detected between isolates (Fig. 9a). Isolate VI (288.2×10^5/g) was the best producer of chlamydospores and the isolate that produced less was Ib (7.3×10^5/g). The viability of chlamydospores was greater than 80% in most isolates, except in the case of isolate Ib, that had a germination rate of less than 70% (Fig. 9b). Isolate Pc10 had the highest germination percentage of chlamydospores (93.1%).

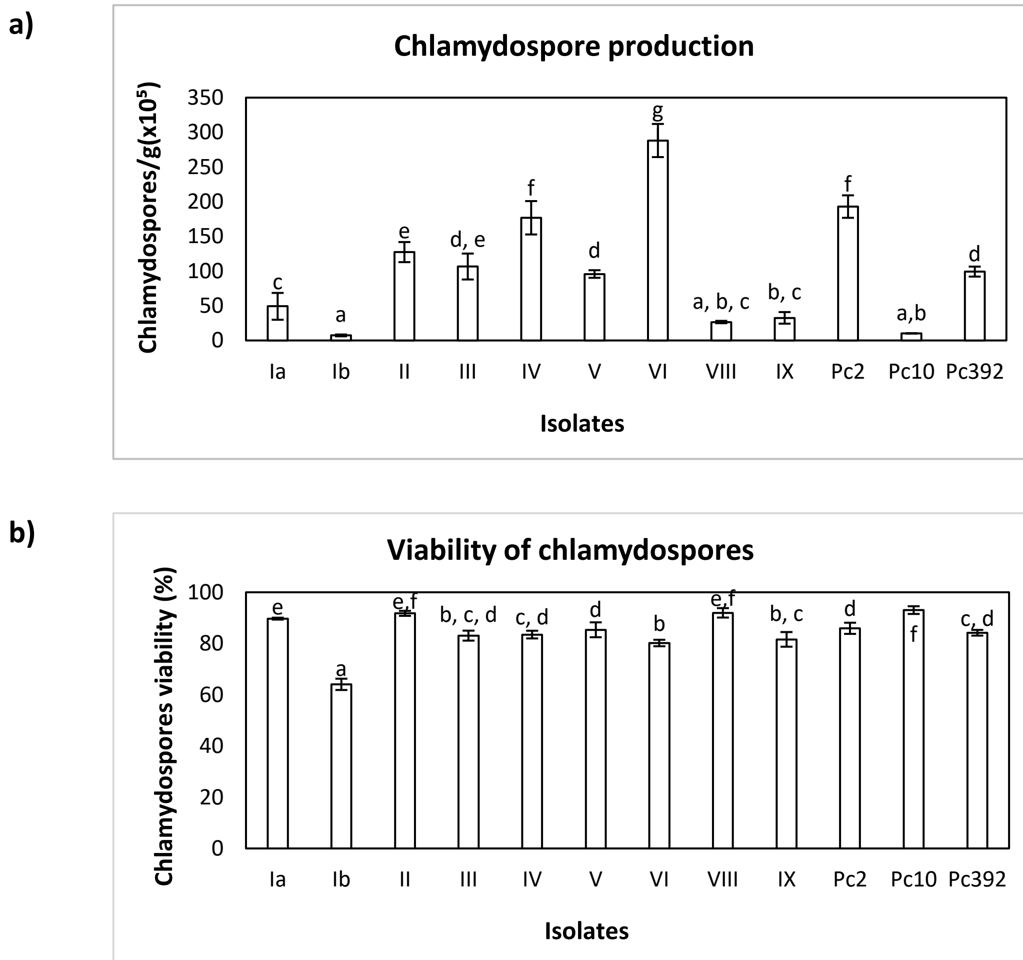


Figure 9 – Chlamydospores production (2nd assay): number of chlamydospores (a) and chlamydospore viability(b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test ($p>0.05$).

3.2.1.3. Third assay

In the third assay, isolates II (216.3×10^5/g) and Pc2 (198.5×10^5/g) produced a significantly higher number of chlamydospores and the isolates that produced significantly less chlamydospores were Ib (1.59x10^5/g), IX (15.5×10^5/g) and Pc10 (4.7×10^5/g) (Fig. 10a). No differences were detected between some isolates (Ia, III and V; IV and VI; Ib, IX and Pc10). Regarding chlamydospore viability, isolate Ia displayed a percentage of germination close to 100% (Fig. 10b). All isolates had a percentage of germination greater than 80%, except isolates Ib (72.4%) and III (76.7%).

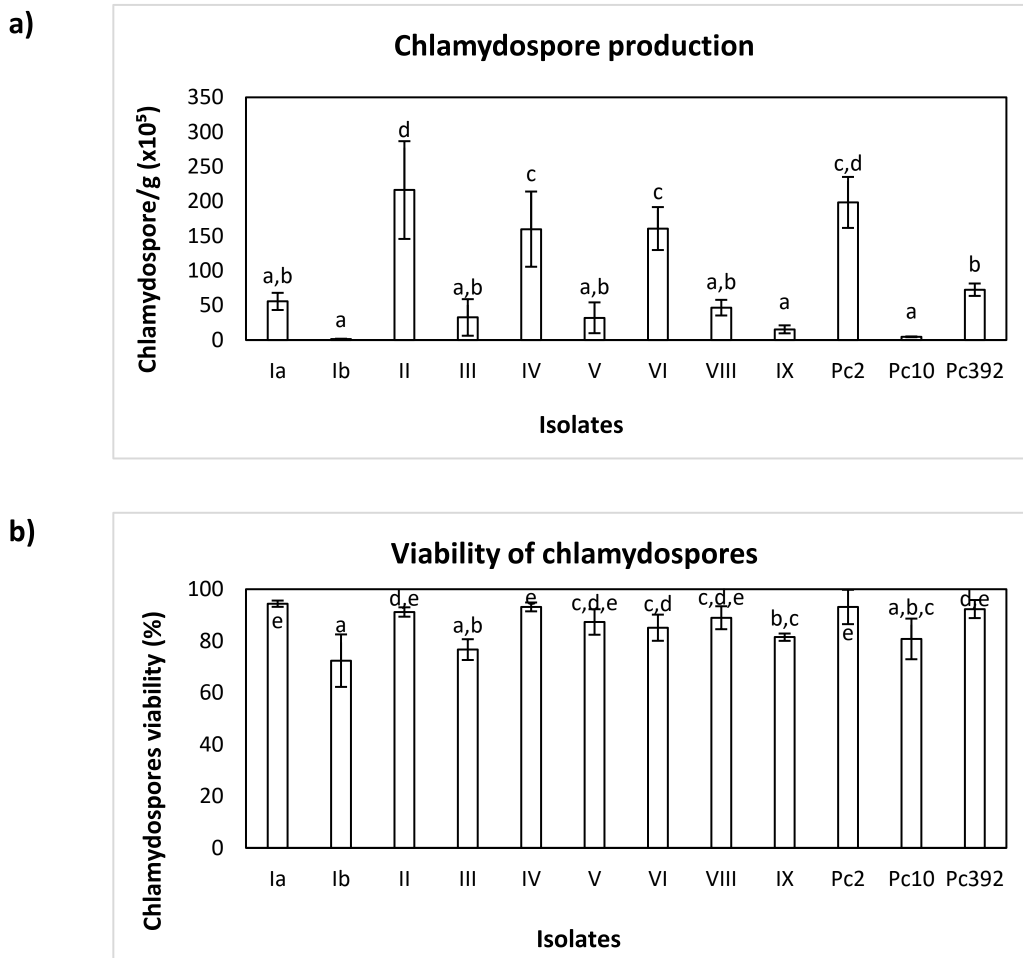


Figure 10 – Chlamydospores production (3rd assay): number of chlamydospores (a) and chlamydospore viability (b) of 12 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2, Pc10 and 392) grown for 21 days on milled barley mixed with sterilized coarse sand (1:1). Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05).

3.2.2. Rhizosphere colonisation

This *in vitro* bioassay was repeated twice and the results were similar between replicates so only the results of the 1st assay are showed. Differences were detected between isolates in their ability to colonise tomato cv. Tiny Tim roots. All isolates colonised more than 80% of the tomato roots except isolates Ib, IX, IV and V. Isolates Ib and IX were poor colonisers (36.8% and 44.3%, respectively), and isolates IV (58.5%) and V (75.4%) also revealed a low percentage of colonisation (Fig. 11). No fungal growth was observed in the controls.

