MOLECULAR IDENTIFICATION, CHARACTERIZATION, VARIABILITY AND INFECTIVITY OF INDIAN ISOLATES OF THE NEMATOPHAGOUS FUNGUS *POCHONIA CHLAMYDOSPORIA*

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Summary. Cataloguing and conservation of beneficial native soil biota play a vital role in sustaining a healthy, productive soil capable of supporting a level of plant growth that is normal for a particular soil and climate. Four geographical strains of *Pochonia chlamydosporia*, a saprophytic fungus antagonistic to plant parasitic nematodes, were isolated from 48 soil samples taken from different crop-soil combinations in Karnataka, India. The strains were designated as PDBC PC, PDBC PC56, PDBC PC57 and PDBC PC69. The four isolates had morphological and molecular (with respect to the ß-tubulin gene) similarity, but differed significantly in their preferences for pH and temperatures for spore germination, mycelial growth, time taken for apparent completion of sporulation and spore production on a corn meal agar medium. The four isolates preferred near neutral pH (6.5-7.7) and moderate temperatures (25-35 ºC) for practically important features, viz., spore germination, mycelial growth and spore production and yield. All four isolates showed great pathogenicity (68-78%) to the eggs of *Meloidogyne incognita* under *in vitro* conditions. PDBC PC56 and PC57 were the most pathogenic (up to 78% egg infection), with the greatest spore yields and least time for sporulation. Identification and quantification of variability of the isolates of the bio-agent with respect to specific features are important for their use under differing agro-climatic conditions and mass production systems. This is the first report to catalogue biodiversity of *P. chlamydosporia* in Karnataka and of studies on the variability of isolates.

Key words: Biological control, *Meloidogyne incognita*, pH, root-knot nematodes, temperature.

Soil health has been defined as "the capacity of the soil to function as a vital living system within ecosystem and land use boundaries, to sustain plant and animal production, maintain or enhance water and air quality and promote plant and animal health" (Doran and Zeiss, 2000). Among the various biotic factors, the abundance, diversity and functioning of soil organisms are key indicators of soil health.

Soil-borne diseases are becoming increasingly problematic in soils that have been cultivated intensively for decades. Such soils lose much of their microbial diversity and biological buffering capacity, which leads to the disappearance of many competitors of microbial pathogens and root feeding nematodes. Thus, cataloguing and conserving the beneficial native soil biota play; a vital role in maintaining a healthy, productive soil capable of supporting a level of plant growth that is normal for a particular soil and climate.

Pochonia chlamydosporia Zare, Gams *et* Evans (formerly *Verticillium chlamydosporium*, Goddard), first reported as a parasite of nematodes by Wilcox and Tribe (1974), belongs to a small group of opportunistic soil-borne fungi that are commonly associated with nematode eggs (Rodriguez-Kabana and Jones, 1988), parasitic to nematode eggs (Morgan-Jones *et al.*, 1983; Morgan-Jones *et al.*, 1984) and distributed in soils worldwide (Domsch *et al.*, 1980). *Pochonia chlamydosporia* is an excellent candidate for biological control of root-knot and cyst nematodes primarily, but also reniform, citrus, burrowing and other nematodes. The fungus is commonly isolated from soil and is able to survive and proliferate in the plant rhizosphere (de Leij and Kerry, 1991). Field efficacy of the isolates depends not only on the plants and their roots, the nematode host and soil biotic and abiotic factors but also on the inherent variability in geographical isolates (de Leij *et al.*, 1993; Bourne *et al.*, 1996). Further, the success of antagonistic fungi for commercial use depends on their amenability for commercial production and formulation, and their inherent antagonistic potential, in which geographical isolates are expected to differ.

In this paper, the results from studies of molecular identity, morphology and variability of four geographical isolates of *P. chlamydosporia* are presented. Our approach involved establishment of the morphological and molecular identity (based on primers designed to amplify a section of the ß-tubulin gene), examination of the variability in pathogenicity against eggs of the root-knot nematode *Meloidogyne incognita* (Kofoid *et* White) Chitw., and assessment of the preferences for temperature and pH of the isolates under *in vitro* conditions.

