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An integrative approach for the selection of *Pochonia chlamydosporia* isolates for biocontrol of potato cyst and root knot nematodes

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Abstract. The nematophagous fungus *Pochonia chlamydosporia*, a natural enemy of plant parasitic nematodes (PPN), has been exploited in the development of sustainable management strategies for PPN control. The intrinsic variation among *P. chlamydosporia* isolates affects biocontrol potential, so sound characterisation is required. Biological, molecular and metabolic analyses can be determinant in the screening and selection of these potential biological control agents. This study aimed to provide integrative characterisation of *P. chlamydosporia* isolates that can support isolate selection for biological control purposes. Eight Portuguese isolates, associated with *Meloidogyne* spp., were used as a case study. Isolates were identified and characterised: i) at the biological level through standard bioassays to evaluate their ability for rhizosphere colonisation, to produce chlamydospores, and to parasitise *Globodera pallida* and *Meloidogyne incognita* eggs; ii) at the molecular level, examining genetic variation using ERIC-PCR; and iii) at the metabolic level, assessing their metabolic profiles using Biolog FF MicroPlates, with concurrent reads of fungal utilisation of 95 different carbon sources. Molecular data and metabolic characterisation were reduced using Principal Component Analysis and compared with the biological characteristics. Molecular profiles could only be related to isolate geographical origin, but the original substrate (eggs or roots), parasitism of *M. incognita* eggs and ability for rhizosphere colonisation were correlated with metabolic profiles, indicative of utilisation of specific carbon sources. *Meloidogyne* egg parasitism and rhizosphere colonisation were related to each other. This integrative characterisation offers novel perspectives on the biology and biocontrol potential of *P. chlamydosporia*, and, once tested on a broader set of isolates, could be used to assist rapid isolate selection.

Keywords. Biolog, isolate selection, metabolic characterisation, nematophagous fungus, rhizosphere interactions.

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INTRODUCTION

Plant-parasitic nematodes (PPN) are widespread pests and are severe threats to agricultural crops (Nicol *et al*., 2011). The most economically important PPN, which have impacts on crops worldwide, are root knot nematodes (RKN), *Meloidogyne* spp., and potato cyst nematodes (PCN), *Globodera* spp. (Jones *et al*., 2013). The facultative nematophagous fungus *Pochonia chlamydosporia* is an endophyte that parasitises sedentary females and eggs of economically important PPN, including cyst nematodes and RKN, and has been found associated with nematode suppressive soils (Manzanilla-Lopez *et al*., 2013).

Growth and proliferation of *P. chlamydosporia* in soil can be slow, and only achieved after multiple applications of the fungus (Vieira dos Santos *et al*., 2014). However, impact of the fungus on nematode reproduction is more affected by the ability to colonise the rhizosphere rather than to proliferate in soil (Manzanilla-Lopez *et al*., 2013). The fungus is rhizosphere competent and proliferates in this niche using nutrients released in root exudates. Although mostly confined to the rhizosphere, an endophytic behaviour has been reported (Maciá-Vicente *et al*., 2009). When the colonising *P. chlamydosporia* contacts nematode egg masses in the rhizosphere, the fungus ceases its saprophytic stage and switches to parasitism. Appressoria formed in response to contact with the egg surfaces enable the attachment to, and penetration of, the eggs, which involves mechanical and enzymatic actions. Secreted enzymes destroy the outer vitelline egg membranes exposing the chitin layers, which are then penetrated by infection pegs. These grow into hyphae destroying egg contents and degrading the egg shells from the inside (Mauchline *et al*., 2004, Monteiro *et al*., 2017). Since RKN and PCN egg shells are constitutionally different, nematode host preference may be involved in the switch between fungal saprophytic and parasitic phases, but nutrient availability, either released by plants or available in nematode eggs, may play an important role (Mauchline *et al*., 2004; Esteves *et al*., 2009). Readily metabolised carbon sources and pH could influence the trophic phase switch as they interfere with the production of an alkaline serine protease (VCP1), known to be involved in the early stages of nematode egg infection (Segers *et al*., 1994; Ward *et al*., 2012).

Isolates of *P. chlamydosporia* differ markedly in their rhizosphere competence and ability to parasitise nematode eggs. The outcome of these variations in the regulation of nematode populations is difficult to predict, increasing the need for discriminative screening and selection of isolates. Standard screening encompasses *in vitro* assays for three criteria evaluating the ability of isolates to produce chlamydospores, colonise the rhizosphere, and parasitise nematode eggs (Abrantes *et al*., 2002).

Chlamydospores are resistant structures that can be produced using different solid substrates. These spores can withstand harsh conditions in soil for long periods without additional nutrients, and initiate fungal growth when in favourable environmental conditions. Chlamydospores are usually regarded as the preferred source of inoculum for biocontrol (Kerry, 2001). Isolates that successfully parasitise nematode eggs are not always competent in establishing in soil, as idiosyncratic results have been obtained for soil establishment (Vieira dos Santos *et al*., 2014). Inability to colonise the rhizosphere may hinder contact with nematode galls and egg masses, and thus affect biocontrol potential of particular isolates (Manzanilla-López *et al*., 2013).

Variation in *P. chlamydosporia* isolates has been related to host nematodes and the soil environment from which particular isolates originated (Zavala-González *et al*., 2015). Field biocontrol efficacy of isolates depends on several factors, including the nematode host, soil biotic and abiotic factors, and geographical origin (Nagesh *et al*., 2007). Molecular studies, required to identify isolates and provide fingerprinting, have been used to characterise isolates and detect genetic variation (Medina-Canales and Rodríguez-Tovar, 2017).

