

Appendix 1 Methodology for studies on *Pochonia chlamydosporia* isolates (Rothamsted Research)

Compare different isolates of the biological control agents being deployed

Culture maintenance

Cultures of *P. chlamydosporia* from infected nematode eggs in soil from different parts of the world have been freeze-dried and retained at Rothamsted Research, prior to use. Eight isolates (five collected from cyst nematode eggs and three from root-knot nematode eggs) were grown on 1.7% corn meal agar (Oxoid, Basingstoke, UK) at 25°C prior to use. Chlamydospores used as inoculum were produced in 500 ml conical flasks containing (200 g) sterile rice. After 3 weeks incubation at 25°C, the cultures were washed through 250 and 53 µm sieves to remove the grains, and the fungal propagules, mainly chlamydospores, were collected on a 10 µm aperture sieve and mixed with coarse sand as a carrier. The concentration of chlamydospores was estimated in diluted samples using a haemocytometer. To check the viability of the chlamydospores, 1 g of the inoculum was diluted in 9 ml of a 0.05% agar solution and 200 µl of dilutions at 10⁻² and at 10⁻³ plated on 9-cm-diam Petri dishes containing sorbose (2 g l⁻¹), agar (12 g l⁻¹) and antibiotics (50 mg l⁻¹ each of streptomycin sulphate, chloramphenicol and chlorotetracycline). The proportion of germinated chlamydospores was estimated after incubation at 25°C for 2 days; germination ranged between 85-95%.

To assess the stability of the fungus in repeated culture, a selection of isolates of *Pochonia chlamydosporia* (Pc 400, Pc 280, Pc 392 and Pc132) with different characteristics that can be distinguished using molecular diagnostic tests were subcultured every week, in two different agar culture media, potato dextrose agar (PDA) and corn meal agar (CMA). Chlamydospores derived from these cultures were freeze dried and kept in vials. These spores from different repeated subcultures produced over the past 12 months (60) will be used as inoculum for tests to detect changes within isolates and therefore be used for inoculum quality assurance.

Estimation of fungal saprotrophic growth: Colonisation in soil of eight isolates of P. chlamydosporia.

Mycelial growth of the eight isolates of *P. chlamydosporia* was estimated in soil of different texture (sandy loam, loamy sand and compost). Sandy loam composed of sand : silt : clay, 67 : 19 : 14; pH 7.9, loamy sand composed of sand : silt: clay, 52 : 36: 12; pH 7.4 and compost comprised of peat : loam : vermiculite : horticultural grit, 75 : 12 : 3 : 10; pH 5.5. Petri dishes (5 cm diam) were filled with sieved (sterilised or non-sterilised) soils adjusted to 30% moisture. The soil surface was smoothed with the help of spatula and nylon mesh (30 µm aperture) was held in close contact with it by means of a plastic ring (Kerry, 1991). A 5-mm-diam disc, taken from the edge of a colony of each isolate growing on corn meal agar, was placed at the centre of the mesh and soil carefully added to the depth of the ring which was then covered with the Petri dish lid. The dishes were placed in a moist chamber and incubated at 18°C for four weeks. The soil and original inoculum (fungal disc) were removed from the nylon mesh, which was then lifted and any remaining soil aggregates carefully shaken off. The mesh was pressed onto a selective medium (de Leij and Kerry, 1991) and removed. The plates were incubated for 15 days at 18 °C and the number of colony forming units (cfu's) estimated. To determine the level of spread of fungal isolates, the plates were divided into three sections (designated as A, B, C, respectively from the source of inoculum to the edge of the plate) by making grids at equal distances (1.66-cm-diam.) and numbers of cfu's on each section were estimated.

