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IVÂNIA ESTEVES

FACTORS AFFECTING THE PERFORMANCE OF *POCHONIA CHLAMYDOSPORIA* AS A BIOLOGICAL CONTROL AGENT FOR NEMATODES

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To my grandfather,
Antonio Joaquim Grazina

ABSTRACT

The work developed in this thesis aimed to increase understanding about the variability and stability in eleven biotypes of *Pochonia chlamydosporia*, a facultative parasitic fungus with potential as a biological control agent against root-knot (*Meloidogyne* spp.), false root-knot (*Naccobus* spp.) and cyst nematodes (*Heterodera* and *Globodera*, spp.).

Differences in performance were assessed by measuring saprophytic and parasitic growth using *in vitro* bioassays. Information on virulence (*in vitro*) was collected for a range of biotypes with the objective to relate *in vitro* parasitic growth with rhizosphere colonisation ability and secretion of extracellular enzymes. Results showed differences between biotypes in their ability to colonise the rhizosphere of plants, parasitise nematode eggs and to produce a range of extracellular enzymes but no significant relationships were found between saprophytic or parasitic growth and enzyme production. For the first time, the specific activity of protease, chitinase, esterase and lipase enzyme production by eleven biotypes of the fungus was examined. Enzymatic activity was shown to vary with the biotype and type of enzyme assayed and biotypes could be ranked according to their similarities in enzyme production

A novel bioassay to estimate egg parasitism using liquid media highlighted the importance of nutrition in infection processes and suggested that all biotypes are able to infect large numbers of eggs rapidly if the conditions are favourable. The assay reliably detected fungal infection in nematode eggs within 48 hours and provided a simple, rapid assay to test the effect of specific nutrients at controlled concentrations on the infection process. Differences in infection rates between biotypes observed in previous tests on agar were not detected in the new assay in which nematode eggs and fungal conidia were added in suspension. Internal colonisation of individual whole *Meloidogyne* spp. eggs by *P. chlamydosporia* was observed using microscopy studies. The destruction of nematode eggs infected with the fungus within seven days, was confirmed.

The *in vitro* formation of appressoria was studied in a range of *P. chlamydosporia* biotypes, for the first time. Biotypes were found to differ in their ability to produce appressoria but this ability was not related to differences in virulence (*in vitro*) against nematode eggs.

In order to monitor the inoculum quality during the production of *P. chlamydosporia*, sensitive tests were developed to examine the stability of the fungus when continuously manipulated *in vitro*. Three biotypes of *P. chlamydosporia* were sub-cultured *in vitro* each week for 60 weeks and tested for their ability to produce chlamydospores, colonise roots, parasitise nematode eggs and produce a range of enzymes. The results confirmed that the performance (as a saprophyte/parasite) of selected but not all biotypes of the fungus was not affected by continuous culturing on agar, with stability observed and confirmed using different approaches.

In the last part of the thesis, the effects of osmotic and matric potential stress on the growth and accumulation of endogenous low and high molecular weight sugar alcohols were studied in three biotypes of *P. chlamydosporia*. Results revealed that mycelial extension was tolerant of both osmotic and matric stress over the range -0.7 to -7.0 MPa. Under water stress (-2.8 to -7.0 MPa), depending on the solute used (glycerol, NaCl, PEG 8000) the fungus accumulated predominantly glycerol, arabitol or erythritol, respectively. This suggested that different polyols are used by the species to tolerate ionic and matric stress for osmoregulation and enzyme activity in the environment.

The research presented in this thesis reinforced the need for careful selection of a potential biocontrol agent from the natural biodiversity that exists in soils and highlighted the possibility of improving inoculum quality by altering culture conditions. Future work on *P. chlamydosporia* should be directed towards a) studying aspects related to the basic biology of the fungus and its infection processes, b) optimising conditions for mass production of the fungus to improve inoculum quality and c) understanding the mechanism(s) responsible for the switch from the saprotrophic to the parasitic phase of the fungus using a range of molecular, physiological and biochemical techniques.

Keywords: *Pochonia chlamydosporia*, biological control, nematodes, saprophytic growth, root colonisation, parasitic growth, egg parasitism, egg infection, appressoria, enzyme activity, water stress.

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LIST OF ABBREVIATIONS

A_w: water activity

BCA (s): biological control agent(s); biocontrol agent(s)

CFU: colony forming units;

CMA: corn meal agar;

HPLC: high performance liquid chromatography

LSF: liquid state fermentation;

Mi: *Meloidogyne* spp.

PCN: potato cyst nematodes;

PCR: polymerase chain reaction;

PDA: potato dextrose agar;

PEG: polyethylene glycol

PPN: plant-parasitic nematodes;

RPM: rotations per minute;

RKN: root-knot nematodes;

SSF: solid state fermentation;

SM: semi-selective media

TCA: trichloroacetic acid;

VCP1: subtilisin-like, extracellular protease from *Pochonia chlamydosporia*;

WA: water-agar media

W/W: weight/weight;

W/V: weight/ volume;

YEM: yeast extract medium;

CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

Plant-parasitic nematodes (PPN) are an important threat to agriculture. The most economically important group are the sedentary endoparasites, which include the genera *Meloidogyne* (root-knot nematodes), *Heterodera* and *Globodera* (cyst nematodes). The use of several nematicides, particularly those products with methyl bromide as the active ingredient, have been restricted or banned in most countries, as they are extremely toxic, and hazardous in the environment, causing the depletion of the ozone layer (Thomas, 1996). Among the control measures available, biological control is regarded as a promising option, when used together with other control strategies (Whitehead, 1997). The anamorphic and facultative parasitic fungus, *Pochonia chlamydosporia* (Goddard) Zare & Gams (synonym: *Verticillium chlamydosporium* Goddard) has been associated with soils which suppress the multiplication of cyst nematode populations (Kerry *et al.*, 1982) and is seen as a potential biological control agent (BCA) against root-knot and cyst nematodes. In order to provide an efficient level of control, *P. chlamydosporia* should become established in the rhizosphere and survive, even in the absence of nematode hosts, and be able to infect (Kerry *et al.*, 1993), parasitise and consume nematode eggs (Kerry & Jaffee, 1997).

Since it was first found to be associated with the decline of PPN (Willcox & Tribe, 1974; Kerry, 1975), the fungus *P. chlamydosporia* has been extensively studied and its potential as a BCA demonstrated (Crump & Irving, 1992; Kerry, 1995; Kerry, 2001; Atkins *et al.*, 2003c; Kerry *et al.*, 2005). Recently, results from field trials in Cuba, where a native biotype of the fungus (*P. chlamydosporia* var. *catenulata,* biotype 392) was applied in a cropping cycle of tomato – tomato – cabbage – bean – tomato, the infection of root-knot nematode eggs increased from 30% to more than 80% in the initial and final susceptible tomato crops, respectively and the numbers of second-stage juveniles in the soil were decreased by more than 90 % in fungal treated soil, compared to untreated soil (Kerry *et al.*, 2005). However, the efficacy of a biological control agent is not only dependent on the ability of the organism to control the target pest. Other factors such as those involved in the production and release of the organism can affect the success of a product based on a living organism. *P. chlamydosporia* can be produced

with relatively low capital and technology inputs, which makes possible its use in resource poor countries, where pesticides are difficult or expensive to obtain. However, there are still aspects related with the production and ecology of this fungus that require research. As a facultative parasite, *P. chlamydosporia* has two important stages in its life cycle – a saprophytic and a parasitic stage. The existence of differences in saprophytic and parasitic competence between biotypes has been reported in the past (Irving & Kerry, 1986; Kerry *et al.*, 1986; De Leij & Kerry, 1991). However, the cues affecting the switch from saprophyte to parasite are still not understood (Kerry, 2001) and the importance of studying the factors affecting the performance of *P. chlamydosporia* as a BCA is, therefore, of crucial importance.

This thesis focuses on the study of saprophytic and parasitic growth of *P. chlamydosporia* in a range of biotypes and its measurement using *in vitro* bioassays. To understand differences in performance, the ability to produced enzymes is studied and its relation with parasitism/saprophytic ability is evaluated. The effects of repeated *in vitro* culture in growth and production of enzymes are also explored. In the last part of the work, the effects of water stress on growth and accumulation of endogenous reserves in spores and mycelia are investigated.

1.2 NEMATODES

Nematodes form a separate phylum of animals, the Nematoda, which is probably the second largest after insects (Dropkin, 1989). It comprises a vast and diverse group of multicellular organisms, bilaterally symmetrical pseudocoelomates, with digestive, nervous, excretory, and reproductive systems, but lack a discrete circulatory or respiratory system (Jenkins & Taylor, 1967). More than 12,000 species have been described but these represent just a small part of their estimated diversity (Wheeler, 1990). Most species are free-living in soil and water, while others are successful parasites of animals and plants.

1.2.1 **Plant-parasitic nematodes (PPN)**

Plant-parasitic nematodes are a severe constraint on agricultural production. They are responsible for annual crop losses estimated at more than 100 billion dollars each year (Sasser & Freckman, 1987) and can affect the yield of a broad range of crops.

The most economically important groups are the sedentary endoparasites, which include the genera *Meloidogyne*, *Heterodera* and *Globodera* (cyst nematodes). The genus *Naccobus* (false root-knot) is also economically important in the Americas (Manzanilla-Lopez *et al.*, 2002).

Plants severely infected by these nematodes show symptoms of nutrient deficiency; appear wilted, stunted, chlorotic or necrotic and defoliated as a consequence of the problems in absorption of water and nutrients that result from the stunted root system caused by nematode infection. Such general symptoms mean that nematode damage is often misdiagnosed as the effects of abiotic soil factors. Also, root diseases can be caused by nematodes in combination with soil organisms (diseases complex) and nematodes can be vectors of plant pathogenic virus (Mai, 1985).

Two orders contain plant parasitic nematodes: Tylenchida includes the great majority of plant parasites, and Dorylaimida includes several vectors of plant virus diseases. Root-knot and cyst nematodes belong to the order Tylenchida, suborder Tylenchina, and family Heteroderidae and have as a main characteristic their sedentary endoparasitic habit and a pronounced sexual dimorphism. Nematodes belonging to the genus *Naccobus* spp. are included in a different family denominated Pratylenchidae but also show sexual dimorphism (Dropkin, 1989).

1.2.1.1 **The genus *Meloidogyne* (root-knot nematodes)**

The genus *Meloidogyne* includes more than one hundred described species, in which *M. incognita* (Kofoid & White), *M. javanica* (Treub) Chitwood, *M. arenaria* (Neal) Chitwood and *M. hapla* Chitwood are the most economically significant (Sasser, 1980; Mai & Abawi, 1987; Roberts, 1995). This genus has a vast range of hosts of more than 2000 plant species (Roberts, 1995). These nematodes are particularly important in tropical agriculture but some species such as *M. hapla* are also damaging in temperate soils.

During their life cycle, the infective 2nd stage juvenile (J2) invades the root, primarily in the region of elongation, close to the meristematic zone and starts to migrate down the plant cortex towards the root tip. After entering the base of the vascular cylinder, the J2 starts to migrate up the root and eventually stops to establish a feeding site (Wyss *et al.*, 1992). Here, it becomes sedentary and causes the

transformation of cells upon which it feeds. These cells increase in number (hyperplasia) and size (hypertrophy) and are termed as giant cells. The formation of these giant cells is induced by a continuous stimulus from the nematode (Bird, 1979). The juvenile undergoes three moults, and matures into vermiform male, not essential for reproduction in many species, which emerge from the roots, or into a pyriform female, that remains attached to the feeding site and produces eggs throughout its adult life (Dropkin, 1989). The eggs are deposited in a gelatinous matrix produced by six rectal glands (Maggenti & Allen, 1960) and are exposed on the root surface. If the environmental conditions become favourable, the eggs will hatch and a new cycle begins. The number of generations per year varies according to the host plant and food availability, but usually *Meloidogyne* spp. has more than one generation per crop (Karssen & Moens, 2006).

1.2.1.2 **The genus *Heterodera* and *Globodera* (cyst nematodes)**

The life cycle of cyst nematodes (*Heterodera* spp. and *Globodera* spp.) has similarities with the *Meloidogyne* life cycle but cyst nematodes usually do not induce the formation of root galls. The second stage juveniles emerge from the eggs, enter the roots and move to the vascular cylinder where, after injecting stylet secretions, establish a feeding site, which formation is characterised by the destruction of the cell walls between the initial feeding site and the neighbouring cells. The result is the formation of a multinucleated syncytium (Wyss & Zunke, 1986). After feeding begins, the juvenile becomes sedentary, three moults occur until sexual differentiation occurs. The elongate and motile adult males, which are usually necessary for reproduction, emerge and leave the roots. The term "cyst nematode" refers to the female's swollen body which becomes thick and hard after death and remains in the soil as a cyst containing embryonated eggs (Dropkin, 1989). Each female can produce 200-600 eggs, most of them being retained within the body (Young, 1992).

The most important species of these nematodes are *Heterodera schachtii* on beet, *Heterodera avenae* on oats, barley and wheat, *Heterodera glycines* on soybean and *Globodera pallida* and *Globodera rostochiensis* on potato (Dropkin, 1989).

1.2.1.3 The genus *Naccobus* (false root-knot nematode)

The nematodes belonging to this genus are indigenous to the Americas and include two valid species – *Naccobus dorsalis* and *Naccobus aberrans*. The first species has minor importance because of its limited geographical distribution, only identified in USA, but *N. aberrans* is considered as a major pest of many crops in North and South America (Manzanilla-Lopez *et al.*, 2002). In Latin America this nematode is an important parasite of bean, chilli pepper, tomato, and sugar beet but the host range is much wider (Manzanilla-Lopez *et al.*, 2002). Recently *N. aberrans* has been described as a species complex, after molecular analysis of several populations (Reid *et al.*, 2003).

The life cycle of *Naccobus* is different from that of other sedentary endoparasites and begins when the second stage juveniles enter the roots. Once inside, they move intracellularly, penetrating the end and side wall of cells causing necrotic lesions, but they can also leave and re-enter the root (Clark, 1967). The 3rd and 4th stage juvenile (J3 and J4) females are motile and migrate through the roots and soil, leaving and re-entering the roots where they can induce galls by feeding upon a spindle shaped syncytium. Eggs are deposited into a gelatinous egg sac and are exposed on the root surface. The males are vermiform and leave the root to move in search of a female (Clark, 1967).

1.3 CONTROL OF NEMATODE PESTS IN TROPICAL AGRICULTURE

The correct evaluation of the scale of the problem is crucial to decide upon the strategy to apply in each situation. Proper diagnosis is essential for the appropriate use of nematode control measures (Whitehead, 1997). Methods to combat diseases caused by PPN include cultural, physical, chemical and biological control methods. The combination of several management strategies are desirable to achieve long term nematode control (Whitehead, 1997). In tropical subsistence agriculture, where chemical inputs are low and high yields are strongly desired, biological control combined with different nematode management strategies has potential to make a major contribution to the management of nematode populations (Davies *et al.*, 1991). The main features of this type of agriculture are the use of small plots of land, where a wide variety of crops are grown, with often low yields and high labour inputs. The exploitation of nematode natural enemies can include the use of methods to increase

microbial activity of indigenous flora and fauna or be achieved by the application of selected organisms as BCAs (Kerry & Hominick, 2002). Methods such as the use of soil amendments that favour microbial activity and can increase the number of nematode antagonists in soil and the rhizosphere (Sikora, 1992). To reduce nematode populations, BCAs can be integrated into crop rotation with poor and resistant crops to nematodes, or be combined with methods that favour the establishment of the BCA (Kerry & Hominick, 2002). The use of soil solarisation or steam sterilisation before application of a BCA can reduce the numbers of micro-organisms in the soil and therefore, are useful methods to avoid competition of other organisms with the introduced BCA. One of the limitations often attributed to the use of BCAs are the high rates of inoculum required to provide control. In subsistence agriculture systems the rates of application of a BCA can be high as long as the substrates for producing inoculum are cheap and locally available (Davies *et al.*, 1991).

1.3.1 Cultural, physical and chemical control

Some cultural and physical methods have been known and practised for centuries by many civilizations (Rodríguez-Kábana & Canullo, 1992) and they are extremely important, as they can provide efficient control without contaminating or polluting the environment, but unfortunately they have limitations.

Cultural practices include crop rotation with antagonistic plants such as switchgrass (*Panicum virgatum*) (Kokalis-Burelle *et al.*, 1995), flooding techniques (Hollis & Rodriguez-Kabana, 1966; Maas, 1987), trap cropping (Whitehead, 1997) and introduction of organic amendments such as chitin (Hallmann *et al.*, 1999; Akhtar & Malik, 2000). However, they cannot be applied in all situations. In intensive production systems, especially when high value cash crops are produced, it is not always possible to change techniques and find a suitable crop rotation because this can mean loss of time and money (Whitehead, 1997).

Physical treatments such as steam sterilisation, soil pasteurisation, and hot water treatments are recognized to be effective but only suitable for small-scale systems, and its application is often expensive because they require large amounts of energy and sophisticated equipment. They are, thus, not suitable for all farmers. Solarisation is seen as a good option, especially when combined with other biological or

chemical control measures, to treat small areas (e.g; gardens, seedbeds). However, this technique depends to a great extent on the local climate and weather conditions. It should be done during the hottest part of the year (possibly interfering with planting schedules) and is most effective near the soil surface. However, this approach does not consistently control certain heat-tolerant pests and those at depth where it is difficult to elevate temperatures sufficiently to kill nematodes (Stapleton, 2000).

Chemical control in contrast to physical and cultural measures, which have been used for many centuries, only became a possibility for nematode management in the 20th century and particularly after the Second World War, when the synthesis of new chemical compounds occurred. The dangers associated with the manufacture and use of these products has become an important issue. Nematicidal treatments have been successfully used, but nematicides are usually expensive and may raise problems of environmental pollution and/or of accumulation of toxic residues in edible plant products. Methyl bromide is one of the most effective fumigants to control a broad range of problems (nematodes, fungi, insects, weeds). Being highly toxic to humans and highly volatile, it must be applied by skilled personnel and under a plastic tarp. For this reason, the use of nematicidal products containing methyl bromide as the active ingredient will be restricted or banned as their use has been associated with the depletion of the ozone layer (Miller, 1996).

The discovery of new classes of nematicides, with novel activity, which are more effective and less toxic to the environment would be very helpful in the control of nematode pests. However, the development of such compounds will require considerable research effort, and if they are specific only to certain nematodes they are unlikely to be considered economic by the traditional agrochemical companies (Hague & Gowen, 1987).

1.3.2 Biological control

According to Stirling (1991), biological control of nematodes is "a reduction of the nematode populations which is accomplished through the action of living organisms other than the used of nematode-resistant host plant, which occurs naturally or through the manipulation of the environment or the introduction of antagonists". Biological control can be, therefore either natural or induced.

In natural control, an equilibrium between the indigenous natural enemies and the nematode host population is established without any human intervention, apart from the intensive cropping of susceptible crops. The existence of suppressive soils, soils that contain microbial communities able to prevent the increase of nematode populations on susceptible crops, has been reported by several authors. Populations of at least six species of *Heterodera* and *Globodera* and one of *Meloidogyne* have been reported to decline under monoculture or perennial crops (Kerry, 1987). Fungal parasites of females and eggs have been considered responsible for this decline, but usually, the evaluation of the individual contribution of each species involved in the suppression is difficult to evaluate (Kerry & Jaffee, 1997). The contribution given by different groups of organisms are however, possible to estimate, for example, by suppressing their activity through the use of selective biocides to estimate the effects on the nematode populations without the response of the potential organism (Kerry, 1988).

Biological control, which will be considered in dept later in this chapter, can also be induced when the organism that is able to provide control is introduced into the ecosystem (Kerry, 1987).

PPN can be a target for many soil organisms. Predators include protozoa, mites, collembola, tardigrates and arthropods, whereas parasites include bacteria and fungi. Interactions between natural enemies and nematodes are influenced differently by several abiotic and biotic factors (Sayre & Walter, 1991). The interactions among different predators and parasites are likely, as different predators/parasites may compete as well as attack the PPN, but these interactions are complex and not well understood (Rosenheim *et al.*, 1995).

Biological control cannot replace the use of pesticides as BCAs of nematodes as they are unlikely to be as fast acting as nematicides. However, they should be used to limit the development of potential pests, as part of an integrated and sustainable strategy of control (Kerry, 1987). Also, their successful use may require advisory support. For PPN control, an understanding of their population dynamics, ecology and biology is essential (Kerry & Hominick, 2002).

1.3.3 Bacteria and nematophagous fungi

1.3.3.1 Bacterial antagonists

The production of metabolites released by some bacteria proliferating in the rhizosphere can interact with the presence of PPN (Akhtar & Malik, 2000). Several bacteria, including *Pseudomonas* spp. (Oostendorp & Sikora, 1989; Oostendorp & Sikora, 1990; Hackenberg *et al.*, 2000), *Bacillus* spp. and *Agrobacterium radiobacter* (Becker *et al.*, 1988; Jonathan *et al.*, 2000) have been found to reduce nematode invasion, gall index, hatch or mobility. However, the mechanisms by which bacteria inhibit PPN are poorly understood. The production of antibiotics, toxins, root exudate degrading compounds or induction of systemic resistance are some of the hypotheses put forward (Kloepper *et al.*, 1999). The nematicidal compounds produced by actinomycetes, especially *Streptomyces* sp., have attracted special interest, since avermectins were identified and successfully commercialised as anthelmintics (Burg *et al.*, 1979).

An important group of antagonists of nematodes are the gram-positive and endospore-forming bacteria included in the genus *Pasteuria* spp. Three important species of *Pasteuria* are parasites of PPN: *P. penetrans* on *Meloidogyne* spp., *P. thornei* on *Pratylenchus* spp. and *P. nishizawae* on cyst nematodes of the genera *Heterodera* and *Globodera* (Chen & Dickson, 1998). All are obligate parasites, producers of endospores that are able to attach to the cuticle of active nematodes. The attachment is an essential prerequisite for infection but different strains of *Pasteuria* have different attachment profiles on different nematode populations (Bird *et al.*, 2003).

The infection process involves the formation of a germ tube which penetrates inside the nematode body wall giving rise to a vegetative microcolony. These microcolonies will eventually proliferate in the pseudocoelom of a developing female and ultimately will mature into spores, and prevent the production of eggs. The mature endospores are released into soil when the plant material containing root-knot females decompose and spores may remain viable in the soil for several years.

Pasteuria penetrans has shown potential for biological control of nematodes in several pot and controlled field experiments (Mankau, 1975; Stirling, 1985; Minton & Sayre, 1989). The commercial exploitation of *Pasteuria* is limited by the lack of methods for large scale production and restricted host range of individual strains. The

current methods of mass culture of *P. penetrans* are restricted to the multiplication of the parasite in its nematode host, and the number of spores produced can be highly variable (Chen *et al.*, 1996; Pembroke *et al.*, 2005). However, *Pasteuria* can be used as an agent for inundation in confined situations such as glasshouses and small field plots and has been associated with natural control (Kerry, 1987).

1.3.3.2 **Fungi**

Fungi are the most widespread microorganisms able to parasitise nematodes. Many species have been found associated with nematodes, but only a few have shown potential as biological control agents (Jatala, 1986; Stirling, 1991). They can be divided into three categories, according to their mode of action: a) endoparasitic fungi, b) nematode trapping fungi, c) parasites of eggs and females (Cayrol, 1983).

(a) Endoparasitic Fungi

Most of the endoparasitc nematophagous fungi are present in the soil mainly as conidia or resting spores which are produced in large quantities, but mycelial growth is sparse or absent outside the host. These organisms are often obligate parasites and poor saprophytes in the soil. The infective propagule can be either a flagellated zoospore produced by Chytridiomycetes and Oomycetes (e.g *Catenaria anguillulae*) that encyst on the nematode host cuticle, or adhesive conidia, produced by a number of hyphomycetes (e.g. *Drechmeria conidiospora*) that stick to the nematode cuticle and after germination penetrate the host body wall. Some others can produce conidia that initiate infection after being ingested by the nematode, lodging in the buccal cavity or gut (e.g *Harposporium* spp.). However, PPN are stylet-bearing and are unable to ingest these conidia (Siddiqui & Mahmood, 1996).

The limitations for use of endoparasitic fungi as BCAs are due to the fact that they are difficult to produce *in vitro*, and to establish in a soil as they are poor saprophytes and the reserves contained in their small spores are not sufficient to initiate colonisation (Cayrol, 1983).

(b) Nematode trapping fungi

This category of parasites is characterised by the existence of a mycelium that forms specialised organs of capture such as constricting and non- constricting rings, or adhesive structures such as rings, hyphae, knobs, branches and networks (Cayrol, 1983). The fungus *Arthrobotrys oligospora* is probably the best known trapping fungus. It forms a specialised network with loops thinly coated by a fibrilallar adhesive material that traps and immobilises the nematode prior to infection (Nordbring-Hertz & Mattiasson, 1979).

The ability of these fungi to capture nematodes varies with the fungal species; species that are good saprophytes and grow quickly *in vitro* are less effective as nematode parasites, while others that have slow growth and less saprophytic ability are more effective parasites (Sayre & Walter, 1991). Trapping fungi are considered poor competitive saprophytes, susceptible to antagonism and non-specific. Another limitation for the successful use of these parasites as BCAs is their short period of trap activity which may not coincide with the period of juvenile emergence and migration in soil (Cooke, 1964; Siddiqui & Mahmood, 1996).

(c) Parasites of eggs and females

The mode of action of this group of fungi differs completely from the two previous types described. The parasitism does not affect active nematodes (juveniles and free living nematodes) but eggs and females of PPN, especially when exposed on the root surface and in soil (Kerry, 1987). Once in contact with females, cysts or egg masses, these fungi are able to colonise them and infect eggs and may provide nematode population control.

A considerable number of fungi have been reported to colonise eggs and cysts but only a few have been studied in detail. Within this group, important obligate parasites (e.g *Nematophthora gynophila* and *Catenaria auxiliaris*) have been recorded from cyst nematode females (Kerry, 1987), but because they are difficult to cultivate and to grow *in vitro*, their use as BCAs is limited. The facultative parasites are usually considered more promising, as they can survive in the soil and rhizosphere in the absence of nematode hosts and they can be readily grown on artificial media. Two important parasites are included in this group, *Paecilomyces lilacinus* (Thom) Samson

1974 and *Pochonia chlamydosporia* (Goddard) Zare & Gams 2001(formerly named as *Verticillium chlamydosporium*). The latter fungus is the subject of this study.

1.4 POCHONIA CHLAMYDOSPORIA AS A POTENTIAL BIOLOGICAL CONTROL AGENT FOR NEMATODES

1.4.1 Importance

The nematode cyst *Heterodera avenae* Woll. has been successfully suppressed in cereal monocultures for more than 13 years in many soils of Northern Europe (Stirling, 1991). In those soils, *H. avenae* females and eggs were parasitised by a range of fungi, of which *N. gynophila* and *P. chlamydosporia* were the most widespread and abundant (Kerry *et al.*, 1982). In contrast with *N. gynophila, P. chlamydosporia* is a facultative fungus that can parasitise eggs and females of cyst and root-knot nematodes and survive in the soil and rhizosphere of healthy plants even without the presence of a nematode host (Kerry *et al.*, 1993). The fungus has been isolated worldwide from soils in forests, grasslands, savannah, arable and garden soils and also from fungal spores and invertebrate eggs (Domsh & Gams, 1972).

Although, the efficacy of the fungus can be affected by several factors, which will be detailed below, *P. chlamydosporia* shows considerable promise as a biological control agent, specially when used as part of an Integrated Pest Management (IPM) regime and in the presence of a poor plant host for the nematode (Kerry, 1995).

1.4.2 Taxonomy and morphological characteristics

The fungus *P. chlamydosporia* is included in a wide anamorphic genus that comprises of a number of saprophytic and parasitic fungi (entomophagous, nematophagous and other invertebrates) - *Verticillium* section *Prostrata*, is characterised by the prostrate conidiophores of many of the species. Due to the complexity of this group, it has been reclassified several times. Gams & Zare (2001) revised this genus and grouped the species into three different genera, based on morphological observations and phylogenetic analysis using the Internal Transcribed Spacer (ITS) region: *Lecanicillium* n. gen. includes the majority of the entomopathogenic and fungicolous species; *Haptocillium* comprises the nematophagous

fungi that produce adhesive conidia and sometimes chlamydospores and *Pochonia*, comprising the egg parasites, in which *P. chlamydosporia* is included.

Pochonia chlamydosporia produces two types of asexual spores, conidia and dictyochlamydospores. It produces vegetative hyaline hyphae which are septate, and fine aerial mycelium. The conidiophores are usually prostrate and formed in ramified hyphae where conidia generating cells are produced. These cells are termed as phialides and can be borne singly or in pairs frequently in whorls of three to five. The conidia have an oval to ellipsoidal shape, depending on the sub species of fungus, and they are borne terminally, either singly or in small clusters or chains. The size can vary between 3-4 µm by 1.5-2.0 µm (Gams, 1988).

The dictyochlamydospores are hyaline and multicellular spores produced abundantly on aerial mycelium or submerged in agar, with thick cell walls. The spore diameter varies between 15 to 30 x 10-20 µm and can be composed of 6-9 cells. These spores are never intercalary like normal chlamydospores, but always produced terminally on more or less well-developed pedicels, and are more similar to aleuriospores (Barron & Onions, 1966). However, as a way to simplify the terms, these spores are termed as chlamydospores in this thesis, as in most of the literature referring the fungus.

The colonies on agar are white or whitish to ochre yellow and cottony, with the same colour on the reverse side, and can reach 3.5-4.5 cm diameter when grown on Czapek's agar for 14 days at 25 °C (Barron & Onions, 1966).

The teleomorph of *P. chlamydosporia* is the fungus *Cordiceps chlamydosporia*, and is known to be a parasite of mollusc eggs (Zare *et al.*, 2001).

1.4.3 **Variability**

Pochonia chlamydosporia may be part of a complex of similar species with considerable variation between biotypes, especially in *in vitro* growth (Kerry *et al.*, 1986), ability to colonise the rhizosphere (Kerry *et al.*, 1986) and in pathogenicity (Irving & Kerry, 1986). Different biotypes, with different characteristics of the fungus can be found existing in the same soil or area. Understanding the importance of such variability and their interactions, is crucial for the successful use of this natural enemy as a BCA (Kerry & Hominick, 2002). In Cuba, two varieties of the fungus, *P.*

chlamydosporia var. *chlamydosporia* and *P. chlamydosporia* var. *catenulata* were isolated from soils and shown distinct morphological differences (Zare *et al.*, 2001). However, the biological activities of both varieties were similar (Atkins *et al.*, 2003c).

1.4.4 **Tritrophic interaction**

The existence of variability between biotypes enforces the need for careful evaluation when selecting potential biotypes for biological control. However, the control of nematode populations in soil is much more complex and not only depends upon the presence of the fungus. The plant plays an important role, linking *P. chlamydosporia* and its nematode host in a strong tritrophic interaction. The susceptibility of the host plant to nematode attack influences the numbers of nematodes invading the roots, their rate of development and fecundity and consequentially, the number of eggs colonised by the fungus. In highly susceptible plant hosts, large egg masses are produced in response to nematode infection and a significant number of egg masses remain embedded inside the root tissue, isolated from the fungus, which is confined to the rhizosphere (Kerry & Bourne, 1996). The tolerance of the plant to nematode attack is, therefore, important.

The extent of fungal growth in the rhizosphere is also affected by the plant species and possibly cultivar, and nematode control is expected to be greatest on roots supporting extensive colonisation by the fungus (Kerry, 1995). There is evidence that fungal growth is stimulated by the presence of nematodes in the roots, an effect thought to be partly systemic, according to results from experiments using split root systems, where nematodes were separated from the fungus (Bourne & Kerry, 1999). The developmental stage of the nematode eggs plays another important role in the control, with immature eggs being more susceptible to nematode infection than those eggs containing second stage juveniles (Irving & Kerry, 1986).

Therefore, reductions in nematode populations are likely to be greatest on plants which are poor hosts for the nematode and that support abundant growth of the fungus in their rhizospheres. The knowledge of such complex interactions is essential for the development of rational biological control strategies (Kerry, 2000).

1.4.5 **Proliferation, dispersal and survival**

Pochonia chlamydosporia is a facultative parasite recorded from nematodes (Willcox & Tribe, 1974), eggs of snails (Barron & Onions, 1966) and soil. However, the fungus does not depend exclusively on parasitism to survive. It uses root exudates to grow along the rhizosphere (Kerry & Jaffee, 1997) and it can colonise other soil organisms including other fungi (Kerry, 1995). The factors which affect the switch of the fungus from a saprophytic to a parasitic phase are not well understood (Kerry, 2001). The fungus is a relatively poor competitor in soil (Bourne & Kerry, 2000) and its proliferation is greater in organic than in mineral soils (Kerry *et al.*, 1984; De Leij *et al.*, 1993).

Temperature and water availability are important factors that influence the survival of the fungus in the soil. The fungus can survive in greater numbers of Colony Forming Units (CFU) at water potentials of -0.2 MPa than at other water potentials, and optimum of temperatures between 25 and 30 °C. However, different biotypes can differ in their optima temperature (Bourne & Kerry, 2000).

To enable the fungus to survive when the hosts are scarce, the fungus produces chlamydospores, which contain reserves that allow the fungus to survive in hostile environments (Kerry & Bourne, 1996). The fungus can be spread by the transport of conidia by water and also carried by soil invertebrates (Kerry & Bourne, 1996).

1.4.6 **Parasitism**

When hyphae encounter the eggs from root-knot or cyst nematodes, it forms an appressorium on the egg surface and penetrates inside the egg. Appressoria are hyphal structures with the specific function of adherence to the host surface (Lopez-Llorca *et al.*, 2002). Once inside, an infection bulb is formed from which hyphae proliferate and consume the egg contents. The egg consumption duration by the fungus is unknown and can be important for studies measuring the impact of the fungus on nematode population dynamics. After consuming the egg contents, the internal hyphae lyse whereas others exit the egg and parasitise other eggs or produce chlamydospores.

The nature of the gelatinous matrix produced by *Meloidogyne* spp. can play an important role in reducing the ability of the fungus to parasitize the nematode eggs.

Different biotypes of *Pochonia* have shown different levels of sensitivity to the antimicrobial activity present in egg masses (Mas & Coosemans, 2002).

Other factors influencing the rate of parasitism by the fungus are the amount of inoculum present in the soil and roots (Kerry *et al.*, 1986) and the specificity of the biotype for the target nematode host (Mauchline *et al.*, 2002).

1.4.6.1 **The structure of the nematode eggshell in tylenchids**

The eggshell in tylenchids is composed of three layers – an outer vitelline layer, a middle chitinous layer and an inner lipid layer. The thickness of each of these layers varies from species to species (Bird & McClure, 1976). In the root-knot nematode, *M. javanica*, for example, the vitelline layer is very thin (10-40 nm), when compared with the 400 nm thick chitinous layer (Bird & Bird, 1991).

The vitelline layer forms the outer layer of the eggshell and derives from the vitelline membrane of the fertilised oocyte. It retains a unit membrane-like structure that may become thickened when fully formed (Bird & McClure, 1976).

The chitinous layer provides structural strength to the eggshell and is often the thickest and the most obvious of the three layers (Bird & McClure, 1976). It is the same thickness over all the eggshell and is composed of a non-helicoidal complex of chitin and protein (Bird & McClure, 1976; Bird & Bird, 1991). The eggshell is the only nematode structure where the presence of chitin has been conclusively demonstrated (Wharton, 1980; Bird & Bird, 1991).

The inner lipid layer contains a series of lipoprotein layers and is the major barrier to permeability, protecting the eggs from harmful and osmotic stress chemicals (Wharton, 1980). It thickens at the poles of the egg and is membranous on its innermost surface (Bird & McClure, 1976). The eggshell becomes less permeable after the lipid layer is formed. During egg development, it becomes remarkably impermeable to chemicals and fixatives such as osmium tetroxide. Even when they are exposed to this fixative for several days, the eggs can still remain viable. Prior to hatch, lipids are hydrolysed and the eggs become more permeable (Bird & Bird, 1991).

The presence of proteins is common to all the three layers and accounts for 50% of the eggshell dry weight in *M. incognita* (Bird & McClure, 1976) and 59% in eggs of

H. rostochiensis (Clarke *et al.*, 1967). In both species, proline is the most abundant amino acid present (Clarke *et al.*, 1967; Bird & McClure, 1976).

1.4.6.2 **Appressoria as infection structures**

Pochonia chlamydosporia produces an appressorium when in contact with the surface of a nematode egg (Segers, 1996). This specialised infection structure enables the fungus to become firmly attached to the egg and to penetrate it. In this process, mechanical and enzymatic forces are known to be involved. Enzymes are secreted to help destroy the outer vitelline membrane of the eggshell and expose the chitin layer, allowing the infection peg, a specialised hypha, to penetrate into the egg. The appressorium gathers the mechanical force and enzymatic degradation in a small area, promoting host penetration (Lopez-Llorca *et al.*, 2002).

The appressorium can be described as a differentiated slightly swollen structure, usually formed at the tip of the germ tube. However, it can also form from hyphae, either at the tips or along the hyphal elements, and can assume various forms and lengths (Dean, 1997). In *P. chlamydosporia*, appressoria are produced either at the ends of long hyphae, from lateral branches or at the tip of the germ tube (Segers *et al.*, 1996; Lopez-Llorca *et al.*, 2002). Appressoria are also produced by phytopathogenic fungi such as *Colletotrichum* spp., *Uromyces* spp., *Magnaporthe grisea* and *Puccinia* spp. and the mechanisms of formation, attachment and function have been extensively studied (Dean, 1997). In this group of fungi, appressoria have a similar function and are produced to enable penetration of the plant cell walls, by pressing against and attaching to the plant surface in preparation for infection. Similar structures are produced by entomopathogenic fungi such as *Metarhizium anisopliae* (St. Leger *et al.*, 1989) and by several nematophagous fungi such as *Dactylella oviparasitica* (Stirling & Mankau, 1979), *P. lilacinus* (Dunn *et al.*, 1982) and *Pochonia suchlasporia* (Lopez-Llorca & Claugher, 1990). Before germination, some fungi produce proteinaceous adhesive compounds – adhesins- which enable spores to attach to the host (Young & Kauss, 1984; Hamer *et al.*, 1988). Adhesins can be released at a single time point in response to an external stimulus (for example, hydration) (Hamer *et al.*, 1988), or at more than one time prior to germ tube emergence (Sela-Buurlage *et al.*, 1991).

The mechanisms by which fungi detect the presence of the host surface and form appressoria are complex and vary with the organism (Mendgen *et al.*, 1996). Chemical and physical signals derived from the host are thought to induce appressorium formation. When conidia from *Colletotrichum gloeosporioides* and *C. musae* were exposed to the fruit-ripening hormone ethylene, germination and production of appressoria were induced (Flaishman & Kolattukudy, 1994). In contrast, transgenic tomato fruits, unable to produce ethylene, did not support fungal growth. Other compounds released or present in the plant surface such as sugars, volatiles, phenolics, potassium and calcium ions induce appressorium formation in phytopathogenic fungi (Mendgen *et al.*, 1996; Dean, 1997; Deising *et al.*, 2000).

The topography of the host surface is another factor affecting appressoria formation in several fungi. For example, *Puccinia graminis tritici* favours penetration close to cell wall junctions, or it grows towards particular targets such as stoma (Johnson, 1934). The mechanism is known as thigmotropism. Artificial structures, *e.g* plastic replicas, are commonly used to mimic the host surface, to determine the importance of thigmotropism in the absence of specific chemical signals (Dean, 1997). Other factors such as hydrophobicity and hardness of the surface have been shown to play an important role in the induction of appressorium formation (Deising *et al.*, 2000). Conidia of *M. anisopliae* bind to hydrophobic surfaces such as teflon, polyvinyl chloride, polystyrene, polypropylene, polyester and aluminium foil to a greater extent than to hydrophilic surfaces (St. Leger *et al.*, 1989). The fungus *P. chlamydosporia* seems to share the same type of preference for hydrophobic surfaces, with polystyrene being the best substrate to induce appressoria in this fungus (Lopez-Llorca *et al.*, 2002).

Segers (1996), suggested that cues from the egg influence the production of the appressorium. The author also noticed the existence of variation in the production of subtilisins by different biotypes, which may be related to differences in the virulence and host preference of the fungus.(Segers, 1996)

1.4.6.3 **Enzymes involved in the infection process**

Particular extracellular enzymes secreted by the *P. chlamydosporia* are thought to play an important role in the infection process of eggs as they enable the fungus to degrade the host's major barrier to infection – the nematode eggshell. A specific

protease and chitinase have been isolated and purified and are considered to be involved in the infection process serving as virulence factors (Huang *et al.*, 2004).

During the infection process, a 33 kDa subtilisin-like serine protease, designated VCP1, is produced by the fungus (Segers *et al.*, 1994). Immunolocalization of this enzyme at the penetration site indicate that VCP1 degrades the vitelline membrane on the surface of the eggshell and exposes the chitin layer (Segers *et al.*, 1996). This enzyme is serologically and functionally related to Pr1, the much studied enzyme produced by the entomopathogenic fungus *M. anisopliae* (Segers *et al.*, 1995). As demonstrated with pr1, and other pr1-like proteases produced by other nematophagous fungi, such as *Pochonia rubescens* (Lopez-Llorca, 1990), *Arthrobotrys oligospora* (Tunlid *et al.*, 1994), *P. lilacinus* (Bonants *et al.*, 1995), *Lecanicillium psalliotae* (Yang *et al., 2005*) and *Clonostachys rosea* (Li *et al.*, 2006), VCP1 seems to play an important role in the infection process. When *M. incognita* eggs were incubated with purified VCP1, the outer vitelline membrane of the nematode eggshell was removed without formation of appressoria (Segers, 1996). The molecular structure of VCP1 differs between biotypes according to their original nematode host. Biotypes isolated from root-knot nematodes have polymorphisms in their binding regions that are different from those biotypes isolated from cyst nematodes. Hence, this enzyme may also be a host range determinant (Morton *et al.*, 2003). Proteases are produced in the first steps of infection, followed normally by chitinase production, and for this reason their role seems to be fundamental in the host penetration process (St. Leger *et al.*, 1986).

Chitinases are produced by a wide range of organisms, including bacteria, algae, fungi, crustaceans, insects and even by plants as part of their defence mechanism against several pathogens (Punja & Zhang, 1993). The quantity and variety of chitinolytic enzymes produced by microorganisms varies from one species to another (Valadares-Inglis & Peberdy, 1997). Chitinases can be generally classified as endo or exochitinases. Endochitinases randomly cleave glycosidic linkages, generating free ends and long oligosaccharides. Exochitinases have a progressive action starting at the non reducing end of chitin with the release of chitobiose and N-acetylglucosamine units. Chitobiosidase and N-acetyl-ß-glucosaminidase are considered exochitinases (Tronsmo & Harman, 1993). The presence of both, exo and endochitinases significantly increase the efficiency of chitinolytic activity (Bolar *et al.*, 2001).

The production of chitinases by fungi is complex and seems to be multifactor dependent; their production is controlled by a repression-induction system, fungal developmental stage, substrate used or even the assay technique adopted (Valadares-Inglis & Peberdy, 1997).

In fungi, chitin is a major structural component of cell walls and chitinases are known to play a role in fungal cell metabolism, hyphal growth and differentiation (Gooday *et al.*, 1992; Yanai *et al.*, 1992; Cabib *et al.*, 2001). Furthermore, the secretion of chitinases can also be associated with host infection in several mycopathogenic, entomopathogenic and nematophagous fungi (St. Leger *et al.*, 1993; Brurberg *et al.*, 1996; Dupont *et al.*, 1999). Multiple chitinases have been detected and purified in several entomopathogenic fungi including *Metarhizium anisopliae*, *Beauveria bassiana* and *Aspergillus flavus* (St. Leger *et al.*, 1993).