Enterobacterial Repetitive Intergenus Consensus sequence (ERIC)-PCR as a technique for PCR-based DNA fingerprinting provides rapid evaluation of isolates genetic variation. ERIC primers act randomly and amplify repetitive sequences in fungus genomes, and produce PCR products with multiple band profiles, and this allows isolate discrimination (Morton *et al*., 2003). However, ERIC-PCR profiling has only been informative for geographical origin and host preference of isolates.

The Biolog FF MicroPlate system (Biolog Inc.) has been used to characterise filamentous fungi according to their metabolic profiles. These profiles are obtained from fungus utilisation of 95 carbon sources from different chemical groups (amines/amides, amino acids, carbohydrates, carboxylic acids, polymers and miscellaneous compounds). This method has been used to identify and aid the definition of new species of fungi with small morphological variation, when molecular techniques are of limited application (Rice and Currah, 2005). In parallel, Biolog FF MicroPlates have been used to assess fungal functional diversity in environmental samples (Lucas *et al*., 2013), and to support fungal isolate characterisation, particularly if they are closely related (Pawlik *et al*., 2015; Singh, 2009). The method can also complement bioassays

in the selection and characterisation of isolates for biological control (Lopes *et al*., 2012). To our knowledge, *P. chlamydosporia* has not been characterised using this system.

The main goal of the present study was to provide an integrative methodology that can assist the selection of *P. chlamydosporia* isolates for the biocontrol of PPN. Here, we focus on *G. pallida* and *M. incognita* as, respectively, representative species of PCN and RKN. We anticipate that knowledge gained by the integration of molecular techniques, metabolic profiling and standard *in vitro* bioassays will support isolate selection, and provide new insights into the isolate functional characters that predict their success as biological control agents.

MATERIALS AND METHODS

Pochonia chlamydosporia isolates

Root knot nematode females from infected tomato (*Solanum lycopersicum* L.) roots, obtained in a greenhouse in Alcochete, Setúbal, Portugal, were identified by esterase phenotyping according to Esbenshade and Triantaphyllou (1985) and Pais *et al.* (1986). *Pochonia chlamydosporia* isolation was carried out by plating nematode eggs and host roots on semi-selective medium (De Leij and Kerry, 1991). After 14 d incubation at 25°C, colonies morphologically similar to *P. chlamydosporia* were transferred onto 1.7% corn meal agar (CMA) (Oxoid) for observation of characteristic diagnostic features (Abrantes *et al*., 2002).

The resulting isolates, from either RKN eggs (PE1, PE2, PE3, PE4, PE5) or tomato roots (PR1, PR2, PR3), were identified by PCR using specific primers. Isolate Pc2, from *G. rostochiensis* eggs (Vieira dos Santos *et al*., 2013), and a non-native isolate, Vc10 (IMI 331547) originally from *M. incognita* eggs from Brazil supplied by Rothamsted Research, United Kingdom, were also included (Table 1). Isolate Vc10 was used as a standard for *P. chlamydosporia* var. *chlamydosporia*. DNA was extracted from mycelium of *P. chlamydosporia* isolates grown for 10 d on 1.7% potato dextrose agar (PDA) (Difco™) using the E.Z.N.A. Fungal DNA miniKit (OMEGA bio-tek) according to the manufacturer's instructions. The identities of all the isolates were confirmed by PCR. DNA was amplified with the ***β***-tubulin primers specific for *P. chlamydosporia* var. *chlamydosporia* (Hirsch *et al*., 2000). PCR products were separated in 1% agarose gels stained with GreenSafe Direct Load (Nzytech genes & enzymes), and visualised in a transilluminator (Vilbert Lourmat). The relative mobilities of the PCR bands were calculated with the Bio-print Mega software version 12.15 for Windows (Vilbert Loumart).

Table 1. *Pochonia chlamydosporia* var. *chlamydosporia* isolates, geographic origin, substrate and associated nematode species.

Pc isolate	Geographic origin	Substrate	Nematode species associated
PE1	Setúbal, Portugal	Eggs	*Meloidogyne hispanica*
PE2		Eggs	*M. javanica*
PE3		Eggs	*M. javanica*
PE4		Eggs	*M. incognita*
PE5		Eggs	*M. incognita*
PR1		Roots	*M. hispanica*
PR2		Roots	*M. javanica*
PR3		Roots	*M. javanica*
Pc2	Guarda, Portugal	Eggs	*Globodera rostochiensis*
Vc10 (IMI 331547)	Brasil	Eggs	*M. incognita*

Biological characterisation

The abilities to produce chlamydospores, to colonise the rhizosphere of tomato plants, and to parasitise PPN eggs were tested, based on the methods reviewed by Abrantes *et al.* (2002), with modifications.

Chlamydospore production

Production and viability of chlamydospores produced by the eight isolates and of isolates Pc2 and Vc10 were assessed using the method described by De Leij and Kerry (1991). Five conical flasks per isolate, each containing 100 g of sterilised barley:sand substrate (1:1), were inoculated, each with three agar plugs from 10 d old colonies on CMA. After 30 d incubation at 25°C, the number of chlamydospores per three 1 g sub-samples of the colonised medium per flask was estimated using a haemocytometer. Chlamydospore viability was assessed in sorbose agar with antibiotics (12 g L^{-1} technical agar, 2 g L^{-1} of sorbose and 50 mg L^{-1} each of streptomycin sulphate, chlortetracycline and chloramphenicol). After 2 d at 25°C, the percentage of germinated chlamydospores was estimated using a stereomicroscope (Abrantes *et al*., 2002). Three biological replicates were carried out.