Estimation of fungal saprotrophic growth: Colonisation of eight isolates of P. chlamydosporia in the rhizosphere

The potential of the eight *P. chlamydosporia* isolates to colonise pea and mustard rhizospheres was tested in sandy loam and compost soils. Pea (*Pisum sativum* L. var. Kelveden Wonder) or white mustard (*Sinapsis alba* L. var. Albatross) seeds were surface-sterilized using 5% NaOCl with a drop of commercial detergent, were shaken at 120 rpm for 30 min at room temperature. The seeds were then rinsed five times (5 min each) in sterile distilled water and blotted dry on sterile filter paper. Prior to planting, the soils were thoroughly mixed with inoculum (5000 chlamydospores g⁻¹ soil) of each isolate of *P. chlamydosporia* and aliquoted into 6 cm diam plastic pots (100 g soil pot⁻¹). Subsequently, three seedlings of pea or mustard were transplanted into fungus-inoculated or un-inoculated (control) soil and after five days, the seedlings were thinned to one per pot. Each treatment was replicated three times and randomised on a bench in the glasshouse (temperature, 22 °C; photoperiod, 16/8 h light/dark). The plants were harvested two weeks after transplanting. The roots with adhering soil were gently shaken and washed with tap water in a beaker. The roots were cut into 5 mm segments and 8-10 pieces were plated onto the selective medium. The plates were incubated for 15-20 days and the proportion of segments colonised by *P. chlamydosporia* was calculated.

Estimation of fungal parasitic growth: virulence of eight isolates of P. chlamydosporia against eggs of Meloidogyne spp.

Autoclaved coarse sand was added to individual wells of a 96-well microtitre plate (Sterilin; Bibby Sterilin Ltd., UK) which was overlaid with a 4-mm-diam disc of 30 µm aperture nylon mesh. A single egg mass of *Meloidogyne* spp. was inoculated on each nylon mesh and a 10 µl inoculum (8-10 chlamydospores µl⁻¹) of each of the eight isolates of *P. chlamydosporia* was inoculated on each egg mass. The multiple-well plate was placed in a chamber containing sterile distilled water to provide humidity. Each fungal treatment was replicated five times and incubated at 20°C. Egg masses without the fungal treatment served as a control. Following one week incubation, the egg masses were removed, physically crushed and eggs plated onto water agar supplemented with appropriate antibiotics, as above. The plates were incubated for three days at 20°C and the proportion of eggs infected with the fungus was calculated.

Develop rapid methods to evaluate inoculum quality of *P. chlamydosporia* to assure quality control of sequential batches of mass-produced inoculum.

Spore production evaluation test

In order to find a quick and easy test to determine changes in chlamydospore production during sub culturing, the conventional method of counting spores using a haemocytometer was compared with a new method using a spectrophotometer to read the absorbance of spore suspensions with different concentrations.

Chlamydospores from four different isolates (Pc 392, Pc 280, Pc 279 and Pc 132) were suspended in different concentrations in a range between 10⁴ and 10⁶ chlamydospores. Because chlamydospores tend to settle very quickly, two types of suspensions were tested in 0.1% Peg, and in 0.1% agar and differences evaluated. Serial chlamydospore suspensions were counted using a haemocytometer and the level of absorbance (600 nm) of the spore suspensions determined using a

spectrophotometer (CaryWin UV). The results were then compared using linear regression.

Saprophytic ability evaluation test

A simple assay aimed to reduce the time necessary to discriminate reliably differences between isolates has been developed. Sterilised maize seeds were inoculated with different isolates of the fungus (Pc10, Pc 400 and Pc 280) in two different ways (by dipping the seed in a chlamyospore suspension and by using a plug of colonised agar) and planted in pots containing approximately 250 ml of sterilised vermiculite. The spores were extracted from flasks containing rice grain media, washed with water and collected on sieves of 20 μm . For maize sterilisation, seeds were dipped in an 8% solution of sodium hypochlorite with one drop of Tween 20 and shaken in a wrist shaker for 1 hour. The seeds were then washed five times in distilled water and dried for 30 minutes inside the laminar flow cabinet.

After 4, 8, 12 and 16 days roots were taken out from the pots, cut in 1 cm sections and plated on water agar with antibiotics (0.05 g/L streptomycin sulphate, 0.05 g/L chloramphenicol and 0.05g/L chortetracycline). The number of colonised roots was determined after two days incubation at 25°C and the percentage of colonisation determined.