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MATERIALS AND METHODS

Selective isolation of the fungal isolates

Soil samples were collected from 48 locations known to be infested severely with root-knot and reniform nematodes on arecanut/betelvine, carnation, tomato and grapevine farms around Bangalore, Kolar and Sirsi in Karnataka, India. Soil samples were air-dried overnight at ambient room temperature (28-34 °C) and *P. chlamydosporia* was isolated from them as described by Stirling *et al.* (1998). One gram of the air-dried soil from each sample was mixed with 10 ml of sterile distilled water in a sterile test tube and vortexed for 5 minutes to dislodge the spores from soil particles. The resulting soil suspension was then diluted to a 10^{-3} concentration. An aliquot of 1 ml was transferred to a sterile Petri plate, followed by pouring 15 ml of *P. chlamydosporia* semi-selective corn meal agar (CMA) medium (Kerry *et al.*, 1993; de Leij and Kerry, 1991) and incubated at 28 ± 1 ºC for 10 days. Pure cultures were established by transferring individual fungal colonies resembling the description of *P. chlamydosporia* colonies to Petri plates containing sterile 2% CMA medium. Further identification was made on the basis of fungal morphology at 40-60× magnification (Tribe, 1977; Domsch *et al.*, 1980; Gams, 1988). Four geographical isolates of *P. chlamydosporia* obtained from the soil samples taken from different agro-climatic situations were designated as PDBC PC, PDBC PC56, PDBC PC57 and PDBC PC69 and deposited at the Technical Documentation Cell, PDBC, Bangalore.

Maintenance of isolates for experimental use

The four isolates of *P. chlamydosporia* were grown on 2% CMA medium at 28 ± 1 °C for 3 weeks, until sporulation was completed. Each of the isolates was inoculated into 0.5 ml of sterile potato dextrose (PD) broth in 1.5 ml Eppendorf tubes separately and incubated at 28 ± 1 °C for 7 days, when 0.5 ml of autoclaved glycerol was added to each tube. These tubes were maintained at -40 °C as stock. Another set of pure cultures of the isolates were maintained on CMA in Petri plates at 4 °C and used for further experiments.

Infection of root-knot nematode

Roots of tomato that had been inoculated with a single egg mass of *M. incognita* were cut into 1-2 cm sections and incubated in 1% sodium hypochlorite for 10 minutes to release the eggs of the nematode. About 100 ml of sterile water was added to the egg suspension, which was then sieved through 60 µm and 25 µm aperture sieves. Eggs were finally collected in a glass beaker in about 50 ml water. Egg masses from monoxenic cultures of *M. incognita* were dissected axenically from roots and placed in 10 ml of 1% NaOCl for 1 minute to dissolve the mucilaginous substance and release the eggs into the suspension. The eggs were then immediately washed on a sieve with large volumes of water. The final egg suspension was transferred immediately to -4 °C for 2 hours, followed by rapid thawing at 37-40 °C. This treatment did not kill the eggs but prevented their hatching during the pathogenicity studies. Aliquots of 50 µl of the nematode egg suspension, averaging 100 eggs per aliquot, were then transferred to Petri plates containing 1% water agar medium pre-colonized with *P. chlamydosporia* at 10 µl of a 10^5 conidia/ml suspension of the *P. chlamydosporia* isolates. After inoculation, four plates per isolate were incubated at 28 ± 1 °C in the dark and were scored daily over 7 days for fungal infection. Eggs surrounded by dense fungal colonies were classified as infected and the percentage of egg infection was then calculated. An overall percentage of infection per fungal isolate was calculated as the average of percentages scored on four plates.

Production of fungal biomass for DNA extraction and PCR studies

Mycelial biomass of each isolate of *P. chlamydosporia* was produced in 100 ml of PD broth in sterile 250 ml Erlenmeyer conical flasks as shaken cultures. The inoculated flasks were incubated in an orbital shaker (Scigenics) at 28 ± 1 ºC and 120 rpm for 7 days. After 7 days of growth, the aqueous phase was drained and the mycelial pellets were harvested by filtering the semi-solid phase on sterile Whatman filter paper. The mycelial mat was then lyophilized (Lyolab 2000, Heto Holden).

DNA extraction. Genomic DNA from pure cultures of each isolate of *P. chlamydosporia* was extracted by the CTAB method (Mauchline *et al.*, 2002). Two hundred mg of lyophilized mycelium were collected and ground to fine powder with liquid nitrogen in a sterile and pre-chilled pestle and mortar. The samples were then thoroughly mixed with 4 ml of extraction buffer (50 mM Tris HCl pH 8.0, 100 mM EDTA, 850 mM NaCl, 1% sodium dodecyl sulphate), followed by the addition of 0.4 ml of 10% CTAB in 0.7 M NaCl and incubated at 65 ºC for 15 minutes; this was extracted with 0.5 volume of Tris-HCl saturated phenol (pH 8.0) followed by 0.5 volume of chloroform-isoamyl alcohol (24 : 1) and an equal volume of chloroform-isoamyl alcohol. DNA was then precipitated with 2.5 volumes of 100% ethanol, rinsed with ice-cold 70% ethanol and dissolved in 4 ml of TE buffer (100 mM Tris, pH 8.0, 1 mM EDTA, with Rnase at 10 mg/ml). The DNA sample was diluted with TE buffer checked for purity and used for PCR reactions.