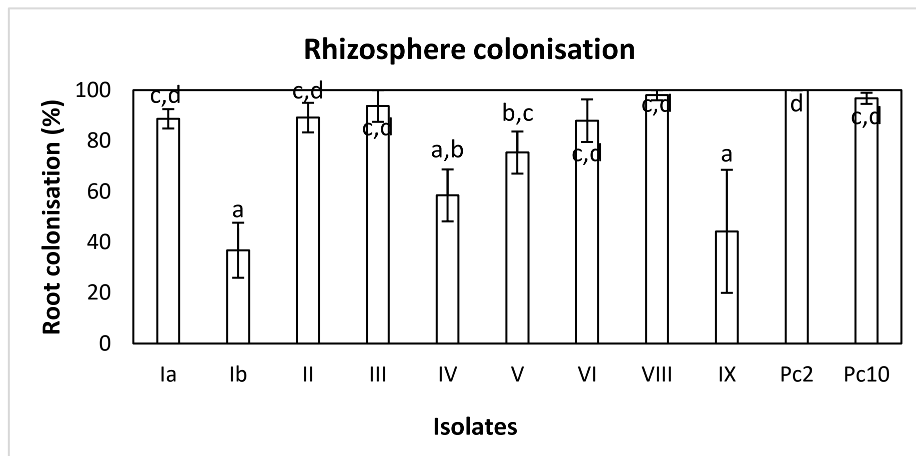


Figure 11 – Percentage of colonised roots of tomato cv. Tiny Tim by 11 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10). Bars represent standard error of means and columns with the same letter are not significantly different according to LSD test ($p>0.05$).

3.2.3. Nematode egg parasitism

Three biological replicates were performed. Although there were some differences between replicates, the ability to parasitise eggs of both nematodes species was very similar for isolate Ia in all three bioassays.

The proportion of parasitised *Meloidogyne* eggs was low for all isolates (more than 65%) and differences were detected among isolates (Figs. 12a, 12b, 12c). Isolate VIII was the best parasite of *M. incognita* eggs, and isolate Ia was the second best. In the three replicates isolate IX was a poor parasite (less than 25%), followed by the isolates V (less than 36%) and VI (less than 36%). For isolate Pc2, in the first and second assays, a contamination was detected in all three plates. Parasitised eggs were detected in the three biological replicates in the controls. However, the fungi observed did not present the morphological characteristics of *P. chlamydosporia*.

In the first assay, isolate VIII was the best parasite of *Meloidogyne* eggs (more than 60%) and the isolate with the lowest percentage of parasitism was isolate IX (less than 30%) (Fig. 12a). No differences were detected between isolates II, VI and Pc10, neither between isolates Ia and III. Significant differences were detected among the

percentages of parasitism of isolates in second assay (Fig. 12b). Isolates Ia and VIII were the best parasites of *Meloidogyne* eggs with more than 50% of eggs parasitised, and isolate IX was a poor parasite (less than 25%). No differences were detected between isolates II, VI and Pc10. Results obtained for the third assay were similar to those of the second assay (Fig. 12c).

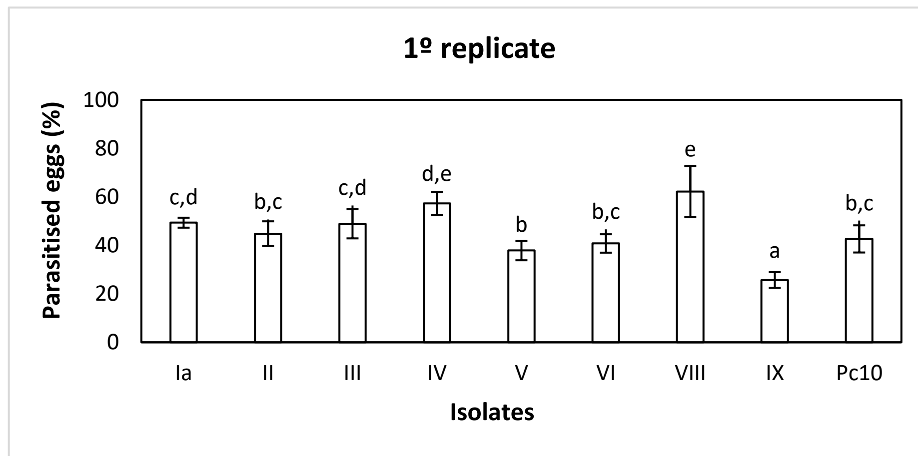
The percentage of *G. pallida* parasitised eggs was low for all isolates in the three biological replicates (less than 60%) (Figs. 13a, 13b, 13c). Some differences were detected between the three replicates. However, isolate Pc2 was the best parasite in the most biological replicates. Isolates IV, VI and Pc10 were consistently poor parasites of *G. pallida* eggs. Control was contaminated in the first assay, 2% of the eggs were parasitised, although no *P. chlamydosporia* characteristics were detected.

In the first assay, no differences were detected for isolates Ia and VIII, as well as for isolates II and IX (Fig. 13a). Isolates V and Pc2 were the best parasites of *G. pallida* eggs (more than 50% of parasitised eggs) and the isolates that parasitised the least percentage of eggs were isolates IV, VI and Pc10 (less than 40%).

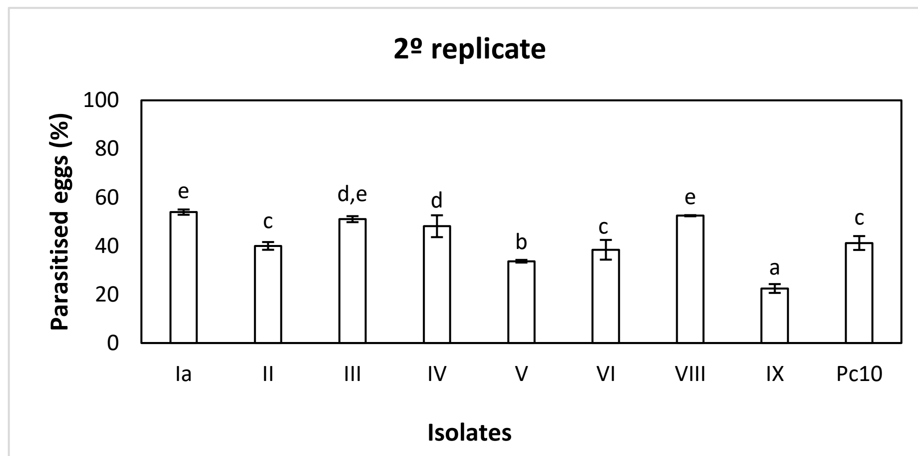
Some differences were detected between isolates in the second assay. The best parasites were isolate Pc2 and isolate III (more than 50% of parasitised eggs) and the worst parasites were isolate VI, IV and Pc10 (less than 40%) (Fig. 13b). No differences were detected between isolates Ia and VIII, neither between isolates II, V and Pc10.

The best parasite in third assay was isolate Ia, which had a percentage of parasitised eggs higher than 55%, and isolates IV and IX were those who parasitised a lower percentage of eggs (less than 40%) (Fig. 13c).

a)



b)



c)

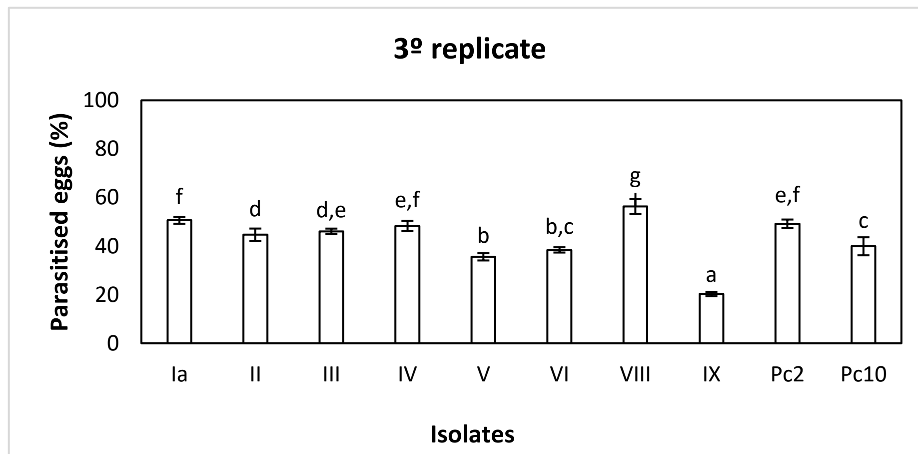
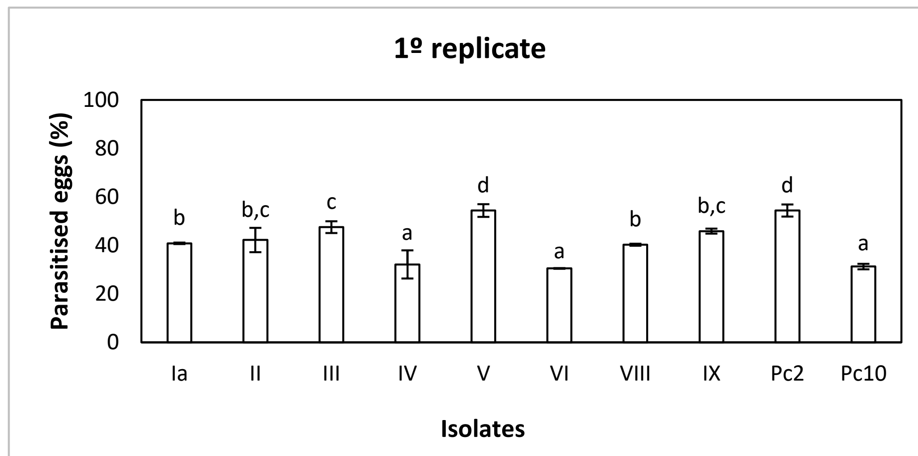
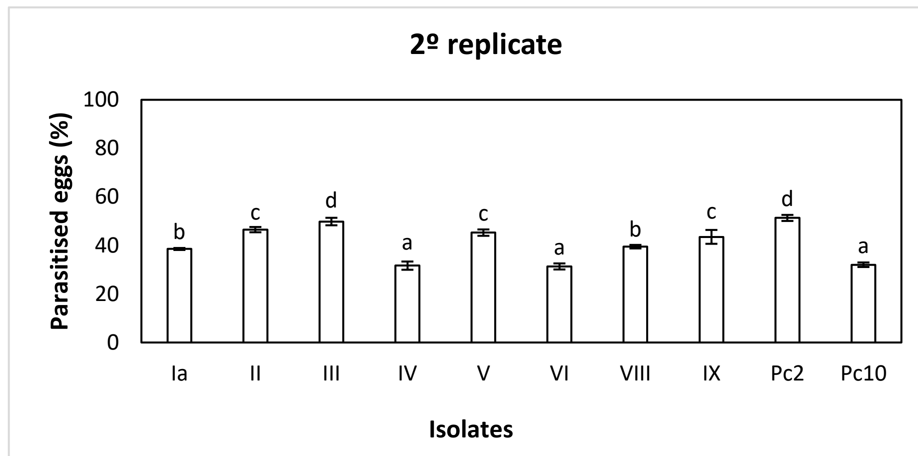


