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Pochonia chlamydosporia: Advances and Challenges to Improve Its Performance as a Biological Control Agent of Sedentary Endo-parasitic Nematodes

Rosa H. Manzanilla-López,[1] Ivania Esteves,[2] Mariella M. Finetti-Sialer,[3] Penny R. Hirsch,[1] Elaine Ward,[1] Jean Devonshire,[4] Leopoldo Hidalgo-Díaz[5]

Abstract: The nematophagous fungus *Pochonia chlamydosporia* var. *chlamydosporia* is one of the most studied biological control agents against plant (semi-) endo-parasitic nematodes of the genera *Globodera*, *Heterodera*, *Meloidogyne*, *Nacobbus* and, more recently, *Rotylenchulus*. In this paper we present highlights from more than three decades of worldwide research on this biological control agent. We cover different aspects and key components of the complex plant-fungus-nematode tri-trophic interaction, an interaction that needs to be addressed to ensure the efficient use of *P. chlamydosporia* as a biopesticide as part of an integrated pest management approach.

Key words: biopesticides, chlamydospores, fungal biotypes, nematophagous fungus.

Pochonia chlamydosporia (Goddard) Zare & W. Gams 2001 (Hypocreales, Clavicipitaceae), was first reported in 1974 as a parasite of nematode eggs by Wilcox and Tribe in the UK, the fungus subsequently becoming one of the most studied potential biological control agents of nematodes. Research conducted on this nematophagous fungus has been reviewed extensively elsewhere (Kerry, 1997, 2000; Lopez-Llorca et al., 2008; Kerry and Hirsch, 2011; Manzanilla-López et al., 2011a; Chen and Dickson, 2012). The present paper aims to highlight key aspects of the bionomics of the fungus, which have paved the way to the use of *Pochonia chlamydosporia* as a biopesticide.

Pochonia chlamydosporia has a worldwide distribution and has been found in nematode suppressive soils to parasitize eggs. The fungus can remain saprophytic in soil in the absence of both plant and nematode hosts. In the rhizosphere, the fungus can colonize the roots of host plants, and several *Pochonia* species have even been reported to show endophytic behavior in some Gramineae and Solanaceae species—a growth habit that may result in benefits to the host plant defense against soil-borne pathogens (Lopez-Llorca et al., 2002a; Maciá-Vicente et al., 2009a, 2009b). The fungus is also a facultative parasite of nematode and mollusk eggs, and a hyperparasite of other fungi (Lumsden et al., 1982; Zare et al., 2001).

Several factors have contributed to target this fungus for nematode biocontrol, including easy laboratory culturing, access to strains (from fungal collections), and effectiveness in nematode control. These factors, and the generally increased interest in alternative control measures because of the phasing out of methyl bromide and nematicides, have led to screening and testing of numerous isolates to find potential biocontrol agents, including *Pochonia*, against major plant endo-parasitic nematode pests. Primarily *Heterodera*, *Globodera*, *Meloidogyne*, and more recently *Nacobbus* and *Rotylenchulus* spp., have been studied intensively as shown by an increasing number of worldwide reports of *Pochonia* native isolates. However, only a few strains are commercially available.

A better understanding of the tri-trophic interaction among plant, fungus, and nematodes is essential to enhance the performance of a biological control agent such as *P. chlamydosporia*. Environment, organisms, and their interaction will affect soil and root colonization by the fungus, the saprophytic or parasitic behavior of fungal isolates on target nematodes (i.e., host preference), and crop health and yield. Among the most important factors that were considered during the stages of screening for isolates and selection of strains for an efficient use of this biocontrol agent (Kerry, 2000) were those related to the properties of the fungal isolates in their potential as biological control agents (BCA).

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[1]Department of AgroEcology, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK.
[2]IMAR-CMA, Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal.
[3]Consiglio Nazionale delle Ricerche, Istituto di Genetica Vegetale (CNR-IGV). Via Amendola 165/A, 70126, Bari, Italy.
[4]Bioimaging–Department of Plant Biology and Crop Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK.
[5]Centro Nacional de Sanidad Agropecuaria, Apartado 10, San José de las Lajas, Mayabeque, Cuba.
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Email: rosa.manzanilla-lopez@rothamsted.ac.uk
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These include their ability to: (i) colonize the rhizosphere of plants and cultivars; (ii) to produce chlamydospores in vitro; and (iii) to infect nematode eggs (i.e., percentage of parasitism).