The purification and characterisation of specific chitinases in nematophagous fungi is recent, when compared with research on entomopathogenic fungi (Huang *et al.*, 2004) and requires further study (Morton *et al.*, 2004).

Chitinolytic activity was detected in *Verticillium* spp. when grown in a solid and a liquid medium containing colloidal chitin as an inducer (Dackman *et al.*, 1989). Dupont *et al.* (1999) detected the presence of both endo and exochitinases in cultures of *P. chlamydosporia* growing in a chitin rich medium and studied the effects of these chitinases on the eggshell of *M. incognita* eggs using fluorescence and scanning electron microscopy. Both enzymes weakened the nematode eggshell and caused denting even when eggs were only exposed to these enzymes for 24 hours. However, Tikhonov *et al.* (2002) were the first to purify and characterize chitinases from *P. chlamydosporia* and *P. rubescens*. In their study, they were able to identify an endochitinase (CHI43) from both fungi when grown in a semi liquid medium containing chitin as the main source of C and N. When eggs of *G. pallida* were treated with CHI43, scars on the surface of the egg were observed but the scaring was more pronounced in eggs treated with both CHI43 and a protease purified from the *P. rubescens* (P32). In this situation, extensive peeling could be observed suggesting that chitinases and proteases may be involved in the breakdown of the nematode eggshell and are both required for infection. Similar results were observed in *M. incognita* eggs treated with proteases and chitinases from *P. lilacinus* treated eggs (Khan *et al.*, 2004). Observations

using transmission electron microscopy revealed splitting of the outer vitelline layer and changes in the chitin layer, in eggs treated with chitinases. In eggs incubated with a combination of proteases and chitinases, destruction of the lipid layer could also be observed, suggesting that for effective penetration of nematode eggs, nematophagous fungi must produce protease and chitinase enzymes at the same time to degrade different eggshell layers (Khan *et al.*, 2004).

The importance of lipases in the infection process is less clear. Lipases are ubiquitous in nature (Hülsmann, 1984) and catalyse the hydrolysis and synthesis of esters bonds. The biological function of lipases is the hydrolytic decomposition of triacylglycerols into glycerol and free fatty acids.

The involvement of lipases in plant infection was reported for several pathogenic fungi (Comménil *et al.*, 1995; Nasser *et al.*, 2001). The plant pathogenic fungus *Fusarium graminearum* secretes a lipase which was shown to serve as a virulence factor required for infection of cereals (Voigt *et al.*, 2005); Knocking out the secreted lipase gene (*FGL1*), virulence was reduced in both wheat and rice (Voigt *et al.*, 2005). Secretion of lipases has been suggested to play a role in the infection process of *M. anisopliae* (Clarkson & Charnley, 1996) but its production *in vitro* was only recently investigated (Silva *et al.*, 2005). The production of lipases by *M. anisopliae* is influenced by the type and concentration of carbon (C) and nitrogen (N) sources, as well as culture pH and temperature. However, the direct effects of this enzyme on the insect host were not tested (Silva *et al.*, 2005).

The production of lipases and other enzymes which are produced extracellularly by nematophagous fungi were detected using semi-quantitative colorimetric enzyme tests (API ZYM®) with the objective of identifying groups of important enzymes and its dependence on the nutrient availability (Mendoza de Gives *et al.*, 2003). Four nematophagous fungi – *Duddingtonia flagrans*, *Arthrobotrys*, sp., *A. musiformis* and *P. chlamydosporia* were grown in a rich soya/peptone medium for 10 and 30 days and were tested for the production of 19 enzymes, such as phosphatase, lipases, esterases, glycosidases and proteases. Additionally, *Duddingtonia flagrans* and *Arthrobotrys*, spp., were cultured in different concentrations (0, 10 and 50%) of Czapek Dox liquid medium. In another experiment, *D. flagrans* and *Arthrobotrys*, sp., were transferred to a medium containing nematode juveniles (50 juveniles of either *C. elegans* or *H.*

contortus ml^{-1} of water) or water, and were incubated for three days. The production of lipases from *A. musiformis* and *P. chlamydosporia* was detected after only 30 days and not at 10 days incubation. However, Olivares-Bernabeu & Lopez-Llorca (2002) found lipolytic activity in different biotypes of *P. chlamydosporia* isolated from Spanish soils, after seven days of growth in solid media, but not before (Olivares-Bernabeu & Lopez-Llorca, 2002). The authors also found that lipolytic activity varied with the fungal biotype and was always lower than protease activity. This can be explained as protein is the major constituent of nematode eggshells (Bird & McClure, 1976). Interestingly, the quantity of lipases secreted by *D. flagrans* was greater at higher concentrations of Czapek Dox medium (50%) but the enzyme was not produced in *Arthrobotrys* spp. (Mendoza de Gives *et al.*, 2003). However, in the absence of nutrients in the media (only water), lipase production was detected in *Arthrobotrys* ssp., at higher levels than in *D. flagrans*. Generally, large variation in the level of enzymes produced by the different fungi was found and the production of extracellular enzymes was thought to be dependent on the availability of nutrients (Mendoza de Gives *et al.*, 2003).

1.4.7 **Fungal detection and quantification methods**

One of the requirements in quality control programmes and registration packages for the commercialisation of BCAs is the development of methods to monitor the organism after its release in the environment. Methods to detect and quantify the presence of the fungus in soil, interactions with other organisms and roots are therefore crucial. These methods can also help to understand nematode regulation in suppressive soils and enable the detection of differences in temporal and spatial distribution of different biotypes in the rhizosphere (Kerry & Hominick, 2002).

The use of plating techniques is an important technique to detect fungi in the soil and has been used successfully to detect *P. chlamydosporia* (Mauchline *et al.*, 2002; Atkins *et al.*, 2003b). The development of a semi-selective medium that contains antibiotics and two benzimidazole fungicides (carbendazim and thiabendazole) to which *P. chlamydosporia* has a relatively high level of resistance (and the majority of fast growing saprophytes are sensitive), has allowed the isolation, identification and quantification of the fungus from the soil, rhizosphere and egg masses (Kerry *et al.*, 1993). Plate counting measures the abundance of viable fungal propagules in terms of

CFU using dilution plates and is a useful tool to monitor changes in the abundance of the fungus. However, it cannot distinguish between the nature of these propagules (hyphal fragments, conidia, chlamydospores) and between different biotypes.

Polymerase Chain reaction (PCR)- fingerprinting has enabled the discrimination between different biotypes of *P. chlamydosporia* grown in pure culture (Arora *et al.*, 1996). Also, the use of specific PCR primers based on the β-tubulin gene enables the diagnosis of the species (Hirsch *et al.*, 2000). PCR is a valuable tool that can detect the presence of the fungus at low levels in soil and rhizosphere (Mauchline *et al.*, 2002; Atkins *et al.*, 2003c) but it is a non quantitative assay and cannot estimate viability. Although techniques such as Real time-PCR are specific and quantitative, the combination of PCR diagnostics with methods such as baiting and plating techniques are a reliable way to provide information about population size and viability of the fungus is environmental samples (Atkins *et al.*, 2003b; Peteira *et al.*, 2005). The combined use of PCR-based methods, bait and culture methods have also enabled the detection of differences in the activity of *Plectosphaerella cucumerina* (Atkins *et al.*, 2003a) and *P. lilacinus* in soil (Atkins *et al.*, 2005), two potential BCAs for nematodes.

To fully understand the interactions between the fungus, plant host and nematode, methods for *in situ* visualisation are also needed. Direct staining and microscopy are useful techniques but can be only used in sterile conditions and not in field tests. Other options could be the transformation of the fungus by including a visible marker gene or the use of fungus-specific fluorescent antibodies, but these techniques are not yet available (Hirsch *et al.*, 2001; Atkins *et al.*, 2004). The combination of monitoring techniques is extremely important to evaluate the survival and growth of the fungus in the soil and root environment (Atkins *et al.*, 2003b).

1.5. ATTENUATION IN FUNGI

When cultured in nutritionally deprived media, some fungi may adapt their growth resulting in morphological or physiological effects (Smith, 1991). Serial sub-culturing methods using agar plating are known to cause loss of genetic material and/or certain important fungal characteristics (Jenkins & Grzywacz, 2000). Numerous fungi which were heavily ovicidal when first isolated lost this property during cultivation on artificial media (Chalupova *et al.*, 1977). Changes in morphology and ability to produce

spores, as result of sector formation in colonies growing on agar plates, have been found in both plant pathogenic and entomopathogenic fungi such as *Fusarium oxysporum* (Wing *et al.*, 1995; Kim, 1997) and *M. anisopliae* (Ryan *et al.*, 2002). The continuous sub-culture of a wild type of the pathogenic fungus *M. anisopliae* has led to the selection of mutants with distinctive morphological, biochemical and genetic characteristics (Wang *et al.*, 2002). Some of these changes were accompanied by changes in physiology, namely in the ability to produce enzymes and to produce secondary metabolites (Ryan *et al.*, 2002).

The potential loss of virulence due to repeated *in vitro* culture has obvious implications for the quality of the product resulting from a particular fermentation batch. However, the effects of continuous *in vitro* culture on the virulence and morphological characteristics of entomopathogenic fungi appear to vary considerably among biotypes and species (Hajek *et al.*, 1990; Brownbridge *et al.*, 2001). For example, *B. bassiana*, *L. lecanii* (formerly *Verticillium lecanii*) and *P. fumoroseus* were reported in some studies as losing virulence with repeated culture (Schaerffenberg, 1964; Nagaich, 1973), while these changes were not detected in numerous other studies (Lefêbvre, 1931; Hall, 1980; Brownbridge *et al.*, 2001; Vandenberg & Cantone, 2004). Discrepancies in results may be due to differences in experimental methodology, e.g. use of enriched media, different fungal material (single spores or multispore transfers) or simply might be variable due to differences in the genetic stability of individual biotypes (Hajek *et al.*, 1990; Brownbridge *et al.*, 2001). Factors that reduce genetic stability in fungi range from spontaneous mutations during DNA replication to induced changes in genome structure and content (Smith, 1999). However, certain characteristics of a fungus may recover after passaging through the original host and may improve virulence and expand the host range (Aizawa, 1971; Ferron, 1985; Prenerova, 1994). Frequent sub-culturing of *P. chlamydosporia* has led to reductions in chlamydospore production but no loss of virulence (Coosemans, 1990). The stability of phenotypic traits, including virulence and sporulation, can vary by biotype and therefore should be monitored in a commercial production setting (Vandenberg & Cantone, 2004).

Maintenance of original cultures must be strongly controlled and monitored to ensure the fungus retains pathogenicity, together with other characteristics important for

biocontrol performance (Ryan *et al.*, 2002). To avoid the problems encountered with serial transfers, preservation methods which can reduce metabolism to an extent that it induces artificial dormancy have proved to be successful for conservation (Smith, 1991). This is usually achieved by dehydration or freezing. Preservation using freeze-drying (liophilization) and liquid nitrogen are two examples of techniques that can suspend the metabolism (Smith & Onions, 1994). The choice of preservation methods depends upon many factors such as fungal species, type of fungal material available (spores or mycelia), cost of the equipment and technical experience.

1.6 PRODUCTION AND QUALITY CONTROL

Most of the success of a BCA in the field depends on the implementation of an effective quality control system that assures that no changes occur after the organism has been selected for mass production (Jenkins & Grzywacz, 2000).

After a formal identification of the agent, using molecular techniques and classic taxonomic procedures, the preservation of the original material is required (Smith & Onions, 1994). The next step is the use of efficient production methods which should be strictly controlled. Records of temperature, pH, moisture content and contaminant levels from each batch should be taken and kept on file. The ecophysiological manipulation of the inocula can improve production and factors that can affect the efficacy of the organism should be carefully evaluated (Magan, 2001).

Since *P. chlamydosporia* is not dependent on nematode eggs for its nutrition it is not surprising that the fungus grows on a wide range of nutrient media. In fact, the fungus grows easily, but relatively slowly, *in vitro* on a wide range of culture media but the production of chlamydospores, the most convenient way to introduce and establish the fungus in the soil, is limited in liquid fermentation (Kerry & Bourne, 1996).

The formulation and storage of the product are important matters and must be considered in quality control programmes. Granular formulations are generally considered to be most suitable for microorganisms that are to be applied to the soil but the dry nature of these formulations requires that the organism should have some resistance to desiccation and ability to spread in the soil (Stirling, 1991). Shelf-life can be a limiting factor for many microbial biocontrol products and, therefore, should be

evaluated probably using predictive models (Jenkins & Grzywacz, 2000). Shelf-life should be as long as possible, in order to compete with chemical products (Stirling, 1991).

Chlamydospores can be directly applied to the soil but, as large quantities of spores are required to provide control (10^3-10^6 g^{-1} soil), the inoculum should be localised close to the target area that needs treatment (Kerry & Hominick, 2002).

Another important issue that needs regular monitoring is the product efficacy. Assays that measure viability (this should be greater than 85%) and virulence/pathogenicity should be standardized and should be sufficiently sensitive to measure differences from batch to batch (Jenkins & Grzywacz, 2000).

After being applied in the field, the performance of the agent should be monitored. The complex microbial community of soils imposes a buffering effect on population increase by individual species and this aspect can markedly affect the establishment of the biological control agent when introduced (Deacon, 1991).

1.6.1 Production of inocula using a biphasic system

Besides biological and ecological factors, the commercial success of a biological control agent depends on the implementation of a cost-effective production system. Production methods must aim for high yields of viable and effective concentrations of spores using the lowest capital input possible. Generally, two methods of spore production can be distinguished – liquid state fermentation (LSF), and solid state fermentation (SSF). Unlike LSF, where substrate components are dissolved in water, in SSF, the water availability is restricted (Ooijkaas, 2000).

The different methods of culturing the fungus influences the number and type of spores produced. High yields of conidia of *P. chlamydosporia* can be obtained in liquid media, but such media are unsuitable for chlamydospore production, while solid substrates, such as barley, wheat bran, wheat straw or cracked rice, favour the production of chlamydospores. This type of thick walled spore is the preferred form of inoculum because it is resistant to desiccation and has sufficient food reserves to establish the fungus in soil without additional energy sources (Kerry *et al.*, 1993). Currently, the fungus is being produced in a biphasic system, where a pre-inoculum (liquid) is used to inoculate a cracked rice grain medium (Hidalgo-Díaz, 2003; Kerry *et*

al., 2005). After 21 days, the spores are ready to be harvested. In this medium the fungus produces up to 10^7 chlamydospores per g of colonised rice (Kerry *et al.*, 2005). The biphasic system has the advantage of reducing the risk of contamination and to shorten the time of incubation (Jenkins *et al.*, 1998).

Agricultural raw products (cereal grains, wheat bran, straw) are frequently applied as substrates for SSF as they are rich in C and N sources and contain other nutrients such as protein and cellulose, which are largely water-soluble. For this reason, often no nutrient additions have to be made (Smits, 1998). The use of these substrates is an advantage of SSF over liquid state fermentation. Other advantages are due to the absence of large amounts of water *i.e.*, less water means less volume of media and smaller bioreactors and consequentially less space required, less liquid waste to dispose off and less risk of contamination by bacteria. The main problem associated with SSF is the difficulty to scale up and to remove the heat generated by metabolic activity of the microrganisms (Nagel, 2002). Although, some authors claim that liquid fermentation would be preferable for commercial development of fungi (Papavizas *et al.*, 1984; Powell, 1993), there are numerous successful examples of the use of SSF in producing fungi in industry (Desgranges *et al.*, 1993; Silman *et al.*, 1993; Ooijkaas, 2000) and some consider that SSF produces BCAs of better quality than LSF (Desgranges *et al.*, 1993; Muñoz *et al.*, 1995; Larena *et al.*, 2004).

1.6.2. **Effects of culture conditions on virulence and ecological fitness**

When released in the environment, the BCA is exposed to fluctuating abiotic factors, which can have a great impact on the establishment of the organism in the field and compromise its efficacy in controlling the target pest. Tolerance to environmental-stress conditions such as water availability, temperature and microclimate can determine the success of an ecologically competent BCA in the field (Magan, 2001).

Physiological manipulation of the inoculum can alter the synthesis and accumulation of sugars and sugar alcohols in a number of BCAs and improve their ecological competence when exposed to less favourable conditions.

Water activity (a_w) is a term which describes the actual availability of water for microbial growth. It is defined as the ratio between vapour pressure of the water in a substrate (P) and vapour in pure water (P_0), at a standard temperature and pressure

(Magan & Lacey, 1988). Thus, $a_w = P/P_0$, with a_w of pure water being 1.0. Another way to measure water availability is by measuring the water potential (Ψ_w) which is defined in terms of energy (MPa, where 1 MPa is equivalent to 9.87 atmospheres, or 10 bar pressure) (Deacon, 2006). Total water potential (Ψ_w) consists of the sum of several different potentials: osmotic potential (solute binding forces, Ψ_Π), matric potential (physical binding forces, Ψ_m), turgor potential (or pressure potential, Ψ_p) and gravimetric potential (Ψ_g). So, the result of summing all forces is: $\Psi_w = \Psi_\Pi + \Psi_m + \Psi_p + \Psi_g$ (Deacon, 2006). In solid substrates, where solute potential is the major force, a_w is commonly used. However, using Ψ_w it is possible to partition turgor, osmotic and matric components, allowing studies on their individual influence on the growth and physiological functioning of organisms. In soil and cereal crop residue, matric potential is the major component of total Ψ_w (Magan & Lynch, 1986) and therefore, matric stress is thought to have greater effects on fungal growth and germination than osmotic potential stress (Brownell & Schneider, 1985).

Microbial cells are able to take up water when their internal water potential is less than that of the environment. The water potential gradient across the cell membrane results in the movement of water from higher potential until, by the development of cell turgor, water potential inside the cell is in equilibrium with that outside the cell (Cook & Baker, 1983). Osmoregulation is an energy-requiring process and may be accomplished internally by the accumulation of compatible solutes. When grown under low environmental water potentials (water-stress conditions), fungi are able to lower their intracellular water potential accordingly by synthesis of polyhydroxyalcohols (polyols), organic acids and sugars (Brown, 1978; Luard, 1982; Meikle *et al.*, 1991; Van Eck *et al.*, 1993; Hallsworth & Magan, 1994a; Hallsworth & Magan, 1994b). These solutes are accumulated in protoplasmic and vacuolar spaces in fungal propagules (Brown & Simpson, 1972) and have individual roles in the regulation of the intracellular osmotic potential (Pascual *et al.*, 2000). For example, the low-molecular-weight polyol glycerol appears to be of crucial importance in many fungi (Luard, 1982), as it protects enzymes from accumulation of sodium and loss of water, both of which may denature the enzymes. High amounts of glycerol were accumulated in conidia of *Aspergillus ochraceus* grown under osmotic stress, when compared with those produced with free available water (Ramos *et al.*, 1999).

Another example of a compatible solute is the disaccharide trehalose. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a versatile non-reducing compound commonly found as a storage carbohydrate in eukaryotic cells and implicated in the response to various environmental stresses (Thevelein, 1984), especially heat and osmotic stress (Meikle *et al.*, 1991). Trehalose preserves membranes from damage during dehydration by replacing water in phospholipids membranes in yeasts and filamentous fungi (Magan, 2001). However, the production of trehalose in response to osmotic stress differs with the organism. When cultures of *Penicillium frequentans* were grown at reduced water activity, there was no enhanced accumulation of trehalose (Pascual *et al.*, 2000). Whereas, elevated amounts of glycerol were accumulated under water stress, with a concomitant decrease in erythritol, mannitol and glucose.

The role of mannitol in stress response has been investigated by Chatuverdi *et al.* (1997) who proved that mannitol is capable of substituting for glycerol as the primary intracellular osmolyte in *S. cerevisiae*. The ability to grow in the presence of high NaCl was restored when a multi-copy plasmid encoding the mannitol -1- phosphate dehydrogenase gene obtained from *Escherichia coli* was inserted into a glycerol-defective mutant of *S. cerevisiae* (Chatuverdi *et al.*, 1997).

The response to water stress differs between organisms - some fungi will be more adapted to adjustments in water potential than others. Moreover, micro-organisms are more responsive to changes in water potential at some stages of their life cycle, such as during reproduction, than at other stages (e.g during vegetative growth)(Cook & Baker, 1983).

Apart from a_w stress, other factors, such as the availability of nutrients, can modify the quantity and quality of endogenous reserves accumulated in yeasts and fungi (Pfyffer & Rast, 1988; Jackson & Schisler, 1992; Teixidó *et al.*, 1998b; Ramos *et al.*, 1999) and sometimes these modifications may result in an increase of spore viability (Teixidó *et al.*, 1998a), germination rate (Hallsworth & Magan, 1994a; Hallsworth & Magan, 1995; Hallsworth & Magan, 1996) and even increase virulence against the host (Lane *et al.*, 1991a; Lane *et al.*, 1991b; Chandler *et al.*, 2005).

Modifications in the nitrogen concentration and availability of nutrients had an impact on the quality the inocula from *M. anisopliae* (Ypsilos & Magan, 2005). Nutrition influenced the C and N composition of conidia, germination rate and levels of

spore bound Pr1 in *M. anisopliae* (Shah *et al.*, 2005).Virulent conidia produced at relatively low C:N ratios had high germination rates, and the authors postulated that starvation conditions can enhance fungal virulence (Shah *et al.*, 2005). Spores of *Colletotrichum truncatum* produced in a medium with a C:N ratio of 10:1 were more effective in causing disease in *Sesbania exaltata* than spores produced in higher C:N concentrations (Jackson & Schisler, 1992). Such results reinforce the importance of physiological manipulation on the efficacy of a microbial pesticide if it improves virulence in unfavourable environments. Therefore, understanding the impact of physiological manipulation on the synthesis and accumulation of sugars and sugar alcohols in a potential BCA, is of crucial importance and may help to improve the organism's ecological competence (Magan, 2001).

1.7 AIM AND MAIN OBJECTIVES

The present study was part of a European Union project, which aimed to develop sustainable alternatives to pesticide use against important pests in urban and peri-urban farms in Latin America, using microbial control agents. A selected biotype of *P. chlamydosporia* was being produced for this project in CENSA (Centro Nacional de Sanidad Agropecuária), Havana, Cuba, and commercialised as KlamiC®. Also in Mexico, biotypes of the fungus were selected and applied in small plot trials against *Nacobbus aberrans*.

The main aim of this research was to increase the understanding of the variability and the stability of different biotypes of the fungus *P. chlamydosporia*. Information about these two aspects is crucially important for the implementation of efficient quality control systems in production. The measurement of differences in the performance (saprophytic and parasitic activity) by different biotypes of the fungus and the effects of continuous manipulation of the fungus *in vitro* are the prime objectives of this thesis. To accomplish these objectives, sensitive bioassays needed to be developed, or optimised, and information about egg infection and the production of enzymes by different biotypes needed to be collected. A diagram of the worked carried out in this thesis is shown in Figure 1.1.

More specifically, the objectives of this study were:

- Measure differences between biotypes in their performance as BCAs and to develop and optimise *in vitro* bioassays to evaluate saprophytic/parasitic ability of *P. chlamydosporia* biotypes (Chapter 3 and Chapter 4).

- Study the stability of the fungus when continuously manipulated *in vitro* and investigate the possible occurrence of attenuation during repeated culture (Chapter 3, Chapter 4 and Chapter 5).

- Increase understanding of the infection of the fungus and the range of enzymes produced by different biotypes, which differ in their virulence and saprophytic competitiveness (Chapter 5).

- Study the effects of water stress on growth and accumulation of endogenous reserves in different biotypes of the fungus (Chapter 6).

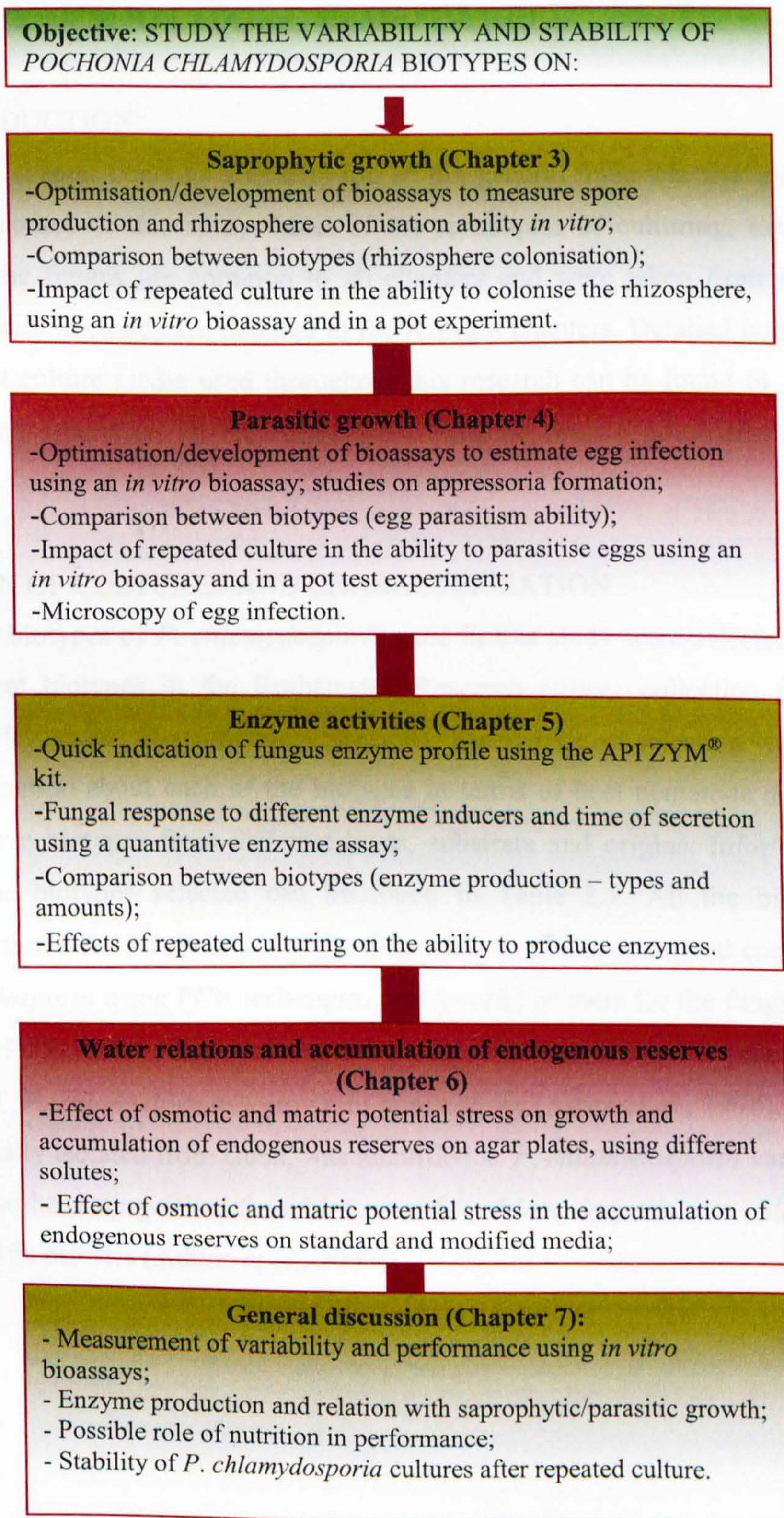


Figure 1.1 Flow diagram of the experimental work carried out in this thesis.

CHAPTER 2: GENERAL MATERIAL AND METHODS

2.1. INTRODUCTION

This chapter contains a description of the general materials and methods used during the course of this study. Most of the techniques of culturing, extraction and storage of the fungus are common to all chapters and were taken from Kerry *et al.* (1998). Specific methods are detailed in the relevant chapters. Detailed composition of the different culture media used throughout this research can be found in Appendix I. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich, England, UK.

2.2. ORIGIN OF CULTURES AND CHARACTERISATION

The biotypes of *P. chlamydosporia* used in this study were selected among the 400 different biotypes in the Rothamsted Research culture collection (Rothamsted Research, Harpenden, Hertfordshire, England). The criteria of selection were based on prior information about each of the biotypes in terms of host nematode and origin, in order to have biotypes from different hosts, substrata and origins. Information about each of the biotypes selected can be found in Table 2.1. All the biotypes were previously tested for the presence of the diagnostic ß-tubulin gene, and confirmed to be *P. chlamydosporia* using PCR techniques and specific primers for the fungus (Hirsch *et al.*, 2000). PCR- DNA fingerprinting has enabled the discrimination between different biotypes of *P. chlamydosporia* grown in pure culture (Arora *et al.*, 1996). The biotype 392, originally isolated from Cuba, was identified as *P. chlamydosporia* var. *catenulata*, and can also be distinguished from biotypes of *P. chlamydospor*ia var. *chlamydosporia* using specific primers (Atkins *et al.*, 2003).

Table 2.1. Biotypes of *Pochonia chlamydosporia* examined

Biotype number	Host nematode	Substratum	Country of Origin	Year of isolation
10	*Meloidogyne incognita*	Eggs	Brazil	1981
16	No information	Soil	Cuba	1996
60	*Heterodera* avenae	Eggs	UK	1985
69	*Heterodera* avenae	Spore	New Zealand	1985
104	*Heterodera schachtii*	Spore	UK	1986
132	*Meloidogyne* spp.	Soil	Kenya	1998
280	*Globodera rostochiensis*	Eggs	UK	2000
309	*Meloidogyne* spp.	Eggs	Zimbabwe	No information
392	*Meloidogyne incognita*	Eggs	Cuba	1996
399	*Meloidogyne* spp.	Eggs	China	No information
400	*Meloidogyne* spp	No information	Bulgaria	No information

2.3 CULTURES

Freeze-dried material from stock cultures of fungi were suspended in sterile distilled water and inoculated onto Petri dishes containing either Potato Dextrose Agar (PDA) (Oxoid) or Corn Meal Agar (CMA) (Oxoid). The plates were incubated at 25 °C for three weeks and then were kept at 4 °C until use in experiments.

2.4 INOCULUM PREPARATION

Chlamydospores were produced using broken rice as a solid substrate following the method described by Hidalgo-Díaz (2003) and were used in tests to evaluate saprophytic ability, impact of repeated culturing and to inoculate soil in the pot experiment. Three 5 mm agar plugs taken from the edge of seven-day-old fungal colonies grown on CMA at 25 °C were transferred to conical flasks (250 ml) containing 100 ml of autoclaved rice broth and these were incubated in an orbital shaker (Gallenkamp) at 28 °C and 120 rpm. After 72 hours incubation, 15 ml of inoculum (obtained from the inoculated rice broth) were added aseptically to conical flasks (500 ml) containing 100 g of pre-cooked and autoclaved rice (10-12 % humidity) and were incubated at 25 °C for 21 days. To separate chlamydospores from the rice, the substrate was washed onto a set of sieves, following the method described in Kerry *et al.* (1998) and the spores extracted were collected on a 10 μm aperture sieve.

2.4.1 **Estimation of the number of chlamydospores and conidia**

To evaluate the quantity of spores per gram of rice, 1 g of inoculum was suspended in nine millilitres of water-agar (0.05%) and chlamydospores were counted using a Neubauer haemocytometer (400 x magnification). Dilutions (10 and 100 times) were made when necessary. The same method was used to quantify numbers of the conidia.

2.4.2 **Viability**

Two hundred microlitres of chlamydospore suspension at a concentration of ca. 500 chlamydospores per ml were spread in Petri dishes containing sorbose agar plus antibiotics (Appendix I). Plates were incubated for 48 hours at 25 °C and the percentage of germinated spores was accessed using a stereo microscope (50 x magnification). Chlamydospores with germ tubes longer than their width or diameter were considered to have germinated. Percentage germination was estimated by examining 100 chlamydospores per replicate and counting how many had germinated.

2.4.3 **Storage**

After extraction, spores were mixed with fine white sand in an approximate ratio of 10:1, sand-chlamydospore (w/w) and were kept at 4 °C, prior to use. Spores were tested for viability before use and were never kept for periods longer than two weeks.

2.5 REPEATED CULTURING

A selection of biotypes of *P. chlamydosporia* (280, 392 and 132, Table 2.1) was sub-cultured every week on CMA. The biotypes were selected in order to have biotypes from different nematode hosts and origins (Table 2.1). One plug of 5 mm diameter (cut from the edge of colonies growing vigorously on agar) was transferred to the centre of a Petri dish (9 cm) containing CMA and was incubated for one week at 25 °C. After this time, another plug taken from the new colony was transferred to a new Petri dish containing fresh media. This procedure was repeated each week for 60 weeks. In the 60th week, two millilitres of autoclaved nutrient broth (Appendix I) were added to the plates, the colonies were gently scraped from the agar, in order to suspend the fungal material in the broth. Five hundred microlitres of this suspension and suspensions

obtained from initial cultures were transferred to small glass vials, separately, in four replicates, and were freeze dried using a lyophilizor (Edwards, EF03).

2.6 NEMATODE EGGS

Root-knot nematode eggs used in enzyme, parasitism and microscopy studies were obtained from egg masses cultured on *Lycopersicum esculentum* L. (tomato cv. Tiny Tim), grown in a temperature controlled glasshouse, at 25 °C.

For studies on enzymes, parasitism and pot test experiment, egg masses were picked off from plant roots using forceps. To release the eggs, egg masses were washed in a solution of 0.5 % commercial bleach (8 % w/v available chlorine) for 1.5 minutes (Bird & McClure, 1976). The egg suspension was then washed five times in sterilised distilled water and poured onto a 125 μm aperture sieve, to trap large debris. The eggs were collected on a 30 μm aperture sieve.

Globodera pallida cysts were separated from infested soil, using the Fenwick can method (Fenwick, 1940). The soil was kindly supplied by Andy Barker (Rothamsted Research, UK). To release the eggs, cysts were crushed using forceps, eggs were suspended in water and passed through a sieve (125 μm aperture) to remove any soil or cyst debris and collected as described above.

2.7 POT TEST EXPERIMENT

Chlamydospores derived from fresh and stored sub-cultures of biotypes 132, 280 and 392 (Table 2.1) were produced, extracted and counted using the method described in 2.4. The fungal inoculum was thoroughly mixed with autoclaved soil substrate (two parts of compost, one part of sand and one part of perlite (w/v) at a rate of 5000 chlamydospores g^{-1} soil. Tomato seedlings (cv. Tiny Tim) were transplanted to pots (15 mm diameter) containing the inoculated soil, and after 10 days, 2000 second stage juveniles of *Meloidogyne* spp. were pipetted in aqueous suspension into four 5 cm deep holes made in the soil around the plants. Juveniles were hatched from eggs derived from stock plants infected with *Meloidogyne* spp., obtained as described in 2.6.

A double pot system was used. The first pot, containing the plant and inoculated soil was inside a second, slightly larger in diameter, containing around 200 ml of soil. The soil and roots that grew in the second pot were not used in the assay. This system

reduces the chances of pots drying out or being over watered whilst restricting the amount of soil to be inoculated. The experiment was conducted in a temperature controlled glasshouse at 25°C, with additional lighting. Water was supplied daily and plants received water from the pot saucer (not directly from the top). Pots were distributed in the glasshouse using a completely randomized block design.
The plants were taken after 65 days of growth and the following parameters were accessed:

2.7.1. **Number of colony forming units (CFU) per gram of root**

Fresh roots were gently washed and cut in one centimetre sections. A sub sample of one gram was selected at random and crushed in nine millilitres of a 0.05% sterile agar solution, using a sterilised pestle and mortar. One ml of this solution was transferred to a new glass vial containing nine millilitres of 0.05% sterile agar solution and two new dilution series were made. Two hundred microlitres of the 10^{-2} and 10^{-3} dilution was plated onto the semi selective medium (Appendix I), in triplicates. The plates were incubated at 25 °C for 10-15 days. After this time, the colonies were counted and the CFU g^{-1} root was calculated.

2.7.2. **Number of colony forming units (CFU) per gram of soil**

The soil contained in each pot was separated from the plant root system, mixed and a 1 g sub-sample was taken at random and transferred into 9 ml of 0.05 % agar solution. The vials were shaken vigorously using a vortex mixer for 20 seconds. Dilutions at 10^{-2} and 10^{-3} of the same solution were plated (0.2 ml) in semi selective medium (composition in Appendix I). Plates were incubated at 25 °C for 10-15 days and after this time colonies were counted to calculate the CFU g^{-1} soil. This value was corrected for dry soil by drying a known amount of soil (100g) for 24 hours at 90 °C and weighed to measure the moisture content.

2.7.3. **Percentage of egg infection**

Ten egg masses were picked off from fresh roots and were crushed between a glass slide and a cover slip. Slides were gently washed using two millilitres of sterile distilled water, the eggs in suspension were counted and the concentration adjusted to

750-1000 eggs ml^{-1}. Two hundred microlitres of the egg suspension (three plates / treatment) were plated in Petri dishes containing water agar with antibiotics (Appendix I). Plates were incubated at 25 °C for three days and the percentage of infected eggs was assessed.

2.7.4. Estimation of the numbers of egg masses per root and number of eggs per egg mass

Egg masses taken from four sub-samples (one gram each) of roots were counted to assess the number of egg masses g^{-1}. To assess the number of eggs per egg mass, 10 eggs masses were picked off the roots and washed in 0.5 % sodium hypochlorite solution (8 % w/v available chlorine) for 1.5 minutes, to dissolve the gelatinous matrix and release the eggs. The eggs were suspended in water and were counted using a light microscope (50 x magnification).

CHAPTER 3: MEASUREMENT OF *IN VITRO* SAPROPHYTIC GROWTH

3.1 INTRODUCTION

There is great variation between biotypes of *Pochonia chlamydosporia* in terms of their ability to grow *in vitro* (Kerry *et al.*, 1986), colonise the rhizosphere (De Leij & Kerry, 1991) and infect nematode eggs (Irving & Kerry, 1986). These three parameters are considered essential when selecting potential BCAs (De Leij & Kerry, 1991; Kerry, 1998; Kerry, 2000; Kerry & Hominick, 2002).

The ability to produce chlamydospores is an important parameter to be measured when selecting biotypes and measuring saprophytic growth. As was mentioned in Chapter 1.6, chlamydospores are the preferred form of inoculum, as they are robust, can be added to the soil without additional nutrients and have a longer shelf life than other propagules (Kerry *et al.*, 1993; Kerry, 2001). Therefore, biotypes producing large numbers of viable chlamydospores should be selected. However, it is important to ensure that the ability to produce spores does not decrease with continued *in vitro* culturing and consequently, spore quantification methods should be applied as part of routine tests in quality control programmes, when mass producing fungal inoculum. Changes in fungal physiology and morphology have been associated with with continuous *in vitro* manipulation of organisms (Hajek *et al.*, 1990; Ryan *et al.*, 2002; Quesada-Moraga & Vey, 2003). In some cases, loss of virulence/pathogenicity had been reported and associated with continuous *in vitro* manipulation of organisms (Schaerffenberg, 1964; Nagaich, 1973; Hajek *et al.*, 1990) but have not been found in others (Hall, 1980; Ignoffo *et al.*, 1982; Brownbridge *et al.*, 2001; Vandenberg & Cantone, 2004; Scully & Bidochka, 2005).

The fungus *P. chlamydosporia* is a saprophyte which can proliferate in the rhizosphere of healthy plants without causing damage or lesions, and does not affect root growth (De Leij & Kerry, 1991). The presence of the fungus in the rhizosphere is essential so that it can colonise the egg masses when they become exposed on the root surface. For this reason it is thought that the effective control of *Meloidogyne* spp. is dependent on the colonisation of the rhizosphere and biotypes which are unable to establish in the root system fail to control these nematodes (De Leij & Kerry, 1991).

Lopez-Llorca *et al.* (2002) examined the rhizosphere of barley plants colonised by *P. chlamydosporia* and confirmed that three weeks after inoculation, the fungus infected and grew within epidermal and cortical cells, spreading longitudinally along the root cells but did not cause any visible symptoms, remaining confined to epidermal and cortical cells. Using a similar approach, Bordallo *et al.* (2002) studied the colonisation of roots of barley and tomato plants by egg parasitic and nematode trapping fungi and found that the fungus *P. chlamydosporia* was still present in the epidermal cells three months after inoculation. The authors also found differences in the extent of root colonisation by *P. chlamydosporia* between tomato and barley plants. Long lignitubers ensheathing hyphae were found only in tomato but not in barley roots. However, the amount of fungus inoculated in both of these two experiments is unlikely to be found under field and soil conditions where the abundance of the fungus is often much less and so, the results under these circumstances may be different. Lesser amounts of *P. chlamydosporia* were isolated from roots of tomato plants grown in non-sterilised soil, compared to those found in the rhizosphere (De Leij & Kerry, 1991).

Studies on the importance of the host plant in the biological control of nematodes *Meloidogyne* spp. by the fungus *P. chlamydosporia* showed that the growth of the fungus in the rhizosphere is affected by plant species and cultivar (Bourne *et al.*, 1994; Bourne *et al.*, 1996). Fungal colonisation is also thought to be affected by factors influencing plant growth, such as soil moisture or light intensity (Bourne, 1995) and sterile and non-sterile conditions (Bourne *et al.*, 1994). The ability to survive and compete with other organisms in the root and soil environment are important factors for the successful use of the fungus as a BCA (Bourne & Kerry, 2000).

When nematodes are present, fungal proliferation increases in the rhizosphere, and is greater than on healthy roots. This increase occurs when egg masses become exposed on the root surface (Bourne *et al.*, 1996). However, in very susceptible hosts where large galls are produced, or when nematode densities are high, the fungus is less effective in providing control, as eggs remain embedded within the root tissue and escape parasitism (Bourne & Kerry, 1999).

The screening of biotypes for the ability to grow in the rhizosphere usually uses a an *in vitro* test (Bourne *et al.*, 1994). It consists of growing seedlings of plants in a sterile and moist substrate to which the fungus is added. After incubation under

controlled conditions, roots are removed, cut in sections and plated onto agar where the proportion of colonised root sections is measured. It is assumed that biotypes that colonise < 80 % of the root segments are not effective colonisers of the rhizosphere in tests in field soil (Kerry *et al.*, 1998).

After the screen of biotypes in the *in vitro* assay, selected biotypes are then tested for their ability to colonise the rhizosphere in non-sterilised soil (Bourne *et al.*, 1994). To isolate and identify the fungus from roots and soil, a selective medium containing antibiotics and two benzimidazole fungicides is used (Kerry *et al.*, 1993).

This Chapter presents research on the development of methods to measure *in vitro* saprophytic growth of the fungus and to compare biotypes in their saprophytic abilities. These methods were later used to study the stability of sub-cultures of the fungus for chlamydospore production and the ability to colonise rhizospheres in both *in vitro* experiments and in a pot test.

The specific objectives were:

- To compare the conventional bioassay of counting chlamydospores using a haemocytometer with a new method using a spectrophotometer to read the absorbance of chlamydospore suspensions (3.3.1.) The objective was to try to find a quick and easy test to determine changes in spore production.

- To optimise the existing method to measure rhizosphere colonisation and to study root colonisation by different fungal biotypes (3.3.2. and 3.3.3). The aim was to reduce the time necessary for the bioassay to detect small differences between biotypes and test different plant roots for fungal colonisation.

- To test saprophytic ability of sub-cultures of the fungus using an *in vitro* bioassay and in a pot experiment, in order to study the effects of repeated sub-culture on the stability of the fungus and the possibility of the development of attenuation (3.3.4.).