Figure 12 – Percentage of parasitised *Meloidogyne incognita* eggs by 10 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10) grown for 10 days on 1.7% corn meal agar (CMA). (a) First assay; (b) second assay; (c) third assay. Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05).

a)



b)



c)

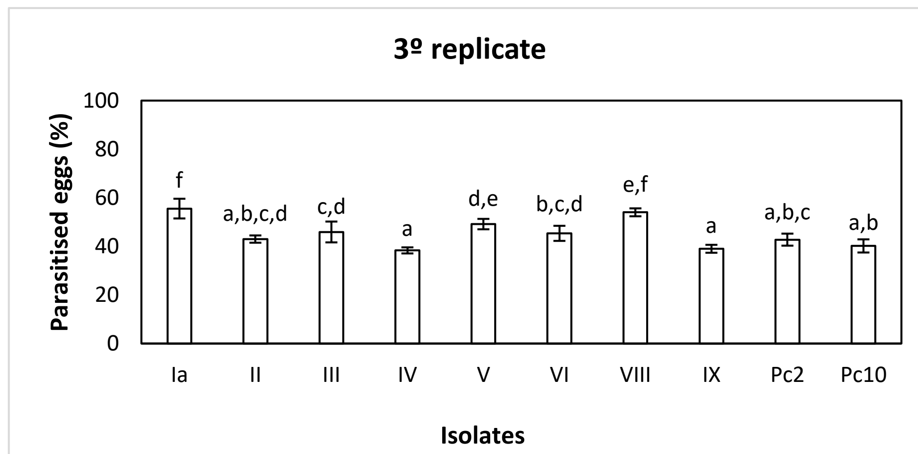
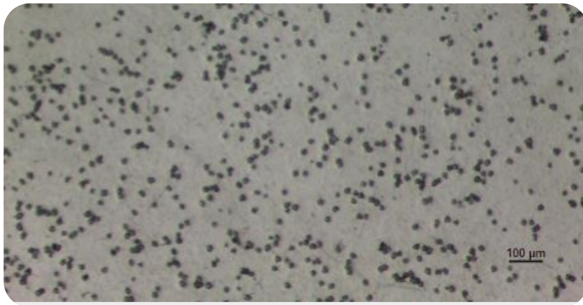


Figure 13 - Percentage of parasitised *Globodera pallida* eggs by 10 *Pochonia chlamydosporia* isolates (Ia, Ib, II, III, IV, V, VI, VIII, IX, Pc2 and Pc10) grown for 10 days on 1.7% corn meal agar (CMA). (a) First assay; (b) second assay; (c) third assay. Bars represent standard error of means and columns with the same letter are not statistically significantly different according to LSD test (p>0.05).



4. Discussion

4. Discussion

Plant-parasitic nematodes (PPN) are among the most widespread pests and are recognized as one of the greatest threats of agricultural crops (Handoo, 1998; Nicol et al., 2011; Haegeman et al. 2012). The most economically important PPN which have a great impact on crops worldwide are root-knot nematodes (RKN), *Meloidogyne* spp., and cyst nematodes, *Heterodera* spp. and *Globodera* spp. (Haegeman et al. 2012, Jones et al., 2013). The facultative nematophagous fungus, *Pochonia chlamydosporia* parasitises sedentary females and eggs of economically important PPN, such as cyst and RKN (Vieira dos Santos et al., 2014a). The efficacy of the use of *P. chlamydosporia* as a biological control agent against several nematodes (*Meloidogyne* spp. and *Globodera* spp.) in tomato plants, has been revealed in *in vitro* assays and under glasshouse conditions. In addition, some studies revealed that the fungus can promote the growth of several crops, such as tomato, potato, cotton, lettuce and eggplant (Zavala-González et al., 2015). The fungus has a worldwide distribution and can easily be isolated from soil, roots or nematode eggs using a semi-selective medium (Leij and Kerry, 1991).

Molecular studies are required to identify and characterise isolates at molecular level and detect genetic variation between isolates (Abrantes et al., 2002). Screening for best isolate selection is an important step for the development of *P. chlamydosporia* as a biological control agent (Manzanilla-López at al., 2013). The standard screening depends on rapid *in vitro* tests for three important criteria based on the ability of isolates to produce chlamydospores, to colonise the rhizosphere, and to parasitise egg nematodes (Abrantes et al., 2002).

Molecular identification and characterization of isolates were performed to obtain isolate specific markers that allow the evaluation of the variability among *P. chlamydosporia* isolates from different substrates and geographic origins (Table 1). Furthermore, this information is needed for the potential registration of a specific isolate as a biological control agent (Abrantes et al., 2002). Many groups of fungi have been studied through PCR-based DNA fingerprinting and sequence analysis (Morton, 2003a). Molecular studies are necessary for the identification of specific isolates of the fungus (Mauchline et al., 2002). Detection and quantification of fungus in soil can be achieved with the use of specific primers which distinguish the two varieties of *P. chlamydosporia*, var. *chlamydosporia* and var. *catenulata* (Hirsch et al., 2001; Mauchline et al., 2002). The β-tubulin gene provided the basis to design specific primers for *P. chlamydosporia* var. *chlamydosporia* and since it has an intron which is not present in other fungi, those primers do not amplify DNA from other species of *Pochonia* or from members of other genera (Mauchline et al., 2002; Kerry and Hirsch, 2011). Identification of *P. chlamydosporia* isolates was confirmed using specific β-tubulin primers. A PCR product of 270 bp was obtained for all isolates, except for isolate 392 (Fig. 1a). This confirms the identity of all Portuguese isolates used in this study as *P. chlamydosporia*

var. *chlamydosporia* (Hirsch et al., 2001). β-tubulin gene primers did not amplify DNA from *P. chlamydosporia* var. *catenulata* (Fig. 1a) (Atkins et al., 2003a; Kerry and Hirsh, 2011). Specific primers for *P. chlamydosporia* var. *catenulata* confirmed identity of isolate 392, which was the only one that presented a band with 370 bp (Fig. 1b). The band size was slightly different from the band size reported by Atkins et al. (2003a), which was 352 bp. This is probably due to to different PCR conditions mainly regarding annealing temperature and Mg^{2+} concentration.

The use of ERIC-PCR as a technique of PCR-based DNA fingerprinting provides a rapid evaluation of the genetic variation of the isolates. ERIC primers act randomly and amplify repetitive sequences in the genome of the fungus and produce a PCR product with a multiple band profile, which allows the discrimination of different isolates (Morton et al., 2003a). ERIC have a highly conserved nucleotide sequences, but their location on the genome differs between isolates. Production of specific diagnostic fingerprinting is due to the different locations of nucleotide sequences in the genome (Atkins et al., 2003b). Patterns obtained from ERIC-PCR (Fig. 2) were used to generate a phylogenetic tree, which grouped to both their geographical and host nematode origin (Fig. 3). Although, these results should be confirmed, because isolate Pc2 is the only one that was isolated from *Globodera* eggs and is being compared with twelve isolates associated with *Meloidogyne* spp. The same happens with the preference of geographical origin, only isolates Pc10 and 392 are exotic isolates compared to other Portuguese isolates. To confirm the results obtained it will be necessary to use more isolates from different geographic origins and associated with different nematode species.

PCR amplification of the ITS region with primers ITS1 and ITS4 resulted in a 614 bp band (Fig. 4a) for all isolates similar to the values obtained by Zare et al. (1999). Amplication of the ITS region using ITS4 and ITS5 primers revealed a single band with 655 bp (Fig. 4b) for all isolates, this band size is similar to the one obtained by Medina-Cannales et al. (2014).