Rhizosphere colonization: The efficacy of the BCA depends on fungal rhizosphere colonization to facilitate colonization of galls, egg masses of root-knot nematodes (RKN), as well as eggs within cysts. Crop selection in a rotation plan can enhance the effective numbers of propagules available in soil for rhizosphere colonization during the next crop, in a plant-fungus compatible interaction. Significant levels of nematode control can be achieved only if fungal isolates are capable of proliferating in the rhizosphere. Plant species and their varieties and cultivars differ in root exudates and rhizodeposits and, therefore, in their ability to support *P. chlamydosporia* growth in the rhizosphere. Crops and plant species that can support more than 200 colony forming units (CFU)/cm^2 of root are considered to be good hosts of the fungus. Good hosts for *P. chlamydosporia* include beans, cabbage, crotalaria, kale, pigeon pea, potato, pumpkin, and tomato. Chili, sweet potato, cowpea, rye, tobacco, and cotton are moderate hosts (100–200 CFU/cm^2 of root), whereas poor hosts (<100 CFU/cm^2 of root), include aubergine, okra, soybean, sorghum, and wheat (de Leij, 1992; Bourne et al., 1996).

Chlamydospores: Conidia and hyphal fragments have low survival rates in soil and are subjected to fungistasis when added to soil without a supplementary energy source. Chlamydospores have sufficient food reserves to enable the fungus to establish without the addition of other energy sources (Kerry et al., 1993; Mauchline et al., 2002). Therefore, in vitro chlamydospore production has become one of the criteria to select fungus strains. An application rate of 5,000 chlamydospores/cm^3 soil is common for experimental purposes but it may vary according to strain and target nematode (de Leij et al., 1992; Kerry et al., 1993; Stirling and Smith, 1998; Viane and Abawi, 2000).

Tri-trophic interactions: Plants may or may not be compatible with nematodes, and this interaction will determine nematode reproduction, as well as the extent of damage and yield loss caused depending on its host status to nematodes (i.e., resistant, susceptible, tolerant). Good hosts for *P. chlamydosporia* can be either good or poor hosts for the plant-parasitic nematode. In general, fungal control of the nematode can be greater in plants that are poor hosts for the nematode but which also support extensive rhizosphere fungal growth. Plant susceptibility or resistance to RKN infection affects root gall size and this may result in eggs being retained inside large galls, thereby preventing them from being exposed in the rhizosphere to *P. chlamydosporia* parasitism (Atkins et al., 2003), thus "escaping" fungal infection. Therefore, application of the fungus to a relatively poor host for the nematode, producing smaller galls so that most egg masses are exposed on the root surface, might provide a more effective control (Kerry, 1997). Thus increasing *P. chlamydosporia* application rate in soil does not necessarily increase the colonization of the rhizosphere, a situation observed in a poor host where the fungal growth in the rhizosphere does not respond to increased soil application doses (de Leij et al., 1992).

Nematode root invasion: It is also important to note that *P. chlamydosporia* does not reduce initial plant invasion by juvenile stages, the main target being eggs, although second-stage juveniles of *Meloidogyne* within egg masses can be colonized and parasitized (Manzanilla-López, unpub. data).

Environmental factors: When released in soil, *P. chlamydosporia* is exposed to fluctuating abiotic factors such as water availability and microclimate, which can have a great impact on establishment in the field and its efficacy in controlling nematodes (Bourne and Kerry, 2000; Esteves et al., 2009b). Another environmental factor to be considered when introducing isolates to control nematodes is temperature. *Pochonia* species show optimal growth at 25°C and are less effective at temperatures above 30°C. However, isolates can differ in their optimal temperature, those obtained from cyst nematodes having lower optima than those derived from RKN (Bourne and Kerry, 2000).

Genetic diversity and fungal abundance: For several decades, ecological studies on nematophagous fungi were hampered by the lack of reliable techniques to determine at a finer level the growth, abundance, and virulence of species in the soil and the rhizosphere (Siddiqui et al., 2009). Some strains of *P. chlamydosporia* are now exploited in commercial formulations, but there is still limited knowledge about the genetic diversity of naturally-occurring populations. Ecological and quantitative studies with greater precision are increasingly becoming available using molecular tools. The fungus can be isolated directly from soil, roots, and nematode eggs using a semi-selective agar media, and identified using PCR primers with specificity at the subspecies (variety) level, based on the β-tubulin (Hirsch et al., 2000) and other genes (Rosso and Ciancio, 2005; Rosso et al., 2007). Intra-specific variation can be determined by polymerase chain reaction (PCR) fingerprinting (Mauchline et al., 2004) with arbitrary primers such as random amplification of polymorphic DNA (RAPD) PCR and enterobacterial repetitive intergenic consensus (ERIC) PCR. Genetic variation among isolates has been related to both the host nematode and geographical origin of fungal strains (Arora et al., 1996; Morton et al., 2003a; Manzanilla-López et al., 2009a, 2009b).