3.2 MATERIAL AND METHODS

3.2.1 **Evaluation of spore production using a spectrophotometer**

Chlamydospores from five different biotypes (60, 104, 132, 280 and 392, Table 2.1.) were suspended in 1 % polyethylene glycol (PEG) 8000 solution in a range of concentrations between 10^5 and 10^7 chlamydospores ml^{-1}. The spores required for this test were extracted from flasks containing rice grain, followed the method described in Section 2.4. Serial chlamydospore suspensions were counted using a haemocytometer and the level of absorbance (600 nm) of the spore suspensions was determined using a spectrophotometer (CaryWin UV). The results were then compared using linear regression in Genstat®. The experiment was duplicated. Additionally, the average size of chlamydospores was recorded for each of the biotypes tested. A suspension of chlamydospores was plated in Petri dishes and examined microscopically (Zeiss Axiovert 135) at x 400 magnification. Ten chlamydospores per biotype were photographed and measured in both directions at right angles using the computer software programme Leica Qwin and Lida®. Statistical analysis of data was performed using analysis of variance in Genstat®. Comparison between chlamydospore average size and absorbance was analysed using linear regression.

3.2.2 **Tests on rate of colonisation**

A simple *in vitro* test was developed to evaluate the rhizosphere competence of the biotypes, by measuring the percentage of root colonisation. Initial work has aimed to reduce the time necessary for the bioassay to discriminate reliably differences between biotypes.

Basically, three biotypes of *P. chlamydosporia* (10, 400 and 280, Table 2.1.) were tested in for ability to colonise rhizosphere of maize roots (*Zea mays*) at different time intervals.

Maize (*Zea mays* L. Katumani) seeds were surface sterilised using 10 % of an 8% sodium hypochlorite solution (Fisher Scientific, UK) with one drop of Tween 20 and shaken at room temperature for one hour. Seeds were then rinsed five times with sterile distilled water and air-dried inside a sterile flow cabinet. The presence of contaminants was checked by plating seeds in germination media (Appendix I) for five days at 25 °C. Surface sterilised seeds were dipped in a suspension of chlamydospores

(3 x10^4 spores per seed) and were sown in Magenta® pots containing 250 ml of moist vermiculite, at 10 mm depth from the surface. The quantity of spores carried by each seed was determined before the beginning of the experiment by weighing wet seeds, dry seeds and seeds dipped in a spore suspension where the concentration of chlamydospores was known. The quantity of spores that were carried by each seed was calculated from the difference in weight between the non inoculated and inoculated seed. The spores required for this test were extracted from flasks containing rice grain, followed the method described in 2.4. Viability of inoculum was checked before inoculation.

After 4, 8, 12 and 16 days roots were removed from the pots, and in sterile conditions were cut in one cm sections and plated on 0.8% water-agar with antibiotics (Appendix I). The number of total and colonised roots was determined after two days incubation at 25°C and the percentage of rhizosphere colonisation determined. The results were analysed using general linear models (GLM) using Genstat®. The output of the analysis can be found in Appendix II.

3.2.3 **Tests on rhizosphere colonisation from different seed treatments**

To measure saprophytic ability *in vitro*, rhizosphere colonisation was tested by applying different biotypes to seeds, with the objective to check if biotypes colonise the rhizosphere of different plant species to the same extent, and also to select a plant where differences between biotypes could be quickly detected, if any differences in colonisation occur. Seeds of maize (*Zea mays* L., cv. Katumani), pea (*Pisum sativum* L., cv. Feltham First) and bean (*Phaseolous vulgaris* L., Kenyan local variety) were inoculated with chlamydospores at a rate of 3x10^4 spores per seed. Eleven biotypes of *P. chlamydosporia* were tested (biotypes 10, 16, 60, 69, 104, 132, 280, 309, 392, 399 and 400, Table 2.1). The experiment was performed under sterile conditions using Magenta® pots and the seeds inoculated with the fungus were sown in moist vermiculite. After eight days, roots were taken and the percentage of rhizosphere colonisation was evaluated, as described in 3.2.2. To adjust the percentage of rhizosphere colonisation to root length, the rhizosphere colonisation index was calculated by dividing the number of root sections which were colonised with fungus by the total length of root.

Immediately after treatment, the inoculation method was tested by assessing the number of spores adhering to the surface of individually treated seeds. Three individual seeds from each plant were inoculated with chlamydospores (3×10^4 spores per seed). After inoculation, seeds were individually washed in 15 ml of sterile distilled water. Two hundred microlitres of this suspension was plated out on 0.8 % water agar plus antibiotics and on the semi-selective medium (three plates per seed). Plates were evaluated after two days in water agar and 14 days in semi-selective medium and the number of CFU was assessed. Analysis of variance was applied to the results using Genstat®. When data showed a clear skewed distribution, it was transformed using logarithm in the following way:

$$\text{Logit} = 0.5 \times \log [C/ (1-C)];\ C = [(\%\ \text{Colonisation} \times 10 + 0.5)]/1001$$

Results of the analysis are presented in Appendix II.

To check that the results were consistent, two pilot experiments were performed prior to this experiment. The output analysis is presented in Appendix II.

3.2.4 Effect of repeated culturing on the ability to produce chlamydospores and to colonise the rhizosphere using an in *vitro* bioassay and in a pot experiment

Sub-cultures derived from three biotypes of *P. chlamydosporia* (280, 392 and 132, Table 2.1) were obtained using the method described in 2.5., and were compared with the initial cultures of the fungus for the ability to produce chlamydospores and to colonise the rhizosphere of plants, in two different experiments. The effects of repeated culturing on spore production and rhizosphere colonisation were studied both in *vitro* and in a pot experiment. Sub-cultures were compared using analysis of variance using Genstat® and transformed as described above (3.2.3). The output of the analysis is presented in Appendix II.

- *In vitro* bioassay:

To evaluate changes in the ability to produce chlamydospores, initial cultures (one week of sub-culture) and sub-cultures (60 weeks of sub-culture) of the fungus growing in Petri dishes, were used to inoculate a rice grain medium, following the method described in 2.4. After 21 days of growth, the number of chlamydospores per g of rice and the percentage of spore germination were assessed for both treatments. Chlamydospores from these cultures were used in the inoculation of disinfected maize

seeds which were sown into Magenta® pots containing moist vermiculite, using the method described in 3.2.2. After eight days growth in sterile conditions, roots were taken and the percentage of root colonisation was evaluated, as described before (3.2.2).

- Pot test experiment:

In a different experiment, chlamydospores were inoculated to soil (5000 chlamydospores g^{-1} soil) and added to pots before transplanting tomato plants. Details of this experiment are detailed in 2.8. The estimation of rhizosphere colonisation was measured by assessing the number of CFU per g of root.

3.3 RESULTS

3.3.1 Evaluation of spore production using a spectrophotometer

A positive, linear and significant relationship between the level of absorbance and chlamydospore concentration was detected, for the five biotypes tested (Figure 3.1). The absorbance level detected was higher when increased concentrations of chlamydospores were suspended in PEG. This relation was valid for concentrations between 10^5 to 10^7 chlamydospores ml^{-1}. No significant differences between biotypes were found at a concentration level of 10^5 and 10^7 chlamydospores ml^{-1}. The size of chlamydospores differed between biotypes (Figure 3.2) but was not related to the levels of absorbance measured at different concentrations (Figure 3.3).

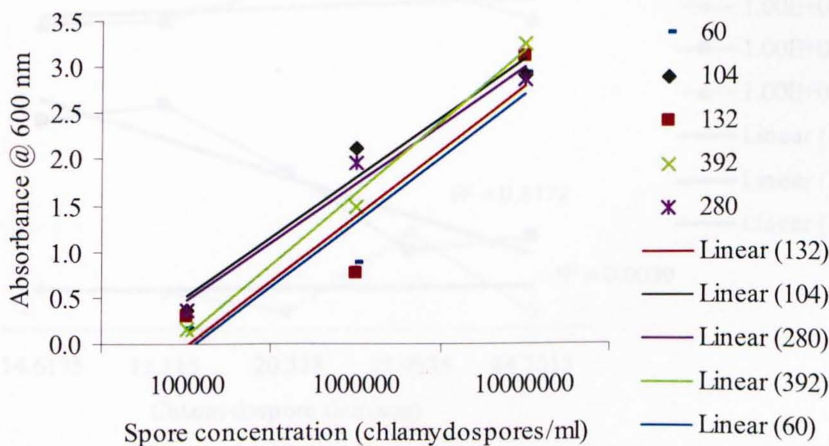


Figure 3.1 - Relation between absorbance level and chlamydospore concentrations of different biotypes – 60, 104, 132 and 392 - of *Pochonia chlamydosporia*. A positive linear regression was obtained for all biotypes ($r^2(60)=0.927$, $r^2(104)=0.952$, $r^2(132)=0.8722$, $r^2(280)=0.9748$, $r^2(392)=0.9937$).

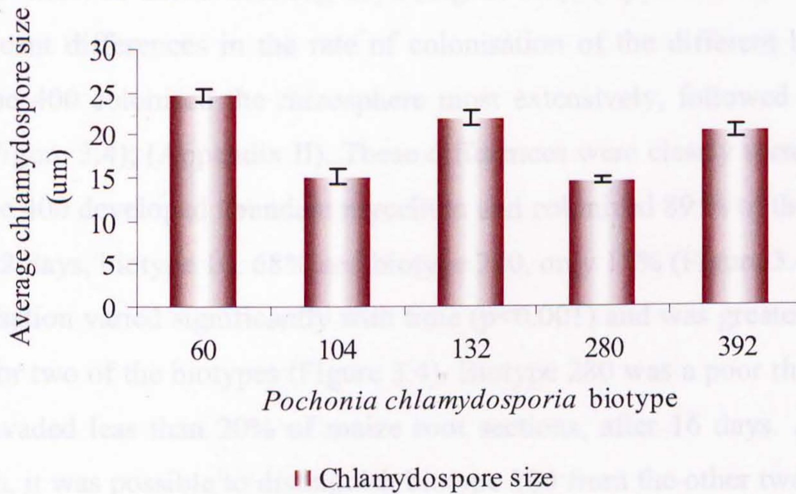


Figure 3.2 – Average size of chlamydospores (μm) for each of the five biotypes studied (60, 104, 132, 280 and 392). Ten chlamydospores per biotype were examined microscopically (Zeiss Axiovert 135) at x 400 magnification and were measured in two directions at right angles using the computer software programme Leica Qwin and Lida®. Differences between biotypes were found (ρ>0.05) using ANOVA single factor analysis. Different letters represent significant differences. I-Standard error bar.

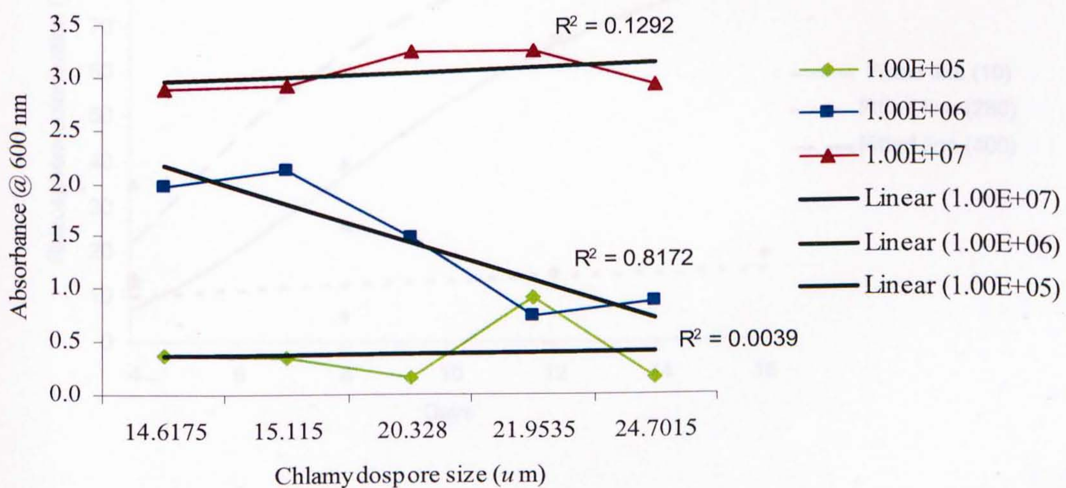


Figure 3.3 – Relationship between absorbance and average chlamydospore size for different concentrations of chlamydospores. No relation between the size of chlamydospores and the level of absorbance was found in suspensions of chlamydospores suspended at 10^5 and 10^7.

3.3.2 **Tests on rate of colonisation**

The GLM analysis used a fitted linear model with binomial distribution and logit link. First, days and biotypes were fitted as factors and treatment means were predicted, then, a line was fitted with log days (Figure 3.4); (Appendix II). The results showed significant differences in the rate of colonisation of the different biotypes ($p<0.001$). Biotype 400 colonised the rhizosphere most extensively, followed by biotype 10 and 280 (Figure 3.4); (Appendix II). These differences were clearly seen on the agar plates; biotype 400 developed abundant mycelium and colonized 89 % of the roots, on average, after 12 days, biotype 10, 68% and biotype 280, only 15% (Figure 3.4). The rhizosphere colonisation varied significantly with time ($p<0.001$) and was greater than 80% after 16 days for two of the biotypes (Figure 3.4). Biotype 280 was a poor rhizosphere coloniser and invaded less than 20% of maize root sections, after 16 days. After eight days of growth, it was possible to distinguish biotype 280 from the other two biotypes, in terms of their ability to colonise the rhizosphere. The differences between this biotype and biotypes 10 and 400 persisted until the end of the experiment, which finished after 16 days of root and fungal growth.

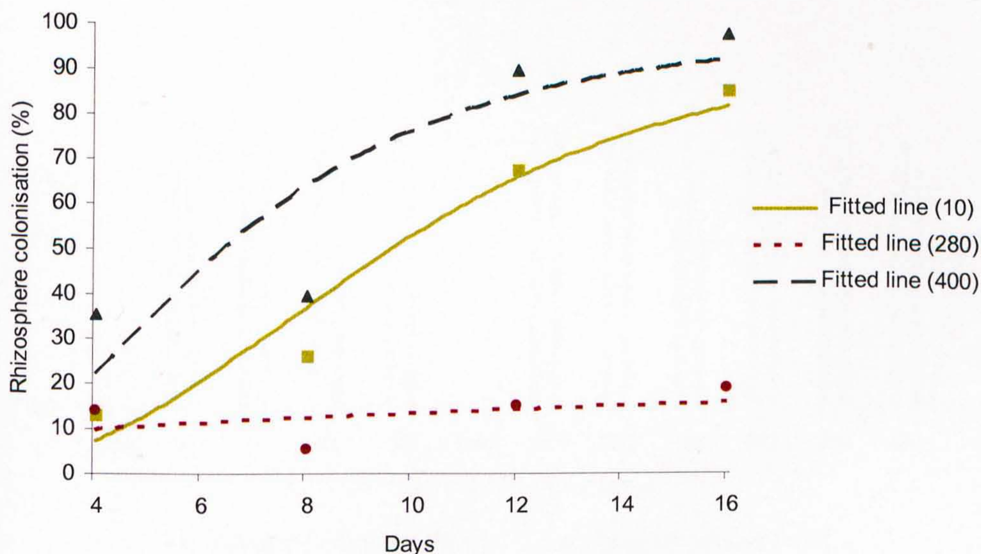


Figure 3.4 - Ability to colonise the rhizosphere of maize plants grown in sterile conditions of three biotypes of *Pochonia chlamydosporia* (biotypes 10, 280, and 400). Biotypes were inoculated using a known concentration of chlamydospores in suspension (3 x10^4 spores per seed of maize). Significant differences between biotypes and days were found. ($p<0.001$). Biotype 280 was significantly different from biotypes 132 and 392 and this difference could be detected after 8 days of growth until the end of the experiment.

3.3.3 Tests on rhizosphere colonisation from different seed treatments

Rhizosphere colonisation was significantly greater on pea and bean than on maize, for all the biotypes tested except for biotype 104, for which it was not possible to obtain results, due to the presence of contaminants on the seed (Figure 3.5); (Appendix II). Pea produced fewer roots to sample after eight days, and this may explain the greater percentages of rhizosphere colonisation (Figure 3.5 - C). However, seeds from maize carried more spores (Figure 3.6), suggesting that biotypes were less able to colonise the rhizosphere of this plant. The type of seed coat influenced the adherence of the chlamydospores to the seeds, and the quantity of spores carried by each type of seed was significantly different ($p<0.001$) (Figure 3.6); (Appendix II).

Significant differences between biotypes were found in all the seeds tested but higher number of significant differences were observed in maize ($p<0.001$) (Figure 3.5-A) than on bean and pea (Figure 3.5 – B and C). Although the results may not correlate with what happens in field conditions, maize should be used as a bioassay, to distinguish biotypes and changes in the quality of inoculum produced by repeated mass culture.

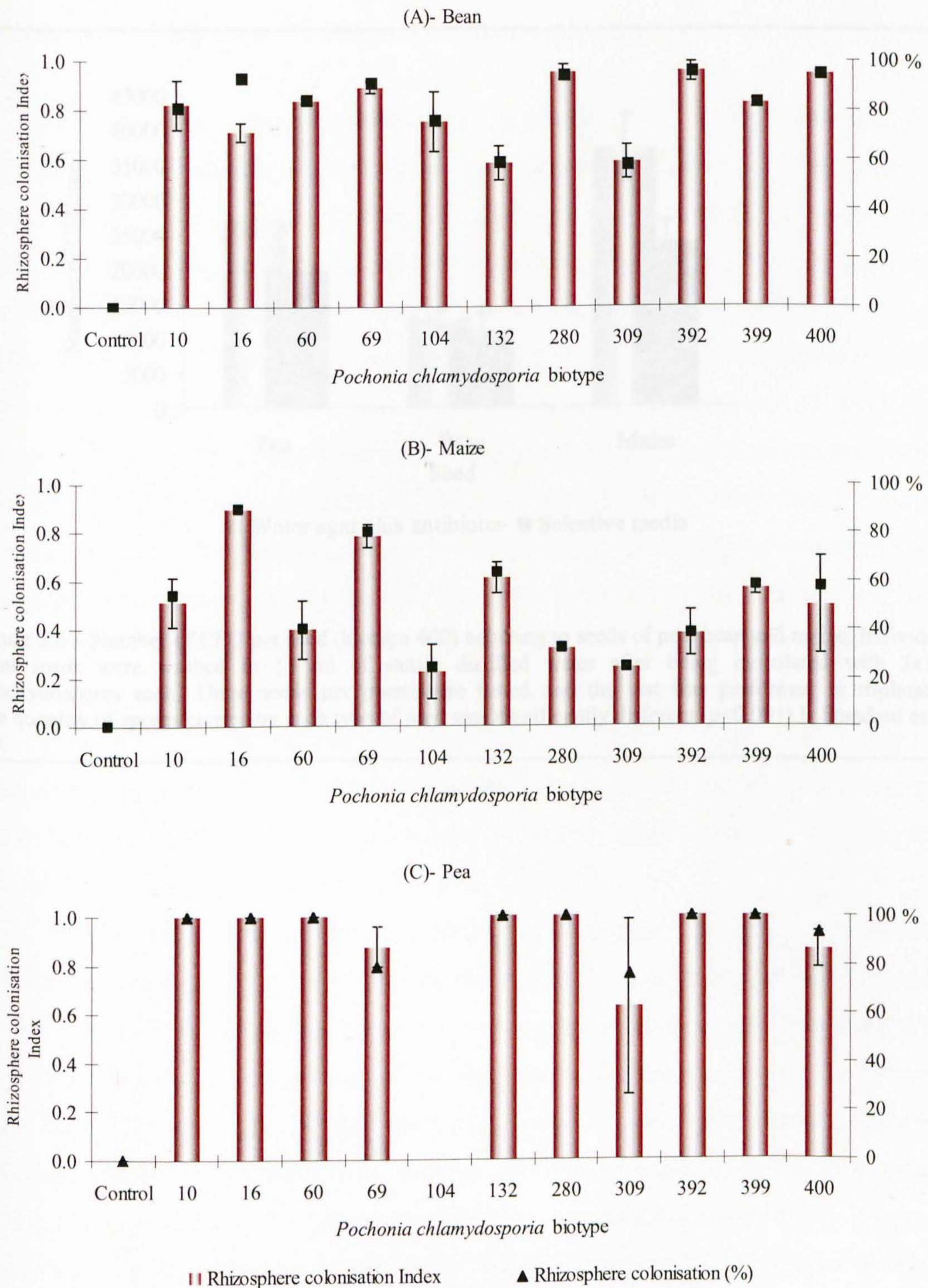


Figure 3.5 – Rhizosphere colonisation index and percentage of rhizosphere colonisation by eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 104, 132, 280, 309, 392, 399 and 400) in bean (A), maize (B) and pea (C), after eight days of growth in sterile conditions. It was not possible to obtain results for biotype 104 on pea due to the presence of contaminants in the seed. The different treatments were fully randomised. A combined analysis of variance was in Genstat. Higher number of significant differences between biotypes were found using maize as host plant ($p<0.001$).

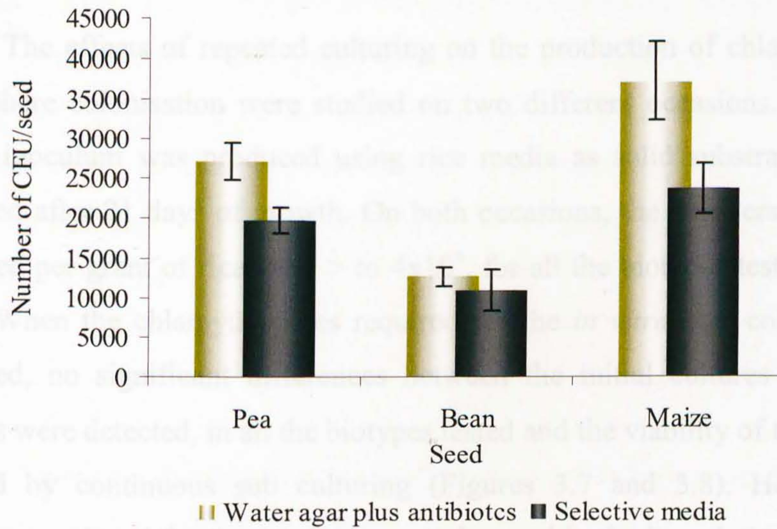


Figure 3.6 – Number of CFU per seed (biotype 400) adhering to seeds of pea, bean and maize. Individual plant seeds were washed in 15 ml of sterile distilled water after being inoculated with $3x10^4$ chlamydospores each. Three seeds per plant were tested and the test was performed in triplicates. The quantity of spores carried by each type of seed was significantly different ($p<0.001$) I- Standard error bar.

3.3.4 Effect of repeated culturing on the ability to produce chlamydospores and to colonise roots using an in *vitro* bioassay and in a pot experiment

The effects of repeated culturing on the production of chlamydospores and on rhizosphere colonisation were studied on two different occasions. At both times, the fungal inoculum was produced using rice media as solid substrate and spores were extracted after 21 days of growth. On both occasions, the numbers of chlamydospores produced per gram of rice were > to 4×10^6, for all the biotypes tested (Figures 3.7 and 3.10). When the chlamydospores required for the *in vitro* root colonisation test were produced, no significant differences between the initial cultures and 60 week sub-cultures were detected, in all the biotypes tested and the viability of these spores was not affected by continuous sub culturing (Figures 3.7 and 3.8). However, the fungal inoculum produced for the second time, to be used in the inoculation of non-sterile soil, significant differences ($p<0.05$) between the initial culture and after 60 weeks of sub-culture were found for biotype 280 (Figure 3.10). Furthermore, the viability of the spores of biotype 280 was also significantly decreased ($p<0.05$) those after 60 week of sub culturing (Figure 3.11). For the remaining two biotypes tested, no changes in chlamydospore production or viability were detected between the initial cultures and sub-cultures ($p>0.05$) but fewer spores were produced, when compared with the first time of production (Figures 3.10 and 3.11).

The ability to colonise the rhizosphere of maize plants, in an *in vitro* bioassay, was not affected by the continuous sub culturing of the fungus after 60 week of repeated culture (Figure 3.9). Using this bioassay, no changes in rhizosphere colonisation were detected for the three biotypes studied but significant differences between the biotypes were found (Figure 3.9). In soil conditions, results were more variable between replicates and biotypes (Figure 3.9). Biotype 280 did not establish in the rhizosphere, whereas biotype 132 colonised it most extensively. As observed in the *in vitro* bioassay, changes in the rhizosphere colonisation by different biotypes of the fungus were not detected between the initial cultures and after 60 week of continuous sub culturing (Figure 3.12). Overall, biotypes 132 and 392 were considered good chlamydospore producers and good root colonisers. Both produced > 10^6 chlamydospores g^{-1} rice (Figures 3.7 and 3.10), colonised up to 80 % of maize root sections after eight days of *in*

vitro growth (Figure 3.9) and were not affected by the continuous *in vitro* sub culturing (Figures 3.7 to 3.12). Biotype 280 was shown to be a poor rhizosphere coloniser of maize and tomato plants, in both *in vitro* and soil conditions (Figures 3.9 and 3.12) The fungus colonised, on average, only 20% of the root sections of maize, in the *in vitro* bioassay and was present in low amounts in the rhizosphere of tomato plants, in the pot test experiment. In addition, this biotype produced less spores than the other two biotypes tested and its ability to produce chlamydospores decreased with continuous sub culturing (Figure 3.10).

- Spore production and *in vitro* rhizosphere colonisation ability

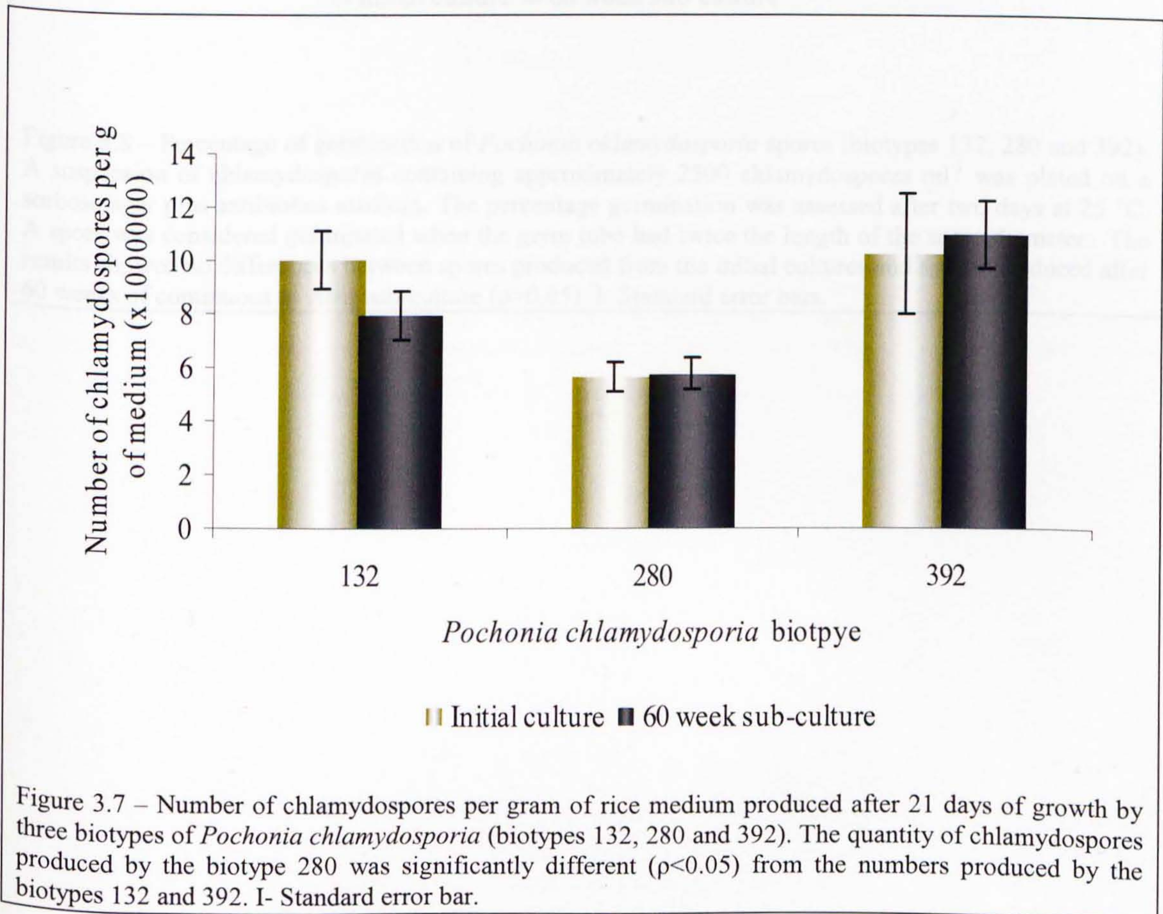


Figure 3.7 – Number of chlamydospores per gram of rice medium produced after 21 days of growth by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392). The quantity of chlamydospores produced by the biotype 280 was significantly different ($\rho<0.05$) from the numbers produced by the biotypes 132 and 392. I- Standard error bar.

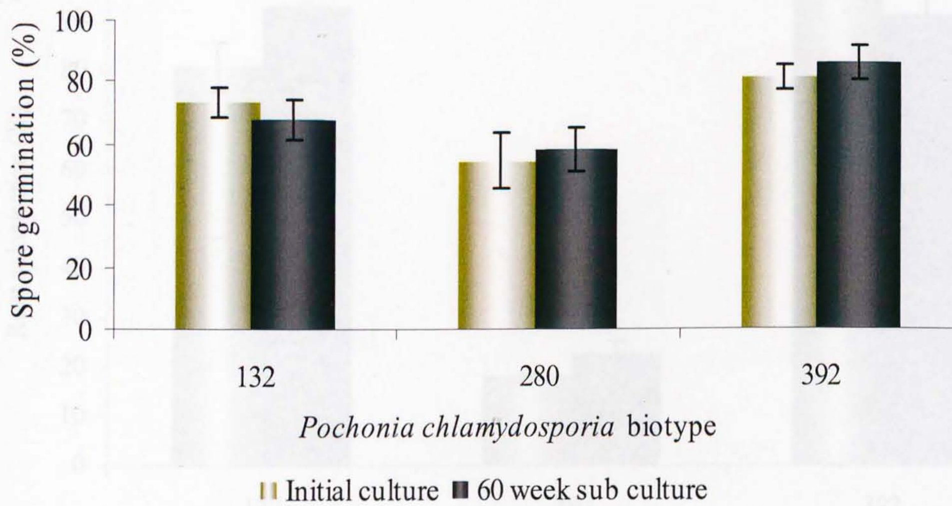


Figure 3.8 – Percentage of germination of *Pochonia chlamydosporia* spores (biotypes 132, 280 and 392). A suspension of chlamydospores containing approximately 2500 chlamydospores ml^{-1} was plated on a sorbose agar plus antibiotics medium. The percentage germination was assessed after two days at 25 °C. A spore was considered germinated when the germ tube had twice the length of the spore diameter. The results showed no differences between spores produced from the initial cultures and spores produced after 60 weeks of continuous *in vitro* sub-culture ($\rho>0.05$). I- Standard error bars.

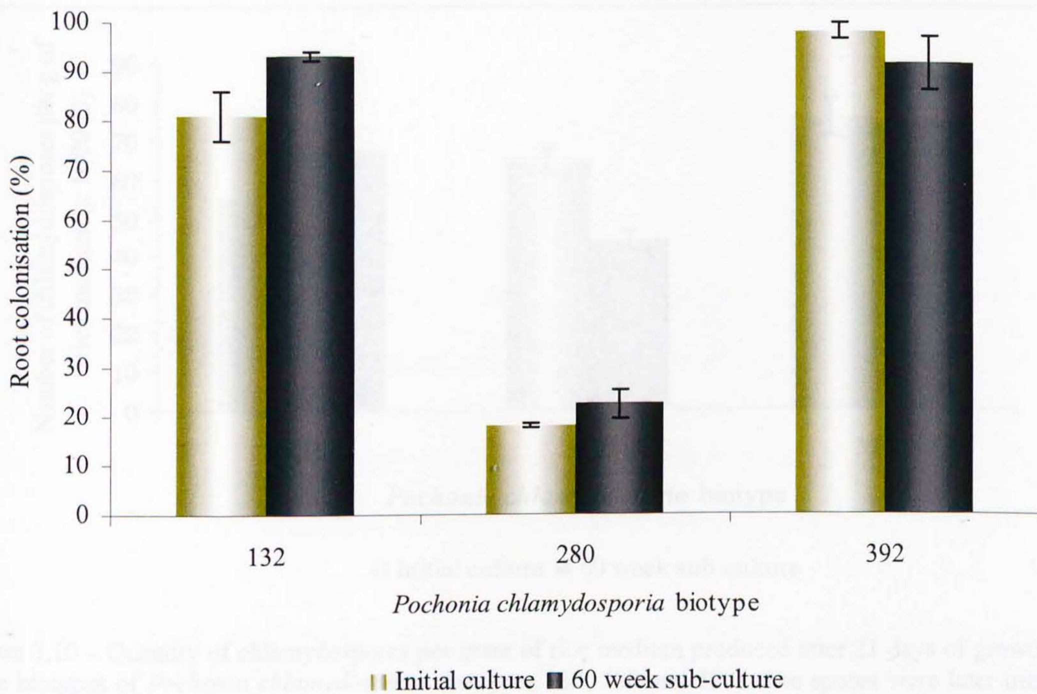


Figure 3.9 - Rhizosphere colonisation ability by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) in maize, after eight days of growth. No differences between spores produced from the initial cultures and spores produced after 60 weeks of continuous *in vitro* sub-culture were found ($\rho>0.05$). Biotype 280 was a poor rhizosphere coloniser of maize plants, when compared with the two other biotypes tested. I- Standard error bars.

Spore production and rhizosphere colonisation ability in soil conditions (pot test experiment)

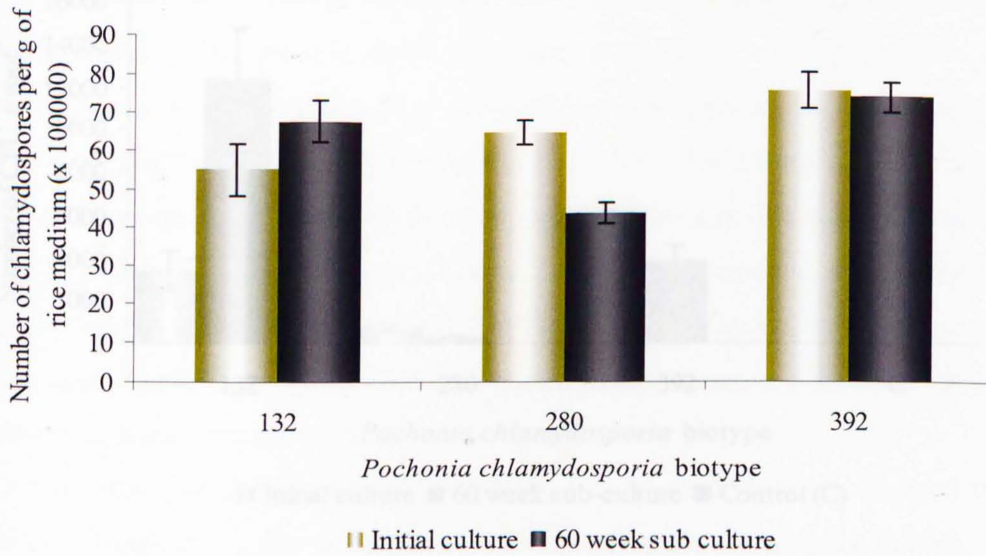


Figure 3.10 – Quantity of chlamydospores per gram of rice medium produced after 21 days of growth by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392). The spores were later used to study rhizosphere colonisation ability in soil conditions, in a pot test experiment. The quantity of chlamydospores produced by the biotype 280 was significantly different from the quantity of chlamydospores produced after 60 weeks ($\rho < 0.05$). I- Standard error bars.

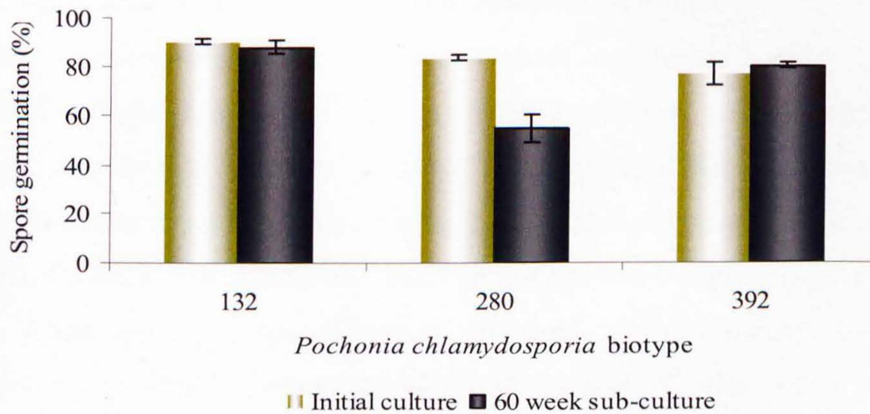


Figure 3.11 – Percentage of germination of *Pochonia chlamydosporia* spores (biotypes 132, 280 and 392). A suspension of chlamydospores containing approximately 2500 chlamydospores ml^{-1} was plated on a sorbose agar plus antibiotics medium. The percentage of germination was assessed after two days at 25 °C. A spore was considered germinated when the germination tube had twice the length of the spore diameter. Significant differences between spores produced from the initial culture and after 60 weeks of continuous *in vitro* sub-culture were found for biotype 280 ($\rho<0.05$). I- Error bars.

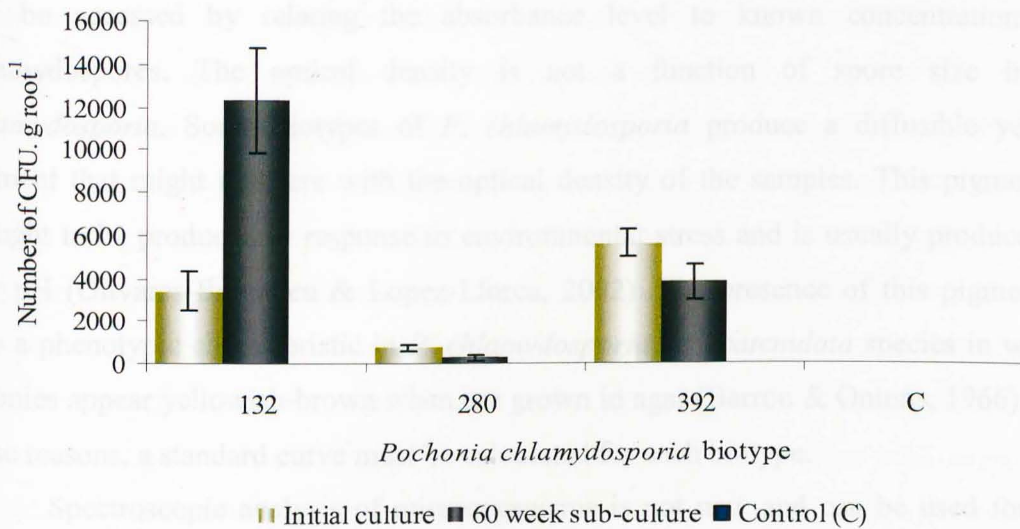


Figure 3.12 - Rhizosphere colonisation ability of three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) on tomato plants, after 65 days of growth. No significant differences between the number of CFU g^{-1} root produced from the initial cultures and after 60 weeks of continuous *in vitro* sub-culture were found (ρ>0.001). Biotype 280 showed little establishment in the rhizosphere when compared with biotypes 132 and 392. I- Standard error bars.

3.4 DISCUSSION

The quantification of numbers of chlamydospores present in a liquid suspension can be accessed by relating the absorbance level to known concentrations of chlamydospores. The optical density is not a function of spore size in *P. chlamydosporia*. Some biotypes of *P. chlamydosporia* produce a diffusible yellow pigment that might interfere with the optical density of the samples. This pigment is thought to be produced in response to environmental stress and is usually produced at low pH (Olivares-Bernabeu & Lopez-Llorca, 2002). The presence of this pigment is also a phenotypic characteristic in *P. chlamydosporia* var. *catenulata* species in which colonies appear yellowish-brown when are grown in agar (Barron & Onions, 1966). For these reasons, a standard curve must be calculated for each biotype.

Spectroscopic analysis of microorganisms is not new and can be used for the detection, identification, and enumeration of microorganisms and cells (Merek, 1969; Alupoaei *et al.*, 2004). Bacterial growth can be estimated using a spectrophotometer (Merek, 1969; Casimiri & Burstein, 1998). By measuring the amount of light absorbed by a bacterial suspension, the numbers of bacteria that are present in the suspension can be estimated. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. Using the same principle, pollen counts can also be estimated using the same method (Bynum & Smith, 2001). This process is quicker than the traditional method of using a cell counting chamber and, therefore, might be useful in production systems where the quantity of spores of a single biotype produced has to be continuously and carefully monitored. However, this method does not distinguish between viable spores and is less likely to be applied in resource poor systems, where cheaper alternatives are preferable to quicker methods. To estimate viable spores, plate techniques must be used.

When producing the fungus to be used in the bioassay, the quantity of chlamydospores slightly decreased to $< 10^7$ spores g^{-1} rice when inoculum was produced for the second time (Figures 3.7 and 3.10). This variation could be due to small differences in the process of production and fermentation of the inoculum. Small changes in temperature, aeration, and quantity of available water can influence and affect fermentation of the inoculum (Nagel, 2002). The protocol of inoculum production that was followed does not allow a rigorous control of all these three parameters and

was developed to produce chlamydospores for laboratory experiments and small field trials. However, for large field trials or for commercial use, fluctuations in the production of inoculum should be avoided and methods of production which can control these parameters should be developed in the future.

Differences in rhizosphere colonisation ability were found between different biotypes of *P. chlamydosporia*. Using an *in vitro* bioassay, differences could be detected as early as eight days, using maize as host. Although this plant is not a susceptible host for some root-knot nematodes, its roots were colonised to a different extent by different biotypes. Compared with pea and bean seeds, maize seeds germinated quickly and produced enough root to be sampled after eight days of growth. Also, seeds could be coated with large numbers of spores because of its size. Pea and bean seeds were also suitable for seed inoculation, but were slower to germinate and were easier to colonise by all the biotypes tested. After eight days, no differences between biotypes could be detected on pea or bean roots, which were fully colonised by the fungus. Differences in rhizosphere colonisation between the three plant hosts tested can be attributed to factors related to the structure of each plant and/or its susceptibility to nematode attack. Bordallo *et al.* (2002) suggested that monocotyledonous/dicotyledonous are colonised differently by nematophagous fungi because of their differences in cell wall composition. Graminaceae cell walls contain less pectin and extensin than other plants and for this reason are thought to be easier to colonise by fungi. However, Persmark & Jansson (1997) found that legumes such as pea (dicotyledonous), favour the proliferation of both nematophagous fungi and nematodes in the rhizosphere, when compared with non legumes such as barley and that this proliferation could be related with the presence of nematodes or with the increase in nutrients present in the rhizosphere and available for fungi. Bourne *et al.* (1996) found that solanaceous plants (tomato and aubergine), which are more susceptible to root knot nematodes, tended to support less fungal colonisation whereas brassicas (kale and cabbage) support the greatest amount of fungus in the rhizosphere. In this experiment, maize supported less colonisation by most of the biotypes when compared with pea and bean, however, biotypes 16 and 69 colonised more than 80% of maize root segments, equivalent to that in pea and bean, showing the extent of rhizosphere colonisation varies with the biotype and plant host. Being a saprophyte allows the fungus to grow in the absence of

nematodes and this characteristic probably confers on the fungus a certain level of tolerance and versatility in colonising different root environments and substrates.