Rhizosphere colonisation

To study the ability of isolates to colonise the rhizosphere, roots of tomato cv. Tiny Tim roots were used,

since tomato is considered to be a good host for *P. chlamydosporia* (Manzanilla-López *et al*., 2013). Tomato seeds were surface sterilised with a sterilisation solution (1.6% sodium hypochlorite with 0.02% Tween) for 10 min, and rinsed three times with sterile distilled water. The seeds were then germinated in Petri dishes containing Gamborg's B5 medium supplemented with 15 g L^{-1} sucrose 1.5% and 8 g L^{-1} agar, adjusted to pH 6.4. The plates were incubated in the dark, at 25°C. Fungus inoculum was obtained by washing 10-d-old colonies grown on 1.7% CMA with 10 mL Gamborg's B5 medium. The number of spores (chlamydospores and conidia) was counted using a haemocytometer. Five 25 mL capacity flasks per isolate, containing 15 ml of autoclaved sand amended with 5 mL of half-strength Gamborg's B5 medium, were each inoculated with 10^5 spores, and planted with a 3 d-old single germinated tomato seed. The flasks were covered with transparent adhesive film and incubated for 21 d at 25°C and 12 h photoperiod. The plants were then up-rooted, shaken free of sand and placed onto 0.8% water agar for 3 d at 25°C. The percentage of roots colonised by each isolate was estimated using a stereomicroscope. Experimental controls consisted of flasks without fungal inoculum. These assays were conducted twice.

Nematode egg parasitism

The ability of each isolate to parasitise nematode eggs was assessed using standard *in vitro* bioassays (Abrantes *et al*., 2002). Ten-d-old colonies of each isolate, growing on 1.7% CMA, were washed with 10 mL of sterile distilled water. The numbers of chlamydospores and conidia per mL were quantified using a haemocytometer. Plates containing 0.8% technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg L^{-1} of each) were each inoculated with a 0.2 mL of a 10^5 spores mL^{-1} suspension of each isolate, and were then incubated for 2 d at 25°C. Five plates per isolate were prepared.

A *G. pallida* population, originally from infected potato fields in Cantanhede, Coimbra, was selected from the collection at the NEMATO-lab, Centre for Functional Ecology (CFE), Department of Life Sciences, University of Coimbra (UC), and was propagated on roots of potato cv. 'Desirée' grown in 900 cm^3 plastic pots with a sterilised mixture of sandy loam soil and sand (1:2). Pots were inoculated with a small polyester bag containing cysts, to give approx. 5 eggs g^{-1} of soil. The number of eggs per cyst was assessed by counting five replicates of a suspension of eggs obtained by crushing 50 cysts in water. The pots were placed in a glasshouse at 20°C, with a 16 h photoperiod, and were watered regularly. After three months, new cysts were extracted from the soil using a modified Fenwick can (Shepherd, 1986). Eggs within cysts were released using forceps, and then suspended in water.

A RKN population of *M. incognita*, originally from potato roots and maintained in pot cultures of tomato cv. 'Coração de Boi' at the NEMATO-lab, CFE, UC, was selected. The population was propagated on susceptible tomato cv. 'Tiny Tim', grown in autoclaved sandy loam soil and sand mixture (1:1) in a greenhouse. The culture was transferred periodically to new tomato seedlings inoculated with ten egg masses per plant, placed beneath the tomato roots. Handpicked egg masses were mechanically disrupted, and the released eggs were suspended in water by agitation.

Egg suspensions were sieved through 75 µm and 20 µm sieves to separate eggs from debris, and were concentrated to obtain 2 eggs per µL. Two hundred and fifty eggs of each nematode species were transferred to each Petri plate colonised by the fungus, and were gently spread using a sterilised glass rod. Plates were incubated at 25°C for a further 3 d, after which the number of parasitised eggs was counted using standard methods (De Leij and Kerry, 1991). Experimental controls consisted of plates inoculated with nematode eggs, without the fungus. The bioassays were repeated three times.

Molecular characterisation

All the *P. chlamydosporia* isolates were characterised by ERIC-PCR, as described by Arora *et al.* (1996), and all reactions were performed three times to assess PCR pattern reproducibility. DNA was extracted from mycelium as described above. PCR products were separated in 2% agarose gels stained with GreenSafe Direct Load and visualised in a transilluminator. ERIC-PCR fingerprints produced distinct bands that were visually scored for presence (1) or absence (0) to produce a binary matrix from gel photographs recorded with the Bio-print Mega software version 12.15 for Windows.

Metabolic characterisation

The global phenotypes and utilisation by each of the isolates of 95 low molecular weight carbon sources (plus a negative control) were evaluated using the Biolog FF MicroPlate (Biolog Inc.), following manufacturer instructions. Pure cultures of each isolate were grown on CMA at 25˚C in the dark for 3 weeks, until spore formation was visible. The inoculum for the 96 well FF Micro-

Plates was prepared by first soaking a sterile swab in inoculating fluid and then gently rolling over the surface of the agar plates. The obtained spores were suspended in FF inoculating fluid supplied by Biolog in glass tubes (Cat. Nº 1006), mixed gently by hand and adjusted to approx. 75 % transmittance at 590 nm using a Biolog Turbidimeter, previously calibrated using a FF Biolog Turbidity standard (Cat. Nº 3426). Spore suspension (100 µL) was added to each well, and the FF MicroPlates were then incubated at 25˚C in the dark. The optical density at 490 nm (mitochondrial activity) was determined using an ASYS UVM 340 microplate reader (Hitech GmbH) for each plate at 24 h intervals over the next 7 d. Carbon sources were considered not utilised in wells in which colour development was less than, or equal to, that of negative controls.