Fungal abundance in soil and the rhizosphere may not be reliably estimated if based solely on culture-based methods. DNA based quantitative PCR (qPCR) with species-specific primers has been shown to improve the estimation of fungal abundance of *P. c.* var.

chlamydosporia although it also detects DNA from nonviable and moribund material (Mauchline et al., 2002; Atkins et al., 2009). Used together, viable counts and qPCR should provide complementary information on fungal abundance.

Compatibility and competition between native and introduced biotypes for biocontrol: Different studies have been conducted to assess abundance, diversity, and competitiveness of fungal isolates in soil, rhizosphere, and eggs in vitro and under glasshouse conditions. Different *P. chlamydosporia* strains were fingerprinted and identified using ERIC PCR, showing that they can occur together in the same niche, and may compete with each other within the rhizosphere; their capabilities as root colonizers or egg parasites can vary when they are applied singly or in combination (Manzanilla-López et al., 2009a). However, the question remains as to whether diversity of isolates can be maintained in shared habitats (i.e., soil, rhizosphere, eggs) with other native/nonnative populations when nonnative populations of the fungus are introduced to soil. Preliminary studies on compatibility or anastomosis of selected isolates of *P. chlamydosporia* biotypes (from *Meloidogyne* sp., *Heterodera* sp., and *Globodera* sp.) to assess vegetative or heterokaryon compatibility suggested that biotypes isolated from different hosts did not anastomose but further studies are required to confirm incompatibility (Manzanilla-López et al., 2011b).

Egg infection: Anatomical and physiological features affect the infection process of the eggs, including enzymatic activity within fungal isolates and nutrient availability for the fungus. Eggs of RKN and cyst nematodes (CN) also differ in their susceptibility to fungal strains, which show a nematode host preference. Eggs in early stages of embryogenesis are more susceptible to parasitism than those containing a second-stage juvenile.

Enzymatic activity: Extracellular enzymes secreted by *P. chlamydosporia* play an important role in the infection process of eggs as they enable the fungus to degrade the nematode eggshell that is the host's major barrier to infection. Specific proteases and chitinases have been isolated and purified and their activity against the nematode eggshell demonstrated (Segers, 1996; Tikhonov et al., 2002; Esteves et al., 2009a). These enzymes are considered to be involved in the infection process and serve as virulence factors (Huang et al., 2004; Yang et al., 2007; Mi et al., 2010; Palma-Guerrero et al., 2010).

Parasitic ability: The serine protease VCP1 of *P. chlamydosporia* is involved in the early stages of the infection process (Morton et al., 2003a, 2003b). Primers for the detection of the *vcp1* gene from *P. chlamydosporia* and a PCR assay for host preference to distinguish between biotypes of the fungus from CN and RKN have been designed. These are based on polymorphism in the substrate recognition site of the serine protease implicated in infection of the preferred nematode host. Although the molecular diagnostic test can confirm the host preference of fungal biotypes, the primers need more extensive testing since CN-biotypes can infect RKN eggs and vice versa (Siddiqui et al., 2009).

Physiological and molecular mechanisms through colonization and penetration: Females of RKN and CN can be parasitized by vegetative hyphae in the rhizosphere with the production of an appressorium but without further specialized infective structures. Appressorium formation and egg infection must be preceded by environmental signaling, a process not yet understood (Lopez-Llorca et al., 2002b). Thigmotropic responses (i.e., toward hydrophobic and hydrophilic surfaces) and adhesion process mediated by glycoproteins, and enzymes (e.g., serine proteases) are involved in prepenetration events (Lopez-Llorca et al., 2002b). Appressoria formation is thought to be a nutritional response in many plant and entomopathogenic fungi. Conducive conditions are provided by nutrient depletion, low levels of complex nitrogen sources, and low C:N ratios (Blakeman and Parbery, 1977; St. Leger et al., 1989; Jackson and Schisler, 1992). Low C:N ratios influence egg pathogenicity by *P. chlamydosporia* (Segers, 1996), whereas production of conidia seems to be favored by media with relatively high C:N (Mo et al., 2005). The change, or "switch," from the saprophytic to the parasitic phase of the fungus may therefore be related to nutrients (including C and N) that are either released by plants into the rhizosphere or available in eggs, egg masses, and galls. VCP1 is an important enzyme in the early stages of parasitism, and the effect of various nutrients and pH on its expression was analyzed, by enzyme assays and qPCR, in the presence and absence of nematode eggs (Ward et al., 2011, 2012). Glucose repressed VCP1 production by *P. chlamydosporia,* and this repression could not be overcome by addition of eggs of the host (*M. incognita*). The effect of ammonium chloride was more complicated; at early stages after transfer to ammonium chloride-containing medium (4 hr), VCP1 levels were repressed but in most cases they were increased after 24 hr. For most strains, VCP1 expression was higher when the fungus was grown in more alkaline pH conditions (E. Ward et al., unpub. data).