After 60 weeks of continuous sub culturing, the three biotypes were able to colonise the rhizosphere of maize to the same extent as the initial cultures and they were also able to colonise the rhizosphere of tomato plants after 65 days of growth in soil. The repeated sub-culture on agar did not stop or decrease rhizosphere colonisation but differences between biotypes were detected. Biotype 280, originally isolated from cyst nematode eggs, performed less well in the rhizosphere colonisation test, both *in vitro* and in pot test experiments after repeated mass culture. This biotype also decreased the ability to produce chlamydospores and spore viability after 60 weeks of sub-culturing, which might indicate that biotype 280 is less stable and more susceptible to change than the other biotypes tested. In these other two biotypes there was no evidence of attenuation, even in these extreme conditions of repeated sub culturing.

CHAPTER 4: MEASUREMENT OF *IN VITRO* PARASITIC GROWTH

4.1 INTRODUCTION

It has been demonstrated that the presence of nematodes in the rhizosphere increases fungal abundance and parasitic activity in *Pochonia chlamydosporia* (Bourne & Kerry, 1999). However, the mechanisms by which the fungus switches from a saprophyte to a parasite are not well understood (Kerry, 2001). The factors affecting parasitism in the rhizosphere are described in Chapter 1, section 1.4 and include aspects related to the presence of nematodes, nematode species (Mauchline *et al.*, 2004), nematode densities in the rhizosphere (Kerry & Bourne, 1996), egg maturity (Irving & Kerry, 1986), and the type of plant and its susceptibility to nematode attack (Kerry & Bourne, 1996), soil temperature (Irving & Kerry, 1986) and inoculum density (Kerry *et al.*, 1986).

The importance or interactions between these factors are usually studied in pots or field experiments but this kind of study involves large amounts of work, time and resources and for this reason it is done only for selected biotypes. The screening of potential biotypes is usually performed in *in vitro* tests. These tests are normally quick and enable many biotypes to be eliminated before more time and resource consuming screens are conducted in pot and field experiments (Kerry *et al.*, 1998). *In vitro* parasitism tests are also useful in quality control systems, when it is necessary to monitor the ability of a selected fungal biotype to parasitise nematode eggs and that attenuation through repeated sub-culturing is not occurring. However, the performance of biotypes in the laboratory tests (Kerry *et al.*, 1986) may be different from soil (Kerry *et al.*, 1984). In previous studies, biotypes that were good parasites of eggs *in vitro*, were shown to be ineffective when tested in soil if they failed to colonise the rhizosphere (De Leij & Kerry, 1991).

A standard *in vitro* test to measure egg parasitism was adapted from Kerry & Crump (1977) by Irving & Kerry (1986). The method used was reliable in determining parasitic growth in cyst nematode eggs by six biotypes of *P. chlamydosporia* and could detect differences between the biotypes after three weeks at 12°C. Later, this method was optimised for root-knot nematode eggs and biotypes isolated from tropical and sub-tropical climates and the time necessary for the bioassay was reduced to three days at 25

°C (Kerry *et al.*, 1998). The proportion of eggs parasitised over a fixed period of time was used as an estimate of virulence from each biotype.

The first part of this Chapter focused on the development of a novel method to estimate parasitism *in vitro* and the results were compared with the standard method developed by Irving & Kerry (1986). Information about egg parasitism ability (*in vitro*) was collected for a range of biotypes with the objective to relate *in vitro* parasitic growth with rhizosphere colonisation ability (Chapter 3) and secretion of extracellular enzymes (Chapter 5). The aim was to determine if biotypes with the best parasitic performance were also good rhizosphere colonisers, enzyme producers or vice versa (Chapter 7). Studies on nematode trapping fungi have shown that attraction intensity is lowest in fungi with moderate and high saprophytic ability (Jansson & Nordbring-Hertz, 1979) and growth rate and good saprophytic ability are accompanied by lower predacious efficiency (Cooke, 1963). In previous studies, the relationship between egg parasitism ability and fungal growth rate, or the ability to produce chlamydospores by *P. chlamydosporia* was not related (Kerry *et al.*, 1986).

In the second part of the Chapter, biotypes were compared for their ability to produce appressoria *in vitro*. As mentioned in Chapter 1, appressoria are specialised structures produced by the fungus that allow penetration of the nematode eggshell. It is not know if biotypes could differ in their ability to produce appressoria *in vitro* and if any differences could be correlated with virulence.

In the third part of this Chapter, sub-cultures of the fungus were tested for egg parasitism ability in an *in vitro* test and in a pot experiment, in order to study the effects of repeated sub-culture on the stability of the fungus and the possibility of the development of attenuation.

Observations on egg infection by the fungus were studied in the last part of the Chapter. The aim was to visualise how the fungus spread inside the nematode.

The specific objectives were:

- To develop a novel bioassay to estimate egg parasitism *in vitro* and to comparison with a standard bioassay;
- To measure *in vitro* appressoria formation of different biotypes of the fungus and possible relation with virulence;

- To study the effects of repeated culturing of three biotypes of the fungus, on the ability to parasitise eggs of *Meloidogyne* spp. using an *in vitro* bioassay and in a pot experiment;
- To visualise *P. chlamydosporia* spread inside single mature nematode eggs.

4.2 MATERIAL AND METHODS

4.2.1 **Development of a novel test to estimate egg parasitism *in vitro* and comparison with a standard bioassay**

Novel method to estimate egg parasitism

A suspension of 100 eggs ml^{-1} of root-knot (*Meloidogyne* spp.) and cyst nematode eggs (*Globodera pallida*) was added to 2.5 ml microtubes with conidia of *P. chlamydosporia* in 0.0125 % yeast extract in water (YEM), at a concentration of $5.5x10^3$ conidia ml^{-1}, to give a total volume of 1.5 ml. Eggs were separated from egg masses using the method described in Chapter 2, section 2.6. Conidia were obtained from colonies of *P. chlamydosporia* growing on Potato Dextrose Agar (PDA). Ten millilitres of media containing 0.0125 % YEM in water were added to Petri dishes colonised by the fungus and the mycelium was gently mixed with the water using an L-shaped glass rod. The suspension was transferred onto a 10 μm aperture sieve to trap mycelium and chlamydospores and the remaining suspension, containing the conidia, was used in the experiment. The number of conidia was counted using a haemocytometer (400 x magnification) and the concentration was adjusted to $5.5x10^3$ conidia ml^{-1}. The extraction of conidia and inoculation of nematode eggs was performed inside a laminar flow hood to avoid contamination. The samples were left in a horizontal shaking incubator at 150 rpm and 25 °C for 48 hours and after this time the proportion of infected eggs was assessed using a stereo microscope (50 x magnification). Nine different biotypes of the fungus were tested (biotypes 10, 16, 60, 104, 132, 280, 392, 399, 400, Table 2.1) and the experiment replicated four times. Controls consisted of suspensions of eggs non-inoculated with fungus. As an additional control, a treatment using conidia of *Botrytis cinerea*, a plant pathogen, was tested against root-knot and cyst nematode eggs. *B. cinerea* was kindly supplied by Professor Isabel Abrantes and L. Fonseca (University of Coimbra, Portugal). The nematode eggs were obtained using the protocol described in Chapter 2, section 2.6.

Effect of conidial concentration on the proportion (%) of *Meloidogyne* spp. and *Globodera pallida* infected eggs by two biotypes of *Pochonia chlamydosporia*

In a second experiment, different concentrations of conidia (ranging from 10 to 10^4 spores ml^{-1}), from biotypes 392 and 400 were tested against *Meloidogyne* spp. and *G. pallida* eggs and the percentage of parasitism was measured for each concentration of spores tested.

Effects of YEM on the percentage of egg hatch and parasitism

To test if the media (yeast extract in water) used in the experiment influenced egg hatch, three solid watch glasses each containing twenty *Meloidogyne* spp. eggs were suspended in water or 0.0125% yeast extract, and incubated at 25°C for 20 days. The percentage of egg hatch was counted every three days for the first 15 days and then after 20 days. The percentage of nematodes hatched was compared in both treatments.

The effects of YEM on the percentage of parasitism were tested for biotypes 280 and 392. *G. pallida* and *Meloidogyne* spp. eggs (100 eggs ml^{-1}) were inoculated with a suspension of conidia (5.5×10^3 spores ml^{-1}) in sterile distilled water and the percentage of egg infection was assessed after three, five and seven days.

Standard bioassay for parasitism

Nine biotypes of the *P. chlamydosporia* (biotypes 10, 16, 60, 104, 132, 280, 392, 399 and 400, Table 2.1) growing on CMA were washed in 2-5 ml of sterile distilled water and 0.2 ml of fungal suspension were spread onto a Petri dish (9 cm diameter) containing 0.8 % water agar with antibiotics (Appendix I). Plates were incubated at 25 °C for two days. Approximately 200 root-knot nematode eggs (*Meloidogyne* spp.) / cyst nematode eggs (*G. pallida*) were added to each plate and spread around the plate which was colonised by the fungus. The nematode eggs were obtained using the protocol described in Chapter 2, section 2.6. The Petri dishes were incubated at 25°C and after 3 days the number of parasitised eggs was counted. Controls consisted of P. dishes inoculated with nematode eggs, without the fungus.

Effect of type and concentration of fungal inoculum on the proportion (%) of *Meloidogyne* spp. eggs infected by using a standard method to estimate egg parasitism

The influence of the quantity and type of fungal inoculum (conidia or chlamydospores) on the rate of parasitism of nematode eggs (*Meloidogyne* spp.) was studied in a separate experiment. Concentrations of spores, either conidia or chlamydospores, ranging from 10 to 10^5 spores per Petri dish (9 cm of diameter) were tested using three biotypes of *P. chlamydosporia* (biotypes 280, 392 and 400, Table 2.1). The proportion of egg parasitised was determined after three days of growth using the standard method described above.

4.2.2 Measurement of appressoria formation of different biotypes of *Pochonia chlamydosporia* using a hydrophobic substrate

To measure differences between biotypes in the ability to form appressoria *in vitro*, a method developed by Lopez-Llorca *et al.* (2002) was tested. Drops of 10 µl of conidial suspensions (1-5x10^3 conidia ml^{-1}) in 0.0125 % YEM were incubated on 15 x 15 mm squares of polystyrene (Petri dish Bibby Sterilin, UK). Conidia were obtained from colonies of *P. chlamydosporia* growing in PDA as described in 4.2.1. Eleven biotypes of the fungus were tested (biotypes 10, 16, 60, 69, 104, 132, 280, 309, 392, 399 and 400, Table 2.1). The number of germ tubes formed from the germinated conidia and the proportion with appressoria was assessed microscopically (400 x magnification) using Calcofluor white M2R, after 24 hours incubation at 25 °C in a moist chamber. Controls were made using water only and plating the spores on agar.

The effects of different solutions on the formation of appressoria *in vitro* and on the germination (%) of conidia were studied using biotype 392. Conidia (1-5x10^3 conidia ml^{-1}) were suspended in water, 0.0125% YEM, Ringer's solution (6 g NaCl, 0.075 g KCl, 0.1 g $CaCl_2$, 0.1 g $NaHCO_3$) and 0.1 % Triton. Ten micro litres of conidial suspension were inoculated on the 15 x 15 mm squares of polystyrene. The numbers of conidia germinated and the number of appressoria formed.was recorded after 24 hours, at 25° C and the proportion estimated and compared between biotypes.

4.2.3 Effect of repeated culturing on the ability to parasitise *Meloidogyne* spp. nematode eggs using an in *vitro* bioassay and in a pot experiment by three biotypes of *Pochonia chlamydosporia*

Sub-cultures from three biotypes of *P. chlamydosporia* (280, 392 and 132, Table 2.1) were obtained using the method described in 2.5, and were compared with the initial cultures of the fungus for the ability to parasitise eggs of *Meloidogyne* spp. The effects of repeated culturing on the level of egg parasitism were studied both in *vitro* using the standard bioassay described in 4.2.1, and in a pot experiment. The numbers of egg masses per root, number of eggs per egg mass, numbers of CFU per gram of soil and the amount of egg infection were assessed. The methodology used is described in Chapter, section 2.8.

4.2.4 Data analysis

The experiments described in 4.2.1, 4.2.2 and 4.23 were analysed using analysis of variance in Genstat®. The data were checked to ensure the normality of variance by plotting histograms of residuals and plotting the residuals against the fitted values, respectively. When data showed a clear skewed distribution, it was transformed using logarithm in the following way:

$$\text{Logit} = 0.5 \times \log [C/(1-C)];\ C = [(\%\ \text{egg parasitism} \times 10 + 0.5)]/1001$$

Additionally, PROBIT analysis was applied to the results described in 4.2.1 and a LD_{50} was calculated and Spearman correlation coefficient was calculated to compare the ability of different biotypes of the fungus to produced *in vitro* appressoria with egg parasitism ability (4.2.2). The Spearman correlation coefficient is a non-parametric measure of correlation – it assesses how well an arbitrary monotonic function could describe the relationship between two variables, without making any assumptions about the frequency distribution of the variables (Bobko, 2001). The output of all analysis is presented in Appendix III.

4.2.5 Microscopy studies in egg infection

Sample preparation

Root-knot nematode eggs containing second-stage juveniles were infected with conidia of the fungus (biotype 392, Table 2.1) suspended in 0.0125 % YEM in water, using the protocol described in 4.2.1, for seven days. Thirty single infected eggs were carefully lifted from the media and were placed in 20 µl of 4% low melting point agarose. Nematode eggs containing second-stage juveniles without fungus were used as controls (30 eggs). Eggs were surfaced sterilised using a solution of 0.5 % commercial bleach (8 % w/v available chlorine) for 1.5 minutes. This technique has proved to keep the eggshell intact and not affect egg physiology (Bird & McClure, 1976).

The samples were fixed in 4 % paraformaldehyde and 2.5 % glutaraldeyde in 0.1 M phosphate buffer (pH 7.2) and were left shaking overnight at room temperature. This primary fixative was washed twice in 0.05 M phosphate buffer (pH 7.2), each wash being 15 minutes. For secondary fixation, samples were fixed in 1% osmium tetroxide in 0.05 M phosphate buffer (pH 7.2) and were left for 2 hours in the dark, at room temperature. After this time, samples were washed in two changes, of 0.05 M phosphate buffer (pH 7.2) for 15 minutes each wash, and were dehydrated in an ethanol series (EtOH) consisting of 10, 20, 30, 40, 50, 60 and 70 % EtOH (2 x 15 minutes, each stage). The samples were left overnight at 4 °C in 70% EtOH and, after this time, the solution was changed from 70% to 80%, 90% and 100% dry EtOH, 1:1 EtOH-propylene oxide and 100% propylene oxide (2 x 30 minutes, each stage). Samples were infiltrated using propylene oxide and Spürr resin (3:1, 1:1 and 1:3 propylene oxide - Spürr resin, respectively). In each of the infiltration steps, samples were left for 1 hour at room temperature until the 100% resin stage. Resin was changed twice daily, for four days before samples were fully polymerised. Samples were polymerised in a pre-warmed embedding oven at 70 °C for 8 hours. Resin was prepared according to the manufacturer's instructions. All reagents and resin were bought from TAAB Laboratories Equipment Ltd., Berkshire, England, UK.

Sample sectioning, mounting and observations

Of the eggs embedded in resin, only eggs oriented longitudinally were cut in sections. Sections from the whole egg were cut to a thickness of 1 µm using glass knives

on an Ultracut microtome (Reichart-Jung, Austria), and collected on multiwell slides (ICN Biomedicals, Ohio, USA). Sections were stained with toluidine blue and were observed using a light microscope attached to a Leica camera linked to the computer at 40 x magnification (Figure 4.1).

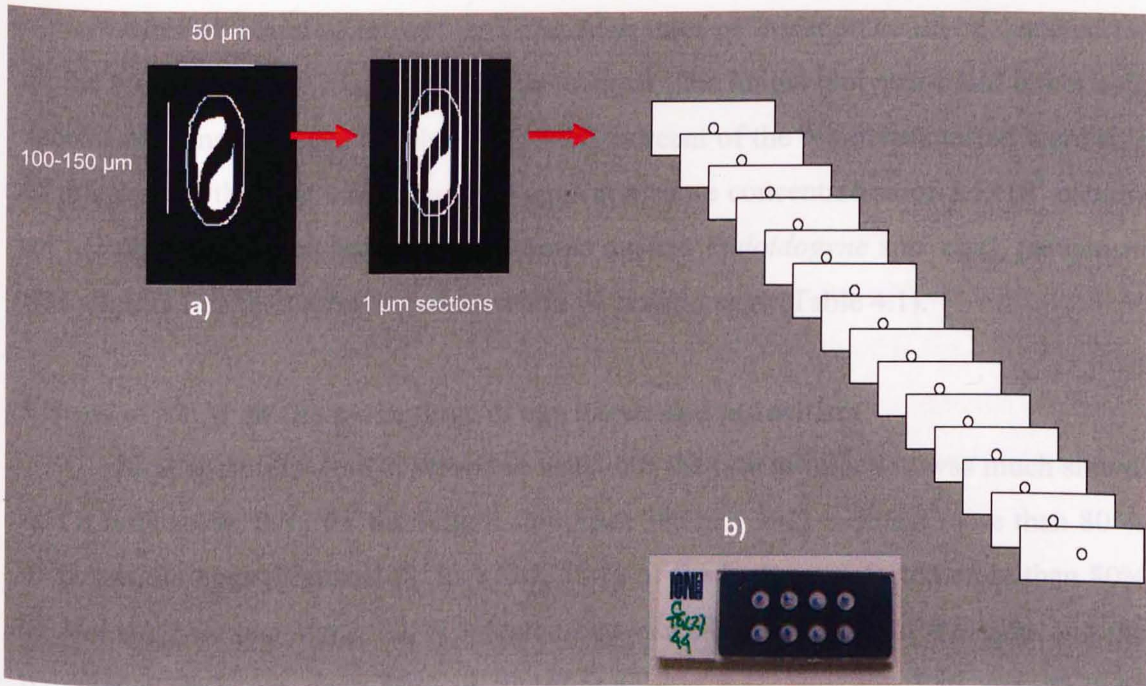


Figure 4.1. Diagrammatic representation of egg sectioning and mounting. (a) Average size of a RKN nematode. (b) Sections mounted in a multiwell slide (ICN Biomedicals, Ohio, USA).

4.3 RESULTS

4.3.1 **Development of a novel test to estimate parasitism *in vitro* and comparison with a standard method**

Novel method to estimate egg parasitism

After 48 hours of growth in YEM, high rates of infection could be detected for all the biotypes tested, compared with the control. The fungal biotypes could infect both species of nematode eggs (Table 4.1). Ninety percent of the 9 biotypes tested were able to infect more than 80% of *G. pallida* eggs at a spore concentration of 5.5×10^3 conidia ml^{-1} (Table 4.1). When biotypes were tested against *Meloidogyne* spp. eggs, parasitism was slightly less than infection observed in *G. pallida* eggs (Table 4.1).

Effects of YEM on the percentage of egg hatch and parasitism

Similar results were observed in water but the rate of infection was much slower and it took seven days for the fungus (biotypes 280 and 392) to infect more than 80 % of *G. pallida* eggs (Figure 4.2). In YEM, 55 % of the biotypes infected more than 80% of *Meloidogyne* spp. eggs, 22 % infected between 50% and 80% of the eggs and the remaining 22 % less than 50% of the eggs (Table 4.1). Biotypes 280 and 16 were significantly different from the rest and only infected 46% and 29% of the eggs, respectively (Table 4.1). Conidia from *Botrytis cinerea* germinated in YEM but this fungus was not able to infect eggs (Table 4.1). The addition of yeast extract to the medium had no significant effect in the numbers of eggs that were hatched (data not shown).

Effect of conidial concentration on the proportion (%) of *Meloidogyne* spp. and *Globodera pallida* infected eggs by two biotypes of *Pochonia chlamydosporia*

When different concentrations of conidia were tested using two biotypes of the fungus (biotypes 392 and 400), egg infection was significantly increased when higher concentrations of spores were used (Figure 4.3). One of the biotypes (392) was only tested for *G. pallida* eggs due to problems of contamination in some of the samples. A

LD_{50} was calculated for each interaction (biotype/nematode specie), but no significant differences were found between the three groups tested (400/*Meloidogyne* spp., 400/ *G. pallida* and 392/*G. pallida*) (Appendix III). The average LD 50 calculated for the three groups can be found in Figure 4.3 and correspond to 621 spores ml^{-1}. However, when data was grouped in two types of interactions (compatible and incompatible), biological and statistical significance were found (Appendix III). Biotype 400, which was originally isolated from root-knot nematode species required less spores to infect 50% of *Meloidogyne* spp. eggs (LD_{50} = 362.4). On contrary, for an incompatible interaction – biotypes 400 and 392 infecting eggs from a cyst nematode specie (*G. pallida*), a higher concentration of spores was required to parasitise 50% of the eggs (LD_{50} = 763.4) (Appendix III).

Although it was not possible to detect host preferences using a concentration of 5.5 $x10^3$ conidia ml^{-1} of suspension, in this method high rates of infection could be detected as early as 48 hours after the fungus was in contact with the egg, earlier than it had been able to measure in previous work. And because eggs were infected rapidly, it was possible to produce eggs with much more precise infection times for studies in the egg colonisation of that would be possible with the agar/ Petri dish bioassay.

Table 4.1 – Proportion of infected eggs (%), in liquid, in two nematode species - *Meloidogyne* spp. and *Globodera pallida* by nine biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 104, 132, 280, 392, 399 and 400). Percentages are average mean from four replicates followed by transformed value using logit (in brackets). SE – Standard error of means.

Biotype	Eggs parasitised (%)			
	Meloidogyne spp.	±SE	*Globodera pallida*	±SE
10	80 (0.78)	9	88 (1.74)	7
16	29 (-0.66)	9	79 (0.68)	4
60	72 (0.48)	4	95 (2.01)	3
104	73 (0.75)	9	94 (1.40)	0
132	91 (1.18)	2	94 (1.47)	2
280	46 (-0.1)	12	99 (3.26)	1
392	92 (1.37)	3	88 (1.19)	5
399	97 (1.71)	1	95 (1.53)	1
400	100 (3.45)	0	100 (3.80)	0
Botrytis cinerea	0	0	0	0
Untreated	0	0	0	0
LSD (5%)	0.84		1.15	

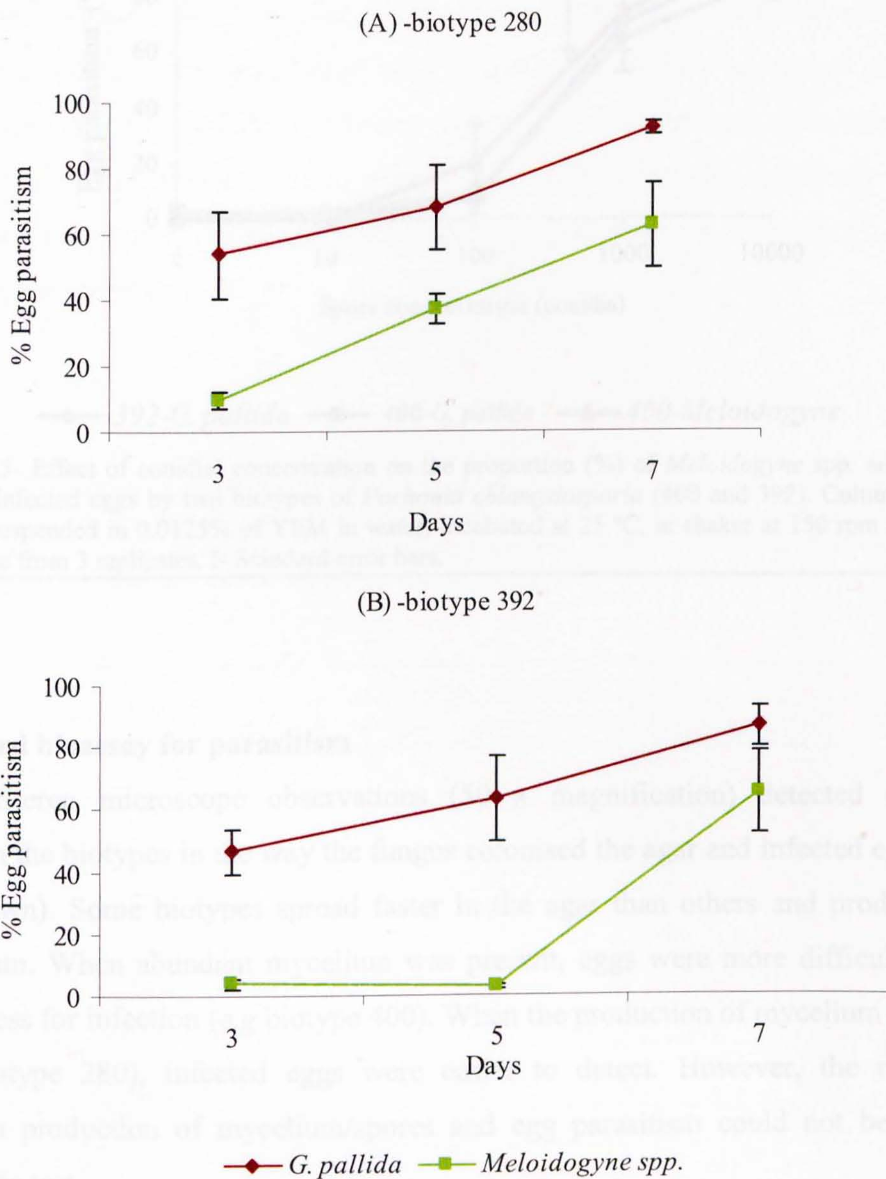


Figure 4.2 – Proportion of eggs (*Globodera pallida* and *Meloidogyne* spp.) parasitised in water by two biotypes of *Pochonia chlamydosporia* (biotype 280 – A; biotype 392 – B). Culture conditions: conidia suspended in sterile distilled water, incubated at 25 ºC, in shaker at 150 rpm for 3, 5 and 7 days. Means are from 4 replicates. I – Standard error bars.

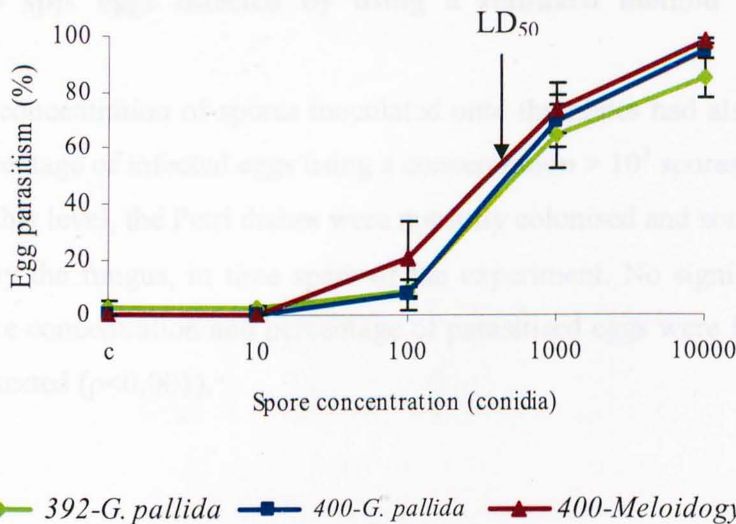


Figure 4.3- Effect of conidial concentration on the proportion (%) of *Meloidogyne* spp. and *Globodera pallida* infected eggs by two biotypes of *Pochonia chlamydosporia* (400 and 392). Culture conditions: conidia suspended in 0.0125% of YEM in water, incubated at 25 °C, in shaker at 150 rpm for 48 hours. Means are from 3 replicates. I- Standard error bars.

Standard bioassay for parasitism

Stereo microscope observations (50 x magnification) detected differences between the biotypes in the way the fungus colonised the agar and infected eggs (image not shown). Some biotypes spread faster in the agar than others and produced more mycelium. When abundant mycelium was present, eggs were more difficult to detect and assess for infection (*e.g* biotype 400). When the production of mycelium was sparse (*e.g* biotype 280), infected eggs were easier to detect. However, the relationship between production of mycelium/spores and egg parasitism could not be measured using this test.

The proportion of eggs parasitised by the fungus was small for most of the biotypes tested; only biotype 280 infected higher numbers of eggs, in both nematode species (Table 4.2). The assay appears to be discriminating only for virulent biotypes. The type of spore (conidia or chlamydospore) had no effect on the quantity of *Meloidogyne* spp. eggs infected by the fungus (Figure 4.4), although parasitism was slightly greater using chlamydospores in biotype 400 (Figure 4.4-B), no differences were found for the other biotype tested (Figure 4.4-A).

Effect of type and concentration of fungal inoculum on the proportion (%) of *Meloidogyne* spp. eggs infected by using a standard method to estimate egg parasitism

The concentration of spores inoculated onto the plates had also no relationship with the percentage of infected eggs using a concentration > 10^3 spores per plate (Figure 4.4). Below this level, the Petri dishes were not fully colonised and some eggs could not be reached by the fungus, in time spam of the experiment. No significant differences between spore concentration and percentage of parasitised eggs were found, for both of the biotypes tested ($\rho<0.001$).

Table 4.2- Proportion of infected eggs (%) on agar, in two nematode species - *Meloidogyne* spp. and *Globodera pallida* by nine biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 104, 132, 280, 392, 399 and 400). Percentages are average means from four replicates followed by transformed value using logit (in brackets), per each nematode specie. SE – Standard error of means.

Biotype	Eggs parasitised (%)			
	Meloidogyne spp.	±SE	*Globodera pallida*	±SE
10	46 (-0.08)	4	20 (-0.71)	2
16	13 (-0.95)	2	12 (-1.02)	1
60	8 (-1.22)	2	16 (-0.85)	2
104	10 (-1.13)	2	14 (-1.01)	6
132	10 (-1.11)	2	9 (-1.20)	3
280	46 (-0.08)	6	91 (1.28)	4
392	13 (-0.97)	1	10 (-1.14)	2
399	42 (-0.16)	1	34 (-0.37)	9
400	10 (-1.14)	2	22 (-0.67)	5
Untreated	0	0	0	0
LSD (5%)	0.30		0.53	

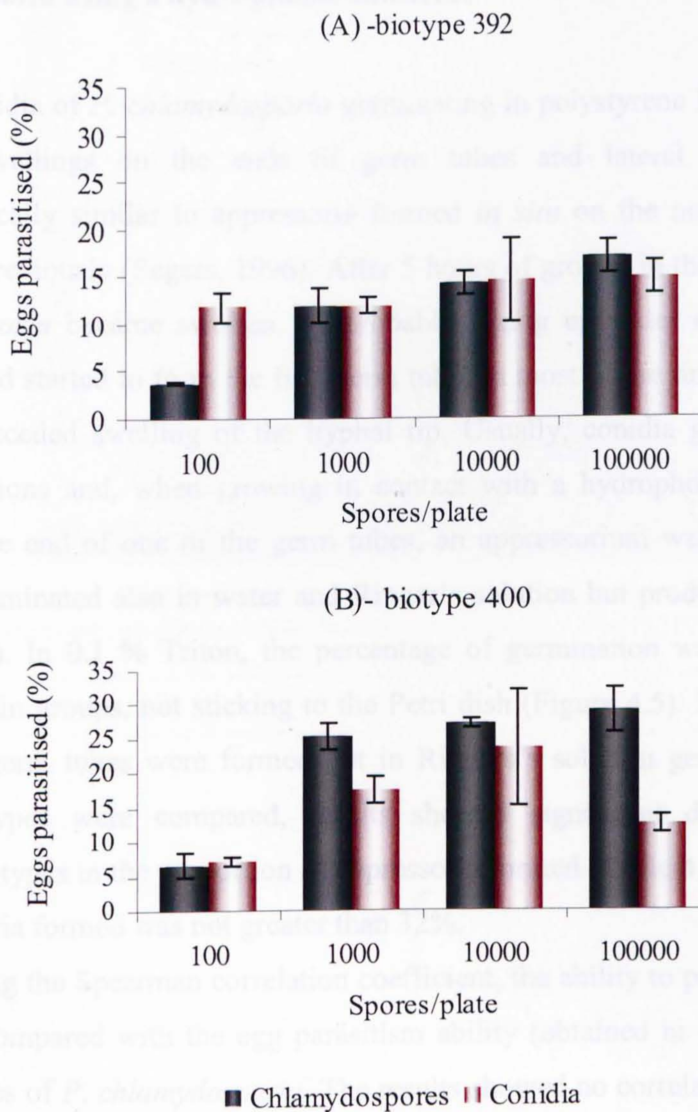


Figure 4.4 – Effect of type (chlamydospores and conidia) and concentration of fungal inoculum on the proportion (%) of *Meloidogyne* spp. eggs infected by using a standard method to estimate egg parasitism. For the two biotypes of *Pochonia chlamydosporia* tested (A – biotype 392, B- biotype 400), the percentage of infected eggs was not affected by the type of propagules or concentrations superior to 10^3 spores per plate. I- Standard error bars.

4.3.2 Measurement of appressoria formation in different biotypes of *Pochonia chlamydosporia* using a hydrophobic substrate

Conidia of *P. chlamydosporia* germinating in polystyrene Petri dishes produced terminal swellings on the ends of germ tubes and lateral branches that were morphologically similar to appressoria formed *in situ* on the nematode eggshell and described previously (Segers, 1996). After 5 hours of growth in the YEM, conidia of *P. chlamydosporia* became swollen, by probably taking up water or nutrients from the medium, and started to form the first germ tube. In most of the times, a second nuclear division preceded swelling of the hyphal tip. Usually, conidia germinated in two or three directions and, when growing in contact with a hydrophobic surface, after 24 hours, at the end of one of the germ tubes, an appressorium was formed (Plate 4.1). Conidia germinated also in water and Ringer's solution but produced few appressoria (Figure 4.5). In 0.1 % Triton, the percentage of germination was small and conidia germinated in groups, not sticking to the Petri dish (Figure 4.5). In water, fine hyphae with long germ tubes were formed but in Ringers's solution germ tubes were short. When biotypes were compared, results showed significant differences ($p<0.001$) between biotypes in the proportion of appressoria formed (Table 4.3) but the percentage of appressoria formed was not greater than 32%.

Using the Spearman correlation coefficient, the ability to produce appressoria *in vitro* was compared with the egg parasitism ability (obtained in 4.3.1, Table 4.2.) for nine biotypes of *P. chlamydosporia*. The results showed no correlation between the two variables (Appendix III). The % of appressoria formed had neither correlation with numbers of *Meloidogyne* sp. parasitised eggs (-0.250) or *G. pallida* eggs (-0.467).

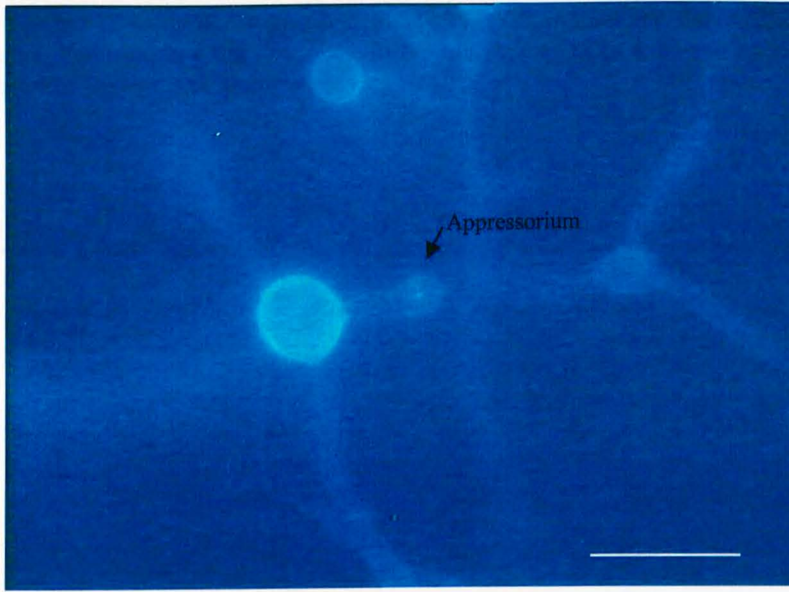


Plate 4.1. - Conidium in YEM (0.0125%) germinated in polystyrene after 24 hours of growth. At the tip of the germ tube an appressorium is visible. Stained with Calcofluor White M2R. Bar =5 μm.

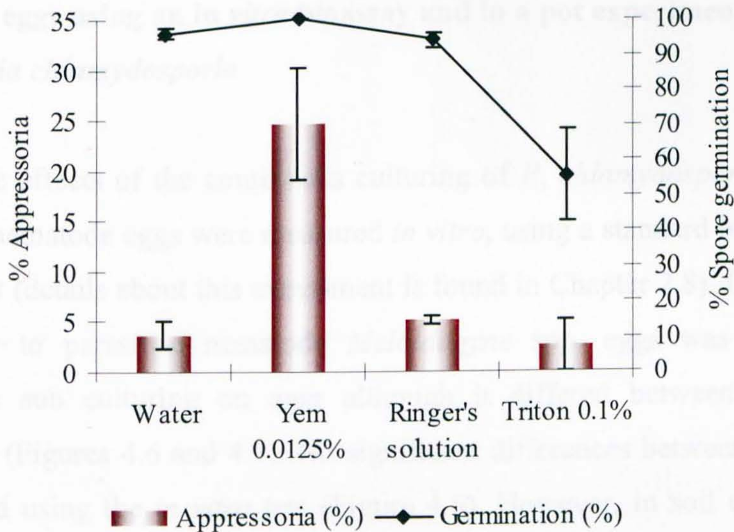


Figure 4.5 - Rate of germination and formation of appressoria *in vitro* when conidia from biotype 392 (1-$5x10^3$ conidia ml^{-1}) was suspended in water, yeast extract media, Ringer's solution and Triton 0.1 %. Means are from eight replicates. The percentage of germination and appressoria formation was determined 24 hours after incubation at 25° C. I – Standard error bars.

Table 4.3 - Formation of appressoria using an artificial substrate (polystyrene) by eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 104, 132, 280, 309, 392, 399 and 400). Conidial suspensions of 10^3 conidia ml^{-1} were suspended in 0.025% YEM, plated in polystyrene and incubated at 25 °C for 24 hours. Percentages are average means from eight replicates followed by transformed value using logit (in brackets). SE – Standard error of means.

Biotype	% Appressoria	± SE	Number of germ tubes/ conidium
10	13 (-0.98)	0.2	2
16	5 (-2.20)	0.6	3
60	17 (-1.44)	0.2	3
69	25 (-1.56)	0.3	2
104	14 (-2.15)	0.2	3
132	32 (-1.08)	0.2	3
280	2 (-3.44)	0.1	2
309	21 (-0.72)	1.0	3
392	25 (-0.63)	2.6	2
399	28 (-0.83)	0.3	3
400	11 (-1.63)	0.5	2
LSD (5%)	1.545		

4.3.3. **Effect of repeated culturing on the ability to parasitise *Meloidogyne* spp. nematode eggs using an in *vitro* bioassay and in a pot experiment by three biotypes of *Pochonia chlamydosporia***

The effects of the continuous culturing of *P. chlamydosporia* on the ability to parasitise nematode eggs were measured *in vitro*, using a standard bioassay and in a pot experiment (details about this experiment is found in Chapter 2.8). In both experiments, the ability to parasitise nematode *Meloidogyne* spp. eggs was not lost with the continuous sub culturing on agar although it differed between soil and *in vitro* conditions (Figures 4.6 and 4.7). No significant differences between the three biotypes were found using the *in vitro* test (Figure 4.6). However, in soil conditions, biotypes differed in their parasitic activity. Biotype 132 was able to parasitise on average 68.7 % of the eggs after 60 weeks of sub-culture (Figure 4.7), was present in large numbers in the soil (Figure 4.9) and in the rhizosphere (Chapter 3, Figure 3.12), but could not infect more than 30% of the eggs in the *in vitro* test (Figure 4.6). On contrary, biotype 280 did not establish in the soil (Figure 4.9) or rhizosphere (Chapter 3, Figure 3.12), infected

less than 2% of the eggs in the pot experiment (Figure 4.7), but performed as well as the other biotypes in the *in vitro* test (Figure 4.6). The same trend was observed for biotype 392, which was more able to parasitise eggs *in vitro* than in soil conditions (Figure 4.6 and Figure 4.8). After 65 days of growth, tomato roots were galled and egg masses were exposed in the root surface. The numbers of egg masses per gram of root were on average of ±18, in the untreated pots. The application of the fungus had no significant effects on the reduction of numbers egg masses per gram of root and numbers of eggs per egg mass (Figure 4.10).

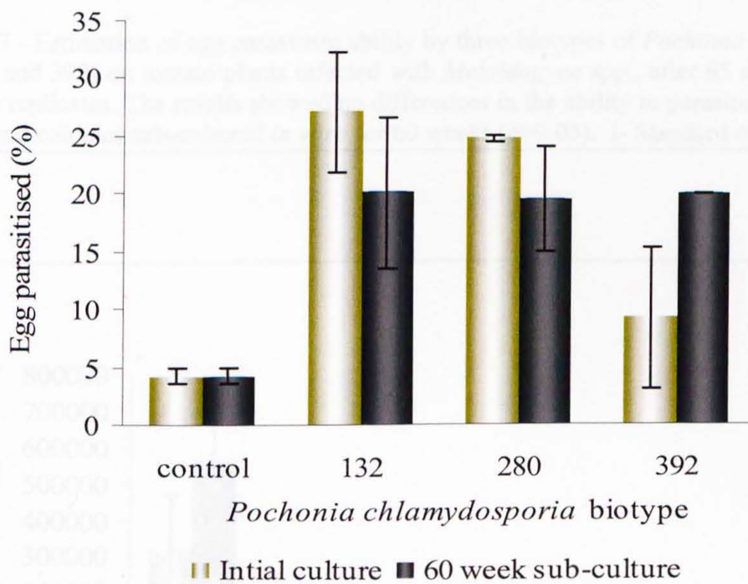


Figure 4.6 – Estimation of egg parasitism ability by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) using an *in vitro* bioassay. The results showed no differences in the ability to parasitise eggs of *Meloidogyne* spp. between the initial cultures and colonies sub-cultured *in vitro* for 60 weeks ($\rho > 0.05$). I- Standard error bars.

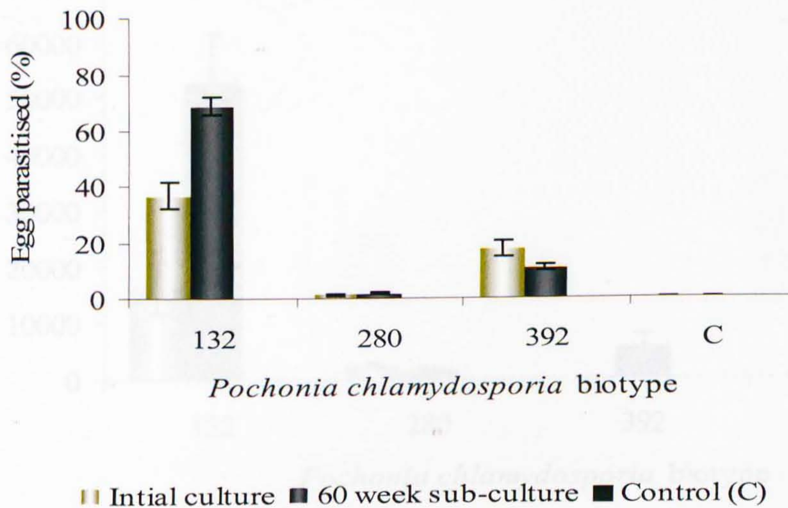


Figure 4.7 - Estimation of egg parasitism ability by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) on tomato plants infected with *Meloidogyne* spp., after 65 days of growth. Means are from five replicates. The results showed no differences in the ability to parasitise eggs between the initial cultures and colonies sub-cultured *in vitro* for 60 weeks ($\rho>0.05$). I- Standard error bars.