The restriction pattern produced using the *Hinf*I enzyme on products from the ITS region consisted of three bands for all isolates (Fig. 7). As expected, the pattern obtained for *P. chlamydosporia* var. *chlamydosporia* isolates could be distinguish from the pattern obtained for var. *catenulata* isolate by a distinctive band fragment as demonstrated by Zare et al. (2001) and Atkins et al., 2003a. ITS-RFLP with *Eco*RI could not differentiate *P. chlamydosporia* varieties. Regarding the restriction pattern obtained with *Hae*III the lack of differentiation between varieties had already been reported (Morton et al., 2003a; Flores-Camacho et al., 2008).

ERIC-PCR can be used to identified intra-specific variation within the species but RFLP-ITS only can discriminate between the two varieties of *P. chlamydosporia* (Morton et al., 2003a).

Rapid *in vitro* tests in laboratory are necessary for carefully selection before introduction of a biological control agent in the soil (Abrantes et al., 2002). The variation between isolates was showed by three standard *in vitro* bioassays. The isolates showed significant variability regarding chlamydospore production, rhizosphere colonisation and nematode eggs parasitism. The inherent variation of *P. chlamydosporia* isolates has been widely described and this variation increases the need to carefully select isolates (Abrantes et al., 2002).

Furthermore, the results showed much variation between biological replicates. Results for most isolates were not consistent in the same bioassay. The largest differences were found in the chlamydospore production bioassay. For example, for isolates III, IV and VI, the results ranged from low production of chlamydospores (less than 100×10^5) to high production. Nonetheless, isolate Ia was the most consistent regarding chlamydospore production and no differences were detected between biological replicates. This bioassay was performed with agar plugs from 10-days old colonies of the fungus, which may explain variability between replicates. 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. For example, it was not possible to assess the ability to parasitise nematode eggs of isolates Ib and 392 as production of spores is very low in 1.7% CMA. Therefore, in order to assess differences among *P. chlamydosporia* isolates an equal amount of spores should be used as inoculum (Vieira dos Santos et al., 2013).

In general, the best chlamydospore producers in the three biological replicates were isolates II, III, IV, VI and the reference isolate Pc2. Isolates Ib and IX were consistently the isolates that produced the least number of chlamydospores (Figs. 8,9,10). Most of the isolates showed a percentage of chlamydospore germination higher than 80%. However, some variability was detected. For example, chlamydospore viability of isolate III in the third replicate was less than 80% (Fig. 10b). In the first and second replicates, isolate Pc10 had the highest percentage of germination, although it was the least producer (Figs. 8,9) . In the third replicate isolate Ia had the highest percentage of germination (Fig. 10b). Isolate Ib had a germination rate of less than 80% in the three replicates. The results obtained for reference isolates Pc2 and Pc10 were lower to the results reported by Vieira dos Santos et al. (2013). Additionally, lower results were reported for isolate Pc10 by Medina-Canales et al. (2014). These differences may be due to the type of substrate used. Mo et al. (2005) suggested that it is necessary to take into account the optimization of the medium for each isolate since it may be the reason for the differences in the production of chlamydospores. The source of carbon used in this study is barley, which is considered a good substrate for chlamydospore production (Mo et al., 2005). Most isolates produce many chlamydospores in this substrate but other isolates, such as isolates Ib and IX that produced fewer

chlamydospores, may have different carbon requirements. Regarding viability, some isolates have a lower germination percentage, which may be due to the incubation time (3 weeks). Some isolates may not withstand the incubation period and although they continue to produce chlamydospores the viability tends to decrease over time (Abrantes et al., 2002).

In the study of Medina-Canales et al. (2014), were the ability of Mexican isolates to produce chlamydospores was evaluated, the lowest number of chlamydospores produced was 1 x 10^6/g of substrate. Nontheless, this is considered an acceptable number for potential commercial scale production (Flores-Camacho et al., 2008; Franco-Navarro et al., 2009). Thus, all isolates evaluated in the present study produced an acceptable number of chlamydospores for potential commercial scale production.

To study the ability of isolates to colonise the rhizospere, tomato cv. Tiny Tim roots were used, since tomato is considered a good host for *P. chlamydosporia* (Manzanilla-López et al., 2013). Differences among isolates were detected. The best coloniser of tomato roots was the standard isolate Pc2 with 100% of colonisation. Some isolates, such as isolates Ib, IV, V and IX colonised less than 80% of tomato roots. Colonisation of the rhizosphere is a crucial factor to select isolates, as it is essential for the control of nematodes by *P. chlamydosporia* (Bourne et al., 1996). Only isolates that colonise more than 80% of plant roots should be selected (Abrantes et al., 2002). The efficacy of the fungus depends on fungal rhizosphere colonisation to facilitate colonisation of galls (Manzanilla-López et al., 2013). The remaining isolates colonised more than 85% and were considered good colonisers of tomato roots. The percentages of colonisation of standard isolates Pc2 and Pc10 were higher for tomato roots than for barley roots (Vieira dos Santos et al., 2013) as plant species differ in their ability to support the fungus (Bourne et al., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999).

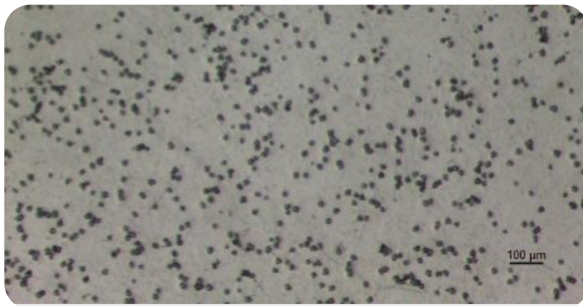
The virulence of the isolates against RKN and PCN eggs was low in all biological replicates and the number of parasitised eggs ranged between 20-60% and 30-55%, respectively. For reference isolates, the percentage of parasitised eggs was lower when compared to previous results (Vieira dos Santos et al., 2013). Many eggs hatched during the assay period for both nematode species, and the low percentage of parasitism can be related with low proportion of immature eggs in this assay. Studies have already confirmed that immature eggs are more susceptible to parasitism than eggs containing J2 (Irving and Kerry, 1986). Juveniles are not parasitised after they hatch (Kerry, 2000). As was expected, reference isolate Pc10 was a better parasite against RKN eggs than against PCN eggs. *Pochonia chlamydosporia* isolates have shown host preference for the host species from which the fungus was initially isolated (Mauchline et al., 2004). The best parasites of RKN eggs in the three biological replicates were isolates Ia and VIII and worst parasites were isolates IX and V. Regarding parasitism against *G. pallida* eggs, results were more inconsistent. In the first two replicates, the reference isolate Pc2 was

the best parasite followed by isolates III and V. However in the third replicate, isolate Pc2 was a poor parasite and isolate Ia was the best parasite. Root-knot and cyst nematodes differ in their susceptibility to specific isolates, but in RKN all species are equally susceptible to the same isolate of the fungus (Kerry, 2000).

Among the reference isolates, the Portuguese isolate Pc2 was the best in chlamydospore production, rhizosphere colonisation and egg parasitism of two nematode species. Isolate Pc2 was used in this study as a reference isolate associated with PCN. Isolate Pc10 was used as a reference isolate as it is an isolate that has been exploited for the development of integrated pest management strategies against RKN (Morton et al., 2003a). However, this isolate was a poor chlamydospore producer but a good rhizosphere coloniser. As already mentioned, the low efficiency of the isolate in this study can be explained by the type of substrate used in the production of chlamydospores. Although, it has been revealed that continuous culture in agar did not affect the ability to colonise the rhizosphere or parasitise nematode eggs, the ability to produce chlamydospores, the viability of those chlamydospores and the *in vitro* enzymatic activity can be affected after 60 weeks of subculturing (Esteves, 2007). Additionally, small changes in temperature, aeration, and quantity of available water can influence and affect fermentation of the inoculum (Nagel, 2002). The reference isolate of *P. chlamydosporia* var. *catenulata*, isolate 392 is being used as a biological control agent developed under the name KlamiC® in Cuba with a high rate of success in controlling RKN population densities in vegetable crops (Kerry, 1987; Hérnandez and Hidalgo-Diaz, 2008). In this study, 392 was a good producer of chamydospores, however this isolate showed a low percentage of germinated chlamydospores. In addition, this isolate produces very low number of spores in 1.7% CMA, which impaired the evaluation of its ability to colonise the rhizosphere and to colonise nematode eggs.

The use of *in vitro* bioassays facilitates the selection process of isolates with potential to be developed as biological control agents. The isolates must be able to colonise the rhizosphere (>80%), to produce chlamydospores *in vitro* as the preferred source of inoculum and to parasitise nematode eggs (Abrantes et al., 2002). Five isolates associated with *Meloidogyne* spp. eggs (Ia, Ib, PII, IV and V), three (VI, VIII and IX) associated with tomato infected roots, one (Pc2) from PCN eggs and two isolates (Pc10 and 392) identified as belonging to two different varieties of *P. chlamydosporia*, were evaluated, by *in vitro* assays, for their ability to produce chlamydospores, colonise the rhizosphere of tomato and parasitise *M. incognita* and *G. pallida* eggs. All isolates revealed marked differences in their performance. Isolate Ia colonised the rhizosphere extensively (89%) whereas IX was a poor coloniser (less than 50%). Isolates II and Pc2 produced the higher numbers of chlamydospores in solid medium (more than $20x10^5$ chlamydospores/g). The proportion of RKN eggs parasitised by the fungus was low (less than 60%) for all isolates. Isolates Ia and VIII were the best parasites against RKN eggs (more than 50%) and isolates V and Pc2 parasitised more than 50% of PCN eggs.