DNA analysis for purity. The DNA from each isolate of *P. chlamydosporia* was checked for purity by determining absorbance at 260 nm and 280 nm, followed by gel electrophoresis. Three µl of the DNA sample in 297 µl TE buffer was transferred to a 0.5 ml capacity quartz cuvette (optically matched with a reference cuvette) and absorbance was determined at 260 nm and 280 nm. The value of A260/280 was calculated. For electrophoresis, 5 µl of DNA was added to 2.5 µl of bromophenol dye [0.25% bromophenol blue (w/v) + 0.25% xylene cyanol FF in 30% (v/v) glycerol in water] mixed thoroughly,

and loaded into wells in 0.8% agarose gel. The gel was run using a BioRad electrophoresis system at a voltage of 60 volts for 3 hours. After the run was complete, the gel was viewed under UV light to detect the DNA.

PCR conditions. DNA samples extracted from the mycelia of *P. chlamydosporia* isolates were tested with a primer set designed to amplify a fragment of the *P. chlamydosporia* β-tubulin gene (Mauchline *et al.*, 2002). These primers are specific for *P. chlamydosporia* and do not amplify DNA from other species of *Verticillium/ Pochonia* or from members of other fungal genera (Hirsch *et al.*, 2000; Mauchline *et al.*, 2002). The primer sequences were as follows: tub1f, 5'-TTT GCA GTA TCT CAG TGT TC-3'; and tub1r, 5'-ATG CAA GAA AGC CTT GCG AC-3'. The PCR mixtures (20 µl) contained 1 µl of sample, each primer at a concentration of 0.1 µM, 1µ PCR buffer (1.5 mM Mg^{2+}; Bangalore Genei, Bangalore, India), 1 mM $MgCl_2$, each deoxynucleoside triphosphate (Genetix) at a concentration of 0.2 mM, 1 U of *Taq* polymerase (Bangalore Genei, India), and 13 µl of PCR grade water. The thermocycling conditions were as follows: 95 °C for 1 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension step of 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide (0.5 µg ml^{-1}). Product sizes were estimated by comparison with a 500 bp DNA ladder (Bangalore Genei, India). Four sequences were compared, based on Clustal analysis using DNASTar software, with the other available gene bank accessions of *P. chlamydosporia*, viz., AJ012713 (Hirsch *et al.*, 2000) and AY646242 (Naik *et al.*, 2003), available in the National Center for Biotechnology Information (NCBI), Maryland, U.S.A.

Effect of temperature and pH on growth parameters

Pure cultures of the four isolates of *P. chlamydosporia* were grown on 2% CMA medium at 28 ± 1 °C for 3 weeks, until sporulation was completed. The conidiospores were then obtained by gently washing the culture plates with 5 ml of sterile distilled water containing 0.05% Tween 60 and the suspensions collected were designated as stock suspensions of each isolate. The concentration of conidiospores in each was counted using a Naeubeur haemocytometer. The spore count was adjusted to about 100 spores (conidia) per 100 µl by dilution and checked with a haemocytometer.

Effect of temperature on the germination of conidiospores and mycelial growth. One hundred µl of stock suspension containing approximately 100 conidia was pipetted into Petri plates containing sterile 2% CMA medium at pH 7.1 and incubated at 15 ± 1, 20 ± 1, 25 ± 1, 30 ± 1, 35 ± 1 or 38 ± 1 °C in a BOD incubator. Four replicates were maintained for each temperature. After 7 days, individual mycelial colonies were counted in each Petri plate and spore germination was estimated.

Effect of temperature on radial mycelial growth. Agar discs of 0.9 cm diameter, taken from the edge of freshly grown cultures of each of the four isolates of *P. chlamydosporia*, were placed at the centre of plates (9 cm diameter) containing 2% CMA at pH 7.1, and incubated at 15 ± 1, 20 ± 1, 25 ± 1, 30 ± 1, 35 ± 1 or 38 ± 1 °C for 14 days. Four replicates were maintained for each temperature. Radial growth was measured after 14 days.

Effect of temperature on apparent completion of sporulation and spore yield. All four isolates of *P. chlamydosporia* were grown individually on 2% CMA at 15 ± 1, 20 ± 1, 25 ± 1, 30 ± 1, 35 ± 1 and 38 ± 1 °C in a BOD incubator until they completed sporulation. The number of days taken by each isolate for apparent completion of sporulation at each temperature was recorded. Inoculated substrates were examined in a laminar flow cabinet at 24-hour intervals after the fifth day from inoculation to record the duration of sporulation (initiation to completion). Apparent completion of sporulation was estimated by (i) visually observing when the mycelial mat (of the *P. chlamydosporia* isolates) on the substrates turned completely to dirty white colour with no fresh growth of mycelium on the surface, and (ii) observing mycelial samples on a glass slide to detect conidiospores under a microscope (100×). To record the numbers of spores per Petri plate of each isolate, 5 ml of sterile distilled water with 0.05% Tween 60 was added to each Petri plate and spores were collected as described earlier.