The method of using colonised agar plugs to inoculate seeds was quicker because spores do not need to be extracted, a procedure that requires much time. However, with this technique it was not possible to guarantee that each plug has the same quantity of fungal material and this can have a great influence on the results and caused much variation between replicates (data not shown). So, the second method where disinfected seeds were inoculated using a known chlamyospore concentration in suspension, was used to investigate differences in rate of rhizosphere colonisation by different isolates.

Virulence test

A standard virulence test was used to assess the rate of infection of eggs of *Meloidogyne incognita* added to water agar and antibiotics (50 mg l⁻¹ each of streptomycin sulphate, chloramphenicol and chlorotetracycline) plates pre-inoculated with different isolates of *P. chlamydosporia*, which were fresh or sub-cultured sixty times on a weekly basis. Each plate was treated with 0.2 ml of fungal spores 2 days before addition of c. 200 nematode eggs and incubated at 25 C for 3 days before the number of infected eggs were counted.

Baiting technique

Sandy-loam or compost soils were autoclaved and allowed to aerate for 48 h. The soils were thoroughly mixed with 5000 chlamyospores (g⁻¹ soil) of each of the eight *P. chlamydosporia* isolates and aliquoted into each cell (6 x 5 cm) of a multiple-celled plastic tray. The soils were baited with either ten *M. hapla* egg masses or ten *G. pallida* cysts held between a nylon mesh of 60- μm pore size (Lockertex) in a 35 mm photographic slide mount (Gepe) (Plate 1). The slide mounts were inserted just below the surface of the soil, and incubated at 21°C for one or four weeks for *M. hapla* egg masses or *G. pallida* cysts, respectively; the soil was watered lightly every two days. Baits were collected, and the egg masses or cysts were crushed with a glass rod, and eggs were collected by standard techniques. Eggs were plated out onto water agar containing antibiotics (0.5 g of technical agar, 50 mg each of streptomycin sulphate, chloramphenicol, and chlortetracycline per litre) and incubated at 25 °C for three days. Baits were added to soil without *P. chlamydosporia* and

incubated as above as a control, to provide the level of background infection in the eggs of both nematode species. Infected eggs were counted after three days, and the percentage of infection was calculated. The proportion of eggs infected from each soil was compared to the control. Fifty infected eggs, with mycelium growing from them, were taken from each bait experiment, and individual eggs were added to 10 ml Czapek Dox liquid medium (Oxoid, Basingstoke) and incubated at 25 °C for 2 weeks. DNA was extracted from the fungal culture as described above and subjected to PCR amplification with the *P. chlamydosporia*-specific primers *Btub1* and *Btub2*, to confirm its identification (Hirsch *et al.* 2000).

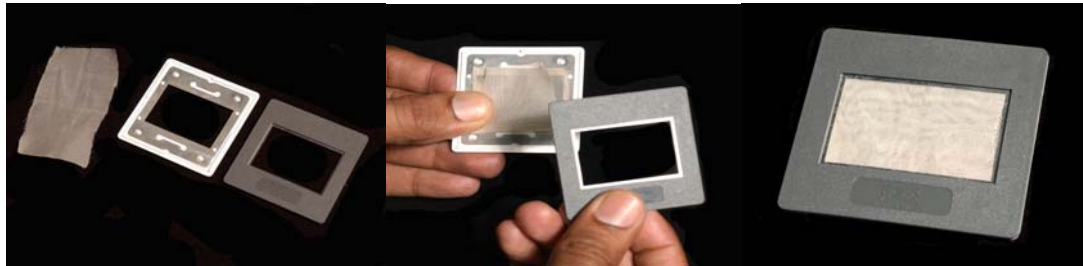


Plate 1 Simple method for baiting soil with root-knot nematode egg masses, which can be easily recovered and the extent of egg parasitism used as a measure of activity of *P. chlamydosporia* in replicate soil samples.

References

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