Isolates Ia and VIII should be considered as good candidates to be developed as potential biological control agents. Although, they were not the best chlamydospore producers, the results were consistent between biological replicates. In addition, they presented a high rate of germinated chlamydospores, were good colonisers of tomato roots and revealed to be good parasites of eggs of both species of nematodes.



5. Conclusion and 6. Future perspectives

5. Conclusion

The potential of Portuguese *P. chlamydosporia* isolates associated *with Meloidogyne* spp. as biological control agents was evaluated using molecular techniques and standard *in vitro* bioassays to assess their ability to produce chlamydospores, colonise the rhizosphere and parasitise nematode eggs.

Results of the molecular analyses revealed that β-tubulin, ERIC-PCR and ITS-RFLP can be used to distinguish different *P. chlamydosporia* varieties. All Portuguese isolates were identified as *P. chlamydosporia* var. *chlamydosporia*. ERIC-PCR can be used to identify intra-specific variation among isolates.

Considering the results obtained in the *in vitro* bioassays, isolates Ia and VIII showed the greatest potential as biological control agents since they revealed desirable traits such as high rhizosphere competence and chlamydospore production, thus having a greater potential to be exploited as biological control agents. However, isolates with a good performance in *in vitro* tests may not be effective in the field due to the influence of biotic and abiotic factors (Abrantes et al., 2002). Further studies, such as pot and fields experiments, need to be performed.

Molecular, biochemical and biological analyses are determinant in the screening of potential biological control agents, particularly in the case of *P. chlamydosporia*, due to the high variability among isolates.

6. Future perspectives

The work described in this study intends to contribute for the development of non-chemical, sustainable and environmentally friendly management strategies of reducing plant-parasitic nematode population densities.

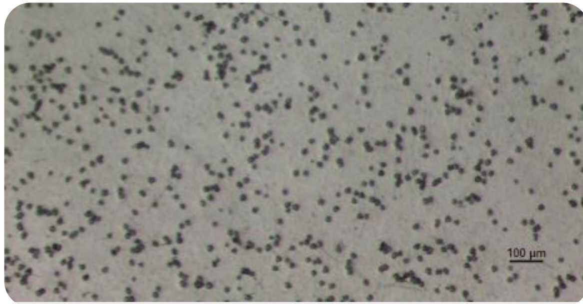
To evaluate genetic diversity of the isolates will be performed other molecular studies using primers designed to amplify intergenic spacers (IGS), and other restriction enzymes, such as *Bam*HI and *Sac*I, will be tested on products from the ITS region (Arora et al., 1996; Peteira-Delgado and Hildalgo-Diaz, 2009).

To a complete and accurate understanding of the potential as biological control agents of the selected isolates, further studies should include pot and field experiments in the presence of the nematode. It has been showed that isolates that perform well in the laboratory may not be effective in soil (Abrantes et al., 2002).

The effect of abiotic factors such as temperature and humidity should also be evaluated. Although, the optimum temperature for *P. chlamydosporia* growth is between 25-30ºC, isolates can differ in the optimum temperature (Bourne and Kerry, 2000; Olivares-Barnabeu and Lopez-Llorca, 2002). The tolerance to temperature

variations can compromise the efficacy of isolates against the target nematode and their establishment in the soil (Magan, 2001). This could be assessed in simple bioassays and in pot experiments in controlled conditions. *In vitro* bioassays could also be performed to study the enzymatic activity of the isolates. Specific enzymes produced by fungus play a key role in the infection process of nematode eggs (Segers et al., 1994; Tikhonov et al., 2002).

As an egg parasite, *P. chlamydosporia* cannot prevent the initial infestation by the infective juveniles, thus the integration of the fungus with other control measures should be considered. Pot assays in controlled conditions could provide important information on the efficacy and behaviour of selected isolates when combined with different control strategies against RKN. Crop rotation in a successful combination with the application of *P. chlamydosporia* could lead to sustainable RKN management, since this traditional control measure can prevent, in some extent, the build-up of large nematode population densities (Abrantes et al., 2002). Other control measure that can be combined with the fungus is to target plant defence mechanisms. The first contact of the parasite with the plant activates the plat natural defences. These defences can be also 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. The application of the fungus (in soil) combined with the use of plant defence activators, through foliar sprays or another method of application, can reduce the susceptibility of crop plants and consequently PPN invasion, development and reproduction. This measure could be a key strategy to increase effectiveness of *P. chlamydosporia* as a biological control agent (Vieira dos Santos et al., 2014a).



7. Bibliography

7. Bibliography

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.-C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E.,Van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.N., Schiex, T., Smant, G., Weissenbach, J. and Wincke, P. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26, 909–915.

Abrantes, I., Santos, S., Bourne, J., Ciancio, A., Lopez-llorca, L., Kerry, B., Mota, M., Tzortzakakis, E. and Verdejo-Lucas, S. (2002). A manual for research on *Verticillium chlamydosporium*, a potencial biological control agent of root knot nematodes (B.R. Kerry and J.M. Bourne, eds.). International Organization for Biological and Integrated Control for Noxious Animals and Plants/West Paleartic Regional Section (IOBC/WPRS), Gent, Belgium, 84 pp.

Abrantes, I., Vieira dos Santos, M.C., Clara, M.I., Fernandes, J.E., Marques, M.L. (2007). Principais doenças e pragas do tomateiro e meios de luta. Instituto do Ambiente e Vida, Coimbra, 176 pp.

Anastasiadis, I.A., Giannakou, I.O., Prophetou-Athanasiadou, D.A. and Gowen, S.R. (2008). The combined effect of the application of a biological agent *Paecilomyces lilacinus*, with various practices for the control of Root-knot nematodes. Crop protection 27, 352-361.

Arora, D.K., Hirsch, P.R. and Kerry, B.R. (1996). PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycological Research 7, 801-809.

Atkins, S.D., Hidalgo-Diaz, L., Clark, I.M., Morton, C.O., Montes de Oca, N., Gray, P.A. and Kerry, B.R. (2003a). Approaches for monitoring the release of *Pochonia chlamydosporia* var. *catenulata*, a biocontrol agent of root-knot nematodes. Mycological Research 107, 207–212.

Atkins, S.D., Hidalgo-Diaz, L., Kaslisz, H., Mauchline, T.H., Hirsch, P.R. and Kerry, B.R. (2003b). Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp.) in organic vegetable production. Pest Managment Science 59, 183–189.

Bailey, D.J., Biran, G.L., Kerry, B.R. and Gilligan, C.A. (2008). Pathozone dynamics of *Meloidogyne incognita* in the rhizosphere of tomato plants in the presence and absence of the nematophagous fungus, *Pochonia chlamydosporia*. Plant Pathology 57, 354-362.

Bent, E., Loffredo, A., McKenry, M.V., Becker, J.O. and Borneman, J. (2008). Detection and investigation of soil biological activity against *Meloidogyne incognita*. Journal of Nematology 40, 109–118.

Bontempo, A.F., Lopes, E.A., Fernandes, R.H., de Freitas and L.G. and Dallemole-Giaretta, R. (2017). Dose-response effect of *Pochonia chlamydosporia* against *Meloidogyne incognita* on carrot under field conditions. Revista Caatinga 30, 258-262.

Bourne, J. and Kerry, B.R. (1999). Effect of the host plant on the efficacy of *Verticillium chlamydosporium* as a biological control agent of root-knot nematodes at different nematode densities and fungal application rates. Soil Biology and Biochemistry 31, 75-84.

Bourne, J. and Kerry, B.R. (2000). Observation on the survival and competitive ability of the nematophagous fungus *Verticillium chlamydosporium* in soil. International Journal of Nematology 10, 9-18.

Bourne, J.M., Kerry, B.R. and Leij, F.A.A.M. De. (1994). Methods for the study of *Verticillium chlamydosporium* in the rhizosphere. Journal of Nematology 26, 587–591.

Bourne, J.M., Kerry, B.R. and Leij, de F.A.A.M. De (1996). The importance of the host plant on the interaction between rootknot nematodes (*Meloidogyne* spp.) and the nematophagous fungus, *Verticillium chlamydosporium* Goddard. Biocontrol Science and Technology 6, 539-548.

Briar, S.S., Wichman, D. and Reddy, G.V.P. (2016). Plant-Parasitic Nematode Problems in Organic Agriculture. In: Organic Farming for Sustainable Agriculture (D. Nandwani, ed.). Springer, US, pp. 107-122.

Brodie, B.B. and Mai, W.F. (1989). Control of the golden nematode in the United States. Annual Review of Phytopathology 27, 443-461.