Effect of pH on spore germination, mycelial growth and sporulation. A citric acid-disodium hydrogen phosphate buffer was used to establish a range of pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0). Double-strength CMA and an equal volume of the buffer were autoclaved separately and mixed after sterilization, when the pH was checked. The pH of the CMA medium in the various mixtures was recorded as 5.5, 6.1, 6.6, 7.2, 7.7, 8.1, 8.8 and 9.2. Conidiospore germination and radial mycelial growth were measured as explained above and spore yield and the time taken for apparent completion of sporulation of the four isolates of *P. chlamydosporia* were compared on the CMA with adjusted pH. All of the studies included four replicates of each treatment.

Statistical analysis

Four replicates were used in all treatments and the mean values were subjected to analysis of variance. The mean values of spore yield were log (base 10) transformed for analysis. The experimental design was completely randomized.

RESULTS

Variation in geographical isolates of *P. chlamydosporia* – morphology, pathogenicity and molecular identification

In the present study, soil samples rather than infect-

Table I. Details of isolates of *P. chlamydosporia,* their morphological characters, pathogenicity and accession numbers.

Isolate	Identity designated	Details of soil sample			Fungal characteristics						
		Location and number of samples screened	Crop and nematode	* Soil type, pH, organic carbon (OC)	Colony on CMA [1]	Pigment in medium	Phialid	Conidia	Chlamydospores	Pathogenicity to eggs of *M. incognita*	NCBI [2] accession number
1	PDBC PC	Bangalore, Karnataka (14 locations)	Grapevine (Root-knot, reniform)	Laterite, pH: 7.8 OC: 1.92%	Creamy white	Light yellow	Slender and long, tapering distally.	Ovoid, smooth, in clusters on aerial mycelia.	Present	70 (66-74)	AY593965
2	PDBC PC56	Kolar, Karnataka (6 locations)	Tomato (Root-knot, reniform)	Laterite pH: 6.8 OC: 2.16%	Creamy white	Dark yellow	Slender and long, tapering distally.	Ovoid, smooth, abundant on aerial mycelia.	Present	68 (66-70)	AY603497
3	PDBC PC57	Sirsi, Karnataka (20 locations)	Arecanut/ betelvine intercrop (Root-knot)	Red pH: 6.2 OC: 2.58%	Yellowish white	Light yellow	Slender and long, tapering distally.	Ovoid, smooth, abundant on aerial mycelia.	Present	76 (74-78)	AY642328
4	PDBC PC69	Polyhouse, Bangalore Karnataka (8 locations)	Carnation (Root-knot)	Sandy loam pH: 7.4 OC: 2.16%	Creamy white	Absent	Slender and long, tapering distally.	Ovoid, smooth, in clusters on aerial mycelia.	Present	72 (70-74)	DQ417603

* Characteristics of soil samples that yielded respective isolates of *P. chlamydosporia.*

[1] CMA = Corn meal agar.

[2] NCBI = National Center for Biotechnology Information, Maryland, USA.

ed/diseased propagules of nematodes and roots were chosen for the isolation of *P. chlamydosporia* in order to study the occurrence of the fungus in different crop-soil conditions, due to the fact that infected eggs and egg masses of root-knot and reniform nematodes perish rapidly in soils and the difficulty of finding typically infected propagules at an appropriate time (in contrast to cyst nematodes in field samples). Of the soil samples collected from 48 locations, four samples from four geographically different locations yielded isolates of *P. chlamydosporia* (Table I). The four isolates of *P. chlamydosporia* obtained from different crop-soil conditions of Karnataka, India, were designated as PDBC PC (from grapevine: red loamy soil, pH 7.8, organic carbon 1.92%), PDBC PC56 (from tomato: laterite, pH 6.8, organic carbon 2.16%), PDBC PC57 (from arecanut/ betelvine intercrop: red, pH 6.2, organic carbon 2.58%) and PDBC PC69 (from carnation in commercial polyhouses: laterite, pH 7.4, organic carbon 2.16%). The isolates are maintained as glycerol stocks at -40 °C at the Nematode Biocontrol Laboratory, PDBC, Bangalore.