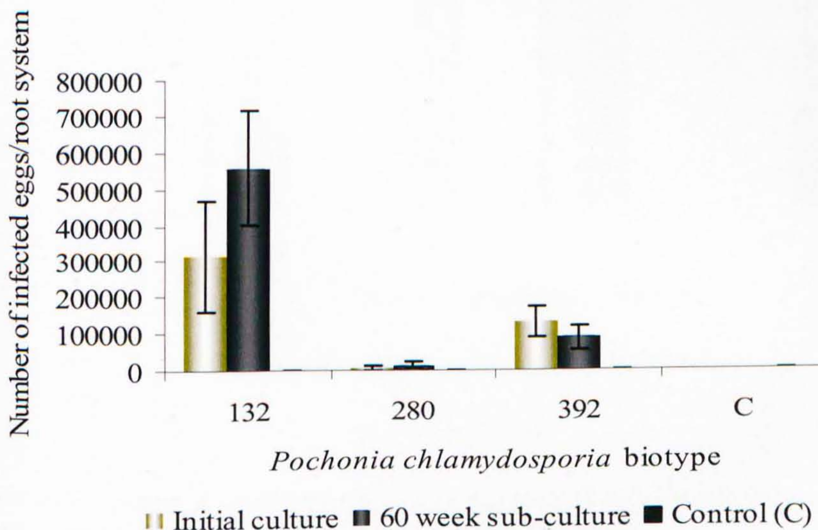


Figure 4.8 – Total number of infected eggs (*Meloidogyne* spp.) per tomato root system after 65 days of growth. Means are from five replicates. The results showed no differences in the ability to parasitise eggs between the initial cultures and colonies sub-cultured *in vitro* for 60 weeks ($\rho>0.05$). I – Standard error bars.

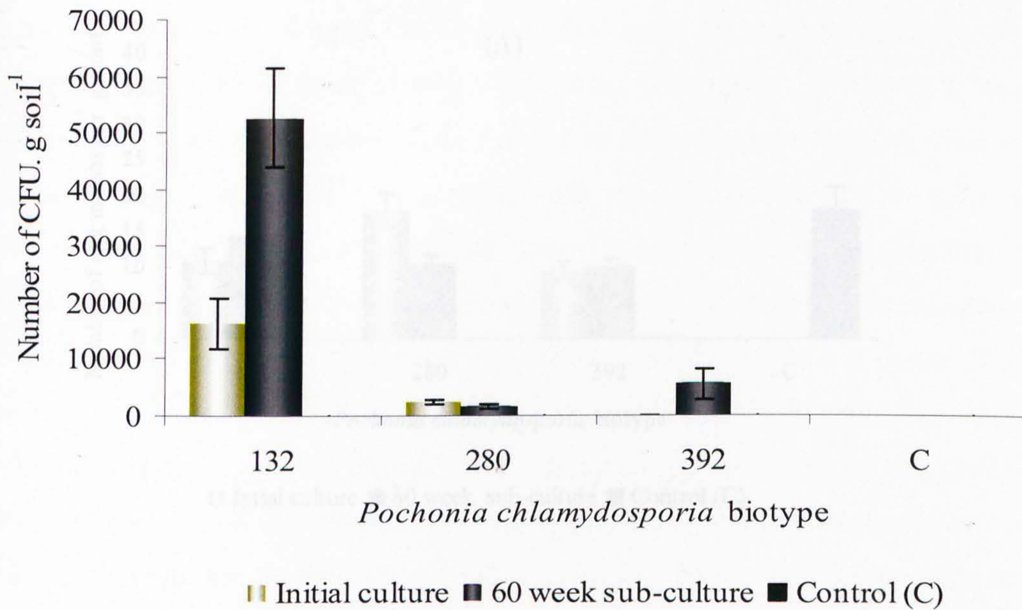
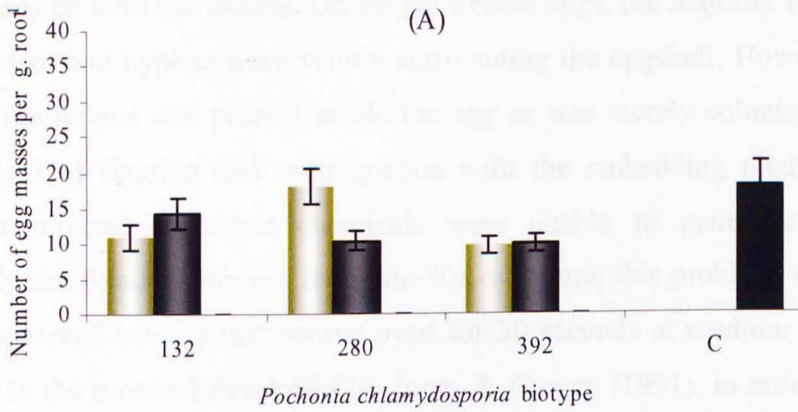


Figure 4.9 – Number of fungal colony forming units present in soil. Means are from five replicates. No significant differences between the number of CFU g^{-1} soil produced from the initial cultures and after 60 weeks of continuous *in vitro* sub-culture were found ($\rho>0.05$). Biotypes 280 and 392 showed little growth in soil when compared with biotype 132. I- Standard error bars.



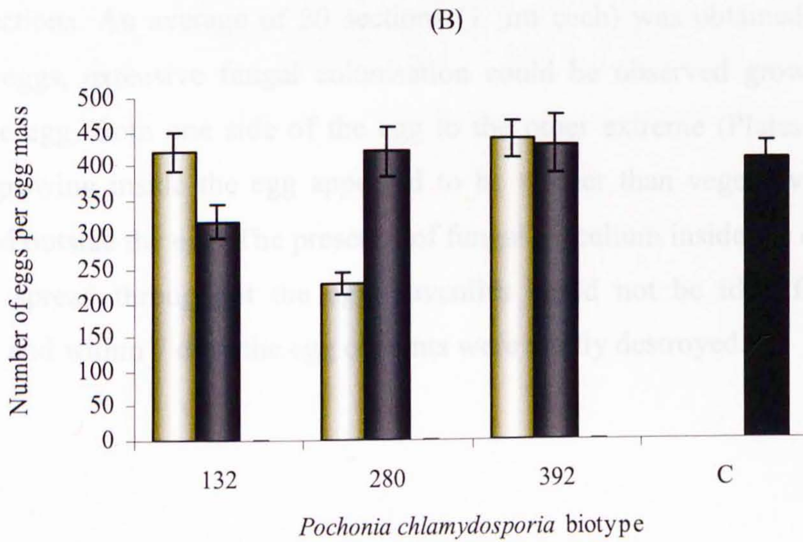


Figure 4.10 – Average of number of egg masses per gram of root (A) and number of eggs per egg mass (B). Means are from five replicates. I- Standard error bars.

4.3.4 Microscopy studies on egg infection

After 7 days of incubation at 25 °C, most juveniles hatched from eggs which were not treated with the fungus. On fungal treated eggs, the majority (>97 %) of eggs were infected, and hyphae were visibly surrounding the eggshell. However, it was not known if the fungus was present inside the egg or was merely colonising the outside. The protocol of fixation and impregnation with the embedding medium gave good results on infected eggs but chemicals were unable to penetrate healthy eggs, particularly the fixative osmium tetroxide. To overcome this problem, uninfected eggs were microwaved using a microwave oven for 30 seconds at medium power (50 °C), according to the protocol developed by Jones & Gwynn (1991), in order to change the permeability of the eggshell. Using this method, the permeability of the shell of a healthy egg was altered and osmium tetroxide was taken up with subsequent darkening of the egg contents and juvenile (Plate 4.2). Due to the reduced size of the eggs and its correct longitudinal orientation in the embedding material, a few eggs were successfully cut in sections. An average of 20 sections (1 µm each) was obtained per egg cut. In infected eggs, extensive fungal colonisation could be observed growing around and inside the egg, from one side of the egg to the other extreme (Plates 4.3 to 4.5) and hyphae growing inside the egg appeared to be thicker than vegetative hyphae which developed outside the egg. The presence of fungal mycelium inside the egg was obvious and was spread throughout the egg; juveniles could not be identified in the eggs observed and within 7 days the egg contents were totally destroyed.

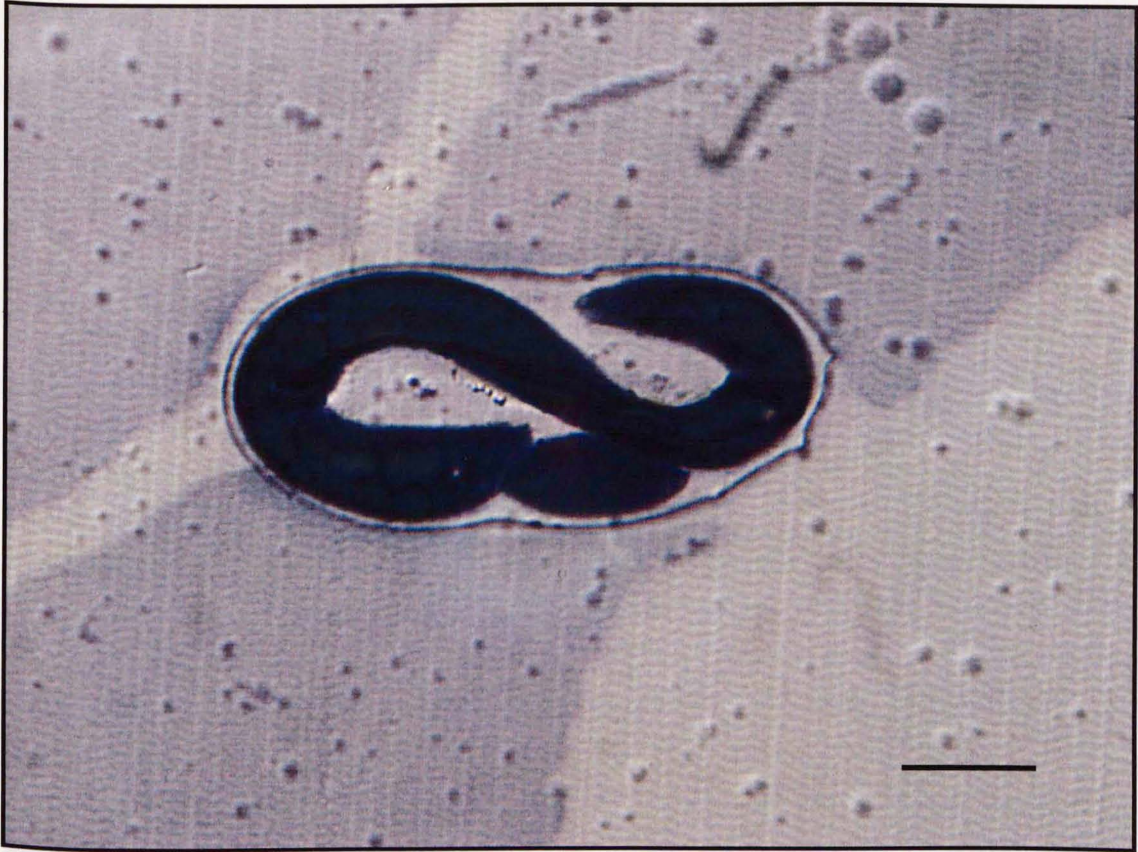


Plate 4.2 – Root-knot nematode healthy egg containing second stage juvenile. Stained with toluidine blue. Bar = 20 μm.

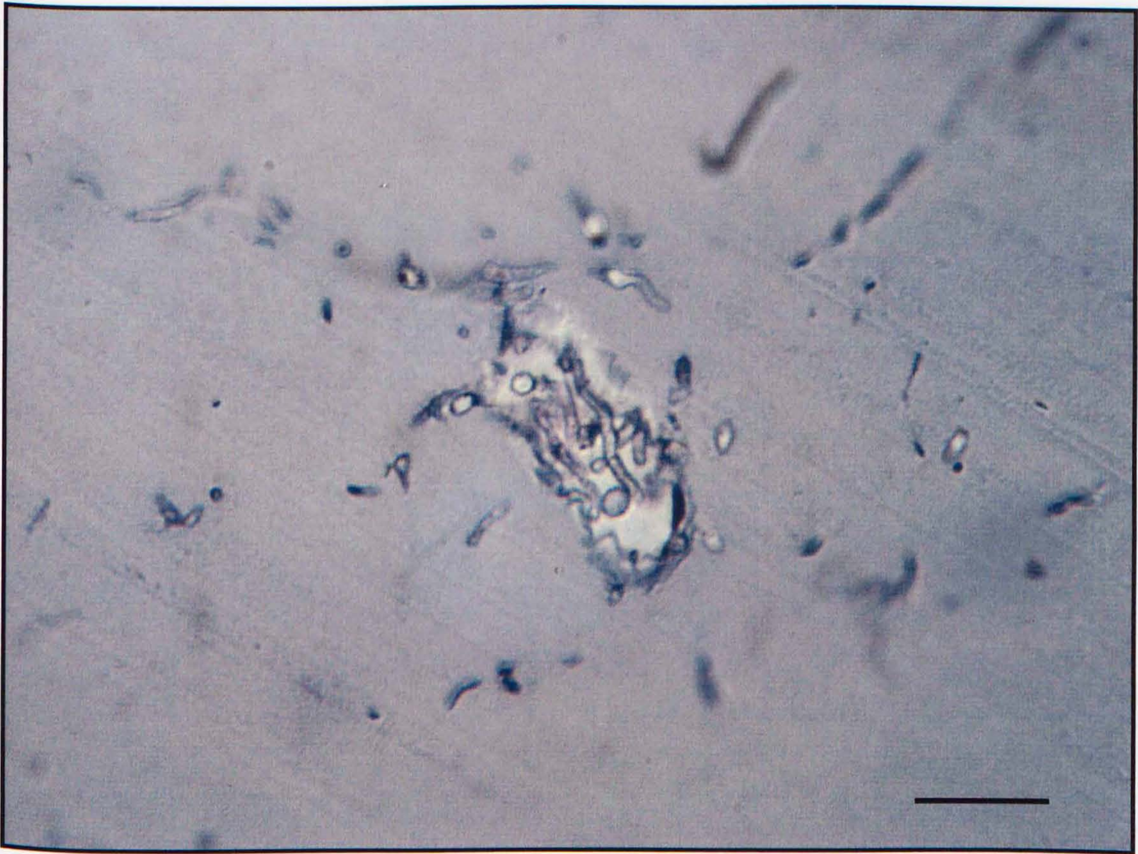


Plate 4.3 – External section (1μm) of a root-knot nematode egg infected with *Pochonia chlamydosporia*, 7 days after fungal inoculation. Hyphal growth is visible outside and inside the egg. Stained with toluidine blue. Bar = 20 μm.

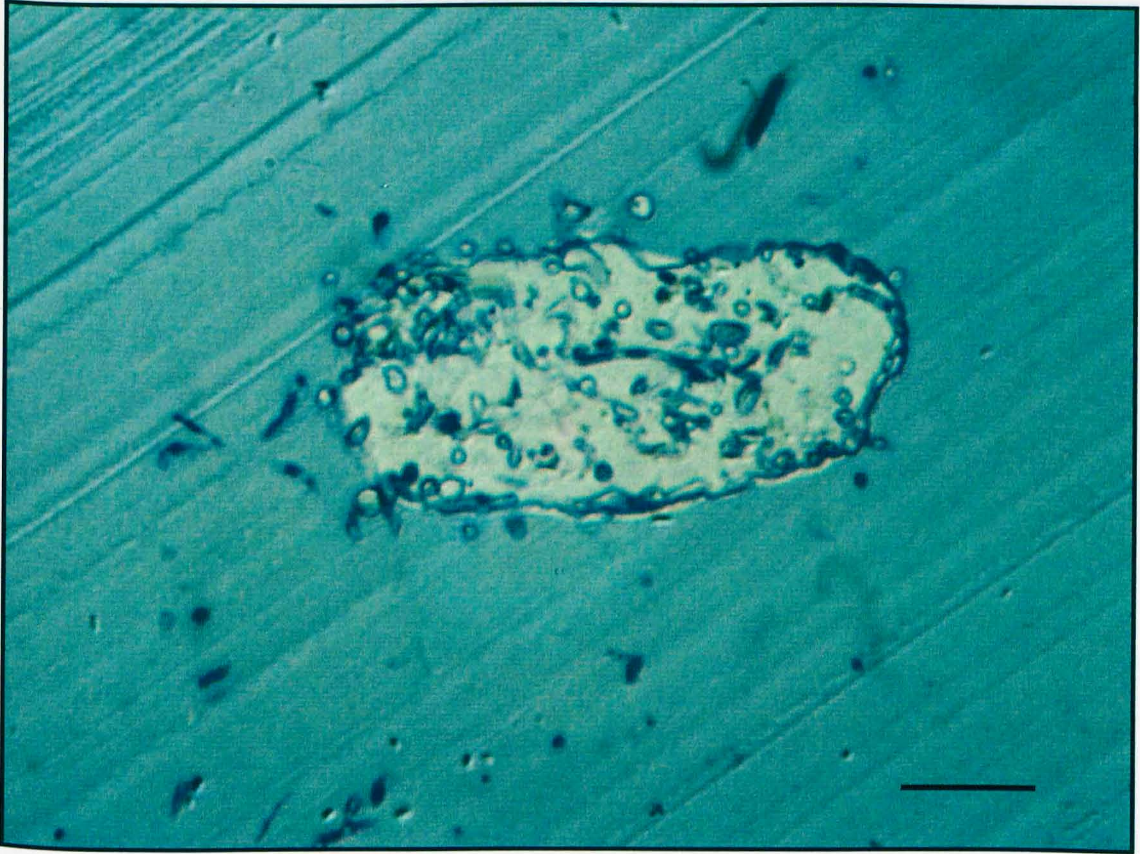


Plate 4.4 – Middle section (1 μm) of a root-knot nematode egg infected with *Pochonia chlamydosporia* (biotype 392), 7 days after fungal inoculation. The presence of hyphae was visible inside the egg. Stained with toluidine blue. Bar = 20 μm.

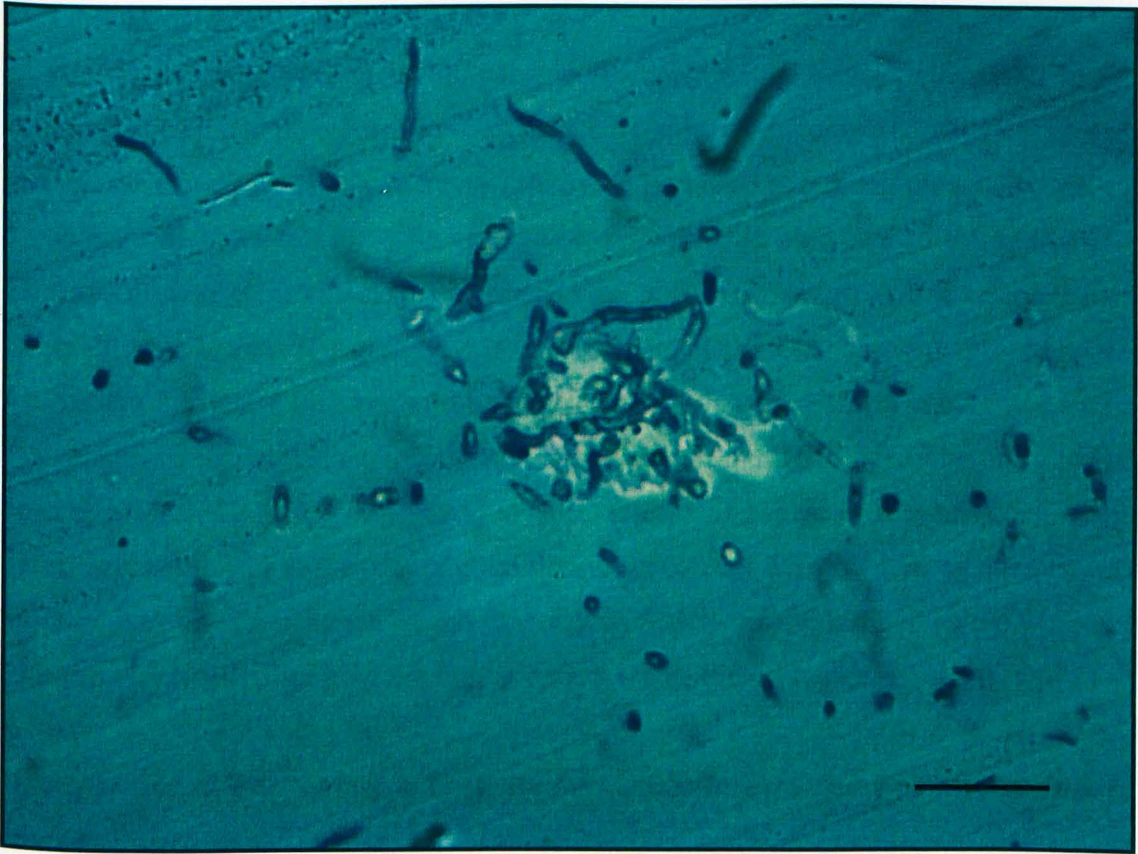


Plate 4.5 - Posterior section (1 µm) of a root-knot nematode egg infected with *Pochonia chlamydosporia* (biotype 392), 7 days after fungal inoculation. Stained with toluidine blue. Bar = 20 µm.

4.4 DISCUSSION

The research present in this Chapter focused on the development of a novel bioassay to estimate parasitism *in vitro* and the results were compared with a standard method developed by Irving & Kerry (1986). Information about egg parasitism ability (*in vitro*) was collected for a range of biotypes with the objective to relate *in vitro* parasitic growth with rhizosphere colonisation ability (Chapter 3) and secretion of extracellular enzymes (Chapter 5). The second part of the Chapter, aimed to compare the ability of different biotypes of the fungus in producing appressoria *in vitro* and study the possible relation between appressoria formation and virulence. The third main objective of this Chapter was to study the effects of repeated sub-culture on egg parasitism ability, in order to study the possibility of the development of attenuation. Observations on egg infection by the fungus were studied in the last part of the Chapter. The aim was to visualise how the fungus spread inside the nematode.

Development of a novel test to estimate parasitism *in vitro* and comparison with a standard method

The proportion (%) of *Meloidogyne* and *G. pallida* eggs parasitised by the fungus using the agar test (standard bioassay) was low for most of the biotypes studied, and was < 20% in six out of the nine biotypes tested, in both nematode species (Table 4.2). Biotype preferences between parasitism and original nematode host could not be detected using this assay, although previous work indicated that host preferences exist and can be detected at the molecular and population level (Mauchline *et al.*, 2002). The relatively few eggs parasitised using this bioassay were also reported by other authors (Stirling & Mankau, 1978; Irving & Kerry, 1986). Using a similar bioassay, Irving & Kerry (1986) found that *H. schachtii* eggs within females and cysts were more readily parasitised than those dispersed on agar, whereas the reverse occurred with *H. avenae*. The reasons for such differences remain unclear. Also, more *Meloidogyne* eggs were parasitised in masses by *Dactylella oviparasitica* than when they were dispersed on the agar surface (Stirling & Mankau, 1978). The mucilage surrounding the eggs is perhaps an important source of nutrition for the fungus to increase its inoculum potential and ensure a high rate of egg colonisation (Irving & Kerry, 1986) and this may explain the low levels of egg parasitism observed in this study. On water-agar (0.8%), the mycelial

growth of a range of biotypes differed; in biotypes with sparse mycelial growth on agar, the hyphae emerging from the eggs could be readily observed, whereas in biotypes with dense mycelial growth, eggs were difficult to score for infection, which was probably underestimated. In previous work, the growth rate was not related to the pathogenicity against nematode eggs *in vitro* (Kerry *et al.*, 1986) and increasing the density of mycelium surrounding the eggs by increasing the concentration of culture nutrient medium had little effect on parasitism (Irving & Kerry, 1986). On agar, no relationship was found between the quantity of fungal inoculum inoculated onto each plate and the percentage of parasitism (Figure 4.4). Increases in the quantity of the fungus added to Petri dishes did not result in a significant increase in the numbers of parasitised eggs. The effect of conidial concentration on the proportion of *H. avenae* eggs infected by five biotypes of *P. chlamydosporia* was also studied by Kerry *et al.* (1986) using a similar test on agar and, although there was a tendency to increase the parasitism when more inoculum was present, this tendency was weak and was not observed for the majority of the biotypes tested. The type of fungal spores (chlamydospores or conidia) required to initiate the colonisation of plates led to similar numbers of parasitised eggs, showing that the nature of the fungal propagules was not important in this type of bioassay. However, in soil the type of spore may have more significance: chlamydospores are more robust, contain more food reserves, and are expected to establish the fungus better in soil (Kerry *et al.*, 1993), whereas fungal conidia and mycelium are often more susceptible to mycostasis in soil and need additional food sources in order to survive and compete with other organisms (De Leij & Kerry, 1991).

The infection of eggs using a liquid suspension of conidia in yeast extract medium proved to be possible and a new protocol to estimate egg parasitism was developed. The proportion of infection was greater than the one observed on agar, on average 75% and 92% of *Meloidogyne* spp. and *G. pallida* eggs were infected, respectively using a concentration of 5.5 $x10^3$ conidia ml^{-1}. Although in this method small differences between biotypes could not be detected, at least at the level of conidial concentration used, infection of eggs could be detected as early as 48 hours using this quick, cheap and reliable method. However, the significance between a compatible interaction (a biotype parasitizing the same species of nematode for which it was originally isolated) and an incompatible interaction (a biotype parasitizing a different

nematode species for which it was originally isolated) was found using smaller concentrations of conidia.

Without a nutrient source, infection of eggs was still possible but eggs took longer to infect and infection levels were lower. The addition of yeast extract as a complex organic source of nitrogen to the medium seemed to stimulate egg infection, suggesting that nutrition was important in parasitism. The formation of appressoria in *Metarhizium anisopliae* is thought to be a nutritional response, with the most conductive conditions being provided by low levels of complex nitrogen sources such as low concentrations of yeast (St. Leger *et al.*, 1989). Low concentrations of YEM (0.0125%) have been shown to induce the formation of appressoria *in vitro* in *M. anisopliae,* but at higher concentrations (0.1%) only occasional sub terminal appressoria were formed (St. Leger *et al.*, 1989). This suggests that not only the source but also the concentration of nutrients are important in the development of infection structures. These amounts may be similar to the nutritional levels that can be found in the rhizosphere.

Surprisingly, in this test, *G. pallida* eggs were easier to infect than *Meloidogyne* eggs. The eggshell of root-knot nematodes is thinner than that of cyst nematodes (O'Hara & Jatala, 1985) and for this reason, *Meloidogyne* eggs were thought to be easier to penetrate and infect by *P. chlamydosporia* than *Globodera* eggs (Segers, 1996). However, if *G. pallida* eggs were in a less developed embryonic stage, they could be more susceptible to infection than *Meloidogyne* eggs containing mature juveniles. Immature eggs and dead eggs are more susceptible to infection than eggs containing second-stage juveniles (Irving & Kerry, 1986).

Both methods have shown limitations and need further optimisation. Despite those limitations, the techniques used demonstrate the parasitic nature of the fungus and should be useful in further studies of its biology and ecology.

Measurement of appressoria formation in different biotypes of *P. chlamydosporia* using a hydrophobic substrate

The ability to produce appressoria *in vitro* was studied in 11 *P. chlamydosporia* biotypes. All the biotypes were able to produce appressoria from germinated conidia, after 24 hours growth, when in contact with a hydrophobic substrate (polystyrene). Differences between biotypes were found but the reasons for these differences were not

further investigated. Presumably, biotypes producing bigger conidia contain more endogenous reserves, are more able to spread and produce appressoria. Morphological changes in the conidia were observed when germination was initiated. This phenomenon is known as isotropic swelling, and involves water uptake and is the second of the four stages which are required for spore germination (d'Enfert, 1997). The other stages are: (i) breaking down of spore dormancy, (iii) establishment of cell polarity and (iv) formation of a germ tube. In water, only a slight increase in spore size was observed and few appressoria were produced, but the germination rate was not significantly different from that of conidia germinated in YEM. This suggests that *P. chlamydosporia* conidia contain enough endogenous reserves and spore germination is not dependent on any additional nutrients. Studies in several plant pathogens, particularly in *Magnaporthe grisea* demonstrated that spore germination and appressorium formation are tightly linked and coordinated with each other through several signal pathways (Dean, 1997). In a recent study using *Colletotrichum gloeosporioides*, a facultative plant pathogenic fungus, it was suggested that pathogenicity was determined by the type of germination – inducing signals already at the onset of spore germination (Barhoom & Sharon, 2003). In the same study, it was also suggested that *C. gloeosporioides* used two different germination strategies for pathogenic and saprophytic growth. The pathogenic germination involved the formation of a single germ tube, whereas the saprophytic germination, induced by fermentable sugars, involved the formation of two germ tubes, which took longer to be formed (Barhoom & Sharon, 2003). Most of the biotypes of *P. chlamydosporia* formed two or three germ tubes from a single conidium, when in contact with polystyrene and supplied with YEM, but not in water. The importance of conidial germination in pathogenicity needs to be further studied for this fungus. In earlier work, Segers (1996) found appressoria of *P. chlamydosporia* (biotype 10) of different shapes and sizes on the surface of *M. incognita* compared to those formed on *G. rostochiensis* eggs. Although appressoria were produced on any part of the egg, only a few appressoria were formed.

In conclusion, it is not known if the ability to form appressoria *in vitro* resembles the situation *in vivo*, if biotypes producing more appressoria *in vitro* are the most virulent. In this preliminary work such relationship was not found.

Effect of repeated culturing on the ability to parasitise Meloidogyne spp. nematode eggs using an in *vitro* bioassay and in a pot experiment by three biotypes of *Pochonia chlamydosporia*

The repeated passage of *P. chlamydosporia*, biotypes 132, 280 and 392 through mycological media (CMA) did not affect virulence. No evidence of attenuation could be detected *in vitro* and in pot tests, although loss or enhancement of virulence due to continuous *in vitro* passage has been reported in fungi, such as the entomopathogenic Hyphomycetes (Hajek *et al.*, 1990; Quesada-Moraga & Vey, 2003; Vandenberg & Cantone, 2004). However, it was suggested by Hall, (1980) that such changes may be uncommon.

Interestingly, in this study, the virulence of biotype 392 was significantly greater after 60 weeks of sub-culture and a similar effect occurred with biotype 132 when tested in soil. The number of passages on agar is a factor that may have an impact on this effect. Schaerffenberg (1964) observed that the maximum virulence of a strain of *B. bassiana* was reached at the third generation on nutrient medium; it was maintained at the same level for up to the 16th generation, and from that point virulence decreased. In the present study, the level of parasitism was maintained, if not increased, after 60 passages in CMA, showing a relatively high level of stability for *P. chlamydosporia*. The culture media are thought to have an influence on the consequences of *in vitro* sub culturing; nutrient rich media have shown to reduce the capacity of a fungal biotype to sporulate and to cause a decline in virulence, compared to growth of fungi on weaker media (Schaerffenberg, 1964; Quesada-Moraga & Vey, 2003).

No significant differences between the use of the initial material and sub-cultures were found in both the *in vitro* experiments and the pot test. However, differences between biotypes were found in soil but not using the *in vitro* test. Biotype 132 had a better performance in soil than it did *in vitro* and colonised a greater percentage of *Meloidogyne* eggs. On the contrary, biotype 280 failed to establish in the soil (Figure 4.9) and rhizosphere (Chapter 3, Figure 3.12) and parasitised few eggs, reinforcing the importance of rhizosphere and soil colonisation in the performance of *P. chlamydosporia* as a biological control agent. However, this biotype was considered a good parasite and performed well in previous tests in soil (Mauchline *et al.*, 2004). Perhaps, during the course of this work, this biotype may have changed its

characteristics due to unknown factors. Environmental factors may had affected this biotype and determined the overcome of the results. Loss of efficacy in field trials was also reported before using biotype 10 (Sorribas *et al.*, 2003). However, such changes might not be related with continuous agar transfer, because the initial cultures were also affected and, therefore, events leading to attenuation may be random (Brownbridge *et al.*, 2001).

Microscopy studies of egg infection

Untreated RKN eggs are extremely impermeable to osmium tetroxide (Bird & McClure, 1976) and for this reason the permeability of the eggshell had to be altered by microwaving eggs for a short period of time. Permeability of the eggshell is said to be directly related to the presence of a lipid layer in RKN (Bird, 1971). According to Jones & Gwynn (1991), the use of a microwave oven to aid fixation of nematodes has many advantages; it speeds up the fixation process and also preserves structures that conventional fixation does not. Therefore, this technique is preferable to the mechanical rupture of the egg, used in the past (Bird & McClure, 1976; Morgan-Jones *et al.*, 1983). On fungal infected samples, fixatives could penetrate inside the egg without problems and this was evidenced by the subsequent darkening of the egg. Therefore, the presence of the fungus infecting the egg changed the permeability of the eggshell.

After seven days in contact with the fungus, eggs were colonised externally and internally by *P. chlamydosporia*. Penetration structures have been found in eggs of *Ascaris lumbricoides*, after three days in contact with *P. chlamydosporia* but internal colonisation was not investigated (Lysek, 1978). In studies using cysts containing immature eggs of *G. rostochiensis*, appressoria of *P. chlamydosporia* were found after 1 day of growth, indicating fungal infection occurred as soon as the fungus reached the cyst surface (Saifullah & Thomas, 1997). Appressoria of *P. suchlasporia* were found in eggs of *H. schachtii*, 4 days after inoculation (Lopez-Llorca & Claugher, 1990), but the fungus did not cover the entire egg. In this study, hyphae were spread inside the egg, colonising it from end to end. The low magnification used on the observations, allowed the entire egg to be seen, which was not possible using higher magnifications. In cut infected eggs, juveniles could not be identified inside the eggs. It is possible that the fungus could have destroyed the egg contents, after this time, lysing the nematode

structures and used the nutrients from the egg material to grow and spread outside the egg. It would be interesting to observe eggs with less time of exposure to the fungus, perhaps after 24, 48 and 72 h. Interestingly, hyphae growing inside the eggs appeared to be thicker, which might indicate that nutrient uptake having occurred. But it would be possible that the eggshell could have been damaged or ruptured during the embedded process, causing the release of the second stage juvenile, although the maintenance of the egg shape does seem to indicate that such "phenomenon" has not occurred.

In this study, internal colonization by *P. chlamydosporia* was observed in individual whole RKN eggs, for the first time. It was possible to visualise and confirm destruction of the egg contents in eggs which were infected with the fungus for 7 days, using the method developed in Section 4.2.1. However, many questions have arisen after this experiment – did the fungus consume the egg material earlier than this time? Did the fungus penetrate the eggshell in one or using several penetration zones? Was the eggshell lysed? Further attempts were made in order to distinguish between fungal and egg material, by immunolocalizing the fungus inside the egg, using a specific antibody for *P. chlamydosporia*. However, this research could not be finished during the time of this thesis and is, therefore, left for future research.

CHAPTER 5: STUDIES ON THE PRODUCTION OF EXTRACELLULAR ENZYMES IN SELECTED BIOTYPES OF *POCHONIA CHLAMYDOSPORIA*

5.1 INTRODUCTION

Nutrition in fungi is strongly dependent on the release of catabolic enzymes, which breakdown complex polymers such as starch, cellulose, lipids or proteins in to smaller constituent molecules that are more readily absorbed. As a result of enzyme activity, simple molecules such as sugars, fatty acids and amino acids are absorbed through the cell wall, transported across the cell membrane and are used for growth, energy, reproduction and other life processes. Regardless of their relationship with the substratum, fungi produce extracellular enzymes, which are typically of a hydrolytic or oxidative nature, to interact with the environment outside the cell or hyphae (Trigiano & Ament, 2003). However, this imposes constraints because enzymes are large molecules, about 20,000-60,000 Daltons (e.g. cellulases), so they do not diffuse far from the hyphal surface (Deacon, 2006). As a consequence, fungi create localized zones of erosion of insoluble substrates, and the hyphae must extend continuously into fresh zones (Deacon, 2006). Also because of their large size, enzymes have to be released in regions of new hyphal wall growth, where pore sizes in the wall are larger. Studies using *A. niger* indicated that enzymes destined for release through the wall are transported to the hyphal tip, where they are released by exocytosis (Deacon, 1997).

Extracellular enzymes may be either constitutive or inducible. Constitutive enzymes are produced continually regardless of substrate conditions. Inducible enzymes are synthesized at a low basal rate in the absence of substrate. When a suitable substrate is present in the medium, the synthesis of the corresponding catabolic enzyme increases until the inducer is removed and the enzyme level returns to the basal rate (Priest, 1984). The types of inducible enzymes secreted are dependent on the organism and substrate availability. Some substrates may induce or repress the synthesis and secretion of extracellular enzymes (Butt *et al.*, 1998). In general, the utilisation of complex forms of carbon or nitrogen is repressed when more convenient nutrient sources are available, such as glucose or ammonium, respectively (Paigen & Williams, 1970; Cohen & Drucker, 1977). The secretion of certain enzymes may indicate the ecological niche that these fungi occupy (Bidochka *et al.*, 1999a; Mendoza de Gives *et al.*, 2003); plant

pathogens would require a variety of carbohydrases, such as cellulases and xylanases to hydrolyze plant cell walls (Kolattukudy & Crawford, 1987; Bidochka *et al.*, 1999a), whereas entomopathogenic fungi would primarily require proteases and chitinases for insect cuticle degradation (St. Leger & Bidochka, 1996). Different enzyme profiles have been found in different species included in the genus *Verticillium* and species have been grouped according to their host-pathogen group (insect or plant pathogen) (Bidochka *et al.*, 1999a). In a similar study, insect, mushroom and several nematode pathogens, belonging to the genera *Verticillium* could be distinguished from plant pathogenic fungi in their ability to produce chitinases (Bidochka *et al.*, 1999b). However, when results were analysed phylogenetically, it was concluded that similar enzyme production and pathogenicity of different fungi did not always translate into phylogenetic relatedness, and vice versa (Bidochka *et al.*, 1999b). Studies on the secretion of enzymes may be useful in distinguishing biotypes in some circumstances but alone they are unlikely to identify biotypes and therefore other analyses are required (Carder *et al.*, 1993).

Enzymes play an important role in egg infection by *Pochonia chlamydosporia.* Specific proteases and chitinases have been isolated from the fungus and have shown activity against the nematode eggshell (Segers, 1996; Tikhonov *et al.*, 2002). Areas of low electron density were found around appressoria, suggesting that enzymes are secreted when appressoria are formed and, therefore, are important in the infection process (Morgan-Jones *et al.*, 1983; Lysek & Krajci, 1987; Lopez-Llorca & Robertson, 1992). The type of enzymes secreted must be able to degrade the nematode eggshell and support the consumption the egg contents. The typical composition of a nematode eggshell and the enzymes which are thought to be important in the infection process were described in Chapter 1, section 1.4.6.3.

In this Chapter, a group of *P. chlamydosporia* biotypes were tested for differences in their ability to produce a range of extracellular enzymes. Biotypes are known to differ in terms of their virulence and ability to colonise the rhizosphere and it was hypothesised that they may also differ in their enzyme production.

The objectives were:

(i) To test biotypes in their ability to produce a wide range of enzymes using a semi-quantitative enzymatic method (API ZYM®) and select important enzymes for further quantitative enzyme studies;

(ii) To study the response of the fungus in the production of enzymes on different media amendments;

(iii) Quantify the amounts of enzymes secreted at different times;

(iv) To assess whether differences existed between biotypes in the production of enzymes (types and amounts);

(v) To study the effects of repeated culturing on the ability to produce enzymes;

Information about enzyme activity was collected for a range of *P. chlamydosporia* biotypes and the results were compared with rhizosphere colonisation ability (Chapter 3) and *in vitro* parasitic growth (Chapter 4) with the objective to determine if biotypes with the best parasitic performance were also good rhizosphere colonisers, enzyme producers or vice versa. This comparison is presented in Chapter 7 (General discussion).

5.2 MATERIAL AND METHODS

Enzyme activity was measured using a colorimetric (API system) and spectrophotometric assays. The API ZYM® system is a semi-quantitative colorimetric micro-method that allows the systematic and rapid study of 19 enzymatic reactions using small amounts of sample. The system is based on the observation that specific enzyme activity can be detected by adding inoculum of certain micro-organisms on particular substrates, even in the absence of growth (Buissière *et al.*, 1967). The idea has been commercialised into a handy kit of chromogenic substrates dried on absorbent microcupules and was first found to be useful in clinical microbiology for the identification of pathogenic bacteria (Humble *et al.*, 1977; Kilian, 1978). Nowadays, the kit is mainly used to detect enzyme activity in a wide range of organisms, including fungi (Kurnatowska, 1998; Bramono *et al.*, 2006).

Spectrophotometric assays provide accurate and reproducible results for large numbers of samples (Dixon & Webb, 1979). This study used an artificial chromogenic substrate linked to ρ-nitrophenyl. In a neutral or alkaline solution the substrate is colourless; on enzymatic hydrolysis ρ-nitrophenol is liberated, which at alkaline pH develops a yellow colour suitable for the quantitative measurement of activity of the enzymes (Huggins & Smith, 1947). The release of ρ-nitrophenol was measured using a spectrophotometer. The range of absorbance and pH conditions will depend on the enzymes assayed.

5.2.1. **Semi-quantitative studies on the production of extracellular enzymes using the API ZYM® system**

Conical flasks (250 ml) containing 100 ml Czapek Dox broth (Appendix I.) were inoculated with 10^3 conidia ml^{-1} and incubated in an orbital shaker at 28 °C and 120 rpm. Eight biotypes of *P. chlamydosporia* (biotypes 10, 60, 69, 104, 132, 309, 392 and 400) and three sub-cultures of biotype 280 (8th, 15th and 60th week) were tested. These biotypes were used in the previous experiments. Information about the origin, substrata and host nematode for each of the biotypes studied is found in Chapter 2, Table 2.1.

After seven days, cultures were transferred to a minimal Czapek Dox broth medium (Appendix I.) and were grown for further three days, at 28 °C and 120 rpm. After this time, cultures were centrifuged (2000 rpm, 5 minutes), the hyphae discarded and the

supernatant was tested for enzyme activity using the API ZYM® system (Biomérieux sa., France). Each strip of API ZYM® contains 20 cupules, 19 of them containing a substrate and its buffer to detect a particular enzyme. The support base allows contact between the enzyme and the insoluble substrate. The enzymes detected by the API ZYM® system and their respective substrates are listed in Table 5.1. Each cupule of the API ZYM® strip was inoculated with 65 μl of the sample and incubated for 4 hours at 37 °C and each biotype tested twice. After incubation, 1 drop of ZYM A reagent (Tris-hydroxymethyl-aminomethane, HCl, sodium lauryl sulphate and water) and an equal quantity of ZYM B reagent (Fast Blue BB and 2-methoxyethanol) was added to each cupule and left for 5 minutes to let the colour develop and eliminate the yellow colour of the ZYM B reagents. Results were recorded using a colour chart supplied with the API ZYM® kit. Numerical values were assigned to different colour intensities ranging from 0 to 5, by which enzyme classifications were made. Colour intensity: 0, no activity; 1 and 2, weak activity; 3, 4 and 5, strong activity ($\geq$ 40 nM of substrate hydrolysed). Biotypes were grouped for the production of the enzymes assayed using cluster analysis, in Genstat® version 9. Data were analysed using two categories: no or weak detection (colour intensity: 0, 1 or 2) and strong activity (colour intensity: 3, 4 or 5). A hierarchical tree of similarities was constructed using cluster analysis in Genstat ®.

Table 5.1 List of the enzymes assayed by the API ZYM® system (taken from API ZYM® manual – BioMériuex sa.)