Burton-Freeman, B. and Reimers, K. (2011). Tomato consumption and health: emerging benefits. American Journal of Lifestyle Medicine 5, 182-191.

Caillaud, M.C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., Abad, P., Rosso, M.N. and Favery, B. (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. Journal of Plant Physiology 165, 104–113.

Calderón-Urrea, A., Vanholme, B., Vangestel, S., Kane, S.M., Bahaji, A., Pha, K., Garcia, M., Snider, A. and Gheysen, G. (2016). Early development of the root-knot nematode *Meloidogyne incognita*. BMC Developmental Biology 16, 1-14.

Capote, N., Pastrana, A.M., Aguado, A. and Sánchez-Torres, P. (2012). Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In: Plant Pathology (C.J.Cumagun, ed.). Plant Pathology, In Tech, pp. 151–202.

Castagnone-Sereno, P. (2006). Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. Heredity 96, 282–289.

Cawoy, H., Bettiol, W., Fickers, P. and Ongena, M. (2011). Bacillus based biological control of plant diseases. In: Pesticides in the modern world—pesticides use and management (M. Stoytcheva, ed.). InTech, Rijeka, pp. 273–302.

Chen, P. and Tsay, T.T. (2006). Effect of crop rotation on *Meloidogyne* spp. and *Pratylenchus* spp. populations in strawberry fields in Taiwan. Journal of Nematology 38, 339-344.

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

Costa, J.M. and Heuvelink, E. (2005). Introduction: the tomato crop and industry. In: Tomatoes (E. Heuvelink, ed.). CABI Publishing, Wallingford, UK, pp 1–19.

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

Dias-Arieira, C.R., Mattei, D., Puerari, H.H. and Ribeiro, R.C.F. (2015). Use of organic amendments in the management of root-knot nematode in lettuce. Horticultura Brasileira 33, 488-492.

Dropkin, V.H. (1972). Pathology of *Meloidogyne*-galling giant cell formation, effects on host physiology. OEPP/EPPO Bulletin 6, 23-32.

Duponnois, R., Mateille, T. and Gueye, M. (1995). Biological characteristics and effects of two strains of *Arthrobotrys oligospora* from Senegal on *Meloidogyne* species parasitizing tomato plants. Biocontrol Science and Technology, 5, 517–526.

Egel D., Foster, R., Maynard, E., Weinzierl, R., Babadoost, M., O'Malley, P., Nair, A., Cloyd, R., Rivard, C., Kennelly, M., Hutchison, B., Piñero, J., Welty, C., Doohan, D., and Miller, S. (2014). Midwest Vegetable Production Guide for Commercial Growers. West Lafayette, Purdue Extension, pp. 54-55.

Ehwaeti, M.E., Fargette, M., Phillips, M.S. and Trudgill, D.L. (1999). Host status differences and their relevance to damage by *Meloidogyne incognita*. Nematology 1, 421–432.

EPPO - European and Mediterranean Plant Protection Organization (2013). PM 7/40 (3) *Globodera rostochiensis* and *Globodera pallida*. Bulletin OEPP/EPPO Bulletin 43, 119–138.

Esteves I. (2007) Factor affecting the performance of *Pochonia chlamydosporia* as a biological control agent of nematodes. PhD Thesis. Cranfield University, UK, pp. 270.

Eurostat (2017). Agricultural production-crops. Table 2: Production of fruit and vegetables, 2015. Accessed on 26 March 2017, in:

http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-_crops#Fruit

Fitzpatrick, J.L. (2013). Global food security – the impact of veterinary parasites and parasitologists. Veterinary Parasitology 195, 233-248.

Flores-Camacho, R., Atkins, S.D., Manzanilla-López, R.H., Cid del Prado-Vera, I. and Martínez-Garza, A. (2008). Caracterización de aislamientos Mexicanos de *Pochonia chlamydosporia var. chlamydosporia* (Goddard) Gams y Zare para el control biológico de *Nacobbus aberrans* (Thorne, 1935) Thorne y Allen, 1944. Revista Mexicana de Fitopatologia 26, 93-104

FAO - Food and Agriculture Organization of the United Nations (2017). FAOSTAT Database- Production quantities of Tomatoes by country. Accessed on 30 March 2017, in: http://www.fao.org/faostat/en/#data/QC/visualize

Franco-Navarro, F., Vilchis-Martínez, K. and Miranda-Damián, J. (2009). New records of *Pochonia chlamydosporia* from Mexico: isolation, root colonization and parasitism of *Nacobbus aberrans* eggs. Nematropica. 39:133-142.

García, L., Bulnes, C., Melchor, G., Veja, E., Miranda, I., Montes, O.N., Hidalgo, L. and Marrero, E., (2004). Safety of *Pochonia chlamydosporia* var. *catenulata* in acute oral and dermal toxicity/pathogenicity evaluations in rats and rabbits. Veterinary and Human Toxicology 46, 248–250.

García, L., Melchor, G., Arévalos, J. and Hidalgo, L. (2008a). Evaluación de la fitotoxicidade de la cepa IMI SD 187 *Pochonia chlamydosporia* var. *catenulata* sobre *Zea mays* L. y *Phaseolus vulgaris* L. Revista de Protección Vegetal 23, 38-42.

García, L., Melchor, G., Domínguez, Y., Rodríguez, H., Pino, O. and Hidalgo, L. (2008b). Ecotoxicological evaluation of *Pochonia chlamydosporia* var. *catenulata* in terrestrial invertebrates. Revista de Toxicologia en Línea, 14, 39-50.

Gerbore, J., Benhamou, N., Vallance, J., Le Floch, G., Grizard, D., Regnault-Roger, C. and Rey, P. (2014). Biological control of plant pathogens: advantages and limitations seen through the case study of *Pythium oligandrum*. Environmental Science and Pollution Research 21, 4847–4860.

Godfray, H.C., Beddington, J.R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C. (2010). Food Security: The Challenge of Feeding 9 Billion People. Science 327, 812–818.

Haegeman, A., Mantelin, S., Jones, J.T. and Gheysen, G. (2012). Functional roles of effectors of plant-parasitic nematodes. Gene 492, 19–31.

Handoo, Z. A. (1998). Plant-parasitic nematodes. Accessed on 23 March 2017, in USDA ARS Publication: http://www.ars.usda.gov/Services/docs.htm?docid=9628.

Hashem, M. and Abo-Elyousr, K.A. (2011). Management of the root-knot nematode *Meloidogyne incognita* on tomato with combinations of different biocontrol organisms. Crop Protection 30, 285–292.

Hernandez, M.A. and Hidalgo-Diaz, L. (2008). KlamiC®: agricultural bionematicida from the fungus *Pochonia chlamydosporia* var. *catenulata*. Revista de Protección Vegetal 23, 131-134.

Hill, N.S. (1988). Cultural practices for the management of plant parasitic nematodes. Ornamentals Northwest Archives 12, 7-9.

Hirsch, P.R., Mauchline, T.H., Mendum, T.A. and Kerry, B.R. (2000). Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. Mycological Research 104, 435–439.

Hirsch, P.R., Atkins, S.D., Mauchline, T.H., Morton, C.O., Davies, K.G. and Kerry, B.R. (2001). Methods for studying the nematophagous fungus *Verticillium chlamydosporium* in the root environmental. Plant Soil 232, 21-30.

Hunt, D.J. and Handoo, Z.A., (2009). Taxonomy, identification and principal species. In: Root-knot nematodes (R.N. Perry, M. Moens and J.L. Starr, eds.). CABI International, Cambridge, US, pp. 55–97.

Irving, F. and Kerry, B.R. (1986). Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. II. Factors affecting parasitism of cyst nematode eggs. Nematologica 32, 474-485.

Jaffee, B. A., Ferris, H. Stapleton, J.J., Norton, M.V.K. and Muldoon, A.E. (1994). Parasitism of nematodes by the fungus *Hirsutella rhossiliensis* as affected by certain organic amendments. Journal of Nematology 26, 152– 161

Jang, J.Y., Choi, Y.H., Shin, T.S., Kim, T.H., Shin, K.S., Park, H.W., Kim, Y.H., Kim, H., Choi, G.J., Jang, K.S., Cha, B., Kim, I.S., Myung, E.J. and Kim, J.C. (2016). Biological Control of *Meloidogyne incognita* by *Aspergillus nige*r F22 Producing Oxalic Acid. PLoS ONE 11, 1-15.

Jones, B.J. (2007). Tomato plant culture: In the field, greenhouse, and home garden. Second Edition. CRC Press, Boca Raton, Florida, pp. 1-48.

Jones, J.T., Haegeman, A., Danchin, E. G., Gaur, H. S., Helder, J., Jones, M.G., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J.E., Wesemael, W.M. and Perry, R.N. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14, 946–961.

Karssen, G., Wesemael, W. and Moens, M. (2006). Root-knot nematodes. In: Plant Nematology (R. N. Perry, M. Moens, eds.). CABI international, Cambridge, US, pp. 59-90.