The four geographical isolates of *P. chlamydosporia* were found to be morphologically similar with respect to colony characteristics on growth medium, conidiospores, mycelia and pigmentation in the medium. Mycelia were thin and creamy white (PDBC PC, PDBC PC57 and PDBC PC69) to yellowish white (PDBC PC56) in appearance (Table I); conidiophores were branched, 4-5 as terminal whorls; phialids were slender, tapering distally; conidia were ovoid, smooth, in clumps and prominent; chlamydospores were abundant and secreted pigmentation in the medium.

In vitro evaluation of the four geographical isolates for pathogenicity revealed that all were pathogenic to the eggs of root-knot nematodes and caused mummification of eggs. There were significant differences between the isolates in terms of per cent of eggs parasitized (Table I). The average percentage of eggs parasitized was 66-78%. A maximum mean parasitization of 76% of the eggs was recorded for isolate PDBC PC57, followed by 72, 70 and 68% for PDBC PC69, PDBC PC and PDBC PC56, respectively.

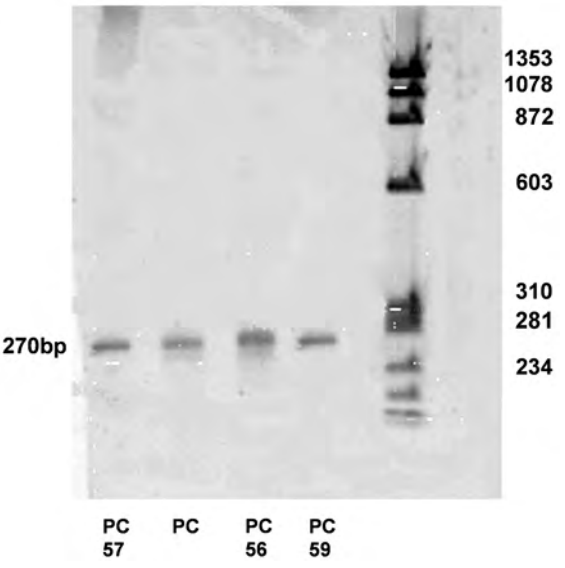

Fig. 1. PCR amplification of ß-tubulin gene for identification of the PDBC isolates of *P. chlamydosporia*.

The results from PCR studies showed that all four geographical isolates of *P. chlamydosporia* contained the ß-tubulin gene, based on the presence of a band at 270 bp (Fig. 1). The sequences of PCR amplicons obtained from each isolate were deposited at the international genbank, NCBI, Maryland, USA. *Pochonia chlamydosporia* isolates, PDBC PC, PDBC PC56, PDBC PC57 and PDBC PC69 were assigned the accession numbers AY593965, AY603497, AY642328 and DQ417603, respectively (Table I). The four geographical isolates of *P. chlamydosporia* (PDBC isolates) exhibited a similarity index of >98% among themselves and the isolate of Hirsch *et al.* (2000), and 94-97% with IIHR isolate VC1 (Naik *et al.*, 2003) based on ß-tubulin gene sequence (Table II).

Table II. Sequence pair distances of PDBC isolates and other isolates of *P. chlamydosporia* reported from India and abroad using the J. Hein method with weighted residue weight table (DNA Star).

		Percentage Identity								
		1	**2**	**3**	**4**	**5**	**6**		Isolates	NCBI No.
Divergence	**1**		98.3	98.6	95.2	98.3	98.4	**1**	PDBC PC	AY593965
	2	1.7		99.6	95.5	98.8	97.4	**2**	PDBC PC56	AY603497
	3	1.4	0.4		97.3	98.2	99.4	**3**	PDBC PC57	AY642328
	4	4.9	4.7	2.7		95.5	94.6	**4**	IIHR VC1	AY646242PC*
	5	1.8	1.3	1.8	4.7		98.9	**5**	VC**	AJ012713**
	6	1.6	2.6	0.6	5.6	1.1		**6**	PDBC PC69	DQ417603
		1	**2**	**3**	**4**	**5**	**6**			

* Naik *et al.*, 2003.
** Hirsch *et al.*, 2000.

Table III. Effect of temperature on spore germination and radial mycelial growth of PDBC isolates of *P. chlamydosporia*.

P. chlamydosporia	Spore germination (%) at 7 days of incubation						Radial mycelial growth (cm) at 14 days of incubation					
Temperature (ºC):	15±1	20±1	25±1	30±1	35±1	38±1	15±1	20±1	25±1	30±1	35±1	38±1
PDBC PC	20	38	68	100	80	54	1.8	3.6	5.8	8.9	8.8	4.0
PDBC PC56	14	30	72	100	72	34	1.6	2.4	5.4	9.0	9.0	4.0
PDBC PC57	24	48	86	100	84	50	2.4	4.0	6.6	9.0	9.0	3.8
PDBC PC69	18	36	82	100	80	48	1.8	3.8	6.2	9.0	9.0	4.0
F-Test	S	S	S	NS	S	S	S	S	NS	NS	NS	NS
S EM	0.3622	0.4201	0.6789	-	0.6678	0.5426	0.0391	0.0646	0.3977	0.1460	0.6378	0.4859
CD (P = 0.05)	1.1585	1.3438	2.1716	-	2.1360	2.8945	0.1250	0.2065	-	-	-	-

S: Significant at 5%.
NS: Not significant at 5%.