Pochonia chlamydosporia gene expression under different nutrient regimes: Gene expression profiles were recently identified by Rosso et al. (2011) through a c-DNA amplified fragment length polymorphism (AFLP) approach. Two isolates (RKN and potato CN biotypes) were assayed at four different times under conditions of saprophytic to parasitic transition under different nutritional stresses (i.e., starvation in presence/absence of eggs or growth media). Expression of a number of genes was compared using electrophoretic profiles of the transcript derived fragments (TDF) of the two isolates. Annotation of basic local alignment search tool (BLAST) analyses showed that most TDFs could be included in functional groups related to metabolic

functions, or were involved in mechanisms of cellular signal regulation, cellular transport, regulation of gene expression, as well as DNA repair. Comparison of treatments ranging from saprophytism to true parasitism showed significant transcriptional reprogramming between treatments (i.e., a "switch" from saprophytic to parasitic phase). Some genes were induced/expressed or repressed thus suggesting a concerted regulation, especially when activated after exposure to eggs. Common amplification profiles reflected expression of basic metabolic genes (constitutive), and were not affected by assay conditions. Statistical analyses applied to the TDFs showed that genes involved in parasitism or other metabolic pathways clustered together (Rosso et al., 2011).

Genome: Recently, *P. c.* var. *chlamydosporia* isolate Pc10 (IMI 331547/CBS 101244) was sequenced (E. Ward et al., unpub. data (Rothamsted) and TGAC, BBSRC sequencing facility, UK), and the sequence will be deposited in a public database. Some relevant data include: 9×10^9 bp genomic DNA sequenced; 3,400 contigs assembled, > 100-fold coverage, and a genome estimate ~ 45 Mb (typical for an ascomycete fungus). Most of the genes analyzed to date show greatest identity to those of the insect pathogen *Metarhizium anisopliae*.

Production of Pochonia-based biopesticides: The success as BCA depends on the method of mass culturing of *P. chlamydosporia*, its formulation, addition of nutrient sources to increase sporulation, shelf life, and storage conditions (Jacobs et al., 2003; Duan et al., 2008; Montes de Oca et al., 2009).

Pochonia mass culturing and formulation: Inoculum produced in liquid shake cultures consists mostly of hyphae and conidia, both of which require an energy source to ensure proliferation in soil. Chlamydospores (resting spores) are not produced in liquid culture. These resting spores can be obtained from a substrate in solid phase, inoculated with conidia/chlamydospores for large-scale production and application (Montes de Oca, 2004). Fungal chlamydospore-based products for soil application (KlamiC®) are available for *P. c.* var. *catenulata* in Cuba where it is successfully used in peri-urban agriculture (Montes de Oca et al., 2009; Fernández-Larrea Vega, 2012; Leopoldo Hidalgo-Díaz, unpub. data). Different strains of the fungus are also produced commercially in the Americas (Freitas et al., 2009), Africa (Dudutech K Ltd.), Europe (Clamitec, Évora University, Portugal), and China as the commercial product Xianchongbike (Mo et al., 2005; see also Table 20.2 in Chen and Dickson, 2012). In Italy, a product based on an oil emulsion containing mycelium, conidia, and chlamydospores of *P. chlamydosporia* isolate IPP-21 has been commercialized for some years, under a registration-free permission, for localized application through drip irrigation water at 1 to 2 liter/ha (Ciancio, pers. comm.). At present, the fungus is mainly applied as chlamydospores on colonized substrates, with application rates to soil ranging from 30 to 400 kg/ha (Stirling and Smith, 1998; Verdejo Lucas et al., 2003) to 3 to 5 ton/ha (Tobin et al., 2008). So far, there has been limited progress in reducing these quantities. Facilities and resources for fungal production as unformulated product for subsistence farming systems can be basic and are not always subjected to regulatory mechanisms. Poor quality control probably accounts for a lack of consistency in efficacy of control, thereby affecting its perceived commercial potential. Hence, there is a need to improve product quality to harmonize standard procedures at different scales of fungus production and formulation (i.e., commercial vs. local, cottage industry). Energy cost and local distribution may also limit production scale and use.