Karuri, H.W., Olago, D., Neilson, R., Mararo. E. and Villinger, J. (2017). A survey of root knot nematodes and resistance to *Meloidogyne incognita* in sweet potato varieties from Kenyan fields. Crop Protection 92, 114-121.

Keating, B.A., Carberry, P.S., Bindraban, P.S., Asseng, S., Meinke, H. and Dixon, J. (2010). Eco-efficient Agriculture: Concepts, Challenges, and Opportunities. Crop Science Society of America 50, 109-119.

Kerry, B.R. (1987). Biological control. In: Principles and practise of nematode control in crops (R.H. Brown and B.R. Kerry, eds.). Academic Press, Sidney, Australia, pp. 233-257.

Kerry, B.R. (1990). An assessment of progress toward microbial control of plant-parasitic nematodes. Journal of Nematology 22, 621–623.

Kerry, B.R. (1997). Biological control of nematodes: prospects and opportunities. In: Plant nematode problems and their control in the near east region (M.A. Maqbool, B. Kerry). FAO Plant Production and Protection Paper-144. Accessed on 3 April 2017, in: http://www.fao.org/docrep/v9978e/v9978e0b.htm

Kerry, B.R. (2000). Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. Annual Review of Phytopathology 38, 423-441.

Kerry, B.R. (2001). Exploitation of the nematophagous fungal *Verticillium chlamydosporium* Goddard for the biological control of root-knot nematodes (*Meloidogyne* spp.). In: Fungi as Biocontrol Agents: Progress Problems and Potential. (T.M. Butt, C. Jackson and N. Magan, eds.). CABI International, Wallingford, UK, pp. 155-168.

Kerry, B.R. and Bourne, J.M. (1996). The importance of rhizosphere interactions in the biological control of plant parasitic nematodes – a case of study using *Verticillium chlamydosporium*. Pesticide Science 47, 69–75.

Kerry, B.R. and Crump, D.H. (1977). Observations on fungal parasites of females and eggs of the cereal cyst nematodes. Nematologica 23, 193-201.

Kerry, B.R., Crump, D.H. and Mullen, L.A. (1982). Studies of the cereal cyst nematode, *Heterodera avenae* under continuous cereals, 1975-1978. II. Fungal parasitism of nematode female and eggs. Annals of Applied Biology 100, 489-499.

Kerry, B.R. and Hirsch, P.R. (2011). Ecology of *Pochonia chlamydosporia* in the rhizosphere at the population, whole organism and molecular scales. Pp. 171–182 In: Biological control of plant-parasitic nematodes: building coherence between microbial ecology and molecular mechanisms (K. Davies and Y. Spiegel, eds.). Springer, London, pp. 171-182.

Khalil, M.S. (2013). Alternative approaches to manage plant parasitic nematodes. Journal of Plant Pathology & Microbiology 4, 1-4.

Kiewnick, S. and Sikora, R.A. (2006). Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. Biological control 38, 179-187.

Kyndt, T., Fernandez, D. and Gheysen, G. (2014). Plant-parasitic nematode infections in rice: molecular and cellular insights. Annual Review of Phytopathology 52, 135–53.

Kyndt, T., Vieira, P., Gheysen G. and de Almeida-Engler, J. (2013). Nematode feeding sites: unique organs in plant roots. Planta 238, 807–818.

Lambert, K. and Bekal, S. (2002). Introduction to Plant-Parasitic Nematodes. The Plant Health instructor. doi: 10.1094/PHI-I-2002-1218-01.

Leij, F.A.A.M. De, and Kerry, B.R. (1991). The nematophagous fungus, *Verticillium chlamydosporium* Goddard, as a potential biological control agent for *Meloidogyne arenaria* (Neal) Chitwood. Revue de Nématologie 14, 157-164.

Leij, F.A.A.M. De, Kerry, B. R. and Dennehy, J.A. (I993). *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M. hapla* in pot and micro-plot tests. Nematologica, 39, 115-126.

Lilley, C.J., Atkinson, H.J. and Urwin, P.E. (2005). Molecular aspects of cyst nematodes. Molecular Plant Pathology 6, 577–588.

Lopes, E.A., Ferraz, S., Ferreira, P.A., Freitas, L.G. and Giaretta, R.D. (2011). Soil amendment with chopped or ground dry leaves of six species of plants for the control of *Meloidogyne javanica* in tomato under greenhouse conditions. Ciência Rural 41, 935-938.

Magan, N. (2001). Physiological approaches to improving the ecological fitness of fungal biocontrol agents in fungi as biocontrol agents. In: Fungi as biocontrol agents – progress, problems and potential (T. Butt, C. Jackson and N. Magan, eds.). CABI Publishing, London, UK, pp. 239-251.

Mankau, R. (1980). Biocontrol: fungi as nematode control agents. Journal of Nematology 12, 244-252.

Manzanilla-López, R.H., Esteves, I., Finetti-Sialer, M.M., Hirsch, P.R., Ward, E., Devonshire, J. and Hidalgo-Díaz, L. (2013). *Pochonia chlamydosporia*: Advances and challenges to improve its performance as a biological control agent of sedentary endo-parasitic nematodes. Journal of Nematology 45, 1-7.

Mauchline T.H., Kerry B.R. and Hirsch P.R. (2002). Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. Applied and Environmental Microbiology 68, 1846-1853.

Mauchline, T.H., Kerry, B.R. and Hirsch, P.R. (2004). The biocontrol fungus *Pochonia chlamydosporia* shows nematode host preferences at the infraspecific level. Mycological Research 108, 161-169.

McSorley, R., (2011). Overview of organic amendments for management of plant-parasitic nematodes, with case studies from Florida. Journal of Nematology 43, 69-81.

Medina-Canales, M.G., Rodríguez-Tovar, A.V., Manzanilla-López, M.H., Zúñiga, G. and Tovar-Soto, A. (2014). Identification and molecular characterization of new Mexican isolates of *Pochonia chlamydosporia* for the management of *Meloidogyne* spp. Biocontrol Science Technology 24, 1–21.

Mitkowski, N. A. and G. S. Abawi. (2003). Root-knot nematodes. The Plant Health Instructor. DOI:10.1094/PHII-2003-0917-01.

Mo, M., Xu, C. and Zhang, K. (2005). Effects of carbon and nitrogen sources, carbon-to nitrogen ratio, and initial pH on the growth of nematophagous fungus *Pochonia chlamydosporia* in liquid culture. Mycopathologia, 159, 381–387.

Moens, M., Perry, R.N. and Starr, J.L., (2009). *Meloidogyne* species e a diverse group of novel and important plant parasites. In: Root-knot Nematodes (R.N. Perry, M. Moens, M., J.L. Starr, eds.). CABI International, Cambrige, US, pp. 1-17.

Moosavi, M.R. and Zare, R. (2012). Fungi as biological control agents of plant-parasitic nematodes. In: Plant defence: biological control, progress in biological control (J.M. Mérillon and K.G. Ramawat, eds.). Springer, New York, US, pp. 67–107.

Morton, C.O., Mauchline, T.H., Kerry, B.R. and Hirsch, P.R. (2003a). PCR-based DNA fingerprinting indicates host-related genetic variation in the nematophagous fungus *Pochonia chlamydosporia*. Mycological Research 107, 198-205.

Morton, C.O., Hirsch, P.R., Peberdy, J.P. and Kerry, B.R. (2003b). Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. Mycological Research 107, 38-46.

Moshe, A., Pfannstiel, J., Brotman, Y., Kolot, M., Sobol, l., Czosneck, H. and Gorovits, R. (2012). Stress responses to Tomato yellow leaf curl virus (TYLCV) infection of resistant and susceptible tomato plants are different. Metabolomics 1, 1-13.

Nagel, F.J.I. (2002). Process control of solid-state fermentation: simultaneous control of temperature and moisture content. PhD Thesis. Wageningen University, The Netherlands, pp. 191

Nagesh, M., Hussaini, S.S., Ramanujam, B. and Rangeswaran, R., (2007). Molecular identification, characterization, variability and infectivity of Indian isolates of the nematophagous fungus *Pochonia chlamydosporia*. Nematologia Mediterranea 35, 47-56.

Nicol, J.M. (2002). Important nematode pests. In: Bread wheat improvement and production (B.C., Curtis, S. Rajaram and M. Gomez, eds.). FAO Plant Production and Protection Series, p. 567.

Nicol, J.M., Turner, S.J., Coyne, D.L., den Nijs, L., Hockland, S. and Maafi, Z.T. (2011). Current nematode threats to world agriculture. In: Genomics and Molecular Genetics of Plant–Nematode Interactions (J.T. Jones, G. Gheysen, and C. Fenoll, eds.). Springer, Heidelberg, Germany, pp. 21–44.

Nordbring-Hertz, B., Jansson, H.-B and Tunlid, A. (2006). Nematophagous fungi. In: Encyclopedia of Life Sciences (J. Willey, ed.). Macmillan Publishers, UK, pp. 1-11.

Nunes, M.C.N. (2008). Solanaceous and other fruit vegetables. In: Color atlas of postharvest quality of fruits and vegetables (M.C.N. Nunes, ed.). Wiley-Blackwell Publishing, Ames, Iowa, US, pp. 239-252.