Table IV. Effect of temperature on apparent completion of sporulation and conidiospore production/Petri plate of PDBC isolates of *P. chlamydosporia*.

P. chlamydosporia	Apparent completion of sporulation (days)						Log_{10} values of conidiospore production/Petri plate					
Temperature (ºC):	15±1	20±1	25±1	30±1	35±1	38±1	15±1	20±1	25±1	30±1	35±1	38±1
PDBC PC	28	28	21	18	16	10	3.4472	5.6628	6.5051	6.7993	7.6812	5.3424
PDBC PC56	28	26	21	18	15	10	3.3868	5.2812	6.8267	7.2836	8.3444	5.6435
PDBC PC57	27	24	20	18	15	10	3.8808	5.9850	7.2041	7.4771	8.3010	5.1559
PDBC PC69	28	26	22	18	16	10	3.7404	5.7709	6.8513	7.1139	8.3424	5.4914
F-Test	NS	S	NS	NS	NS	NS	S	S	S	S	S	S
S EM	0.6124	0.449	0.6455	0.4564	0.4564	0.5401	0.0571	0.0188	0.0152	0.0300	0.0721	0.0488
CD (P = 0.05)	-	1.3737	-	-	-	-	0.1825	0.0603	0.0485	0.0960	0.2307	0.1561

S: Significant at 5%.
NS: Not significant at 5%.

Effect of temperature on growth parameters

Spore germination. Conidiospores of all four isolates of *P. chlamydosporia* germinated at 15 ± 1, 20 ± 1, 25 ± 1, 30 ± 1, 35 ± 1 and 38 ± 1 °C (at pH 7.1 on CMA), although the germination (%) was low at the extreme temperatures (15 ± 1 and 38 ± 1 °C) (Table III). There were significant differences in spore germination among the isolates at all temperatures except at 30 ± 1 °C. Spore germination was less than 50% at 15 ± 1 and 20 ± 1 °C, and more than 50% at 25-35 °C with a maximum of 100% at 30 ± 1 °C, indicating that the temperature optima for all four isolates for spore germination were between 25 ± 1 and 35 ± 1 °C.

Radial mycelial growth. Radial mycelial growth was normal for all four isolates of *P. chlamydosporia* between 15 ± 1 and 38 ± 1 °C, with a maximum growth of 8.8-9.0 cm diameter at 30 ± 1 and 35 ± 1 °C on CMA at pH 7.1. Radial mycelial growth among the isolates significantly differed at 15 ± 1 and 20 ± 1 °C, but not at more than 25 ± 1 °C. At 38 ± 1 °C, radial mycelial growth of all isolates was only 40-45% of that 35 ± 1 °C (Table III). Isolate PDBC PC57 recorded greater growth than the other isolates at 15 ± 1 and 20 ± 1 °C.

Apparent completion of sporulation. There was no significant difference in the number of days for completion of sporulation among the four geographical isolates of *P. chlamydosporia* under study at all the temperatures excepting 20 ± 1 °C (Table IV). The greatest number of days (24-28) to complete sporulation was observed at 15 ± 1 and 20 ± 1 °C, while at 38 ± 1 °C the isolates needed only 10 days to complete sporulation. As the temperature increased from 15 ± 1 to 38 ± 1 °C, the number of days for apparent completion of sporulation decreased (28 to 10 days). Although the time taken for apparent completion of sporulation was least (10 days) at 38 ± 1 °C for all four isolates, this temperature was not considered favourable as other characteristics, viz., spore germination, spore yield, radial mycelial growth, were comparatively poorer than at 35 ± 1, 30 ± 1 and 25 ± 1 °C on CMA at pH 7.1.

Spore production. Conidiospore production on CMA per Petri plate, expressed in log (base 10) values, was normal between 15 ± 1 and 38 ± 1 °C, with the greatest values at 35 ± 1 °C followed by 30 ± 1 °C and 25 ± 1 °C (Table IV). There were significant differences in conidiospore production on CMA among the four isolates at all the temperatures. Greatest production of conidiospores in all four isolates was at 35 ± 1 °C followed by 30 ± 1 and 20 ± 1 °C. As observed for spore germination and radial mycelial growth, isolate PDBC PC57 recorded a greater yield of conidiospores at temperatures of 15 ± 1, 20 ± 1, 25 ± 1 and 30 ± 1 °C compared to the other three isolates, but a lower spore yield at 38 ± 1 °C.