Statistical analyses

Data of rhizosphere colonisation, numbers and viability of chlamydospores and nematode parasitism variables were analysed using IBM SPSS Statistics v.22. Descriptive statistics were used to explore data and identify extreme outliers that were, in most cases, due to technical error, and five data points were subsequently removed from analyses. Generalised linear mixed models (GLMM) were run for all variables using a normal distribution and an identity link function to establish statistical differences between treatments ($P < 0.05$). 'Isolate' was a fixed factor, and biological repeats and model hierarchy were both included as random factors. Satterthwaite approximations were used due to the small number of data points. Pairwise contrasts between estimated means per isolate adjusted for multiple comparisons were obtained by Least Significant Differences. A similar model structure was used to compare the parasitism of PCN eggs with the parasitism of RKN eggs by each isolate, combining results and adding parasitised PPN egg genus as a fixed factor. Estimated means per isolate for each variable resulting from the GLMM were explored with paired-data Pearson correlation analysis using PAST v.3.18. The highly significant correlation between rhizosphere colonisation and parasitism of *M. incognita* eggs was further analysed using Reduced Major Axis (RMA) analysis.

Multivariate analyses were performed in PAST v.3.18 (Hammer *et al.*, 2001) to reduce the number of variables resulting from the molecular (ERIC band matrix) and metabolic (Biolog- carbon source utilisation) profiles, using Principal Component Analysis (PCA). For the metabolic profiles, the average well colour development (AWCD) of the different isolates were calculated over the 7 d incubation, where AWCD equals the sum of the difference between the OD of the blank well (control) and substrate wells, divided by 95 (the number of substrate wells in the FF MicroPlates). The AWCD values were stabilised only at 7 d, therefore OD readings obtained in each of the 95 carbon sources at this incubation time were then reduced to a smaller, easily interpretable, number of explanatory variables with PCA.

The relationships between synthetic gradients on molecular and metabolic profiles obtained through PCA and the GLMM-estimated means of biological data were assessed through correlation analysis in PAST v.3.18. Pearson correlations were used for the comparison between the PC axes containing over 5% of the variance, and the number and viability of chlamydospores produced, rhizosphere colonisation and parasitism of PCN and RKN eggs. Spearman correlations were used for the comparisons of the same axes with nominal data on isolate geographical origin, substrate (eggs or roots) and nematode species associated.

RESULTS

Pochonia chlamydosporia *isolates*

Eight *P. chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2 and PR3) were obtained, associated with three RKN species: *M. hispanica*, *M. incognita* and *M. javanica* (Table 1).

All isolates presented a specific band (ca. 270 bp) for *P. chlamydosporia* var. *chlamydosporia* (data not shown).

Biological characterisation

Chlamydospore production

The ability to produce chlamydospores *in vitro* was variable among the isolates (Figure 1a). Isolates PR1 and Pc2 produced significantly greater numbers of spores (respectively, 171×10^5 g^{-1} and 187×10^5 g^{-1}) when compared with isolates PE1, PE3, PE5, PR2, PR3 and Vc10 (Figure 1a). Chlamydospore viability was less than 85% for isolates PE3, PR1 and PR3 (Figure 1b).

Rhizosphere colonisation

Root colonisation varied from 60% (PR3) to 92% (Pc2). Root colonisation by isolates PE1, Pc2 and Vc10 was significantly greater compared with root colonisation by isolate PR3 (Figure 2).

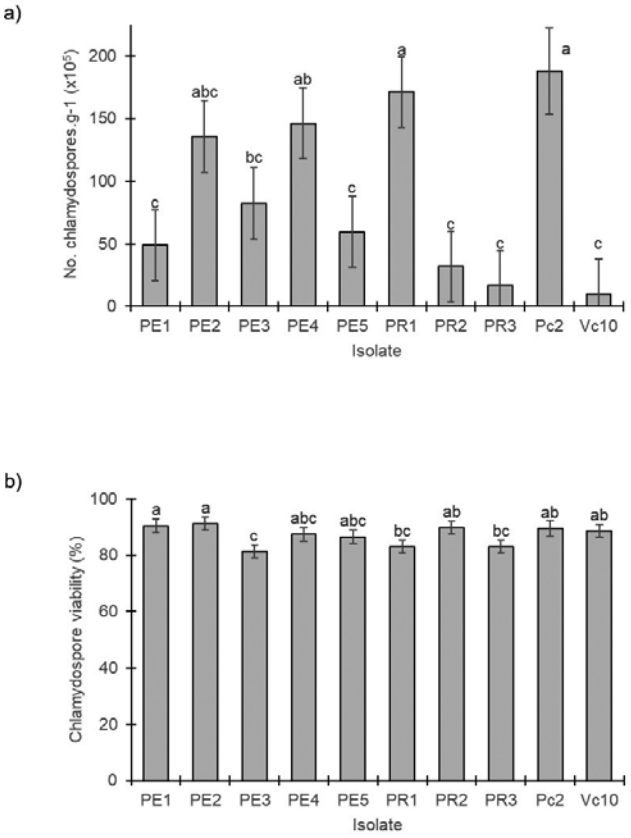

Figure 1. Number of chlamydospores (a) and chlamydospore viability (b) of ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) grown for 30 d on barley:sand substrate (1:1) (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by the same letter are not significantly, different (LSD test; $P < 0.05$).