Enzyme	Substrate	Cupule number	Result Positive	Result Negative
Esterase (C4)	2-naphthyl butyrate	3	Violet	
Esterase lipase (C8)	2-naphthyl caprylate	4	Violet	
Lipase (C14)	2-naphthyl myristate	5	Violet	
Leucine arylamidase	L-leucyl-2-naphthylamide	6	Orange	
Cystine arylamidase	L-valyl-2-naphthylamide	7	Orange	
Valine arylamidase	L-cystyl-2-naphthylamide	8	Orange	
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	9	Orange	
Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	10	Orange	
Alkaline phosphatase	2-naphthyl phosphate	2	Violet	Colourless
Acid phosphatase	2-naphthyl phosphate	11	Violet	
Phosphohydrolase	Naphthol-AS-BI-phosphate	12	Blue	
α-galactosidase	6-Br-2-naphthyl-αD-galactopyranosidae	13	Violet	
β-galactosidase	2-naphthyl- βD-galactopyranosidae	14	Violet	
β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	15	Blue	
α-glucosidase	2-naphthyl- αD-glucopyranosidae	16	Violet	
β-glucosidase	6-Br-2-naphthyl-βD-glucopyranosidae	17	Violet	
N-acetyl-β-glucosaminidase	1-naphthyl-N- acetyl-βD-glucosaminide	18	Brown	
α-mannosidase	6-Br-2-naphthyl-αD-mannopyranosidae	19	Violet	
α-fucosidase	2-naphthyl-αL-fucopyranosidae	20	Violet	

5.2.2 Quantitative studies on the production of extracellular enzymes

Using the information obtained from the API ZYM® system, enzymes deserving further study were selected. Eleven *P. chlamydosporia* biotypes (10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400, Table 2.1) and sub-cultures (initial and 60th week sub-culture) from biotypes 132, 280 and 392 were cultured in liquid minimal medium (Appendix I.) and liquid minimal medium supplemented with:

a) 0.2 % gelatine (from porcine skin); gelatine was filtered through a Millipore filter (45 μm) before it was added into the autoclaved medium.

b) 1 % (w/v) chitin (from crab shells, practical grade); chitin was sieved through a 30 mesh aperture sieve before use. This medium had to be poured in constant agitation to assure its homogeneity (Segers, 1996).

c) 2 % (v/v) extra virgin olive oil and 0.25 % sodium dodecyl sulphate (SDS) (w/v). Stock solutions of SDS and olive oil were prepared and were added to the autoclaved medium individually. The olive oil was sterilised before use.

d) 0.1 % (w/v) *Meloidogyne incognita* eggs; due to the large numbers of eggs required for the preparation of this medium, only biotypes 280, 392 and 309 were tested

in this variant of the experiment. Eggs were surface sterilised using a solution of 0.1 % Hg_2Cl_2, following the protocol described by Manzanilla-Lopez (1997). To check the effects of surface disinfection on egg viability, eggs were plated on agar plates (PDA) and were observed for 15 days. Egg hatch was not affected and no contaminations could be observed, and therefore, this method was considered suitable to obtain surface-sterile, viable nematode eggs for this experiment.

The experiment had different aims. The first aim was to study the response of the fungus in the production of enzymes on the different medium amendments. The media in which enzyme activity was greater for each enzyme was selected to grow the fungus for different time periods (3, 5 and 7 days) in order to study changes in enzyme activity and time of secretion. After five days of growth, biotypes of *P. chlamydosporia* were compared for the production of different enzymes (types and amounts produced) and, finally, the last objective was to study the effects of repeated culturing on enzyme production.

Experimental conditions and fungal inoculation: Twenty millilitres of each medium were poured into 50 ml plastic tubes (Corning) and were inoculated with four agar plugs (5 mm) colonised with the fungus (three replicates per biotype, per medium and per each day of sampling). Samples were incubated in the dark, at 28 °C, in an orbital shaking incubator at 120 rpm (Gallenkamp). After 3, 5 and 7 days, the supernatant was collected and filtered using filter paper (Whatman N° 1). In order to reduce the volume of each sample, the supernatant was freeze dried and re-suspended in 1 ml of sterile distilled water, to be finally measured for enzyme production.

Total protein concentration was measured according to Bradford (1976) using a protein assay kit (Bio-Rad). A standard curve was calculated using bovine serum albumin (BSA) as standard at a concentration between 1.42 to 10 µg/ml, from a standard solution of 0.1 mg/ml BSA. Absorbance was read in a multicsan MRX plate reader (Dynex technologies Ltd, UK), at 495 nm. Enzyme activity was determined by the use of different enzyme assays:

I. **Lipase, esterase and exochitinase activity** was accessed using *p*-nitrophenyl substrates (15 mM of 4-nitrophenyl palmitate, 15 mM of 4-nitrophenyl acetate, and 2mM of 4-nitrophenyl-N-acetyl-D-glucosaminide, respectively).

Enzyme extract (10 μl), substrate solution (40 μl) and the appropriate buffer (20 μl; 25 mM l^{-1} acetate, pH 4.2) were pipetted into the wells of a 96 well microtitre plate (Bibby Sterilin, UK) and incubated at 37 °C for 1 h, using a boiled (100 °C, 10 minutes) enzyme extract as a control. The reaction was stopped by the addition of 5 μl of 1 mol l^{-1} sodium carbonate solution and left for 3 minutes. The enzyme activity was estimated using an MRX multiscan plate reader (Dynex technologies Ltd, UK), by measuring the increase in optical density at 405 nm caused by the liberation of ρ-nitrophenol by enzymatic hydrolysis of the substrate. Total enzyme activity was calculated from the calibration curve of absorbance at 405 nm against *p*-nitrophenol concentration and expressed as nmol *p*-nitrophenol released $min^{-1}ml^{-1}$. Specific activity was expressed in nmol *p*-nitrophenol.min^{-1} ml^{-1} μg of protein.

Total and specific activity was calculated in the following way:

i) Total enzyme activity:

1. Sample absorbance reading – control absorbance reading = actual absorbance reading of sample

2. Calculate the ρ-nitrophenol concentration (nmol ml^{-1}) present in the sample from the ρ-nitrophenol calibration curve

$$\rho\text{-nitrophenol concentration} = \frac{\text{Actual absorbance reading of sample}}{\text{Gradient of standard } \rho\text{-nitrophenol curve}}$$

3.

$$\text{Total activity} = \frac{[\rho\text{-nitrophenol conc. (nmol ml}^{-1}\text{)*dilution factor] x volume extract}}{\text{Time (min)}}$$

ii) Specific enzyme activity

1. Calculate the total protein present in the sample using the BSA standard curve

Total protein = Absorbance of sample/gradient of BSA standard curve

$$\text{2. Specific activity} = \frac{\text{Total activity (nmol } \rho\text{-nitrophenol released min}^{-1}\text{)}}{\text{Total protein (}\mu\text{g)}}$$

II. **Proteolytic activity** was determined using azocasein, a chromogenic substrate:

Enzyme extract (20 µl) and sulphanilamide Azocasein (1% in 0.2 M Tris-Hcl buffer, pH 7.5) were pipetted into the wells of a 96 well microtitre plate and incubated at 37 °C for 1 h using a boiled enzyme extract as a control. The reaction was stopped by the addition of 150 µl of trichloroacetic acid (TCA) (10% w/v) and neutralised by adding 50 µl of 1M NaOH. Plates were centrifuged (3000 rpm, 10 minutes) and supernatants (150 µl) transferred to a 96 well half size enzymoimmunoassay (EIA) plate

(175 µl cavities). Blank samples were prepared similarly but with an inactivated (100 °C, 10 min.) enzyme solution, and absorbance read at 440 nm in a MRX multiscan plate reader (Dynex technologies Ltd, UK). A standard curve was calculated using commercial protease from *Aspergillus oryzae* (500 Units/g; 10 µl = 0.0148 g), at a concentration between 0.5 to 50 U. Total enzyme activity was calculated from the standard curve and was expressed as units of proteases/ml (U/ml). One unit of protease activity is defined as the amount of enzyme that produces an increase in absorbance of 1.0 in 1h at 440 nm.

III. **VCP1 activity** was assayed using N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, following the method described by Morton (2002).

Enzyme extract (2 µl), substrate (100 µl) and buffer (98 µl of 0.1 M Tris Hcl pH 7.9) were mixed in micro tubes (500 µl) and absorbance was immediately and continuously read at 410 nm for 3 min at room temperature, using a spectrophotometer (CaryWin UV). One unit of activity is defined as the amount of enzyme that releases 1 µmol *p*-nitroanilide $min^{-1}ml^{-1}$.

Design and statistical analysis: To compare the effects of different media amendments, time of secretion, biotypes and effects on repeated culture, analysis of variance (ANOVA) was applied to the results using Genstat®. The data were checked to ensure the normality of variance by plotting histograms of residuals and plotting the residuals against the fitted values, respectively. Where data showed a clear skewed distribution, they were log transformed in the following way: [log10 (specific enzyme activity +1)]. Standard errors were generated from data sets by dividing the standard deviation by the square root of n. To rank the ability of the different biotypes in producing proteases, chitinases, lipases and esterases, Kendall's coefficient of concordance was used. This test measures the degree of correspondence between two or more rankings and assesses the significance of this correspondence (Kendall & Gibbons, 1990). The output of these analyses can be found in Appendix IV.

5.3 RESULTS

5.3.1 Semi -quantitative studies on the production of extracellular enzymes using the API ZYM® system

From the 19 enzymes assayed using the method, esterases and esterase lipases were the enzymes found more commonly in the different biotypes (Table 5.2). These two enzymes were detected in large amounts (≥ 40 nM of substrate hydrolysed) in all the biotypes of *P. chlamydosporia* tested, with the exception of biotype 69, in which the activity was weak. Lipase activity was detected in 5 biotypes of the fungus but at a low level (biotypes 10, 104, 132, 280 and 400). Most of the proteases tested were not secreted by any of the biotypes. Only biotype 104 showed strong activity (≥ 40 nM of substrate hydrolysed) in producing the valine arylamidase but none of the biotypes produced the serine proteases trypsin and chymotrypsin. Phosphatases were produced by most of the biotypes but in small amounts, with a few exceptions. High levels of acid phosphatase were detected in biotypes 10, 104 and 400 and phosphohydrolases in biotypes 10 and 104. The production of glycosidases was the most variable among biotypes. Although ß-glucuronidase, α-glucosidase, α-mannosidase were not secreted by any of the biotypes, other enzymes such ß-galactosidase and N-acetyl-ß-glucosaminidase were secreted in large amounts by some biotypes whereas they were not secreted by others (Table 5.2). The reproducibility of the assay gave good results. Although biotypes could be only tested twice, the results were very consistent (data not shown).

Using a cluster analysis, biotypes were grouped in a dendrogram according to their similarities on the ability to produce extracellular enzymes in a non inductive medium. Only colour intensities superior to 3 were used in the analysis, as these indicate positive results. Values lower than 3 were ignored to avoid the risk of recording false positives. The total numbers of enzymes secreted by individual biotypes varied markedly. Biotype 104 produced the greatest range of enzymes (7 out of 19). In contrast, biotype 69 produced none of the enzymes which were detected by the substrates used in the test. The analysis confirmed the existence of different groups of biotypes based on the production of extracellular enzymes by *P. chlamydosporia* (Figure 5.1). All of the three sub-cultures of *P. chlamydosporia*, biotype 280 (8th, 15th

and 60th weeks) produced esterases (cupule number 3) and esterase lipases (cupule number 4). However, after 15 and 60 weeks, N-acetyl-ß-glucosaminidase (exochitinase) was also detected (Plate 5.1).

From the results found in this assay, the enzymes that were secreted in greater amounts by the majority of the biotypes were esterases, lipases and the N-acetyl-ß-glucosaminidase. These enzymes selected for further studies on enzyme activity, using a quantitative method.

Table 5.2 Enzyme activities of nine *Pochonia chlamydosporia* biotypes detected with the API ZYM® system.

Enzyme	Number of biotypes tested [a]		
	Negative	Weakly positive	Strongly positive
Esterase (C4)	0	1	8
Esterase Lipase (C8)	0	1	8
Lipase (C14)	4	5	0
Leucine arylamidase	8	1	0
Valine arylamidase	7	1	1
Cystine arylamidase	8	1	0
Trypsin	9	0	0
Chymotrypsin	9	0	0
Alkaline Phosphatase	1	8	0
Acid phosphatase	1	5	3
Phosphohydrolase	0	7	2
α-galactosidase (Melibiase)	2	7	0
ß-galactosidase (Lactase)	2	6	1
ß- glucuronidase (Hyaluronidase)	9	0	0
α-glucosidase (Maltase)	9	0	0
ß-glucosidase (Cellulase)	4	5	0
N-acetyl-ß-glucosaminidase	4	1	4
α-mannosidase	9	0	0
α-fucosidase	7	2	0

[a] Numerical values were assigned to different colour intensities ranging from 0 to 5, by which enzyme classification was made. Colour intensity: 0, no activity; 1 and 2, weak activity (5-10 nM of substrate hydrolysed); 3, 4 and 5, strong activity (≥ 20 nM of substrate hydrolysed).

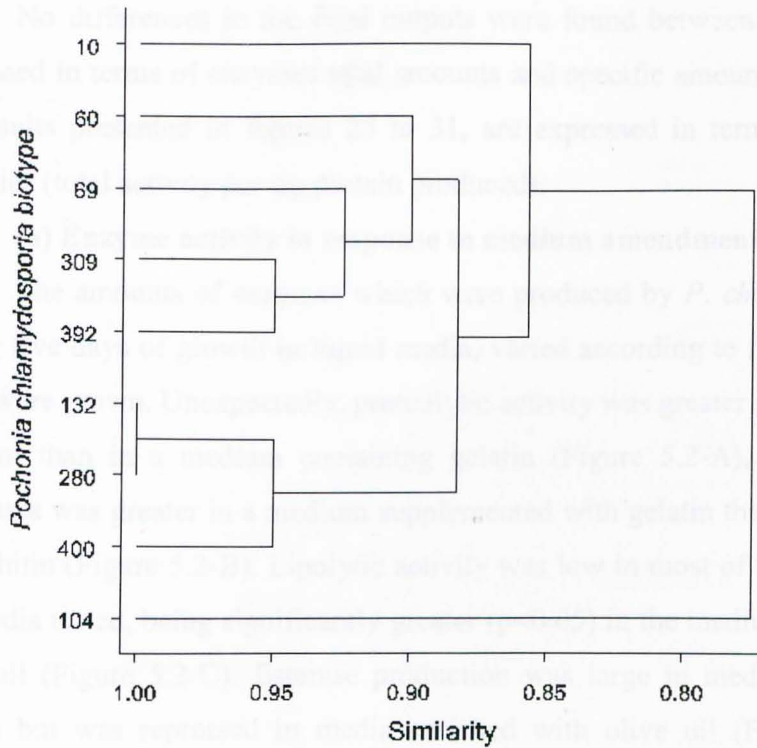
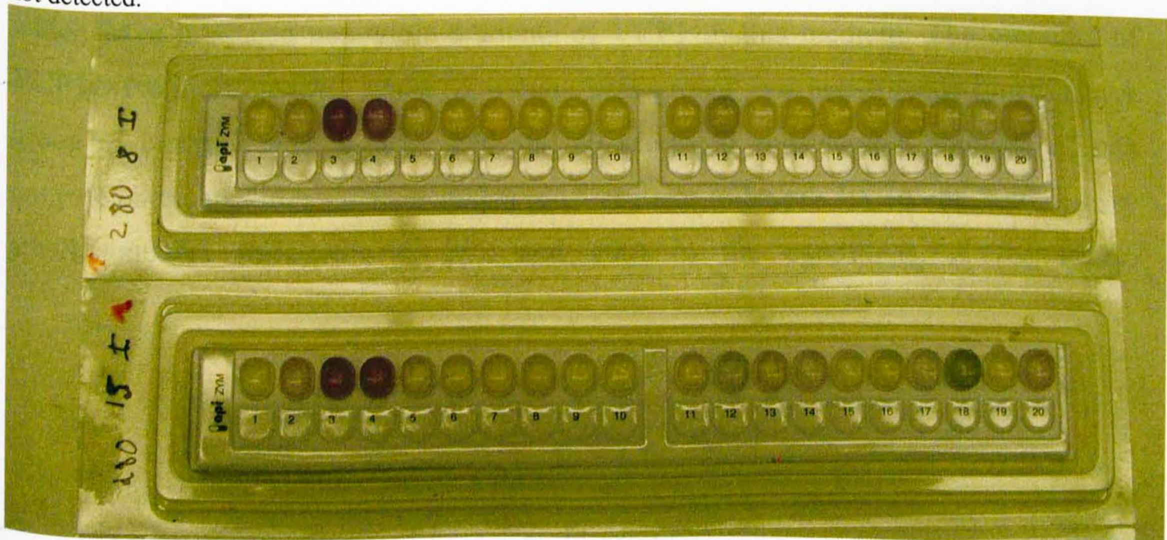


Figure 5.1. Dendrogram showing groups of biotypes of *Pochonia chlamydosporia* grouped according to their similarities in producing extracellular enzymes. Results are the average values of strong enzyme activity (≥20 nM substrate hydrolysed) between two replicates of each biotype. The dendrogram was constructed using cluster analysis (group average).

Plate 5.1 API ZYM® strips showing enzyme activity in two sub-cultures of *Pochonia chlamydosporia*, biotype 280 [8th (above) and 15th (below) week sub-culture]. Strong activity of esterases (well n.3) and esterase lipases (well n.4) was detected in both sub-cultures (≥40 nM of substrate hydrolysed). Activity of N-acetyl-ß-glucosaminidase (exochitinase) was detected for the 15 week sub-culture (well n. 18) and also in the 60th week sub-culture (not shown in the picture). Colourless wells mean that the enzyme was not detected.



5.3.2 Quantitative studies on the production of extracellular enzymes

No differences in the final outputs were found between presenting the results expressed in terms of enzymes total amounts and specific amounts secreted. Therefore, the results presented in figures 25 to 31, are expressed in terms of enzyme specific activities (total activity per μg protein produced).

(a) Enzyme activity in response to medium amendments

The amounts of enzymes which were produced by *P. chlamydosporia* biotypes during five days of growth in liquid media, varied according to the media in which the fungi were grown. Unexpectedly, proteolytic activity was greater in a non-supplemented medium than in a medium containing gelatin (Figure 5.2-A), whereas secretion of chitinases was greater in a medium supplemented with gelatin than in medium enriched with chitin (Figure 5.2-B). Lipolytic activity was low in most of the biotypes and in all the media tested, being significantly greater ($p<0.05$) in the medium supplemented with olive oil (Figure 5.2-C). Esterase production was large in media supplemented with gelatin but was repressed in media enriched with olive oil (Figure 5.2-D). Due to constraints in the numbers of samples that could be processed, it was not possible to measure the activity of esterases and lipases in media supplemented with chitin. For the same reason, chitinolytic activity was not measured in the medium containing olive oil.

VCP1 activity was detected in all the biotypes when grown in the medium supplemented with chitin (Figure 5.3). No activity was found in this medium when not inoculated with the fungus (data not shown). Low levels of activity were found in the medium supplemented with a source of protein, particularly in biotypes 60, 69 and 392 which have produced negligible amounts of VCP1 in this medium. The amounts of VCP1 were more variable when the fungus was grown in a non-amended medium. A group of biotypes such as 132, 280 and 60 produced considerable amounts of this enzyme in this medium, whereas others produced less (biotypes 10, 16 and 309) or very small amounts (biotypes 69, 392 and 400) in the conditions of the assay.

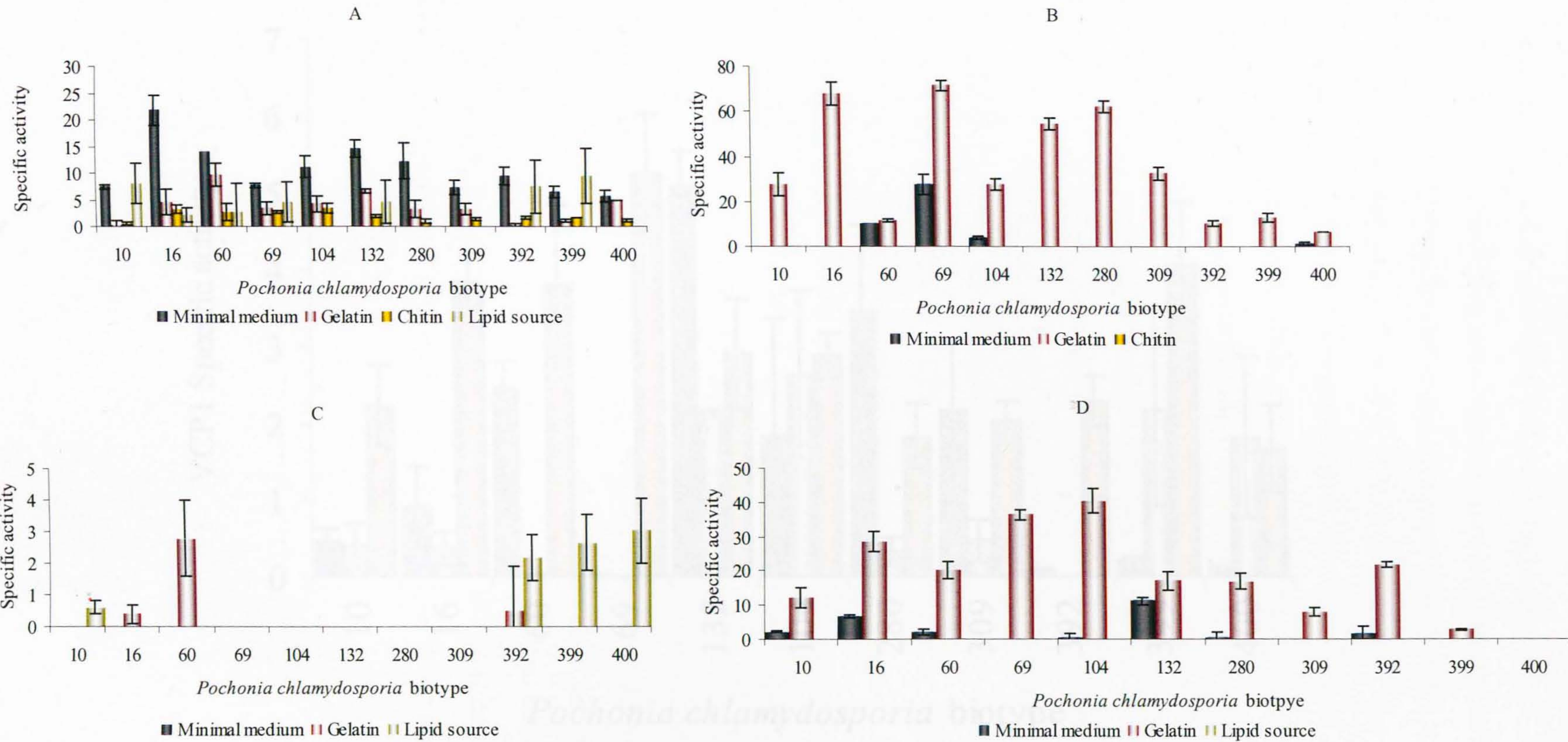


Figure 5.2 – Proteases (A), chitinases (B), lipases (C) and esterases (D) specific activities (nmol ρ-nitrophenol $min^{-1}ml^{-1}\mu g$ protein) produced by eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after five days of growth in different media (minimal medium and medium supplemented with gelatin, chitin and olive oil). I – Standard error bars.

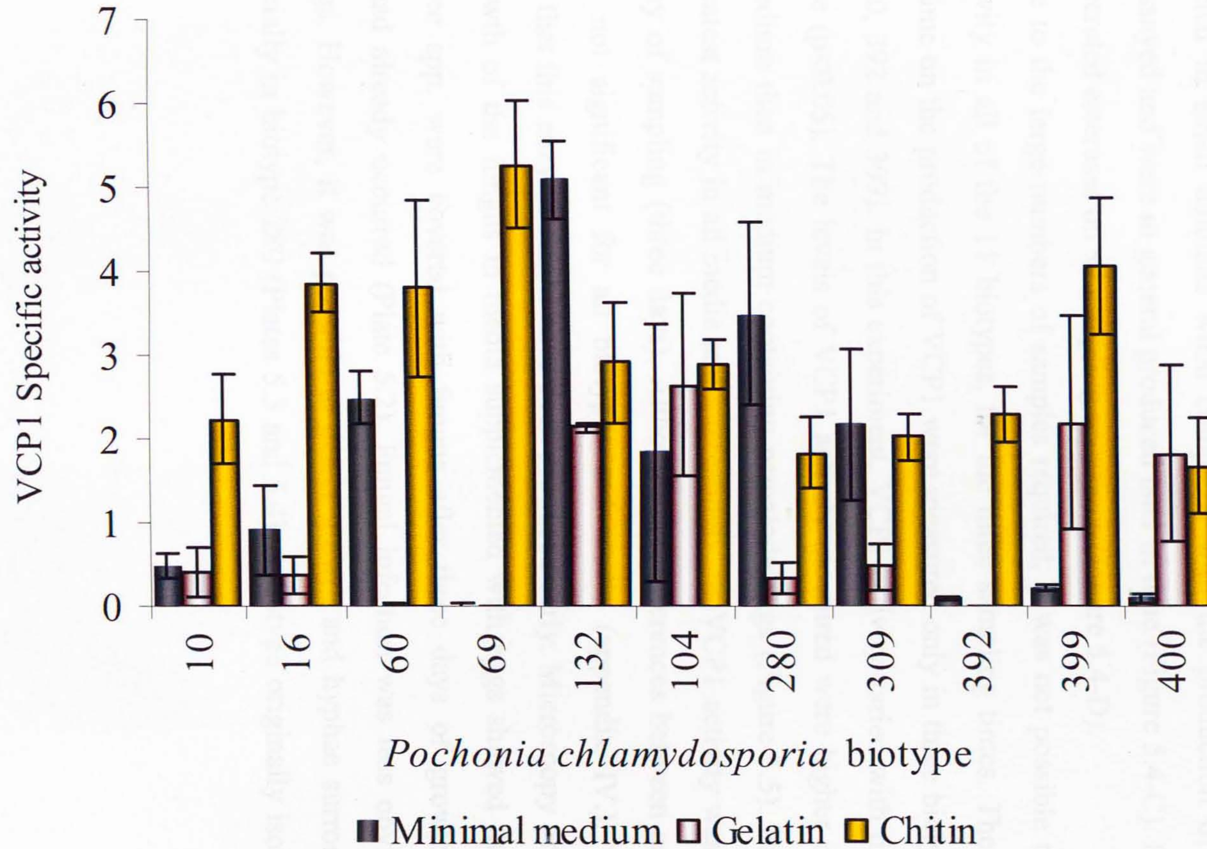


Figure 5.3 – Measurement of VCP1 activity (μmol p-nitroanilide $min^{-1}ml^{-1}\mu g$ protein) in eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after seven days of growth in minimal medium (A) and medium supplemented with gelatin and chitin. Standard error bars.

(b) Enzyme activity and time of secretion

Enzyme activity varied with time and with the biotype of *P. chlamydosporia*. The production of proteases in non supplemented medium did not differ significantly between the first two sampling occasions but decreased significantly by day 7 (Figure 5.4-A). Chitinolytic activity was greater after five days of growth, for the majority of the biotypes, and then significantly decreased after this time (Figure 5.4-B). Lipases were secreted in small amounts when compared with the production of the other enzymes assayed and were in general produced later in time (Figure 5.4-C). In contrast, biotypes secreted esterases on all sampling occasions (Figure 5.4-D).

Due to the large numbers of samples required, it was not possible to measure VCP1 activity in all of the 11 biotypes, for the three sampling times. Therefore, the effects of time on the production of VCP1 were measured only in three biotypes of the fungus (280, 392 and 309). In this experiment, VCP1 activity varied with time, media and biotype ($p<0.05$). The levels of VCP1 activity measured were higher in minimal Czapek medium than in medium containing nematode eggs (Figure 5.5). Biotype 280 had the greatest activity in all media and interestingly, its VCP1 activity was greater in the first day of sampling (three days). Although the differences between activity and days were not significant for all biotypes and media (appendix IV.), there is a suggestion that this enzyme might have been produced early. Microscopy observations on the growth of the fungus in media supplemented with eggs showed that eggs of *Meloidogyne* spp. were covered with fungus after three days of growth and that infection had already occurred (Plate 5.2). Fungal infection was less obvious in *G. pallida* eggs. However, it was possible to detect growth and hyphae surrounding the eggs, especially in biotype 280 (Plates 5.3 and 5.4), a biotype originally isolated from *G. pallida*.

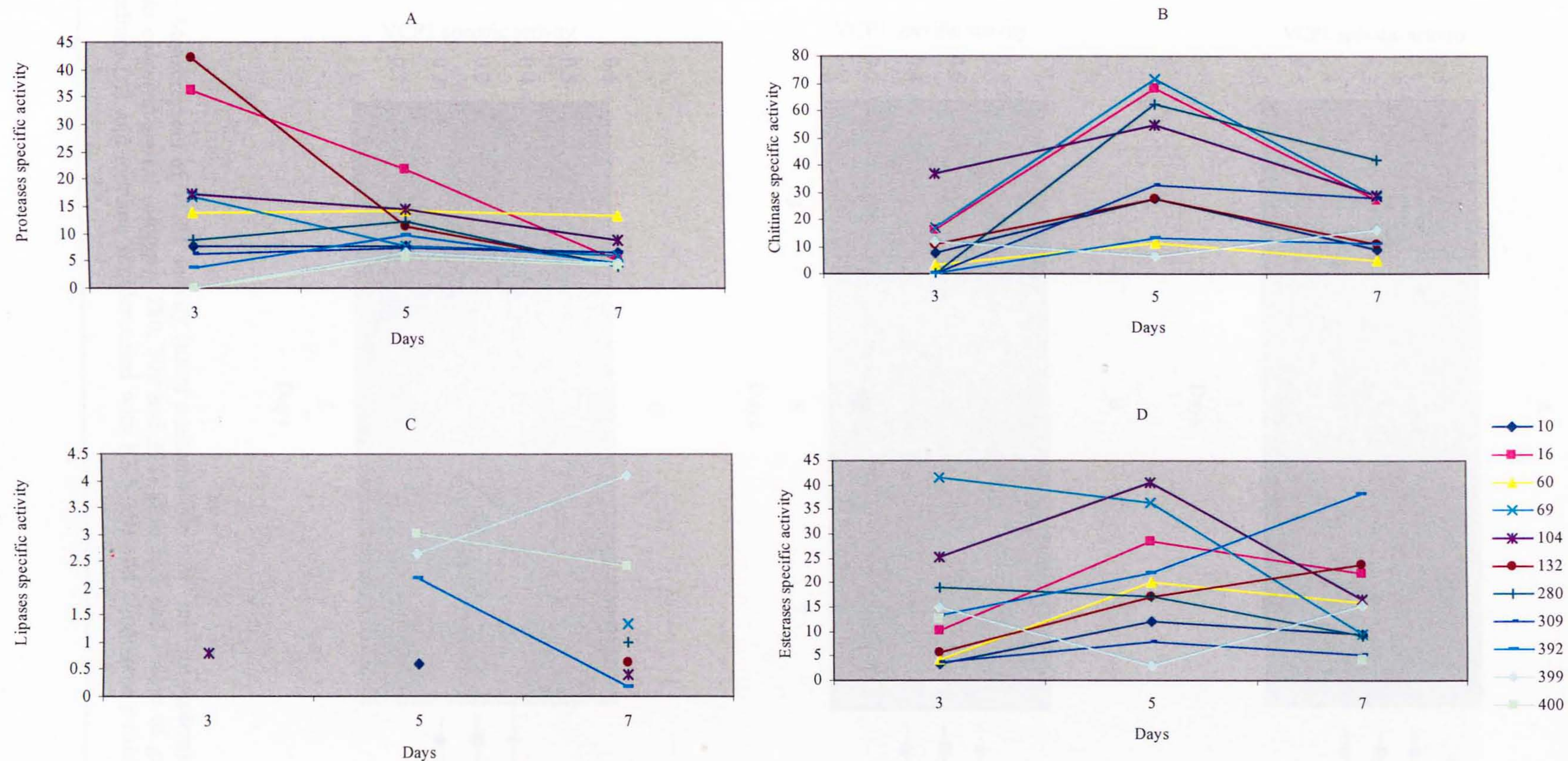


Figure 5.4 – Proteases (A), chitinases (B), lipases (C) and esterases (D) specific activities (nmol ρ-nitrophenol min^{-1} ml^{-1} μg protein) produced by eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after 3, 5 and 7 days of growth in non supplemented medium (A) and medium supplemented with gelatine (B and D), and olive. I – Standard error bars.

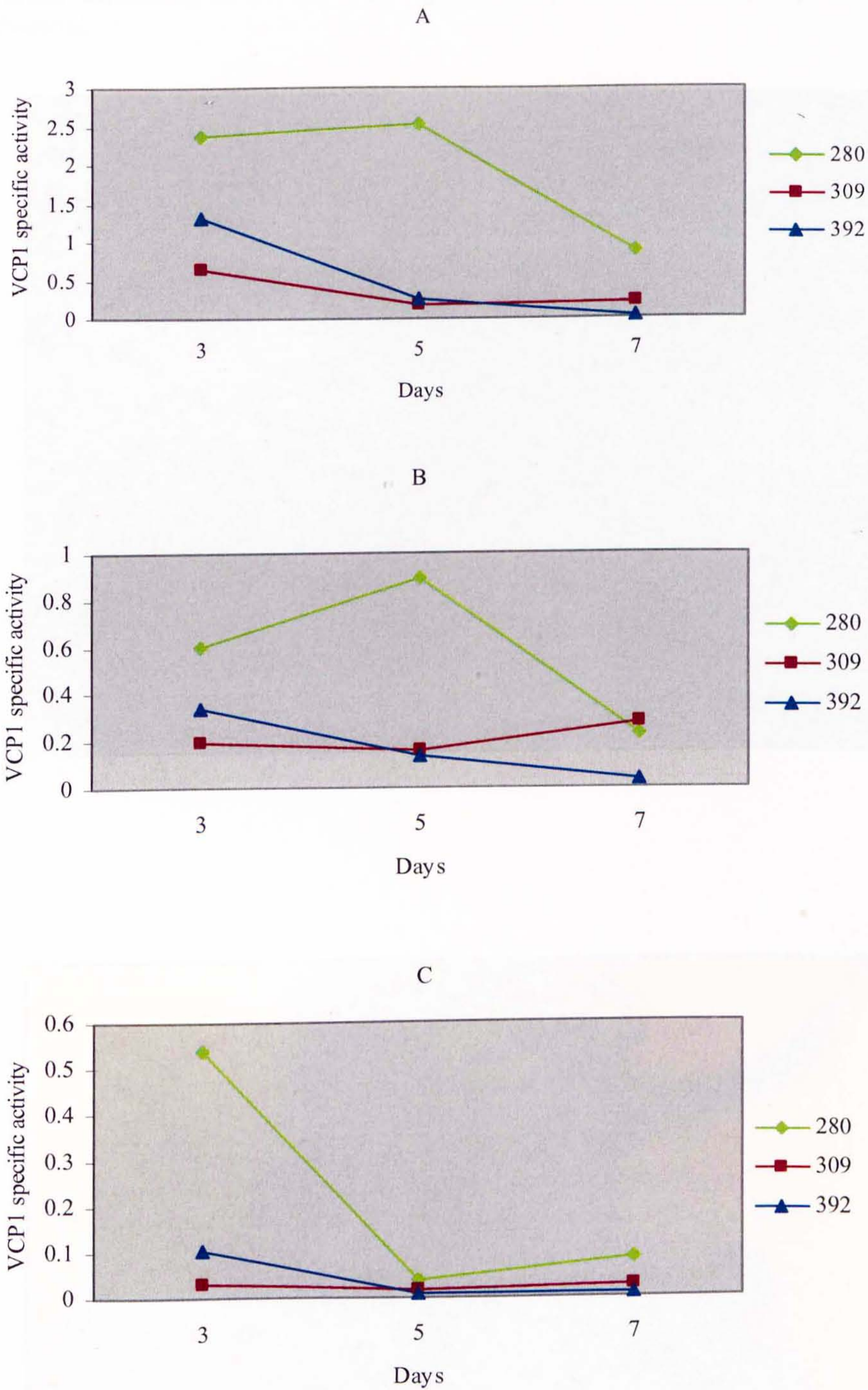
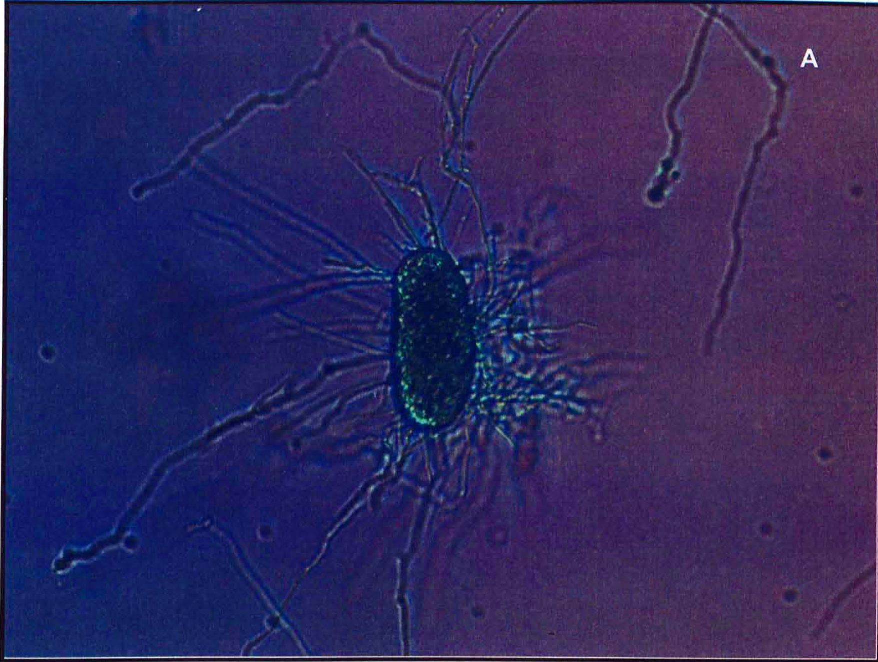


Figure 5.5– Measurement of VCP1 activity (μmol p-nitroanilide min^{-1} ml^{-1} μg protein) in three biotypes of *Pochonia chlamydosporia* (biotypes 280, 309 and 392) after 3, 5 and 7 days of growth in minimal Czapek medium (A) and medium supplemented with RKN (B) and *Globodera pallida* nematode eggs (C).

Plate 5.2 (A) - Immature stage of an egg of *Meloidogyne* sp. infected with *Pochonia chlamydosporia* biotype 280, after three days of growth in liquid medium (x 200 magnification). (B)- Extensive mycelial growth was found surrounding *Meloidogyne* spp eggs and the presence of chlamydospores was noticed (x 50 magnification).



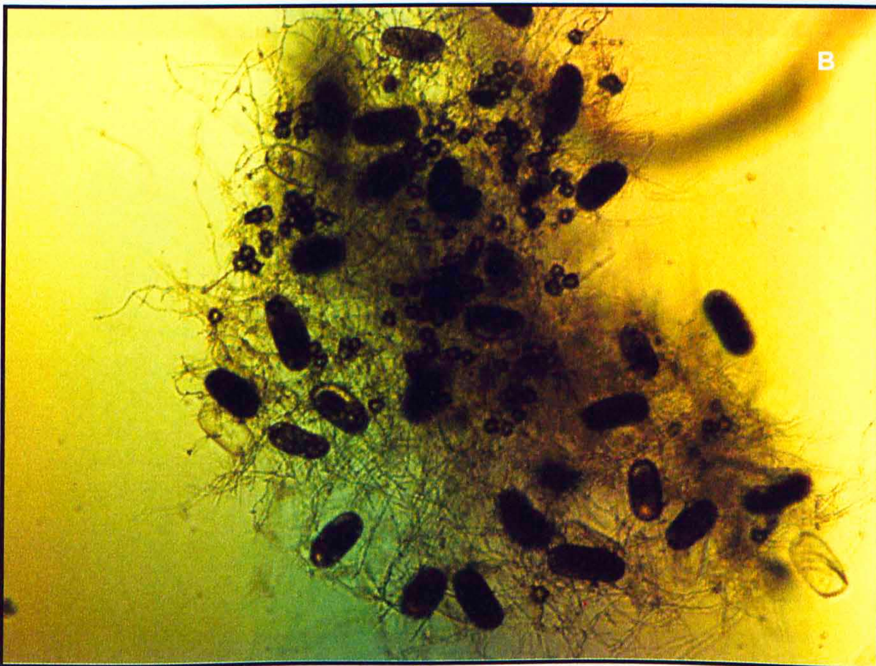


Plate 5.3 *Globodera pallida* egg infected with *Pochonia chlamydosporia* biotype 280, after three days of growth in a liquid medium (200x magnification). Infection was less visible in *Globodera pallida* eggs than in *Meloidogyne* spp. eggs in all of the biotypes. However, chlamydospores and mycelium surrounding the eggs was visible.