Effect of pH on growth parameters

Behaviour of the four geographical isolates of *P. chlamydosporia* in terms of spore germination, radial mycelial growth, spore yield and sporulation at different pHs was examined at 30 ± 1 °C on CMA.

Spore germination. There were significant differences in spore germination among the isolates at pHs of 5.5, 6.1, 6.6, 8.2, 8.8 and 9.2. The isolate PDBC PC57 showed the greatest spore germination (45, 60 and 100%) at pHs of 5.5, 6.1 and 6.6, respectively, and least at pHs 8.8 and 9.2 compared to the other three isolates (Table V). Spore germination of each isolate was normal between pH 6.1 and pH 9.2 through pHs 6.6, 7.2, 7.7, 8.2 and 8.7. Spore germination at the extremes of pH (<6.1 and 9.2) was lower than that at other pHs in all the isolates of *P. chlamydosporia* under study. The greatest spore germination (100%) was at pHs 7.1, 7.7 and 8.2.

Radial mycelial growth. There were significant differences of radial mycelial growth among the four isolates of *P. chlamydosporia* at pHs of 6.1, 6.6, 7.1, 7.7, 8.2 and 9.2, but not at 5.5 and 8.8 (Table V). Mycelial growth was greatest in all four isolates at pHs 7.7 and 8.2. Among the four isolates, PDBC PC57 showed greater mycelial growth than the other three isolates at pHs 5.5, 6.1, 6.6, 7.1 and 7.8, and less at pHs 8.8 and 9.2.

Apparent completion of sporulation. There were significant differences in number of days for apparent completion of sporulation among the four isolates at all pHs except for 7.1 and 7.7 on CMA at 30 ± 1 °C (Table VI). All four isolates took the greatest number of days (20-23) for completion of sporulation at the extreme pHs (5.5 and 9.2), and the least number (15-16) at pHs 7.1 and 7.7. At pHs 6.6 and 7.1, isolate PDBC PC57 took less days than other isolates for completion of sporulation.

Conidiospore production. The four isolates of *P. chlamydosporia* differed significantly in conidiospore production (log values) at all pHs except 7.1. At pH 7.1 there was no significant difference in spore production among the four isolates (Table VI).

DISCUSSION

Detection of the four isolates of *P. chlamydosporia* from different crop-soil-geographic situations with different soil types, pH and organic carbon demonstrated the wide occurrence of *P. chlamydosporia* in different soils and crop rhizospheres. The number of locations from which we detected *P. chlamydosporia* was low (4 out of 48) and a more intensive survey might yield a higher number of isolates. Although the isolations were made from soils of different pH and carbon content under natural conditions, the pH preferences of the iso-

Table V. Effect of pH on spore germination and radial mycelial growth of PDBC isolates of *P. chlamydosporia*.

P. chlamydosporia isolate	Spore germination (%) at 7 days of incubation								Radial mycelial growth (cm) at 14 days of incubation							
pH:	5.5	6.1	6.6	7.1	7.7	8.2	8.8	9.2	5.5	6.1	6.6	7.1	7.7	8.2	8.8	9.2
PDBC PC	20	50	80	100	100	100	60	44	1.6	1.6	3.4	5.8	7.6	6.8	5.0	3.6
PDBC PC57	30	58	70	100	100	100	68	44	2.0	2.4	3.6	6.9	9.0	8.2	4.8	2.2
PDBC PC56	45	60	100	100	100	90	54	20	1.8	2.0	3.0	6.0	8.6	8.0	5.0	3.0
PDBC69	30	50	66	100	100	100	60	26	1.6	1.8	3.2	6.6	8.8	8.8	5.0	3.6
F-Test	S	S	S	NS	NS	S	S	S	NS	S	S	S	S	S	NS	S
S EM	0.7758	0.4774	0.8077	-	-	0.4380	0.3838	0.5376	2.1437	0.0565	0.0312	0.0412	0.0565	0.0677	-	0.0697
CD (P = 0.05)	2.4815	1.5269	2.5836	-	-	1.41010	1.2276	1.7196	-	0.1808	0.0997	0.1410	0.1807	0.2166	-	0.2230

S: Significant at 5%.
NS: Not significant at 5%.

Table VI. Effect of pH on apparent completion of sporulation and conidiospore production/Petri plate of PDBC isolates of *P. chlamydosporia*.