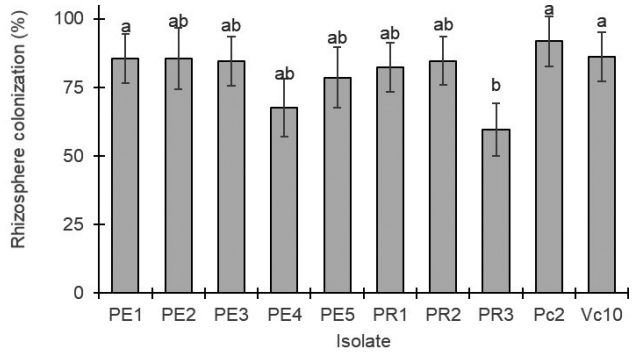

Figure 2. Mean percentages of colonised tomato roots for ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) grown for 21 d on sterilised sand (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by same letter are not significantly different (LSD test; $P < 0.05$).

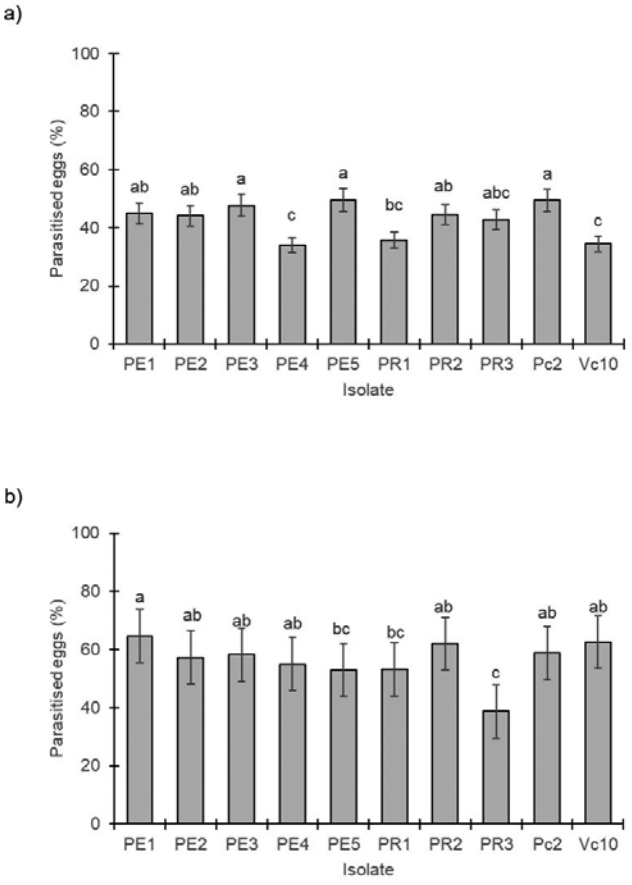

Figure 3. Mean percentages of parasitised eggs of *Globodera pallida* (a) and *Meloidogyne incognita* (b) by ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) after incubation at 25°C for 3 d (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by the same letter are not significantly different (LSD test; $P < 0.05$).

Nematode egg parasitism

After 3 d at 25°C, the percentages of parasitised eggs of PCN and RKN were low for all isolates (Figure 3). Pathogenicity of the isolates varied between 34 and 49 % against *G. pallida* eggs (Figure 3a) and 38 to 65 % against *M. incognita* eggs (Figure 3b). Parasitism of PCN eggs was less than parasitism of RKN eggs ($P < 0.01$), with the exception of isolate PR3, which had a greater parasitism rate of PCN eggs than of RKN eggs. Statistically significant differences in egg parasitism between PPN genera were not found for isolates PE1 and PE4. RMA analysis revealed a highly significant positive linear relationship ($r = 0.818$; $P < 0.01$) between parasitism of RKN eggs and rhizosphere colonisation by the fungus.

Molecular characterisation and integration with biological characterisation

Isolates associated with *Meloidogyne* spp. had very similar ERIC patterns. A different band pattern was observed for isolate Pc2 associated with *G. rostochiensis* eggs (Supplementary Material). The PCA analysis separated the fungal isolates into four quadrants, with isolates Pc2 and Vc10 clearly separated from other isolates along PCERIC2 and from each other along PCERIC1 (Figure 4). Isolates PE1, PE2 and PR3 were also separated by synthetic gradient PCERIC1 from isolates PE3, PE4, PE5, PR1 and PR2. Although significant associations were not found between biological characteristics and PC axes PCERIC1 and PCERIC2 (loadings presented in Table 2), the geographical origins of the isolates were correlated with PCERIC1 ($r = 0.707$, $P < 0.05$).

Metabolic characterisation and integration with biological characterisation

Altogether, the ten isolates used the 95 substrates, but only 72 substrates were used by all the isolates: these included four out of six amines/amides; nine of 13 amino acids, 38 of 44 carbohydrates, 12 of 17 carboxylic acids, three of five polymers and six of ten miscellaneous compounds. Six of the substrates, namely n-acetyl-d-mannosamine, sedoheptulosan, d-lactic acid methyl ester, α-cyclodextrin and β-cyclodextrin, were used by fewer than half of the isolates. Isolate PE5 was the only isolate able to utilise all 95 carbon sources, followed by Vc10 (92 carbon sources), isolate PR2 (91) and isolate Pc2 (90). Isolate PR3 utilised the least, with only 77 wells developing colour, as corrected to the water control (Figure 5). The different profiles of carbon source utilisation positioned