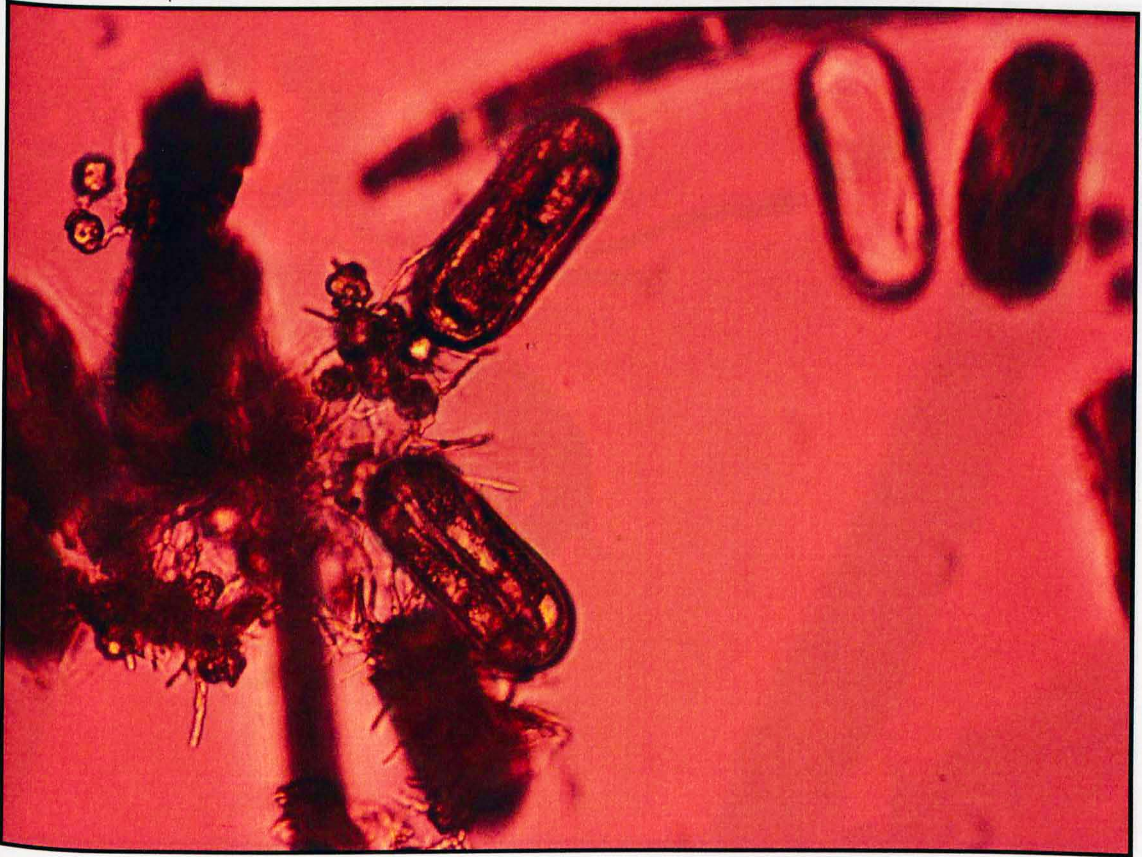
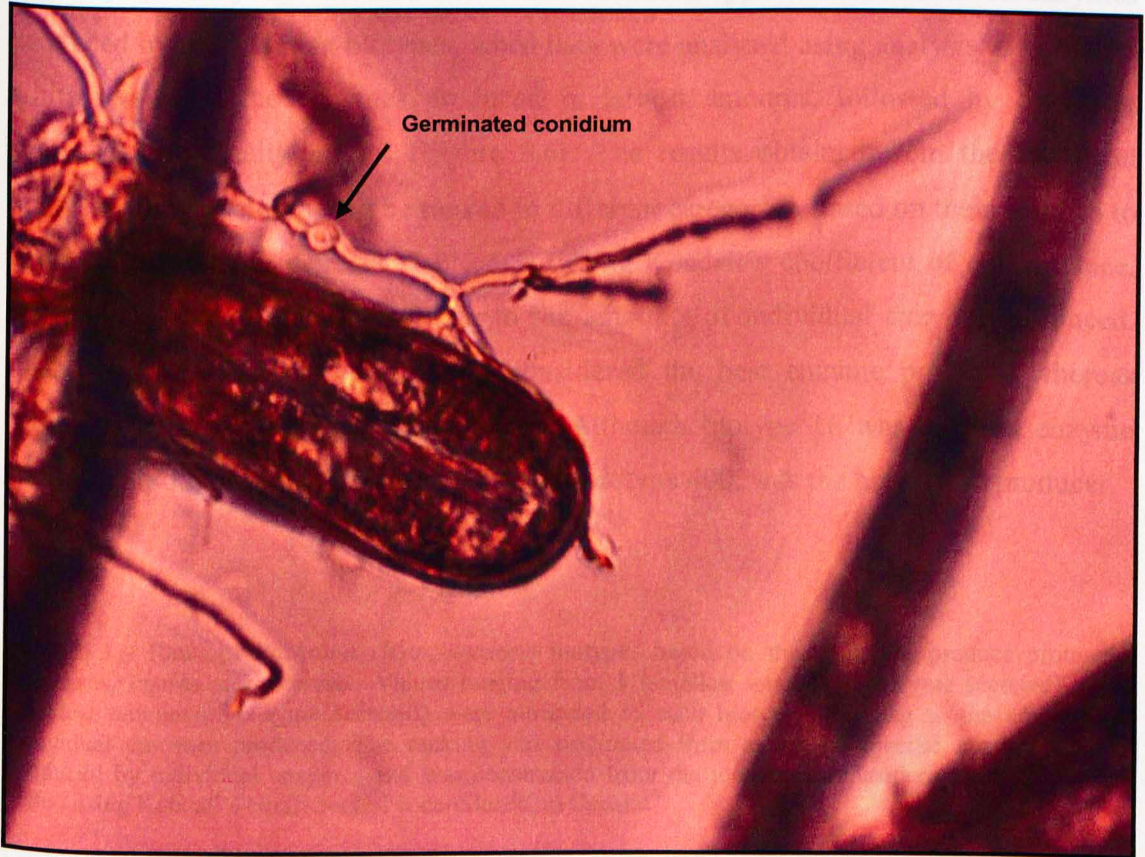


Plate 5.4. A *Globodera pallida* egg surrounded by *Pochonia chlamydosporia* biotype 392, after three days of growth in a liquid medium (400x magnification). The picture shows a germinated conidium attaching to the egg. Fungal growth is less evident in *G. pallida* eggs when compared with growth on *Meloidogyne* spp. eggs.



(c) Enzyme activity in different biotypes of *Pochonia chlamydosporia* after five days of growth

The comparison between biotypes of *P. chlamydosporia* on the production of extracellular enzymes revealed differences between types and amounts of enzymes produced by the different biotypes, when data were analysed using analysis of variance. Chitinases were the enzymes produced in largest amounts, followed by esterases, proteases and finally lipases (Figure 5.6). The results obtained from the statistical analysis allowed biotypes to be ranked in different categories, based on their abilities to produce enzymes in general (Table 5.3). Using Kendall's coefficient of concordance, the biotypes were ranked according to the amounts of individual enzymes produced. Using this analysis biotype 16 was considered the best enzyme producer whereas biotype 400 was the lowest (Table 5.3). Although biotype 16 was the best enzyme producer, it did not produce lipases, whereas biotype 400, was the best lipase producer.

Table 5.3 - Rank of *Pochonia chlamydospo*ria biotypes based on the ability to produce proteases, chitinases, lipases and esterases. Values ranging from 1 (smallest amount of enzyme secreted) to 11 (greatest amount of enzyme secreted) were attributed to each biotype according to the amounts of individual enzymes produced. The ranking was originated from means of specific enzyme activity produced by individual enzymes and was constructed from the total mean considering the 4 enzymes tested using Kendall's coefficient of concordance, in Genstat®.

***P. chlamydosporia* biotype (ranked)**	**Proteases**	**Chitinases**	**Lipases**	**Esterases**	**Mean**
16	11.0	10.0	4.0	9.0	8.5
69	5.0	11.0	4.0	10.0	7.5
104	7.0	6.0	4.0	11.0	7.0
132	10.0	8.0	4.0	6.0	7.0
280	8.0	9.0	4.0	5.0	6.5
392	6.0	2.0	9.0	8.0	6.2
60	9.0	3.0	4.0	7.0	5.8
10	4.0	5.0	8.0	4.0	5.2
399	2.0	4.0	10.0	2.0	4.5
309	3.0	7.0	4.0	3.0	4.2
400	1.0	1.0	11.0	1.0	3.5

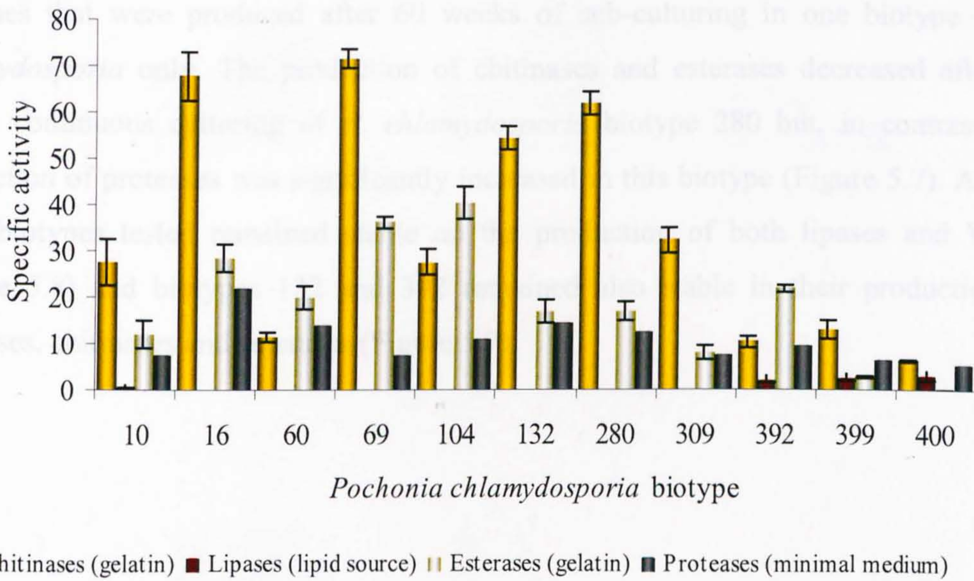


Figure 5.6 – Comparison between eleven biotypes of *Pochonia chlamydosporia* (biotypes 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) on enzyme specific activities (nmol ρ-nitrophenol min^{-1} ml^{-1} μg protein). Chitinases, lipases and esterases were measured after five days of growth. Proteolytic activity was measured in non amended medium, chitinase and esterase activity were measured in medium induced with gelatin and lipase activity was measured in medium containing olive oil. I-Standard error bars

(d) Effects of repeated culturing on the ability to produce enzymes

The continuous sub-culturing on agar had effects on the amounts of extracellular enzymes that were produced after 60 weeks of sub-culturing in one biotype of *P. chlamydosporia* only. The production of chitinases and esterases decreased after 60 weeks continuous culturing of *P. chlamydosporia* biotype 280 but, in contrast, the production of proteases was significantly increased in this biotype (Figure 5.7). All the three biotypes tested remained stable on the production of both lipases and VCP1 (Figure 5.8) and biotypes 132 and 392 remained also stable in their production of proteases, chitinases and esterases (Figure 5.7).

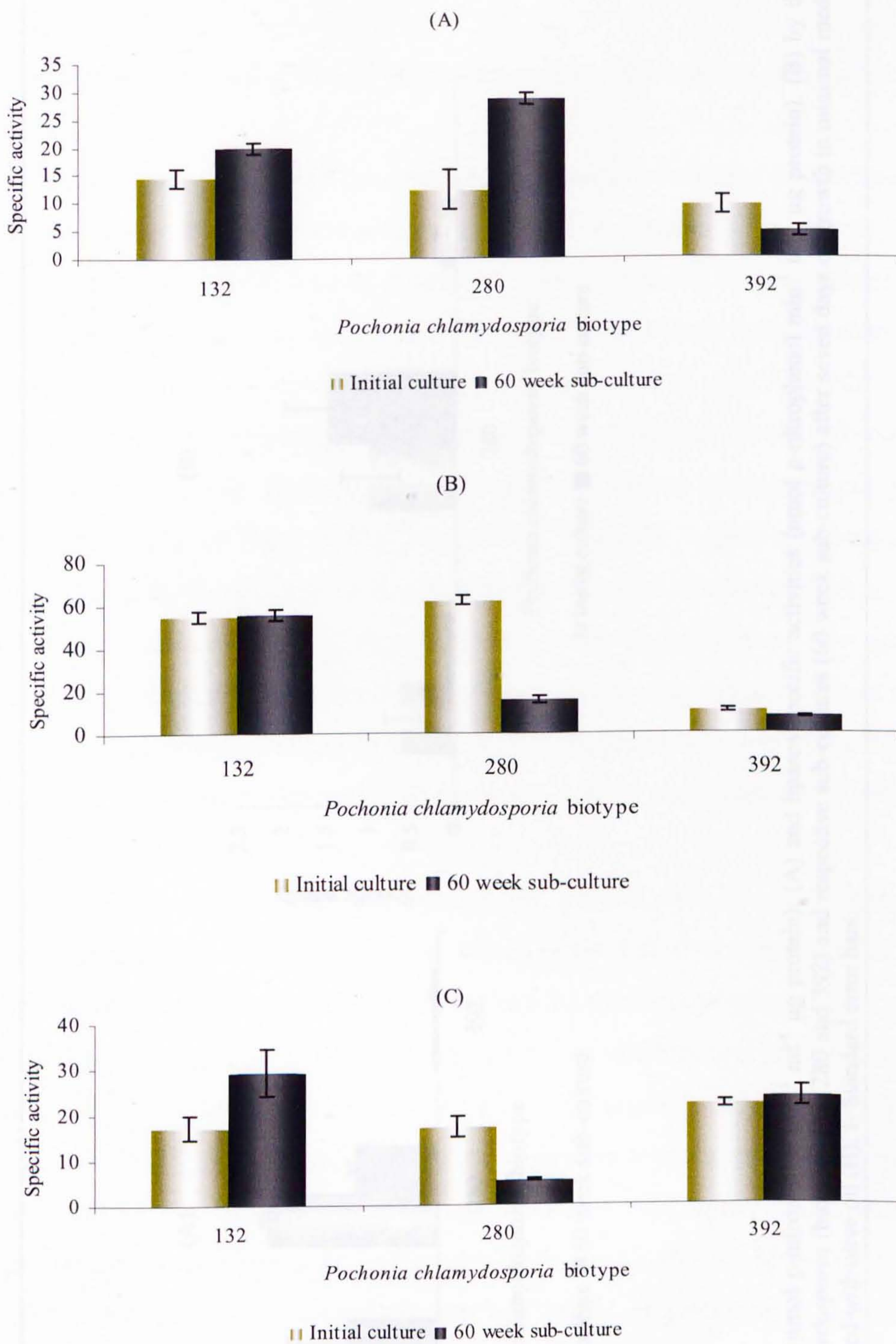


Figure 5.7- Proteases (A), chitinases (B) and esterases (C) specific activities (nmol ρ-nitrophenol min^{-1} ml^{-1} μg protein) produced by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) and respective sub-cultures (60 week sub-culture) after five days of growth in minimal medium (A) and medium supplemented with gelatin (B and C). I- Standard error bars.

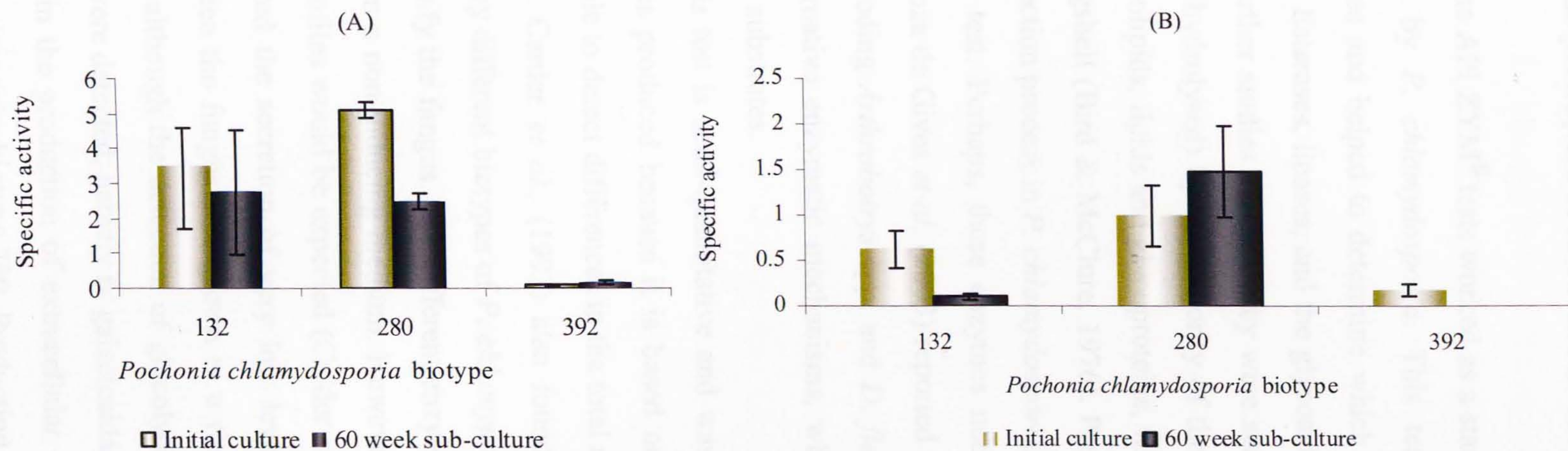


Figure 5.8– VCP1 activity (μmol p-nitroanilide min^{-1} ml^{-1} μg protein), (A) and lipases specific activities (nmol ρ-nitrophenol min^{-1} ml^{-1} μg protein), (B) by three biotypes of *Pochonia chlamydosporia* (biotypes 132, 280 and 392) and respective sub-cultures (60 week sub-culture) after seven days of growth in minimal medium (A) and medium supplemented with olive oil (B). I- Standard error bars.

5.4 DISCUSSION

Studies on the production of extracellular enzymes using *p*-nitrophenyl substrates

The use of the API ZYM® tests worked as a starting point for further studies on enzyme production by *P. chlamydosporia.* This test detects a group of general extracellular enzymes and helped to determine which enzymes were important to be further investigated. Esterases, lipases, and the glycosidase N-acetyl-ß-glucosaminidase were selected for further studies because they were secreted in significant amounts ($\geq$ 20 nM of substrate hydrolysed) by the majority of the biotypes. These enzymes have activity against glycolipids, lipids and glycoproteins, which are constituent components of the nematode eggshell (Bird & McClure, 1976). Proteases, which are thought to be important in the infection process in *P. chlamydosporia,* were not detected in any of the biotypes using this test. Perhaps, these enzymes need to be induced for secretion. Interestingly, Mendoza de Gives *et al.* (2003) reported the same observation in a wider range of fungi, including *Arthrobotrys* spp. and *D. flagrans* and suggested that these fungi may use alternative enzymatic mechanisms, which may alter their production strategy on different substrates.

Although this test is semi-quantitative and was not able to quantify the exact amounts of enzymes produced because it is based on visual observations of colour intensities, it was able to detect differences in the total numbers of enzymes secreted by individual biotypes. Carder *et al.*, (1993) also found differences in the number of enzymes produced by different biotypes of *P. chlamydosporia* in studies using different enzyme kits to classify the fungus. The different enzyme profiles were found when the fungus was grown in a non-inducing medium. However, if different media were used, different enzyme profiles would be expected (Carder *et al.*, 1993). Mendoza de Gives *et al.* (2003) reported the secretion of very low levels of glycolytic enzymes in *P. chlamydosporia*, when the fungus was grown in a rich soya-peptone medium for 30 days. In this study, although the detection of glycolytic enzymes was also low, a few other glucosidases were detected, namely ß- galactosidases and ß- glucosidases.

Differences in the production of extracellular enzymes were detected in sub-cultures of *P. chlamydosporia*, biotype 280. Production of esterases and esterase lipases

were detected in the initial and in the three sub-cultures tested (8, 15 and 60 weeks). However, after 8 weeks of sub-culture, the production of N-acetyl-ß-glucosaminidase was also detected. This enzyme is thought to help degrade the chitinase polymer and was detected in most of the biotypes tested in the experiment. Interestingly, this enzyme was also detected in large amounts in different biotypes of the fungus, by Carder *et al.* (1993).

Shah & Butt (2005) found differences in enzyme profiles using the API ZYM® system between parent and sub-cultures in two biotypes of *M. anisopliae*. In their study, the activity of some enzymes increased whilst others decreased or were lost altogether. In general, *P. chlamydosporia* appears to be more stable – no enzymes were lost, although differences in the amounts of enzymes produced were detected.

Quantitative studies on the production of extracellular enzymes

Pochonia chlamydosporia biotypes produced enzymes in different amounts and responded differently when different inducers were added to the medium. Gelatin showed to be a good inducer for the production of chitinases and esterases but surprisingly did not increase the production of proteases and VCP1. It is possible that the type of gelatin used, obtained from porcine skin has favoured the production of other enzymes apart from proteases. In a previous study, the use of a higher concentration of gelatin (1% instead of 0.2% used in this study) strongly repressed VCP1 activity, as did albumin, whereas fibrous collagen enhanced protease production (Segers, 1996). It was concluded that the inductive effect of protein was not a generic response and the response depended on the source of protein used.

Similarly, chitinase activity was not induced in the medium amended with chitin but increased VCP1 activity. The type of chitin used was of practical grade (from crab shell) and although was washed and sieved before use, may have contained other nutrients apart from chitin which could have induced other enzymes such as VCP1. Chitin is insoluble in water and in this experiment was used untreated, which means that is possible that this molecule was not accessible to the fungus, which was unable to degrade it and therefore to produce chitinases. Nevertheless, the physical presence of chitin in suspension, absent in other media tested, may have provided physical support for fungal to growth, and this may have been another reason why the production of the

serine protease VCP1 was favoured. High VCP1 titres were also found by Segers (1996) using a similar source of chitin in suspension. Furthermore, in the same study, the combined use of chitin and collagen, both insoluble, resulted in an increased VCP1 activity.

Interestingly, all the biotypes tested showed activity for VCP1 in this medium. In contrast, the cyst nematode biotype isolated from New Zealand (biotype 69) and the root-knot nematode biotype isolated from Cuba (biotype 392) which is a variant, *P. chlamydosporia* var *catenulata,* had significantly lower VCP1 activity in the minimal medium and the medium amended with gelatin. The apparently lower activity of the enzyme in these two biotypes could be due to reduced substrate affinity rather than a less active serine protease and therefore the results may have been influenced by the substrate used in the assay [Suc-$(Ala)_2$-Pro-Phe-pNA]. Morton (2002) showed differences in the structure of VCP1 enzyme between biotypes isolated from root-knot and cyst nematodes. Differences were observed on the rim of the substrate-binding region, where a glycine in the enzyme from biotypes from root-knot nematodes is replaced by a larger alanine in biotypes from cyst nematodes. Polymorphisms were also found at the position 57, where a glutamic acid in the enzyme from biotypes from root-knot nematodes is replaced by a glutamine in biotypes isolated from cyst nematodes. Therefore, it is possible that the serine proteases produced by the two biotypes, 69 and 392, are substantially different from proteases produced by other biotypes. Another possibility is that this enzyme could have been secreted in larger amounts earlier than 7 days and then was repressed. Although VCP1 was detected in very small amounts in biotype 392 after 7 days of growth, the activity was significantly greater three days earlier (Figure 5.5). Biotype 280, which was isolated from cyst nematode eggs, also secreted higher levels of VCP1, 3 days post-fungal inoculation in a medium amended with *G. pallida* eggs. The production of VCP1 decreased later with time but other proteases continued to be secreted. This may indicate that VCP1 is secreted in the early stages of infection. Microscopy observations confirmed infection of *Meloidogyne* spp. and *G. pallida* eggs added in the medium, 3 days after inoculation with the fungus although fungal infection was less visible in *G. pallida* eggs (Plates 5.3 and 5.4). Differences in the extent of fungal colonization on the surface of eggs from different nematodes species were also noticed by Segers (1996). In his study, external

colonization of *M. incognita* eggs by *P. chlamydosporia* was faster and more extensive than of G. *rostochiensis* and infection rates appeared to be correlated with the amount of fungal growth on the egg surface. Appressoria also differed in their numbers and shape.

Like the serine protease VCP1, the production of other enzymes in amended and non-amended media varied with time. Lower levels of chitinases have been detected in cultures of *M. anisopliae*, where chitinase activity was not detected for the first 5 days (Nahar *et al.*, 2004). Although the enzyme activities were detected using artificial substrates, they might mimic the response of *P. chlamydosporia* when in contact with nematode eggs. As the first layer of the nematode eggshell contains mainly protein, proteases may be the first to be secreted but they are also required through time in order to degrade the middle and inner eggshell layers as they are also composed of protein, apart from the chitin and lipids. The time of secretion of these two enzymes is also considered to be important in entomopathogenic fungi, in which proteases are secreted in the initial stages of infection, followed by chitinases (St. Leger *et al.*, 1986). The production of exochitinases in the late stage of infection may play a role in the inhibition of the development of other chitin microbial competitors in mycopathogens (Wattanalai *et al.*, 2004). Although lipolytic activity was low, there was the suggestion that lipases might have been produced later in time, with most of the biotypes increasing activity for degradation of lipids after seven days of growth in the medium amended with olive oil. Extra virgin olive oil was chosen among other types of lipid sources because it was shown to increase lipolytic activity in *Fusarium solani* (Maia *et al.*, 1999) and *Metarhizium anisopliae* (Silva *et al.*, 2005). It is possible that different results could be achieved if a different source of lipid or substrate had been used. Also, perhaps lipolytic activity would increase if the duration of the experiment had been longer.

Enzyme activity varied with the biotype and the type of enzyme assayed and biotypes could be ranked according to their similarities in the production of proteases, chitinases, esterases and lipases. Variability in the production of proteases and chitinases has been reported in the past, using different substrates (Segers *et al.*, 1999; Olivares-Bernabeu & Lopez-Llorca, 2002). However, the comparison between biotypes on the production of esterases, lipases and interaction with chitinases and proteases were studied here for the first time.

Chitinases were the enzymes secreted with greatest specific activity, followed by esterases and proteases. The eggshell layer which contains chitin is the thickest of the three layers, and is probably the reason why the fungus produces large amounts of this enzyme.

The role of esterases in the physiology of the fungus is not clear. Segers (1996) detected high esterase activity in culture filtrates of *P. chlamydosporia* and in pure VCP1 enzyme and found that VCP1 was highly active on the hydrolisation of short (C4-C6) and medium (C7-C10) chain esters whereas Pr1, a serine protease secreted by *M. anisopliae* was active on short chain esters only. The ability to degrade both long and short chain of esters may reflect the nutritional versatility of *P. chlamydosporia*.

The production of enzymes was little affected by the continuous sub-culturing of the fungus on agar. The activity of most enzymes assayed remained stable, with the exception of chitinases and esterases which were produced by biotype 280. This biotype produced less of these two enzymes after 60 weeks of sub-culture but its proteolytic activity increased. A similar result was obtained by Shah & Butt (2005) in sub-cultures of *Metarhizium anisopliae*; activities of some enzymes decreased whereas others were increased. No changes on enzyme production were detected in the other two biotypes of *P. chlamydosporia* tested, suggesting that the degree of stability of the fungus may vary with biotype, possibly due to differences in the genetic stability of each individual (Hajek *et al.*, 1990; Brownbridge *et al.*, 2001).

The selection of biotypes for potential biocontrol of nematodes and insects has included studies on enzyme production (Barranco-Florido *et al.*, 2002; Olivares-Bernabeu & Lopez-Llorca, 2002). Such studies might be important and may help to differentiate biotypes to some extent (Carder *et al.*, 1993) but other parameters such virulence and spore production should always be considered in the selection of potential BCAs. In this study, differences in enzyme production were found between biotypes of *P. chlamydosporia*. However, the amounts of enzymes secreted by individual biotypes were shown to vary with time, media and type of enzyme secreted and therefore, culture conditions have an important effect in the results obtained and must be standardised for meaningful comparisons to be made.

CHAPTER 6: WATER RELATIONS, POLYHYDROXYALCHOLS (POLYOLS) AND SUGAR ACCUMULATION IN *POCHONIA CHLAMYDOSPORIA*

6.1 INTRODUCTION

Water availability plays a vital role in fungal growth and survival, by its involvement in the metabolic reactions within the cell. Most fungi require very high water availability, and rapidly dry out or senesce in dry conditions. However, some fungi are able to tolerate much lower water potentials than other organisms and this can have major economic significance, particularly in the spoilage of stored food products, including cereal grains (Magan & Lacey, 1984). In soil pores, however, the relative humidity is > 98 %, even at the permanent wilting point for plants, and soil fungi usually are not exposed to these extreme drying conditions. However, the efficacy of some fungal BCAs in soil has not been consistent because it has been markedly influenced by environmental conditions, including the availability of water, which affects their survival and performance (Cook & Baker, 1983). The shelf-life of a formulated BCA can also be much affected by its ability to withstand drying.

When grown under osmotic water stress, the quantity and/or proportion of the endogenous reserves (Ramos *et al.*, 1999), the spore germination rate (Hallsworth & Magan, 1995) and the virulence of several fungi is changed (Chandler *et al.*, 2005). Culturing *M. anisopliae* under water-stress conditions increased the concentration of the compatible solute erythritol, in conidia, which was accompanied by an increase in spore germination and virulence against the cotton aphid *Aphis gossypii* (Chandler *et al.*, 2005).

The optimal water potential for *Pochonia chlamydosporia* occurs between -0.1 to 0.2 MPa. Its minimum potential for survival appears to be -8 to -10 MPa (Bourne & Kerry, 2000). *Pochonia chlamydosporia* biotypes were more susceptible to low water potentials than other *Pochonia* species, and growth was greatest when water was freely available (Kerry *et al.*, 1986; Bourne & Kerry, 2000). Less information is available on whether there are any differences in tolerance to solute and matric stress. The latter is important as an indicator of the capacity for active growth and survival in soil. This fungus is also found in a wide range of climates, from tropical, to dry and semi-arid climates (Stirling & Kerry, 1983) and is tolerant to a range of conditions. It is to be less

dependent on soil moisture conditions than some other nematophagous fungi such as the zoosporic fungus, *Nematophthora gynophila*, which requires water-filled soil pores for the motility of its infective spores (Kerry *et al.*, 1986). It has been suggested that the induction of chlamydospore formation occurs only under stress conditions unfavourable for growth (Sykes, 1994). In *P. chlamydosporia* chlamydospore production *in situ* is much greater in the parasitic, rather than the saprotrophic phase, of the fungus but the effects of water stress on spore quality and germination have not been previously studied.

Thus, this part of the thesis examined (a) the effect of solute (ionic, non-ionic water stress) and matric potential stress on growth and (b) impact on accumulation of endogenous low and high molecular weight sugar alcohols in response to such environmental stress in three biotypes of *P. chlamydosporia*.

6.2 MATERIAL AND METHODS

6.2.1 Effects of osmotic and matric potential on growth and accumulation of endogenous reserves in three biotypes of *P. chlamydosporia*

Fungal biotypes

Pochonia chlamydosporia biotypes 10, 280 and 392 were used in the study. Information about the origin, substrata and host nematode for each of the biotypes studied is found in Chapter 2, Table 2.1.

Media

The medium used in this study was potato dextrose agar (PDA). The medium was prepared according to the instructions of the manufacturer (Oxoid). PDA was modified osmotically by the addition of the ionic solute KCl or the non ionic solute glycerol (Dallyn & Fox, 1980) to -0.7, -1.4, -2.8 and -7.1 MPa (= 0.995, 0.990, 0.980 and 0.950 water activity, respectively)(Magan, 1997).

For modification of the matric potential, PDA was omitted and known amounts of PEG 8000 were used, resulting in matric potentials of -0.7, -1.4, -2.8 and -4.2 MPa (= 0.995, 0.990, 0.980 and 0.970 water activity, respectively). The water potential generated by PEG 8000 is mainly (99%) due to matric forces (Steuter *et al.*, 1981). The medium was enriched with peptone (10 g l^{-1}) and glucose (40 g l^{-1}) and was autoclaved prior to use. Sterile disks of capillary matting (8.5 cm diameter) were placed in sterile 9 cm Petri dishes to which 20 ml of the cooled medium was added. The matting was overlaid with a sterile disk of black polyester lining cloth and then a cellophane disk (Ramos *et al.*, 1999), to provide support for fungal growth in the liquid medium.

Inoculation and measurement

Petri dishes were inoculated centrally with 5 mm diameter agar plug from the edge of seven days old colonies growing in PDA (Ramos *et al.*, 1999). Five dishes per treatment (solute x water potential) were inoculated and incubated at 25 °C; those at the same water potential were sealed in polyethylene bags (Marín *et al.*, 1995). The growing colonies were measured along two diameters at right angles after 5, 7, 10, 12,

14, 18 and 25 days, until the colony reached the edge of the dish. The radii of the colonies were plotted against time for each replicate, and linear regression was applied to obtain the growth rate (mm day^{-1}). Data of growth rates were subjected to analysis of variance (ANOVA) using Genstat 8.2®.

Quantification of polyols and sugars

Samples of 100 mg of fresh mycelia from all the above 25 day-old-cultures, were mixed with 1 ml of HPLC grade water in a 2 ml microtube (Eppendorf) and sonicated for 2 minutes using Soniprep 150 (Fisher Scientific UK), at 28 μm amplitude. After immersion in a boiling water bath for 5.5 minutes, the samples were left to cool (Hallsworth & Magan, 1994) and 667 μl of acetonitrile were added to maintain a 40:60 acetonitrile:water ratio, the same ratio as the mobile phase of the HPLC. The tubes were then centrifuged for 10 minutes at 1150x g (Microfuge). The supernatant was filtered through 0.2 μm aperture membrane filters (13 mm diameter, Whatman) and injected into a Gilson HPLC for quantification of solutes. The HPLC was fitted with a Hamilton HC-75 Ca^{2+} column, and a refraction index detector. The mobile phase was 40:60 acetonitrile:water (40:60) and the flow rate was 1 ml min^{-1}. The solutes analysed were four polyols (glycerol, erythritol, arabitol and mannitol) and one sugar (glucose). Solute quantification was measured using the Gilson software adapted to the HPLC equipment. Peak areas were integrated by the software and amounts expressed as mg g^{-1} fresh biomass based on the calibration curves and the pure sugar alcohols or sugars. Data on quantification of endogenous reserves were subjected to analysis of variance (ANOVA) using Genstat® 8.2.

6.2.2 Analysis of chlamydospore endogenous reserves in standard cracked rice and modified media

Fungal biotype

The experiment was performed using *P. chlamydosporia* biotype 10 on which extensive work has been carried out previously (De Leij & Kerry, 1991; Bourne & Kerry, 1999; Bourne & Kerry, 2000; Hirsch *et al.*, 2000; Noreen *et al.*, 2001; Atkins *et*

al., 2003; Morton *et al.*, 2003; Sorribas *et al.*, 2003). Information about the origin of this biotype is found in Chapter 2, Table 2.1.

Production of inoculum and analysis

Fungal inoculum (chlamydospores) was produced using a standard protocol developed by Hidalgo-Díaz (2003), as described in Chapter 2, section 2.4. Basically, moist rice (10-12 % water content, w/w) was inoculated with a liquid culture of the fungus. To modify the medium, water moisture used in the standard protocol was replaced by the addition of 10 and 20 % of glycerol, to obtain two lower water potentials: one between -1.4 to -2.8 and another between -4.2 to -7.1 MPa, respectively. Eight flasks containing 50 g of cracked rice inoculated with the fungus with were set up per treatment and were incubated at 25 °C for 21 days. Chlamydospores were washed from rice using tap water, as described in Kerry *et al.* (1998). Spores extracted were collected on a 10 µm aperture sieve.

The quantity and viability of chlamydospores produced in each treatment was estimated using the method described in Chapter 2, sections 2.4.1 and 2.42. The quantification of polyols and sugars in the chlamydospores were made following the protocol described previously for the estimation of these compounds in fungal mycelium.

6.3 RESULTS

6.3.1 Effects of osmotic and matric potential on growth and accumulation of endogenous reserves in three biotypes of *P. chlamydosporia*

Changes in the growth rate of colonies in relation to osmotic (glycerol and KCl) and matric (PEG) treatments were studied in three biotypes of *P. chlamydosporia* (biotypes 10, 280 and 392). Significant differences ($p<0.05$) were found between biotypes in response to osmotic stress but not in response to matric stress ($p>0.05$) (Figure 6.1; Appendix V). Biotype 10 had a higher growth rate in the medium modified with the non-ionic solute but was more sensitive to changes in the osmotic potential when the medium was amended with the ionic solute, KCl (Figure 6.2-E and 6.2-F). On the contrary, biotypes 280 and 392 were more tolerant to the ionic than the non-ionic

solute (Figure 6.2-A and 6.2-B). Similarities in growth rates in different solutes, at similar water potentials were found in biotypes 280 and 392 (Figure 6.1; Appendix V). At -1.4 MPa, the growth rate of biotype 280 in PEG was similar to that in KCl ($p>0.05$). At the same potential, biotype 392 grew at a similar rate in KCl and glycerol ($p>0.05$). And at -2.8 Mpa, growth rates of biotype 392 were similar in PEG and glycerol (Figure 6.1; Appendix V). Although similarities between PEG and the osmotic solutes were observed, the estimated limit of Ψ for growth in PEG was the lowest among the three solutes, varying between -6 to -7 MPa, depending on the biotype (Appendix V., Table 6.1.; Figure 6.2). The fungus was in general more tolerant to the ionic solute KCl, with an estimated limit for growth at approximately -11.8 MPa (biotype 392) (Appendix V; Table 6.1.; Figure 6.1). The optimum mycelial growth rate (1.4-1.8 mm d^{-1}) occurred when water was freely available (-0.7 MPa), for all biotypes and treatments and not at lower osmotic/matric potentials (Figures 6.2 and 6.3).

None of the *P. chlamydosporia* biotypes tested grew after 5 days in the medium modified with glycerol, at – 7.1 MPa, but continued growing at the same water potential in the medium modified with KCl (Figure 6.2).

In general, the total amounts of polyols (glycerol, erythritol, arabitol and mannitol) accumulated intracellularly, significantly increased in osmotically modified media, when compared with endogenous reserves accumulated in unmodified media, with the exception of media modified with KCl, at -1.4 and -2.8 MPa (biotype 280) (Table 6.2; Appendix V). Similarly, the quantity of polyols accumulated by the fungus in media modified with PEG was significantly greater ($p<0.05$) than that accumulated in unmodified media, in all the biotypes tested (Table 6.3). Significant differences ($p<0.05$) in the amounts of total polyols were found between biotypes in response to osmotic stress but not in response to matric stress ($p>0.05$) (Appendix V). The total amounts of polyols accumulated were greatest when the fungus grew at water potentials of -4.2 MPa, in media amended with PEG (Table 6.3).

Significantly greater amounts of glucose were detected in media modified with glycerol and PEG, when compared with the amounts accumulated when water was freely available in the media (Table 6.2; Table 6.3; Appendix V). The amounts were, in general, greatest at low water potentials, in media modified with PEG (Table 6.3).

However, fungal growth in media modified with KCl, did not result in a significant increase in the accumulation of glucose (Figure 6.1, Appendix V).

The proportion of polyols accumulated by the fungus varied with the type of solute present in the media. When grown in media osmotically modified with glycerol, the fungus accumulated large amounts of this solute, in significantly greater amounts than in the control medium (Table 6.4; Figure 6.4). In media osmotically modified with KCl, the accumulation of arabitol was significantly greater than in unmodified media (Table 6.4), in particular at -2.8 MPa (Figure 6.5). In matrically modified media, accumulation of large amounts of erythritol was particularly noticed in all the biotypes, and increased with the reduction of the water potential (Table 6.4; Figure 6.6).

Table 6.1 - Limits of Ψ (MPa) for *P. chlamydosporia* growth in different solutes estimated using linear regression

Biotype	**Solute**	**Minimum water potential for growth (Mpa)**
10		9.28
280	KCl	10.94
392		11.85
10		7.60
280	Glycerol	7.42
392		7.68
10		6.00
280	PEG	6.44
392		7.04

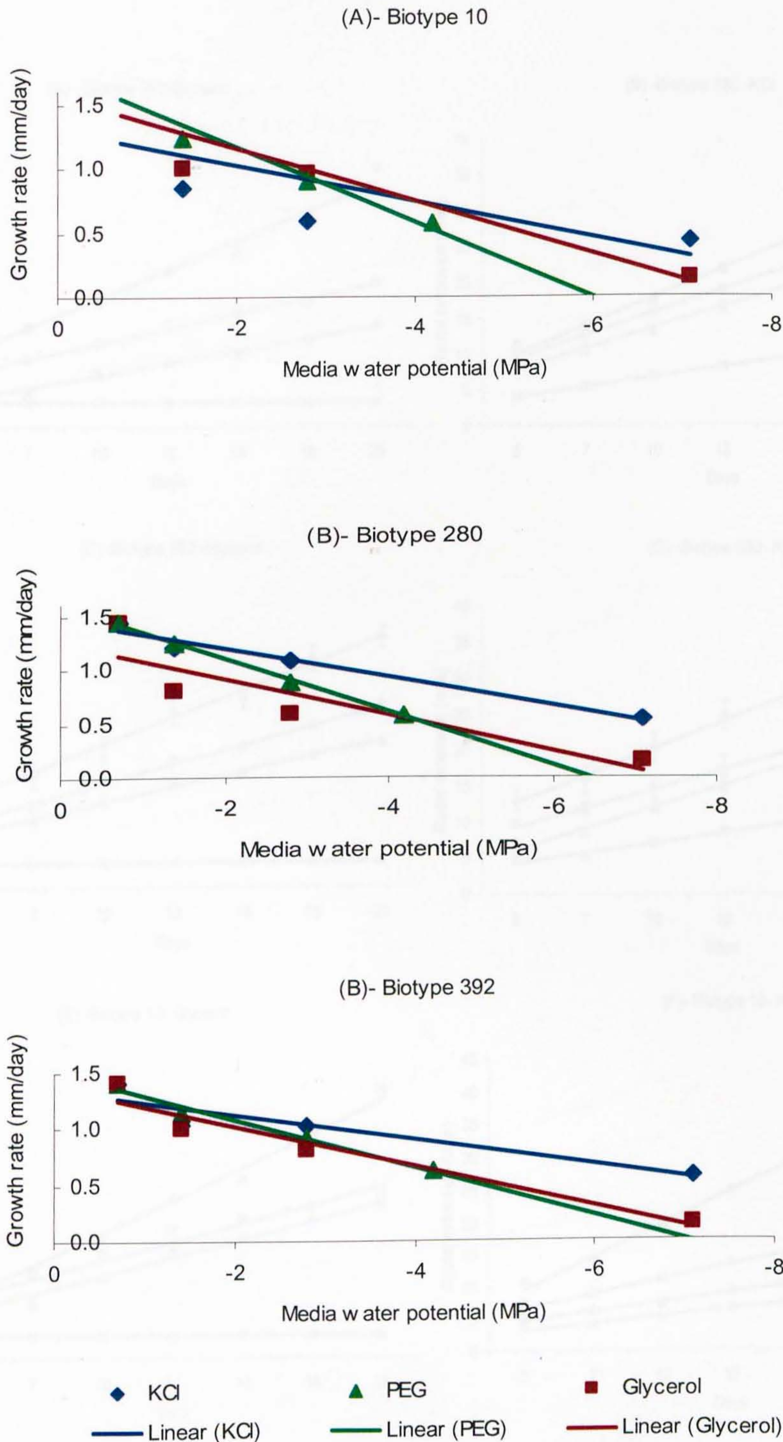


Figure 6.1- Regressions of the effects of osmotic potential (glycerol and KCl modified media) and matric potential (PEG) on the growth rate of three biotypes of *Pochonia chlamydosporia* (A- biotype 10, B- biotype 280, C- biotype 392).

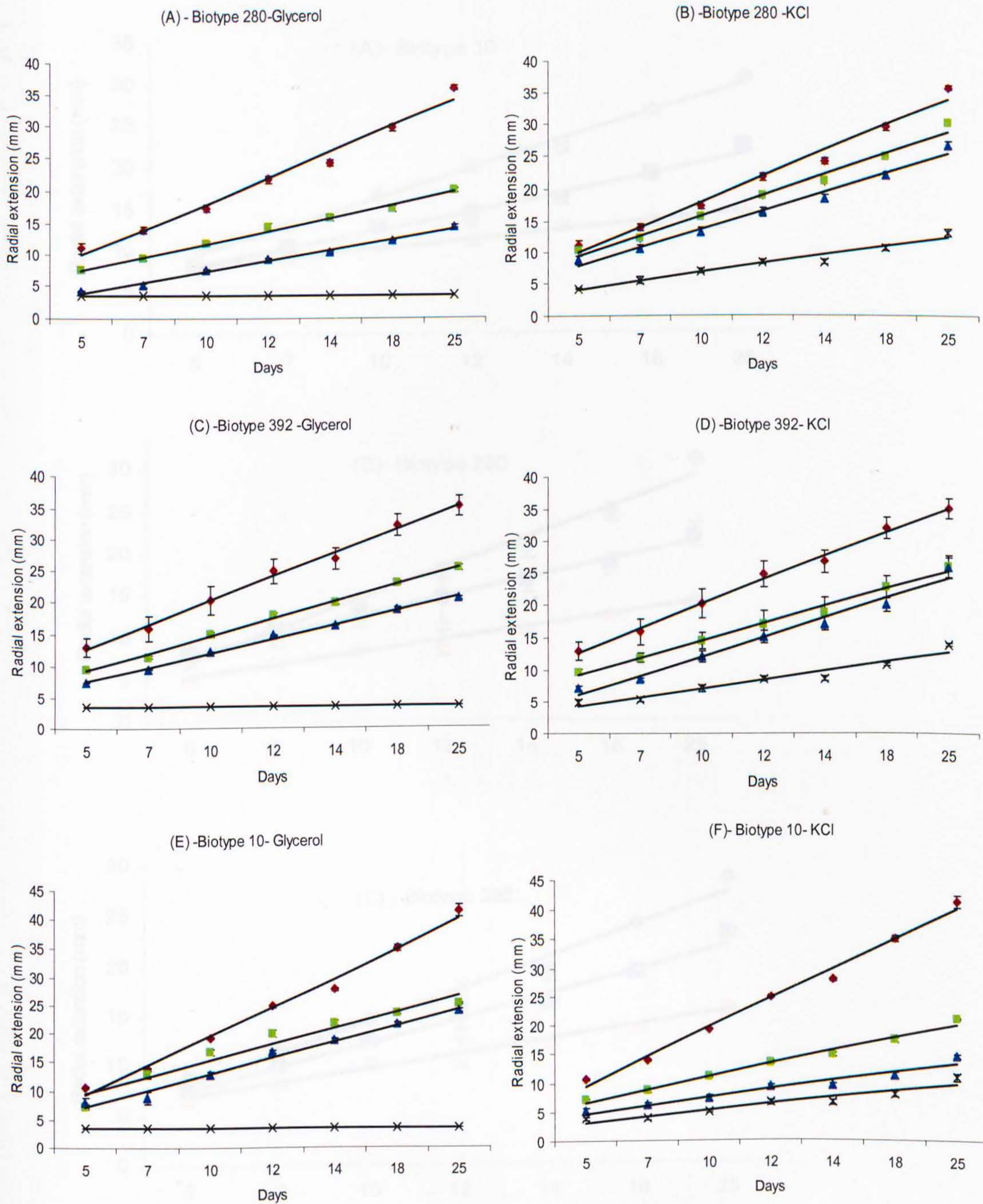


Figure 6.2- Regressions of the effect of osmotic potential (glycerol and KCl modified media) on colony diameter (mm) of three biotypes of *Pochonia chlamydosporia* [biotypes 280 (A, B), 392 (C, D) and 10 (E, F)], at 25 °C. I- Standard error bars.
(Key to osmotic potential :[(– MPa) X,- 7.1 ; ▲ ,- 2.8; ■ , -1.4; ◆ ,- 0.7 (non-modified)].