P. chlamydosporia	Apparent completion of sporulation (days)								Log_{10} values of conidiospore production/Petri plate							
pH:	5.5	6.1	6.6	7.1	7.7	8.2	8.8	9.2	5.5	6.1	6.6	7.1	7.7	8.2	8.8	9.2
PDBC PC	21	18	16	16	16	19	19	22	2.9823	3.8751	6.6335	7.6628	7.5682	6.5784	5.7878	3.4842
PDBC PC56	22	20	18	16	15	16	16	23	3.6989	4.9590	7.2950	7.9294	8.3617	6.3844	5.4448	3.0185
PDBC PC57	20	18	15	15	16	18	19	22	3.7924	4.7634	7.3879	7.7782	8.6021	5.8774	4.6872	2.3989
PDBC PC69	20	20	18	16	16	16	18	21	3.4990	4.9731	7.1353	7.9912	8.6532	6.8974	5.5464	3.4520
F-Test	S	S	S	NS	S	S	NS	S	S	S	S	NS	S	S	S	S
S Em	0.4082	0.5000	0.4208	0.4564	0.3536	0.4564	0.4564	0.4208	0.0348	0.0775	0.0681	0.1239	0.1058	0.0923	0.1031	0.0844
CD (P=0.05%)	1.2579	1.5406	1.2966	-	1.0893	1.4063	-	1.2964	0.1112	0.2480	0.2177	-	0.3385	0.2466	0.3298	0.2268

S: Significant at 5%.
NS: Not significant at 5%.

lates under *in vitro* conditions (CMA, incubation temperature of 30 ± 1 °C) were similar, i.e., maximum radial mycelial growth was observed between pH 7.7 and 8.8. The morphological characters of our isolates matched with the taxonomic description of *P. chlamydosporia* (Tribe, 1977; Domsch *et al.*, 1980; Gams, 1988), confirming their identity. All the PDBC isolates produced thick walled chlamydospores in culture as observed by Zare *et al.* (2001), a feature identified as an essential requirement for *P. chlamydosporia* to be effective in the field (Kerry and Jaffee, 1997; Bernabeu and Lopez-Llorca, 2002). The pathogenicity of the four PDBC isolates on *M. incognita* eggs ranged from 66-77% in the given set of test conditions of 28 ± 1 °C on CMA for 7 days. The data on the pathogenicity of these isolates is likely to be realistic as the estimation was done on eggs that were inactivated through freezing and thawing at mild temperatures, unlike previous studies where per cent parasitization was observed in eggs that were inactivated through heating at higher temperatures. Stirling and Mankau (1978) and Freire and Bridge (1985) reported increased colonization by *Dactylella oviparasitica* Stirling *et* Mankau and *V. chlamydosporium* (= *P. chlamydosporia*), respectively, on eggs of *M. incognita* killed by heat, while Irving and Kerry (1986) reported that dead and immature eggs were those most readily colonized by six strains of *V. chlamydosporium* (= *P. clamydosporia*). Irving and Kerry (1986) suggested that the *in vitro* screening of isolates for pathogenicity against nematode eggs that were heat-killed would give higher levels of parasitization due to alterations in properties of egg shells due to heating. Further, Bernabeu and Lopez-Llorca (2002) reported that the pathogenicity to *M. javanica* (Treub) Chitw. eggs of seven strains of *P. chlamydosporia* isolated from Spanish soils was close to 70-80%.

Kerry *et al.* (2004) were of the opinion that *P. chlamydosporia* demonstrated nematode-host preferences at the sub-specific level and isolates of *P. chlamydosporia* grouped more strongly on the basis of their original host rather than by their geographic origin. Morton *et al.* (2003) observed host-related genetic variation in VCP1 (an alkaline serine protease) between isolates of *P. chlamydosporia* from different nematode hosts, suggesting that such differences were important in exploitation of *P. chlamydosporia* as a nematode biocontrol agent.

Our results have indicated that the agricultural soils of Karnataka contain strains of *P. chlamydosporia*, which were observed to be morphologically and genetically similar. The strains exhibited growth and virulence differences at different pHs and temperatures, in terms of spore production, mycelial growth and pathogenicity. The geographical isolates with a high degree of pathogenicity (>70%) under *in vitro* conditions have potential as bio-agents against economically important root-knot, cyst and reniform nematodes. This is the first effort to catalogue the biodiversity of *P. chlamydosporia* in Karnataka and study the behavioural variability among the isolates. Based on spore yield and number of days for sporulation under *in vitro* controlled conditions on CMA at different temperatures and pHs, PDBC isolate PC57 followed by PC56 were comparatively more superior.

Establishment of the identity and cataloguing the variability in isolates of beneficial organisms, in terms of their biomass production, enzymatic contents and activities, and pathogenicity on different media and in different crop-soil conditions, are essential for successful exploitation of these organisms, both in terms of mass production and ease of field application.

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