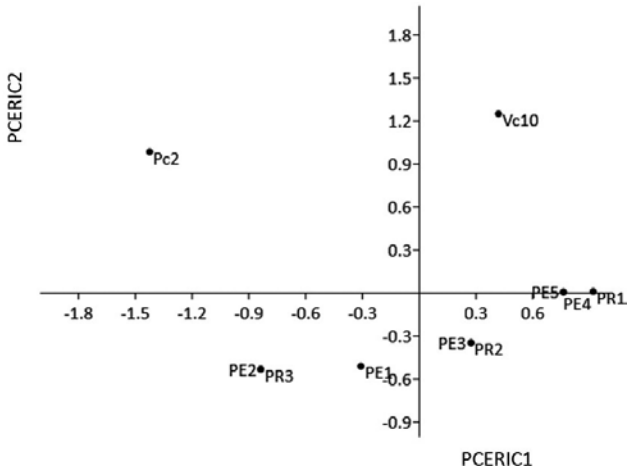

Figure 4. Principal Component Analysis of Enterobacterial Repetitive Intergenus Consensus sequence (ERIC) PCR profiles obtained from *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10; refer to Table 1 for isolate information). PCERIC1 eigen value 0.651; PCERIC2 eigen value 0.400.

Table 2. Synthetic gradients generated by Principal Component Analysis of molecular and metabolic profiling of *Pochonia chlamydosporia* isolates.

Axis	Variance (%)	Positive loading	Negative loading
PCERIC1	49.22	ERIC bands 2, 4 and 6	---
PCERIC2	30.25	ERIC band 8	ERIC band 7
PCBLG1	45.484	Utilisation of most carbon sources; prominent: 2-aminoethanol (amines/amides) glycyl-L-glutamic acid L-glutamic acid L-ornithine L-serine L-threonine (amino acids) β-hydroxy-butyric acid succinic acid (carboxylic acids) adenosine-5'-monophosphate amygdalin succinic acid mono-methyl ester uridine (miscellaneous)	D-ribose (carbohydrates)
PCBLG2	28.58	α-methyl-D-glucoside β-methyl-D-galactoside n-acetyl-D-galactosamine n-acetyl-D-mannosamine (carbohydrates) D-galacturonic acid D-glucuronic acid L-lactic acid (carboxylic acids) D-lactic acid methyl ester (miscellaneous)	2-aminoethanol (amines/amides) L-aspartic acid L-glutamic acid (aminoacids) α-D-glucose L-rhamnose maltose maltotriose (carbohydrates) L-malic acid (carboxylic acids) glycerol succinic acid mono-methyl ester (miscellaneous)