OMAIAA - Observatório dos mercados agrícolas e das importações agroalimentares (2011). A comercialização do tomate em Portugal. Accessed on 18 March 2017, in: http://www.observatorioagricola.pt/item.asp?id_item=141.

Oka, Y., Koltai, H., Bar-Eyal, M., Mor, M., Sharon, E., Chet, I. and Spiegel, Y. (2000). New strategies for the control of plant-parasitic nematodes. Pest Management Science 56, 983–988.

Olivares-Bernabeu, C.M. and Lopez-Llorca, L.V. (2002). Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. Revista Iberoamericana de Micología 19, 104-110.

Onuorah, S. and Orji, M.U. (2015). Fungi associated with the spoilage of post-harvest tomato fruits sold in major markets in Awka, Nigeria. Universal Journal of Microbiology Research 3, 11-16.

Prakash, A., Rao, J., Berliner, J., Pokhare, S.S., Adak, T. and Saikia, K., (2014). Botanical pesticides for the management of plant nematode and mite pests. In: Advances in Plant Biopesticides (D. Singh, ed.). Springer, Cuttack, India, pp. 89–118.

Peteira-Delgado, B. and Hidalgo-diaz, L. (2009). VCP1 protease detection in *Pochonia chlamydosporia* var. *catenulata* strain IMI SD 187. Revista de Protección Vegetal 24, 166-172.

Radwan, M.A., Farrag, S.A.A., Abu-Elamayem, M.M. and Ahmed, N.S. (2012). Biological control of the root-knot nematode, *Meloidogyne incognita* on tomato using bioproducts of microbial origin. Applied Soil Ecology 56, 58-62.

Raiola, A., Rigano, M.M., Calafiore, R., Frusciante, L. and Barone, A. (2014). Enhancing the health-promoting effects of tomato fruit for biofortified food. Mediators of Inflammation, pp 1-16.

Ravichandra, N.G. (2008). Nematode management. In: Plant Nematology (N.G. Ravichandra, ed.). I.K. International Publishing, New Delhi, India pp. 504-590.

Riggs, R.D. and Schuster, R.P. (1998). Management. In: The Cyst nematodes (S. B. Sharma), Springer, Rotterdam, Netherlands, pp. 388-416

Savary, S., Ficke, A., Aubertot, J.-N. and Hollier, C. (2012). Crop losses due to diseases and their implications for global food production losses and food security. Food Security 4, 519–537.

Schwarz, D., Thompson, A.J. and Kläring, H.-P. (2014). Guidelines to use tomato in experiments with a controlled environment. Frontiers in Plant Science 5, 625.

Segers, R., Butt, T.M., Kerry, B.R. and Peberdy, J.F. (1994). The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins in situ. Microbiology 140, 2715- 2723.

Sellito, V.M., Curto, G., Dallavalle, E., Ciancio, A., Mariantonietta, C., Pietrantonio, L., Bireescu, G., Stoleru, V. and Storario, M. (2016). Effect of *Pochonia chlamydosporia*-based formulates on the regulation of root-knot nematodes and plant growth response. Frontiers in Life Science 9, 117-181.

Seo, Y. and Kim, Y.H. (2014). Control of *Meloidogyne incognita* using mixture of organic acids. Plant Pathology Journal 30, 450–455.

Sorribas, F.J., Ornat, C., Galeano, M., Verdejo-Lucas, S. (2003). Evaluation of a Native and Introduced Isolate of *Pochonia chlamydosporia* against *Meloidogyne javanica*. Biocontrol Science Technology 13, 707–714.

Tian, B., Yang, J. and Zhang, K. (2007). Bacteria Used in the Biological Control of Plant-Parasitic Nematodes: Populations, Mechanisms of Action, and Future Prospects. FEMS Microbiology Ecology 61, 197–213.

Tikhonov, V.E., López-Llorca, L.V., Salinas, J. and Jansson, H.-B. (2002). Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fungal Genetics and Biology 35, 67-78.

Timper, P. (2014). Conserving and enhancing biological control of nematodes. Journal of Nematology 46, 75–89.

Trainer, M.-S., Pognant-Gros, J., Quiroz, R.D.L.C., González, C.N.A., Mateille, T. and Roussos, S. (2014). Commercial biological control agents targeted against plant-parasitic root-knot nematodes. Brazilian archives of Biology and Technology 57, 831-841.

Trudgill, D.L. and Blok, V.C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic rootpathogens. Annual Reviews Phytopathology 39, 53–77.

Turner, S.J., Martin, T.J.G., McAleavey, P.B.W., and Fleming, C.C. (2006). The management of potato cyst nematodes using resistant Solanaceae potato clones as trap crops. Annals of Applied Biology 149, 271–280.

Turner, S.J. and Rowe, J.A. (2006). Cyst nematodes. In: Plant Nematology (R.N. Perry and M. Moens, eds.) CABI Publishing, Wallingford, UK, pp. 91-122.

Tzortzakakis, E. A. 2009. Variation in colonization of field soil and associated roots by the nematophagous fungus *Pochonia chlamydosporia*. Nematologia Mediterranea 37:181-186.

Tzortzakakis, E.A. and Petsas, S.E. (2003). Investigation of alternatives to methyl bromide for management of *Meloidogyne javanica* on greenhouse grown tomato. Pest Management Science 59, 1311-1320.

Verdejo-Lucas, S., Ornat, C., Sorribas, FJ. and Stchigel, A. (2002). Species of root-knot nematodes and fungal egg parasites recovered from vegetables in Almería and Barcelona, Spain. Journal of Nematology 34, 405–8.

Verdejo-lucas, S., Sorribas, F.S., Ornat, C. and Galeano, M. (2003). Evaluating *Pochonia chlamydosporia* in a double-cropping system of lettuce and tomato in plastic houses infested with *Meloidogyne javanica*. Plant Pathology 52, 521-528.

Viaene, N., Coyle, D.L. and Kerry, B.R. (2006). Biological and cultural management. In: Plant Nematology (R.N. Perry and M. Moens, eds.). CABI Publishing, Wallingford, UK, pp. 346-369.

Vieira dos Santos, M.C., Esteves, I. and Abrantes, I. (2012). *In vitro* water stress bioassays with the nematophagous fungus *Pochonia chlamydosporia*: Effects on growth and parasitism. Biological Control 63, 310-319.

Vieira dos Santos, M.C., Esteves, I., Kerry, B. and Abrantes, I. (2013). Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolated from potato cyst and root-knot nematodes. Nematology 15, 493-504.

Vieira dos Santos, M.C., Curtis, R.H.C. and Abrantes, I. (2014a). The combined use of *Pochonia chlamydosporia* with plant defence activators - a potential sustainable control strategy for *Meloidogyne chitwoodi*. Phytopathologia Mediterranea 53, 66-74.

Vieira dos Santos, M.C., Esteves, I., Kerry, B, Abrantes, I. (2014b). Interactions between *Pochonia chlamydosporia* and *Meloidogyne chitwoodi* in a crop rotation scheme. Nematropica 44, 37-46.

Wani, A.H. and Bhat, M.Y. (2012). Control of root-knot nematode, *Meloidogyne incognita* by urea coated with Nimin or other natural oils on mung, *Vigna radiata* (L.) R. Wilczek. Journal of Biopesticides 5, 255–258.

White, T.J., Bruns, T., Lee, S. and Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods

and Applications (M.A., Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds.). Academic Press, Inc., New York, US, pp. 315-322.

Widmer, T.L., Mitkowski, N.A. and Abawi, G.S. (2002). Soil organic matter and management of plant-parasitic nematodes. Journal of Nematology 34, 289–295.

Yang, J., Loffredo, A., Borneman, J. and Becker, J.O. (2012). Biocontrol efficacy among strains of *Pochonia chlamydosporia* obtained from a root-knot nematode suppressive soil. Journal of Nematology 44, 67–71.

Zakaria, H.M., Kassab, A.S., Shamseldean, M.M., Oraby, M.M. and El-Mourshedy, M.M.F. (2013). Controlling the root-knot nematode, *Meloidogyne incognita* in cucumber plants using some soil bioagents and some amendments under simulated field conditions. Annals of Agricultural Sciences 58, 77–82.

Zare, R., Gams, W. and Evans, H.C. (2001). A revision of *Verticillium sect. Prostrata*. V. The genus *Pochonia*, with notes on *Rotiferophtora*. Nova Hedwigia 73, 51-86.

Zare, R., Kouvelis, V.N., Typas, M.A. and Bridge, P.D., (1999). Presence of a 20 bp insertion/deletion in the ITS1 region of *Verticillium lecanii*. Letters in Applied Microbiology 28, 258-262.

Zavala-González, E.A., Escudero, N., Lopez-Moya, F., Aranda-Martinez, A., Exposito, A., Ricano-Rodriguez, J., Naranjo-Ortiz, M.A., Ramirez-Lepe, M. and Lopez-Llorca, L.V. (2015). Some isolates of the nematophagous fungus *Pochonia chlamydosporia* promote root growth and reduce flowering time in tomato. Annals of Applied Biology 166, 472–483.