Practical issues related to P. chlamydosporia use: Farming systems have a marked effect on the way a BCA is used and applied. Growers operating in the agricultural systems of developed countries require formulated products with a good shelf-life, low application dosage, and would possibly prefer application as seed treatments. By way of contrast, in subsistence farming systems of developing countries where quality control is minimal or absent, large application rates of an unformulated agent can be used as long as the BCA is produced cheaply and locally (Kerry, 1997).

Soil amendments: Pochonia chlamydosporia is usually added to soil in a colonized rice substrate used as an energy source for the fungus. Soil amendments can improve organic matter levels and increase nematode and pathogen control. However, in some fungal species, high C:N levels can repress infection-related genes compromising parasitic ability. Other factors to be considered when selecting a commercial BCA include soil receptivity to nonnative isolates, biosecurity and local regulations including monitoring after introduction and establishment in the rhizosphere, a requirement now achievable thanks to molecular methods. Only when these factors are applied in an appropriate and integrated manner will the full biocontrol potential of this organism be realized.

Pochonia chlamydosporia secondary metabolites and soil multitrophic interactions: For a BCA to be combined successfully with other integrated pest management (IPM) strategies, it is important to understand, in a more holistic approach, that production of mycotoxins or other secondary metabolites by fungal biopesticides may have detrimental biological effects to soil biota through interactions with the resident microbiota, including nontarget pathogens, and other potential biocontrol agents (i.e., multitrophic interactions). Although tested extensively to EPA standards for registration purposes and with no environmental impacts detected (RIU, 2008), it is documented that *P. chlamydosporia* produces antifungal and toxic compounds to various species of *Puccinia* (Leinhos and Buchenauer, 1992). Other compounds reported from *P. chlamydosporia* and *P. c.* var. *catenulata* (Khambay et al., 2000; Stadler

et al., 2003) include pochonins, and antiviral and antiparasitic resorcylic acid lactones, which are structurally related to monorden (syn. radicicol) (Rosso et al., 2011). Aurovertins are among the major constituents produced by *P. chlamydosporia*. Aurovertin-type metabolites (A3 and A4), obtained from the fungus, have proved to be toxic to *Panagrellus redivivus* in vitro assays (Niu et al., 2010). The chemical structures of aurovertin-like metabolites have been shown to be partly similar to phomalactone, a compound with nematicidal properties. Aurovertin 1 (A1) is the first natural aurovertin identified in *P. chlamydosporia* (Niu et al., 2010).

Conclusions

Pochonia chlamydosporia is an important natural enemy in certain nematode suppressive soils. Rhizosphere interactions between *P. chlamydosporia*, plant, and nematode are complex. Fungal strains from RKN and CN are different and should be considered as biotypes. By contrast, fewer fungal strains, with affinity for *Nacobbus* and *Rotylenchulus*, have been isolated from these nematodes in comparison with RKN and CN. These strains remain to be assessed for *vcp1* gene polymorphism to distinguish biotypes. Consistent and predictable biocontrol will depend on a careful selection of strains. There is a need to better understand the biology of the fungus, reduce application rates, optimize production and formulation methods, and increase validation assays under field conditions. From the ecological perspective, it is necessary to increase studies related to the long-term effect of *Pochonia* on other micro-organisms (multitrophic interactions). Although the parasitic potential of the fungus has been mainly targeted to nematode egg parasitism, recent studies on the endophytic behavior of the fungus have opened research avenues on its effect on plant pathogens such as *Gaemannomyces graminis* var. *tritici* (Monfort et al., 2005; Maciá-Vicente et al., 2009a) and migratory nematodes such as *Radopholus similis* (Freitas et al., 2009). Similarly, the fungus also appears as a promising BCA useful in animal health, as shown by studies on the toxic, ovicidal, and larvicidal effects on animal parasitic nematodes (Braga et al., 2011; Ferreira et al., 2011). Nutrition has a key role in determining parasitic activity (e.g., its effect on VCP1 expression) and an understanding of the nematode-fungus interactions at the molecular scale could provide novel methods for chemical or genetic intervention. Genome sequencing and other "-omics" technologies will open new research avenues for *P. chlamydosporia*, including discovery of new genes involved in the host-parasite interaction. It is expected that a better understanding of the biology and ecology and metabolism from whole organism to molecular scales (Kerry and Hirsch, 2011), will lead to an efficient and effective use of this biocontrol agent.

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