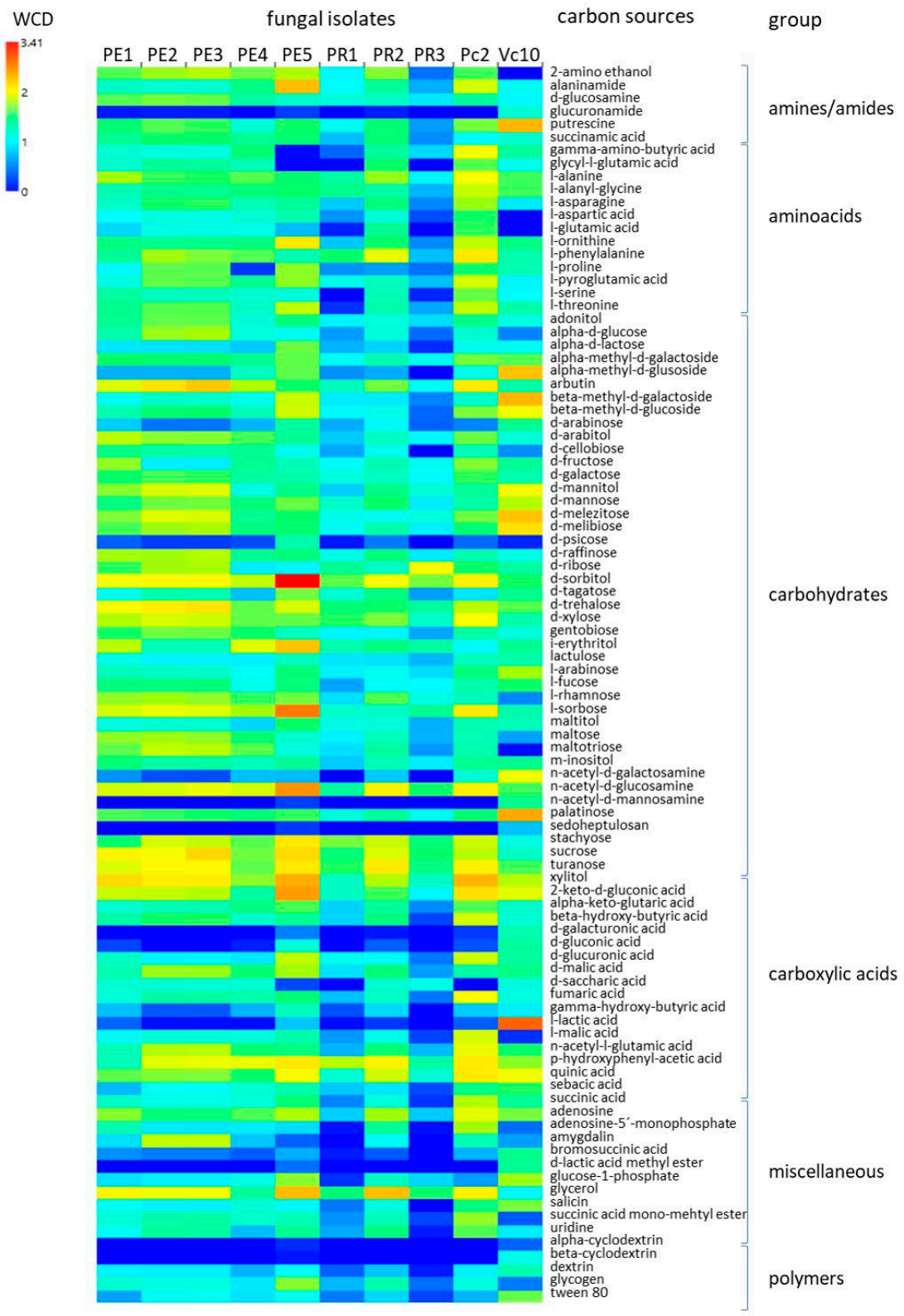

Figure 5. Heat map of carbon source utilisation by *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10; refer to Table 1 for isolate information), assessed through Well Colour Development (WCD) at 7 d, corrected to control, of each of 95 carbon sources/chemical group, using Biolog FF MicroPlates.

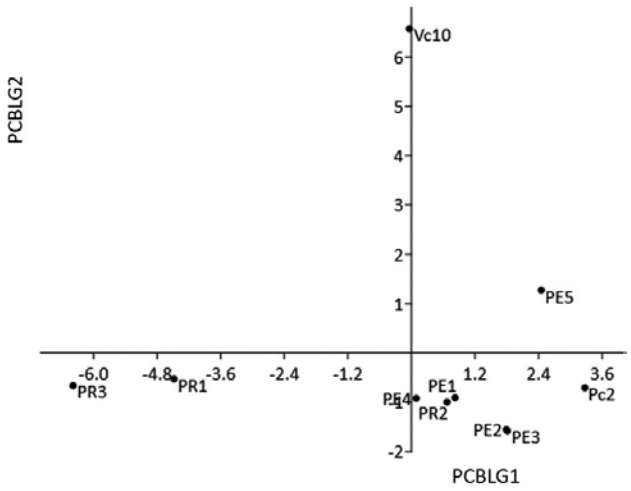

Figure 6. Principal Component Analysis of carbon utilisation profiles obtained through Biolog FF MicroPlates for *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10, refer to Table 1 for isolate information). PCBLG1 eigen value 9.470; PCBLG2 eigen value 5.950.

isolates Vc10 and PE5 in different quadrants to all other isolates, along the two PCA axes of the metabolic profile (PCBLG1 and PCBLG2; Table 2 and Figure 6). This was particularly evident for isolate Vc10, depending mainly on separation obtained by synthetic gradient PCBLG2. Isolates PR1 and PR3 also each had a distinct metabolic profile from all other isolates, being separated by PCBLG1. Significant correlations were detected between the synthetic gradient PCBLG1 and the isolate substrate of origin (eggs or roots; $r = -0.646$, $P < 0.05$), and also with parasitism of RKN eggs ($r = 0.700$, $P < 0.05$) and rhizosphere colonisation ($r = 0.666$, $P < 0.05$).

DISCUSSION

The potential of eight *P. chlamydosporia* isolates associated with *Meloidogyne* spp. as biological control agents was evaluated using integrative characterisation, based on biological, molecular, and metabolic analyses.

Although few statistically significant differences were detected between the evaluated isolates, molecular identification and characterisation allowed evaluation of their variability, affected by different geographic substrate origins (Table 1). This information could be helpful for the potential registration of a specific isolate as a biological control agent (Abrantes *et al.*, 2002). Detection and quantification of particular fungi 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.*, 2000; Mauchline *et al.*, 2002). The β-tubulin gene allowed design of specific primers for *P. chlamydosporia* var. *chlamydosporia*, and since it has an intron that is not present in other fungi, specific primers do not amplify DNA from other species of *Pochonia* or from other fungal genera (Mauchline *et al.*, 2002; Kerry and Hirsch, 2011). This confirmed the identity of all Portuguese isolates used in this study as *P. chlamydosporia* var. *chlamydosporia*.

Rapid *in vitro* bioassays are necessary for precise selection before introduction of a biological control agent in the soil. Although few statistical differences were found, variation between eight isolates was detected using three standard *in vitro* bioassays, including chlamydospore production, rhizosphere colonisation, and nematode egg parasitism. The inherent variation of *P. chlamydosporia* isolates has been previously described, and this variation stresses the need for precise isolate selection (Abrantes *et al.*, 2002).

Ability to produce chlamydospores *in vitro* was variable among the eight isolates. 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 isolates, depending on individual growth rates and sporulation. Therefore, in order to assess differences among *P. chlamydosporia* isolates, equal amounts of spores should be used as inoculum (Vieira dos Santos *et al.*, 2013). The results obtained for reference isolates Pc2 and Vc10 were less than those reported by Vieira dos Santos *et al.* (2013). Additionally, fewer chlamydospores were reported for isolate Vc10 by Medina-Canales *et al.* (2014). Differences in chlamydospore production between isolates may be due to the type of substrate used, as chlamydospore production is greater in substrates with low available carbon and nitrogen, and no sulphur (Mo *et al.*, 2005). Although ability to produce chlamydospores is assessed in the standard characterisation, to ensure success in large-scale inoculum production, this may be variably affected by culture conditions, including carbon and nitrogen contents of the culture substrate (Liu and Chen, 2003; Mo *et al.*, 2005; Vieira dos Santos *et al.* 2012). Moreover, the success of a commercially developed product based on an oil emulsion containing chlamydospores, mycelium and conidia was recently reported (Sellitto *et al.*, 2016). Regarding viability, some isolates have low germination proportions (< 85%), which can be due to the incubation time (3 weeks). Some isolates may not withstand this incubation period, and although they continue to produce chlamydospores, the viability tends to decrease with time (Abrantes *et al.*, 2002). In the

present study, all isolates produced adequate numbers of chlamydospores for potential commercial scale production ($\geq 1 \times 10^6$ g^{-1} of substrate) (Medina-Canales *et al.*, 2014). Therefore, we anticipate that, in isolates that produce numbers of chlamydospores greater than accepted threshold of 10^6 g^{-1} of substrate, the ability to produce viable chlamydospores, rather than the exact number of chlamydospores produced, may be an important characteristic for the selection of these biological control agents.

Differences among isolates were detected in their abilities to colonise tomato rhizospheres. The best coloniser of tomato roots was the standard isolate Pc2 with 92% of colonisation. Isolates PE4, PE5 and PR3 colonised less than 80% of tomato roots, failing to achieve the 80% colonisation threshold for selection for biocontrol (Abrantes *et al.*, 2002). Rhizosphere colonisation is a key feature for these biocontrol agents, as it is critical for the control of nematodes by *P. chlamydosporia* (Bourne *et al.*, 1996). The biocontrol efficacy of the fungus depends on fungal rhizosphere colonisation to facilitate colonisation of galls (Manzanilla-López *et al.*, 2013). This assumption has now gained experimental support by our finding of a strong relationship between rhizosphere colonisation and parasitism of RKN eggs. A link between tomato rhizosphere colonisation and parasitism of PCN eggs was not detected. The proportions of colonisation of tomato roots by reference isolates Pc2 and Vc10 were greater than previous results for colonisation of barley roots (Vieira dos Santos *et al.*, 2013). It has been established that plant species differ in their ability to support the fungus (Bourne *et al.*, 1996; Kerry and Bourne, 1996). Whether this can be further related to fungal parasitism of eggs of nematodes that are able to parasitise those plant species is unknown. We speculate that the fungal isolates might emulate nematode host plant preferences, i.e. a virulent parasite of a given nematode population could be a good root coloniser of the host plant preferred by the nematode. This, to our knowledge, has not been investigated.

The low proportion of egg infection obtained through the standard bioassay has also been observed by other authors. This may be related to changes in the mucilage surrounding the eggs, which are considered as important sources of nutrition for the fungi and ensure high rates of egg colonisation. Additionally, isolates parasitise immature eggs more actively than mature eggs containing second-stage juveniles (Irving and Kerry, 1986). Spontaneous hatching detected in both nematode species during the bioassays could also contribute to the low parasitism levels observed. Our results do not support previous findings suggesting that *P. chlamydosporia* isolates have host preferences for host species from which the isolates were initially isolated (Mauchline *et al.*, 2004). Although isolate PR3 was originally associated with RKN, it was more virulent to PCN than RKN; conversely, Pc2, isolated from PCN, was more virulent to RKN eggs than to those of PCN. Seven of the ten isolates tested were more virulent to RKN eggs than to PCN eggs.

Principal Component Analysis of ERIC profiles obtained for *P. chlamydosporia* isolates separated them according to their geographic origin (Figure 4). Isolate Pc2 was the only one from PCN eggs, and was here compared with nine isolates associated with *Meloidogyne* spp. This may have hindered the detection of relationships among ERIC-PCR profiles and nematode origins. Genetic variability among isolates revealed that isolates can be grouped according to their geographical and the host nematode species. The importance of this genetic variation in the regulation of a host population is, however, unknown (Morton *et al.*, 2003).

Isolates PE5, Vc10, PR2 and Pc2 were able to utilise most of the carbon sources (≥ 90), whereas isolate PR3 utilised the least (77) (Figure 5). Isolate PR3 clearly had different carbon requirements as it produced the fewest chlamydospores in barley. Growth and sporulation are influenced by carbon and nitrogen sources, but are also determined by isolate preferences (Mo *et al.*, 2005; Liu and Chen, 2003). Isolate original substrate (eggs or roots), parasitism of *Meloidogyne* eggs, and rhizosphere colonisation ability were significantly correlated with metabolic profiles, indicating that specific carbon sources (amines/amides, amino acids, carboxylic acids and miscellaneous carbon sources) could be used to predict isolate ability for rhizosphere colonisation and for RKN egg parasitism. High proline contents are characteristic of RKN egg shells (Bird and McClure, 1976). For PCN, although proline was the most abundant, 64% of the total amino acids were aspartic acid, glycine and serine (Clarke *et al.*, 1967). Molecular studies have also found that genes encoding proteases known to be involved in nematode egg penetration processes are also highly expressed during endophytic colonisation of barley roots (Lopez-Moya *et al.*, 2017).

The inherent variation of *P. chlamydosporia* isolates has been widely described, and this variation increases the need to carefully select potential biocontrol isolates. Laboratory *in vitro* tests, that evaluate the three biological characteristics considered fundamental for a good fungus performance as biological control agents, enable rapid and economical selection of isolates, before testing their soil activity in pot tests ahead of field trials (Abrantes *et al.*, 2002).

Pochonia chlamydosporia isolate variability was assessed using Biolog FF MicroPlates for the first time.

It is not clear how the metabolic profiles of fungal isolates, or their ability to use particular carbon sources, ultimately affect biocontrol performance in the field. Nevertheless, use of this metabolic approach may facilitate future isolate selection, as the use of specific carbon sources provides complete information on isolate ability for rhizosphere colonisation, and for nematode egg parasitism. These studies may lead to new perspectives for understanding the intricate multitrophic life styles characteristic of *P. chlamydosporia*.

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