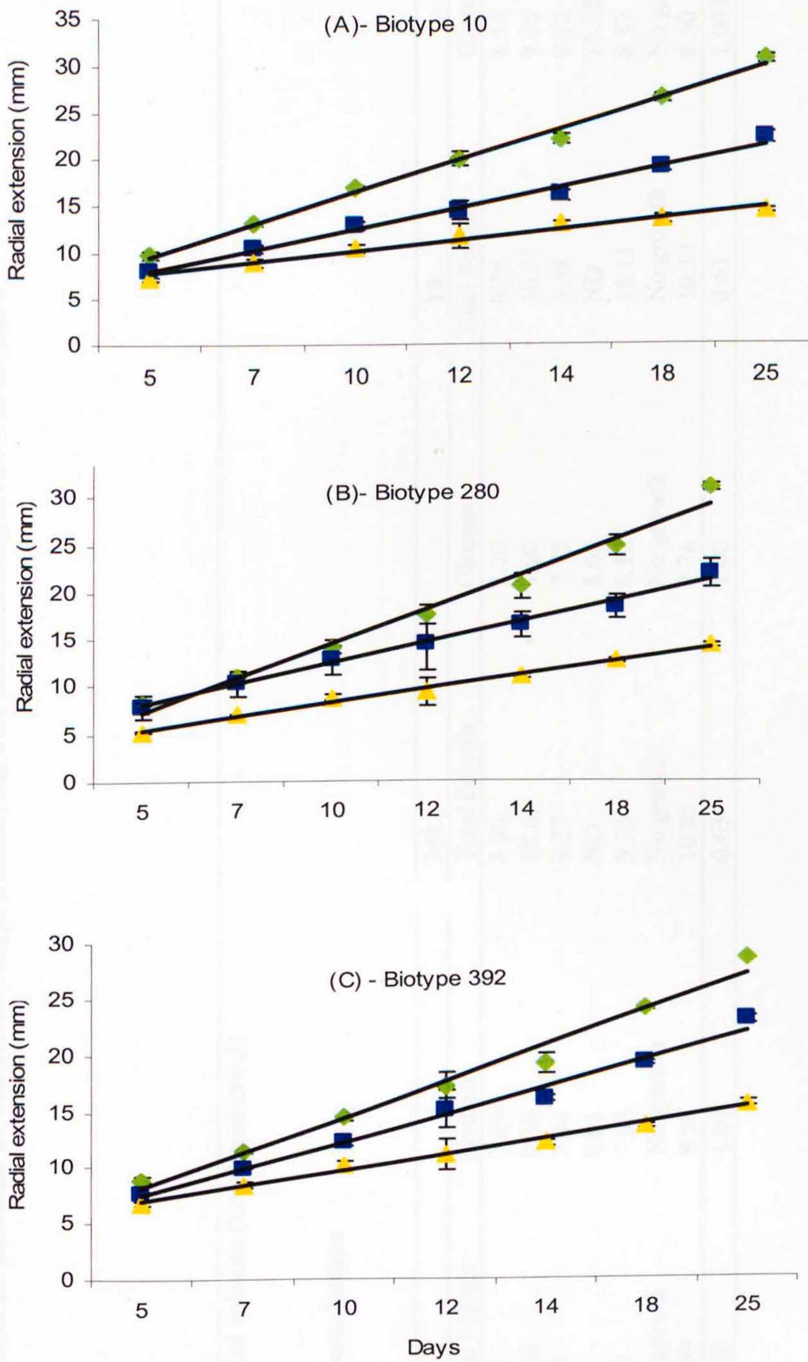


Figure 6.3 – Regressions of the effect of matric potential (PEG 8000) on colony diameter (mm) of three biotypes of *Pochonia chlamydosporia* [biotype 10 (A), 280 (B) and 392 (C)] at 25 °C. (Key to osmotic potential: (–MPa) ▲, -4.2; ■, -2.8; ◆,-1.4). I- Standard error bars.

Table 6.2. Effect of ionic (KCl) and non ionic (glycerol) osmotic potential on the intracellular accumulation of total polyols and glucose in three biotypes of Pochonia chlamydosporia, at 25 °C. Data are means of three replicates per treatment and was transformed using logarithm. Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for the interaction (biotype x media) and were calculated using ANOVA in Genstat ®.

Amount (mg/g) mycelial colonies (Log-transformed)

Pochonia chlamydosporia biotype

		280		**392**		**10**	
Water potential (MPa)		Total Polyols	Glucose	Total Polyols	Glucose	Total Polyols	Glucose
-0.7	Unmodified	9.07	7.69	8.96	7.70	9.26	8.14
-1.4	Glycerol	10.88	9.56	10.63	9.60	10.31	9.86
-1.4	KCl	9.19	7.94	9.27	7.75	9.38	9.22
-2.8	Glycerol	ND	8.91	ND	8.63	ND	10.38
-2.8	KCl	9.50	7.98	9.75	8.12	10.11	8.57
-7.1	Glycerol	No growth	No growth	No growth	No growth	No growth	No growth
-7.1	KCl	10.09	8.58	10.85	9.76	10.30	8.50
LSD (0.05)		0.626	1.00	0.63	1.00	0.63	1.001

ND- Not determined

Table 6.3. Effect of matric potential (PEG) on the intracellular accumulation of total polyols and glucose in three biotypes of Pochonia chlamydosporia, at 25 °C. Data are means of three replicates per treatment and was transformed using logarithm. Least significant differences (LSD) (P<0.05) between values from different treatments are shown for the interaction (biotype x media) and were calculated using ANOVA in Genstat ®.

Amount (mg/g) mycelial colonies (Log- transformed)

Pochonia chlamydosporia biotype

		280		**392**		**10**	
Water potential (MPa)		Total Polyols	Glucose	Total Polyols	Glucose	Total Polyols	Glucose
-0.7	Unmodified	9.07	7.69	8.96	7.70	9.26	8.14
-1.4	PEG	9.89	8.56	9.45	8.99	10.39	9.77
-2.8	PEG	11.09	10.35	11.30	9.92	11.27	10.81
-4.2	PEG	12.27	10.81	11.62	9.54	12.48	9.49
LSD (0.05)		0.422	0.80	0.422	0.80	0.422	0.80

Table 6.4. Amounts of sugar alcohols (mg compatible solute/g *Pochonia chlamydosporia* fresh weight) detected in mycelia of three biotypes of *P. chlamydosporia*, grown in unmodified media (-0.7 MPa) and modified with glycerol, KCl and PEG, at different water potential, after 25 days, at 25°C. Data are means of three replicates per treatment and was transformed using logarithm. Least significant differences (LDS) ($P<0.05$) between values from different treatments are shown for each solute.

Biotype	Water potential (MPa)	Solute	Glycerol	Erythritol	Arabitol	Mannitol
10	-0.7	Control	7.43	7.68	7.46	8.47
	-1.4	Glycerol	9.73	7.75	7.49	9.06
		KCl	5.43	7.87	8.06	8.34
		PEG	7.38	10.12	7.97	7.83
	-2.8	Glycerol	12.8	9.45	8.49	9.73
		KCl	7.86	8.09	9.58	8.19
		PEG	8.36	11.21	7.69	7.74
	-4.2	PEG	8.38	12.43	8.41	8.36
	-7.1	KCl	7.87	10.49	8.63	8.19
LSD (0.05)			2.80	0.54	0.92	0.50
280	-0.7	Control	7.75	7.72	7.46	7.76
	-1.4	Glycerol	10.46	8.07	7.78	8.75
		KCl	7.91	7.64	7.80	7.77
		PEG	7.47	8.68	7.47	9.20
	-2.8	Glycerol	12.19	8.79	8.20	9.06
		KCl	7.59	7.69	8.81	7.79
		PEG	3.39	10.91	8.17	8.52
	-4.2	PEG	7.69	12.23	7.80	7.96
	-7.1	KCl	7.9	9.39	8.24	8.31
LSD (0.05)			0.97	0.71	0.66	0.80
392	-0.7	Control	7.38	7.66	7.49	7.73
	-1.4	Glycerol	10.40	7.95	7.63	8.07
		KCl	8.03	7.68	7.83	7.77
		PEG	7.45	7.81	8.26	8.31
	-2.8	Glycerol	12.61	8.73	7.79	8.10
		KCl	7.62	7.93	9.21	8.08
		PEG	7.51	10.75	10.03	9.02
	-4.2	PEG	7.46	10.87	10.48	9.99
	-7.1	KCl	8.60	9.14	10.14	8.98
LSD (0.05)			0.73	0.60	0.56	0.58

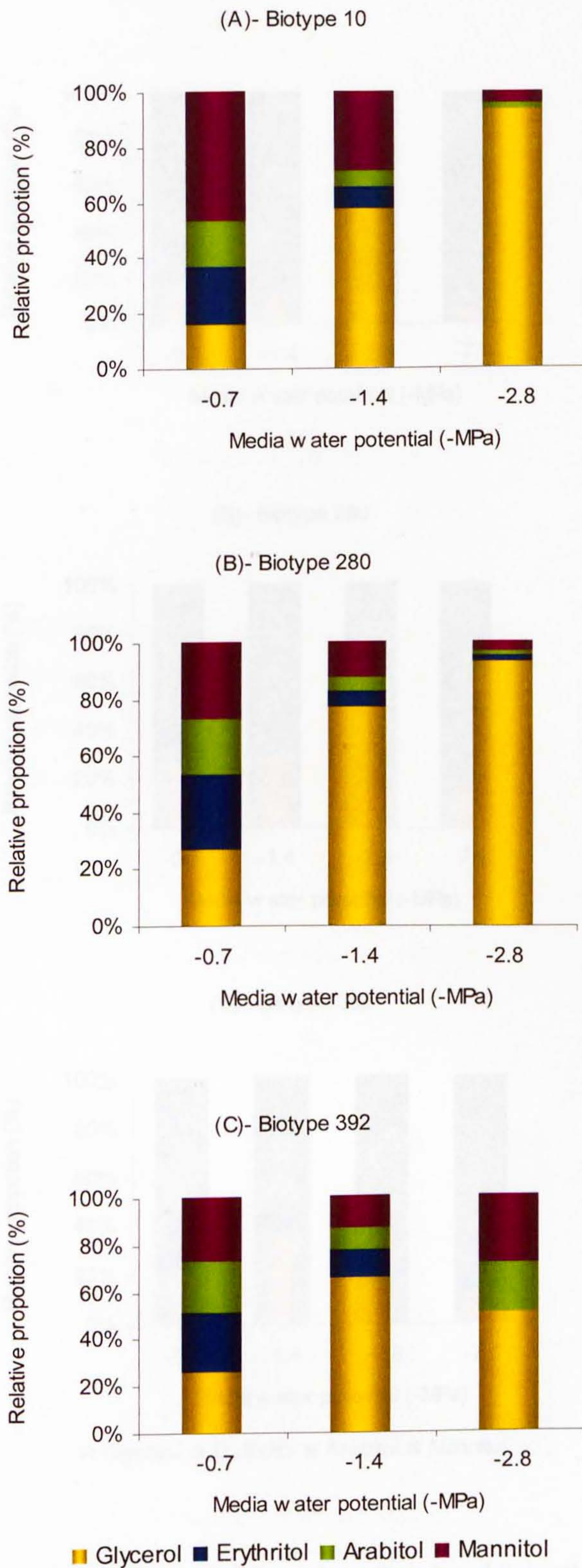


Figure 6.4- Relative proportion (%) of polyols (glycerol, erythritol, arabitol and mannitol) accumulated in biomass of three biotypes of *Pochonia chlamydosporia* (A- biotype 10, B- biotype 280 and C- biotype 392) grown in unmodified agar media (-0.7 MPa) and modified with glycerol to achieve -1.4 and -2.8 MPa,, after 25 days of growth at 25 °C. No growth was observed at -7.1 Mpa.

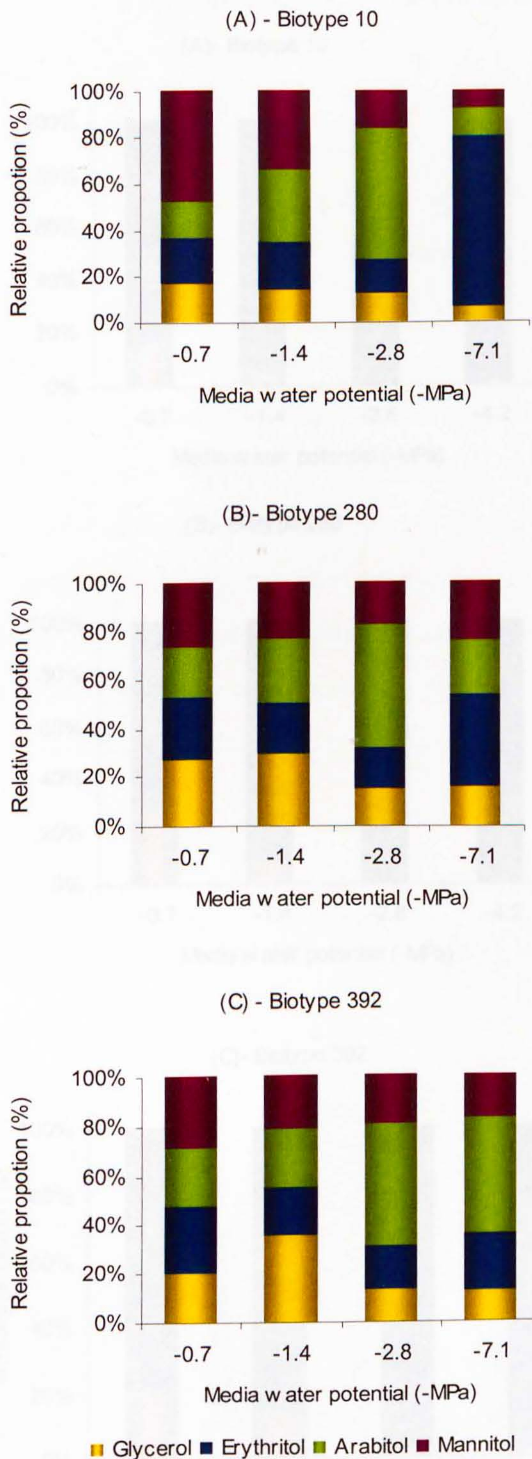


Figure 6.5- Relative proportion (%) of polyols (glycerol, erythritol, arabitol and mannitol) accumulated in biomass of three biotypes of *Pochonia chlamydosporia* (A- biotype 10, B- biotype 280 and C- biotype 392) grown in unmodified agar media (-0.7 MPa) and modified with KCl to achieve -1.4 , -2.8 and -7.1 MPa,, after 25 days of growth at 25 °C.

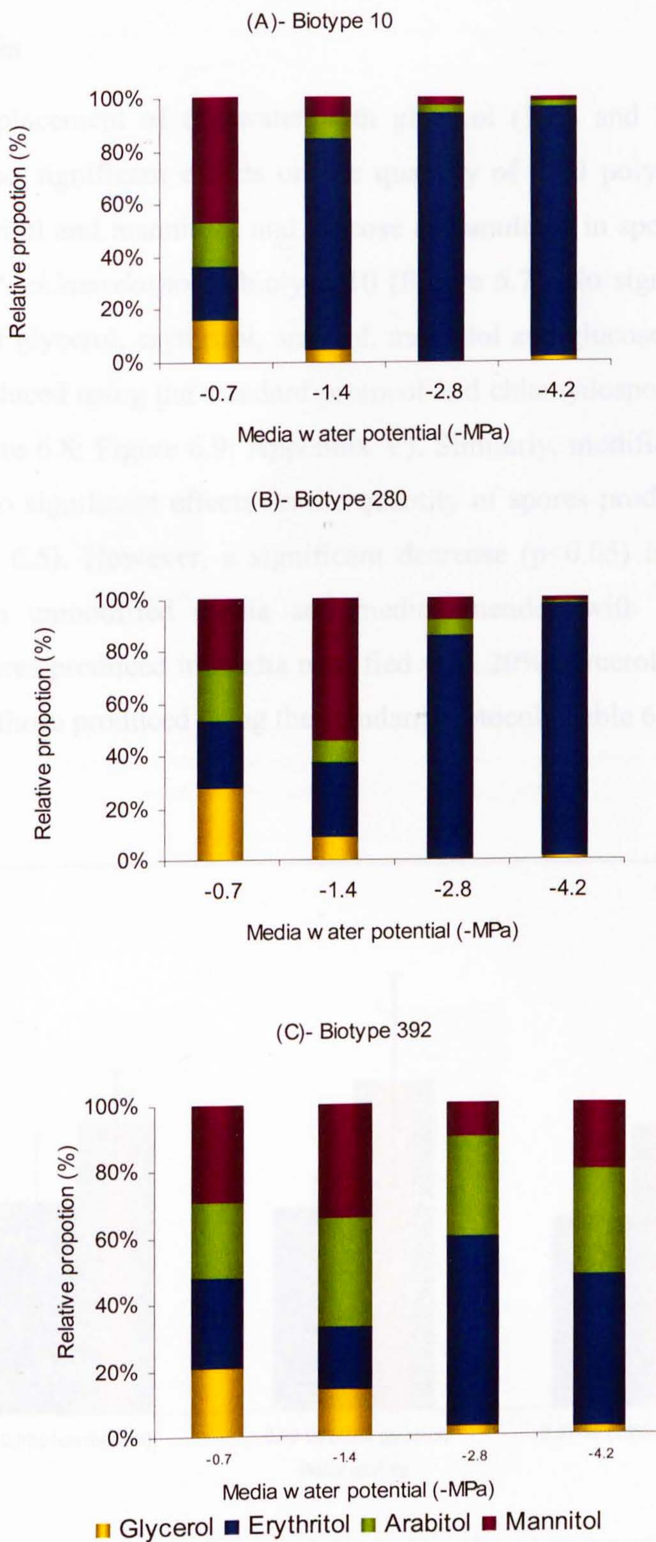


Figure 6.6- Relative proportion (%) of polyols (glycerol, erythritol, arabitol and mannitol) accumulated in biomass of three biotypes of *Pochonia chlamydosporia* (A- biotype 10, B- biotype 280 and C- biotype 392) grown in unmodified media (-0.7 MPa) and modified with PEG to achieve -1.4 , -2.8 and -4.1 MPa,, after 25 days of growth at 25 ºC.

6.3.2 Analysis of chlamydospore endogenous reserves in standard cracked rice and modified media

The replacement of the water with glycerol (10% and 20 %) in the growth medium had no significant effects on the quantity of total polyols (sum of glycerol, arabitol, erythritol and mannitol), and glucose accumulated in spores (chlamydospores) produced by *P. chlamydosporia* biotype 10 (Figure 6.7). No significant differences in the amounts of glycerol, erythritol, arabitol, mannitol and glucose were found between the spores produced using the standard protocol and chlamydospores produced at lower a_w levels (Figure 6.8; Figure 6.9; Appendix V). Similarly, modification of the a_w using glycerol had no significant effects on the quantity of spores produced after 21 days of growth (Table 6.5). However, a significant decrease ($p<0.05$) in spore viability was found between unmodified media and media amended with glycerol (10%). The quantity of spores produced in media modified with 20% glycerol was not significantly different from those produced using the standard protocol (Table 6.5; Appendix V).

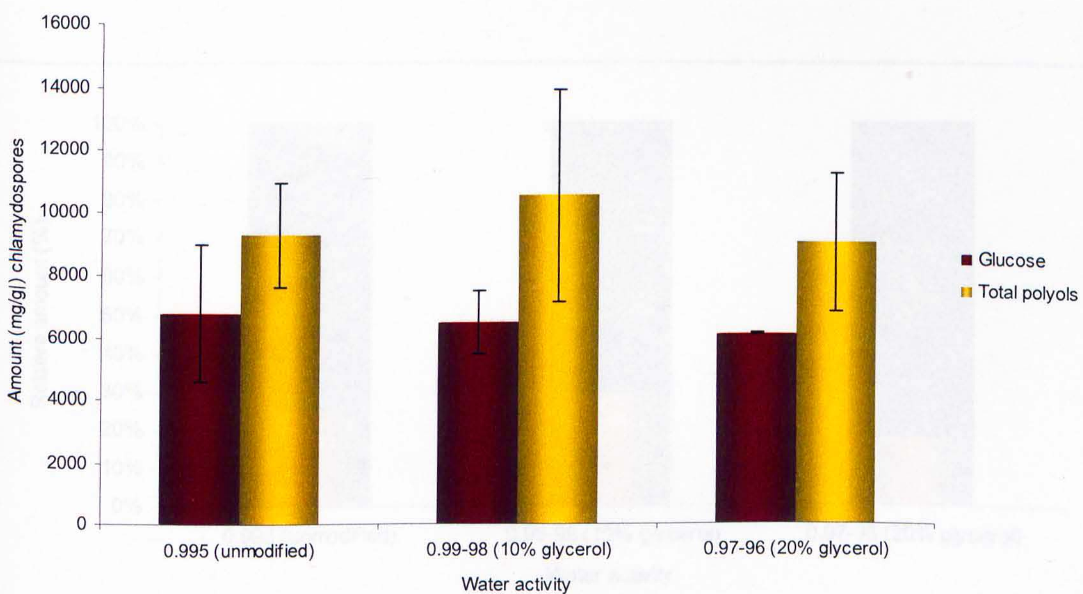


Figure 6.7- Total of polyols and glucose (mg/g) detected in chlamydospores of *Pochonia chlamydosporia* biotype 10, produced in standard (0.995 w_a) and modified conditions, by replacement of water in the growth media with glycerol (10 and 20%). I – Standard error bar.

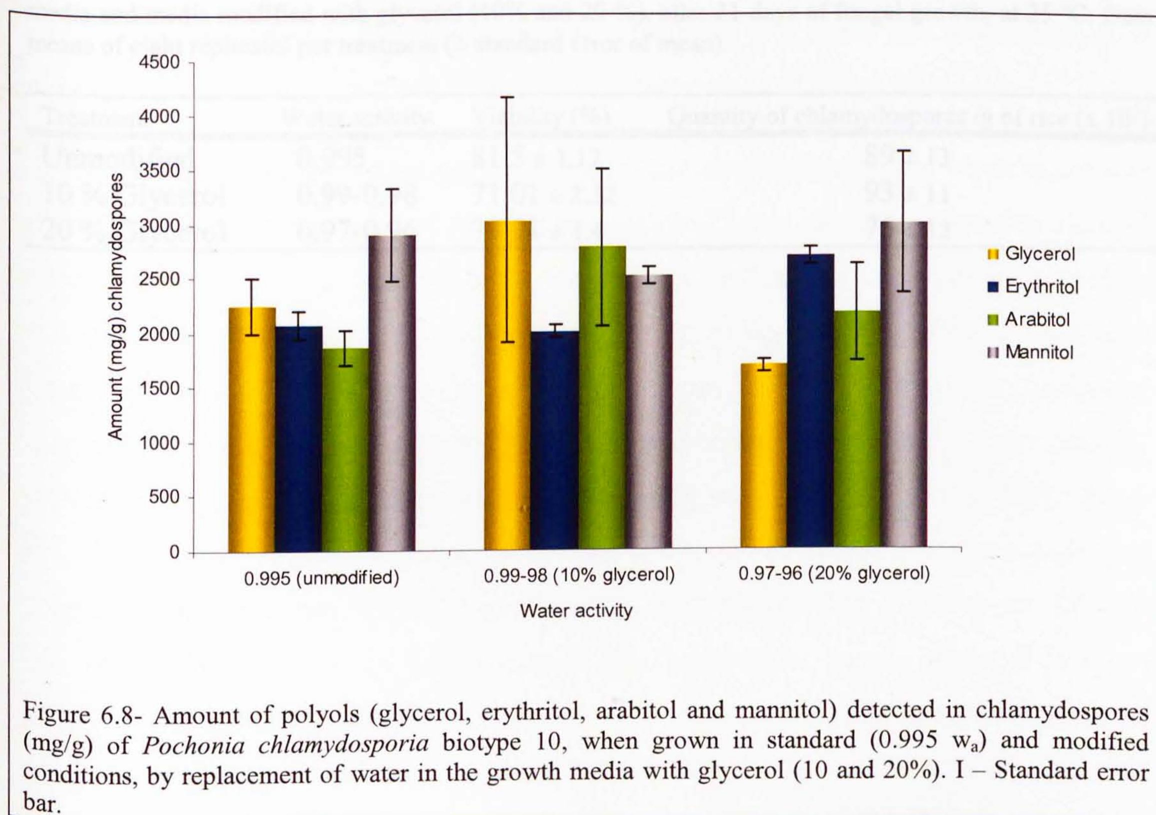


Figure 6.8- Amount of polyols (glycerol, erythritol, arabitol and mannitol) detected in chlamydospores (mg/g) of *Pochonia chlamydosporia* biotype 10, when grown in standard (0.995 w_a) and modified conditions, by replacement of water in the growth media with glycerol (10 and 20%). I – Standard error bar.

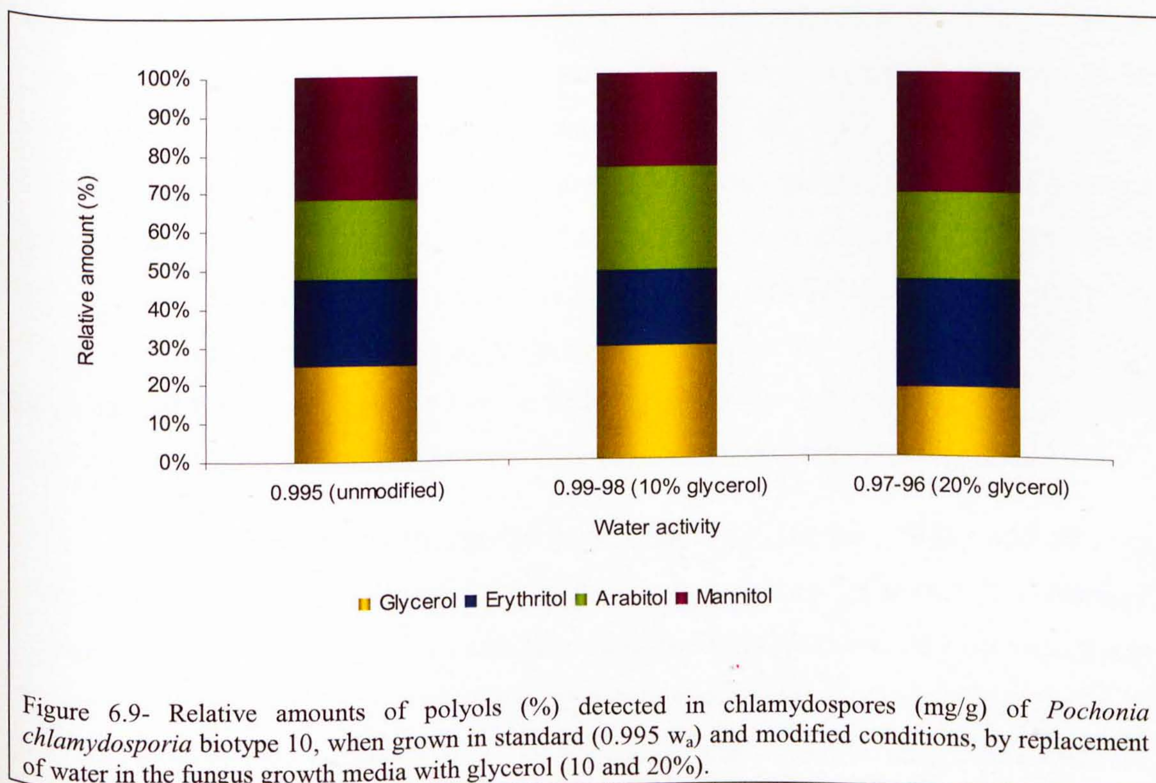


Figure 6.9- Relative amounts of polyols (%) detected in chlamydospores (mg/g) of *Pochonia chlamydosporia* biotype 10, when grown in standard (0.995 w_a) and modified conditions, by replacement of water in the fungus growth media with glycerol (10 and 20%).

Table 6.5 - Viability (%) and quantity of chlamydospores produced per gram of rice media, in unmodified media and media modified with glycerol (10% and 20 %), after 21 days of fungal growth, at 25 °C. Data means of eight replicates per treatment (± standard error of mean).

Treatment	Water activity	Viability (%)	Quantity of chlamydospores /g of rice (x 10^5)
Unmodified	0.995	81.5 ± 1.17	89 ± 13
10 % Glycerol	0.99-0.98	71.01 ± 2.32	93 ± 11
20 % Glycerol	0.97-0.96	79.01 ± 1.4	75 ± 12

6.4 DISCUSSION

For the first time, the effects of matric potential on growth and accumulation of polyols were studied in colonies from different biotypes of *P. chlamydosporia*.

In this study, the fungus grew faster in a non-modified medium, when water was freely available (-0.7 MPa), and reached on average 37 mm of radial extension after 25 days of growth in PDA. Similar results were found by Kerry *et al.*(1986), who studied the effects of pH and water potential on the growth of six biotypes of the fungus on a basal medium amended with a salts mixture or sucrose. However, they never considered matric stress which is more relevant to growth and colonisation in soil. Under osmotic or matric stress, growth rates significantly decreased with the increase of stress (Figures 6.1, 6.2 and 6.3). Although growth rates were lower in PEG, the fungus was relatively tolerant to matric stress, as similar growth rates were observed in glycerol and KCl for two of the biotypes (Figure 6.1; Appendix V). Furthermore, in this study, differences between biotypes were found in response to osmotic stress but biotypes responded similarly when subjected to matric potential stress. This might suggest that that tolerance to matric stress might be important in *P. chlamydosporia*, in order to survive in soil. Some fungi are thought to be more sensitive to matric stress than others. For example, basidiomycetes are more sensitive to matric than osmotic stress, whereas xerophilic fungi, such as *Aspergillius ochraceus*, *A. flavus* would be expected to be more tolerant of matric than osmotic stress (Ramos *et al.*, 1999; Nesci *et al.*, 2004). Moreover, significant differences in the amounts of total polyols were found between biotypes in response to osmotic stress but not in response to matric stress. Tolerance to matric stress may have been related to the accumulation of high amounts of erythritol in PEG media to enable enzyme systems to work effectively (Figure 6.6). The low-molecular weight erythritol is known to efficiently regulate intracellular water potential and is accumulated by xerophylic fungi in response to water stress (Hallsworth, 1995).

All the biotypes were in general more sensitive to the non-ionic solute glycerol than KCl. At -7.1 MPa, the fungus was able to continue growing in a medium amended with the ionic solute, but not in a medium amended with glycerol. Low concentrations of ionic solutes, e.g. KCl, can stimulate growth in some fungi (Larsen, 1986) as K ions can aid transport across the mycelial cell walls to enable better intracellular osmotic

adjustment. Thus, it is possible that KCl may have been utilised from the medium and increased tolerance to osmotic stress.

In media modified with glycerol, at -2.8 MPa, *P. chlamydosporia* accumulated large amounts of this solute, which were significantly greater than in unmodified media, possibly to regulate intracellular osmotic potential. As glycerol was used to modify the medium, it may have been passively accumulated by the fungus to help tolerate the imposed changes in solute stress. Glycerol appears to be of crucial importance in many fungi, as it protects enzymes from accumulation of sodium and loss of water, both of which may denature the enzymes (Luard, 1982). Differences in the growth rate of *P. chlamydosporia* were also found between osmotically modified media and biotypes. Biotype 10 had a higher growth rate in the medium modified with glycerol but it was more sensitive to changes in the osmotic potential when the medium was amended with KCl. In contrast, biotype 280 was more tolerant to KCl than glycerol. Perhaps, different biotypes have different tolerance levels to KCl, which is thought to be toxic in some fungi at high concentrations (Brown, 1978).

The quantities of the major sugar alcohol accumulated in whole colonies of *P. chlamydosporia* depended on the solute used to generate the water stress. Whereas arabitol and glycerol were the main polyols accumulated in osmotically modified media with ionic and non-ionic solutes, respectively, erythritol was the main sugar accumulated in media amended with PEG. These results suggest that the fungus may use different mechanisms to overcome osmotic and matric stress. The versatility to accumulate different polyols and perhaps different sugars under different stress conditions might bring advantage and allow the fungus to survive in soil when environmental conditions are less favourable or variable. In some other fungi, enhanced accumulation of compatible solutes contributed to a significant improvement in viability (Hallsworth & Magan, 1995; Mokiou, 2005; Teixidó *et al.*, 2005) and even virulence (Chandler *et al.*, 2005). Therefore, the viability of the spores produced under different stress regimes might have been different but this aspect was not covered in the study, as few chlamydospores were produced on PDA plates, after 25 days. Future research to study *in vitro* differences in accumulation of polyols and sugars needs to be continued to investigate whether the accumulation of different solutes results in improved physiological quality under water stress/matric conditions. This has been demonstrated

to be improved in other biological control agents (Teixidó *et al.*, 1998; Mokiou, 2005; Ypsilos & Magan, 2005).

Comparison between the quantity of polyols and glucose accumulated in chlamydospores produced in cracked standard rice media and media modified with 10% and 20 % glycerol showed no significant differences between the treatments and the control. It is possible that the amounts of the glycerol used were not sufficient to decrease the water potential of the media and cause significant osmotic stress. Possibly, greater amounts of glycerol were required to produce an effect on spore quality. Future research is required to test a wider humidity range for solid substrates. In addition, no significant differences were found in the quantity of chlamydospores produced or in spore viability, between treatments. However, spore germination was assessed only in unmodified media. It is possible that evaluation of spore germination on water stress media, e.g, 0.95 a_w, would have produced more interesting results. Another important aspect, concerns the extraction of the spores from the media. To separate the chlamydospores from rice, the media were washed using water, following a standard protocol. It might be possible that endogenous reserves were washed out during the extraction of the spores. In previous studies, osmoprotection of blastospores from *M. anisopliae* produced under water stress by using isotonic solutions resulted in a significant increase in the retention of polyols such as erythritol (Ypsilos & Magan, 2004). Detailed trials are needed on the effects of environmental stress on the germination of spores and the physiological impact on inoculum quality.

To conclude, the presence of free water available in the agar media favoured the growth of *P. chlamydosporia*, when compared to growth rates in media where water was restricted. Fungal growth was affected by both osmotic and matric stress, although this species can tolerate matric stress levels relevant to those that occur in soil, effectively. Under water stress, the fungus accumulated a combination of different sugar alcohols, possibly important in osmoregulation, which were dependent on the solute used. In the rice medium currently used to mass produce the fungus, such changes could not be detected using the treatments described.

The research presented in this chapter provided some new information on the potential mechanisms of osmoregulation in *P. chlamydosporia* and the effects of osmotic and matric stress on mycelial growth and the accumulation of endogenous

reserves in spores. However, more research is needed to study the effects of such changes on spore germination and the virulence of the fungus against nematode eggs.

CHAPTER 7: GENERAL DISCUSSION

7.1 INTRODUCTION

The work developed for this thesis aimed to increase understanding about the variability and stability of different biotypes of the fungus *Pochonia chlamydosporia*. Differences in performance were assessed by measuring saprophytic and parasitic growth using *in vitro* bioassays. The measurement of these two aspects is considered essential for the successful use of the fungus as a biological control agent (De Leij & Kerry, 1991; Kerry, 1998; Kerry, 2000; Kerry & Hominick, 2002).

The three main areas important for discussion which have arisen from this research are:

- Measurement of variability and performance using *in vitro* bioassays;
- Relationship between saprophytic/parasitic growth and enzyme production;
- Possible role of nutrition in fungal growth and the infection of nematode eggs;
- Stability of *P. chlamydosporia* cultures after repeated culture.

7.1.1. Measurement of variability and performance using *in vitro* bioassays

Research on microbial biological control agents has revealed large intraspecific variation within and between populations of the same species (Cooke, 1962; Stirling, 1991; Smith & Goodman, 1999; Kerry, 2000; Ross *et al.*, 2000). The importance of this variation in the regulation of host population is unknown but it does mean that potential BCAs need careful selection.

In vitro bioassays are fundamental tools which together with other important assessments are central to the development of fungi as microbial control agents (Butt & Goettel, 2000). These tests are normally quick and enable many biotypes to be eliminated before more time and resource consuming screens are conducted in pot and field experiments (Kerry *et al.*, 1998). A range of standardised bioassays must be tailored according to the organism, target and objective to identify particular biotypes with the characteristics of a potential BCA. For example, bioassays on fungal egg parasites require the presence of nematode eggs, if the objective is to study parasitic

ability, whereas bioassays on nematode trapping fungi will require the presence of healthy and motile nematodes to stimulate the formation of capture organs (Duddington, 1951). If the objective is to study other aspects such as fungal spore viability or saprophytic ability, suitable bioassays have to be designed.

Studies on a facultative fungal egg parasite such as *P. chlamydosporia* rely on the use of *in vitro* bioassays to evaluate two main trophic stages of the fungus. Saprophytic growth is generally measured by studying the ability to colonise rhizosphere and parasitic growth is studied by measuring the ability to parasitise nematode eggs. *In vitro* bioassays have been developed to screen and compare differences in saprophytic and parasitic growth between biotypes of the fungus (Irving & Kerry, 1986; Kerry *et al.*, 1986; Kerry *et al.*, 1998) but their use can be also important in production systems, where it is necessary to monitor the quality of the inocula of a specific biotype during repeated mass production and/or after storage, often requiring quick and reliable methods.

Bioassay designs must be continually improved, applying new techniques and equipment to provide more meaningful information (Butt & Goettel, 2000). In the light of this thought, a novel method relating absorbance levels with different spore concentrations was developed to quantify concentrations of chlamydospores in suspension (Figure 3.1). The method was quicker than the traditional method of using a haemocytometer and therefore might be useful for quick quantification of spores. However, a concentration curve needs to be calculated for each biotype as different biotypes gave different results.

Variability of biotypes exists not only in their morphology and at molecular levels but in terms of their performance as potential BCAs. Differences in rhizosphere colonisation ability were found between different biotypes of *P. chlamydosporia* (Figure 3.5 - B) using maize as the host plant. Although this plant is not a susceptible host for some root-knot nematodes, its roots were colonised to a different extent by different biotypes and the time necessary for the bioassay to distinguish significant differences in the growth on roots by the biotypes could be reduced to eight days (Figure 3.4). Differences in egg parasitism ability were also found between biotypes using a standard and a novel bioassay (Chapter 4.3.1). It is important to point out the importance of this new bioassay, which solved some of the limitations posed by the

existing method using agar plates to evaluate *in vitro* parasitism. Using this method, eggs were infected more quickly, a higher rate of egg infection could be detected (Table 4.1) and an LD50 for each biotype could be estimated (Figure 4.3). However, the bioassay needs further optimisation, as it was not able to detect small differences between biotypes, at least at the conidial concentrations used.

Genetic variation might allow the fungus to adapt to variable conditions and nutrient sources during each of its trophic phases. Recently, different biotypes of the fungus were found colonising the same root environment, in suppressive soils for RKN, and this might confer some advantage to the fungus (Rosa Manzanilla-Lopez, Penny Hirsch and Brian Kerry, unpublished results). However, this might not mean that the coexistence of several biotypes in the same root environment would be advantageous for parasitism; it can perhaps simply confer advantage in terms of survival or saprophytic competence with other organisms. Variation in biotypes is also thought to be related with selection pressures imposed by the nematode host, which are also subjected the selection pressures, such as the need to overcome plant defence mechanisms, agrochemicals, etc (Morton, 2002). Therefore, the development of bioassays requires knowledge about both host and pathogen requirements and their ecology. Such interactions between organism, nematode host and plant are complex and extremely difficult to study using *in vitro* bioassays, as most bioassays are performed under controlled conditions. However, bioassays can provide useful information in studying the contribution of individual factors in performance by eliminating the effect of variable factors. Ideally, the ultimate challenge is to develop bioassays that can be used to predict field efficacy (Butt, 2002).

Biotypes 132 and 392 performed better in soil than they did *in vitro*, colonising a greater percentage of *Meloidogyne* eggs (Chapter 4.3.3), whereas biotype 280 was not able to establish in the soil and rhizosphere and consequentially failed to infect significant numbers of eggs, but performed as well as the other biotypes when infecting eggs *in vitro* (Figures 4.6 and 4.7). Biotypes which were considered good egg parasites when screened *in vitro*, were ineffective in soil if they did not also colonise the rhizosphere (De Leij & Kerry, 1991). Thus, it is necessary to combine results from different bioassays in order to predict results under soil conditions. Even though, efficacy under field conditions can be particularly difficult to predict, even when relying

on information from bioassays. Facultative parasites with a wide range of hosts such as *P. chlamydosporia* are often ubiquitous soil saprophytes (Goettel, 1995), nutritionally versatile (Liu & Chen, 2003) and are more prone to change their survival strategy than obligate parasites, which depend much more on their hosts to survive (Kerry & Hominick, 2002). Therefore, the efficacy of a certain potential BCA, is influenced by several factors and the use of bioassays to predict field efficacy is limited.

In conclusion, *in vitro* bioassays are useful tools to mass select biotypes, for studies on host range, virulence and barriers to infection, but their use to predict performance under field conditions must treated with extreme care. Effort has been made to optimise and develop new bioassays to measure important parameters affecting performance in *P. chlamydosporia*. However, optimisation of the current bioassays available must continue to be developed.

7.1.2. **Relationship between saprophytic/parasitic growth and enzyme production**

The question that arises after collecting data from a range of *P. chlamydosporia* biotypes on their saprophytic and parasitic growth is that can such information be related to the ability of each biotype to produce particular enzymes. Are biotypes with the best parasitic performance, also good rhizosphere colonisers and enzyme producers or vice-versa? Can the *in vitro* production of certain enzymes be related to saprophytic/parasitic *in vitro* growth?

Enzymes are known to be involved in the infection process and they have been suggested to serve as virulence factors in nematophagous fungi including *P. chlamydosporia* (Huang *et al.*, 2004). Proteases may play an important role in virulence of *P. chlamydosporia* but a sequential production of enzymes may be involved in the infection process (Segers, 1996; Tikhonov *et al.*, 2002), as happens in entomopathogenic fungi such as *M. anisopliae* (St. Leger *et al.*, 1986). In Chapter 5, a range of enzymes, which might be important in the parasitic phase of the fungus, was studied in several *P. chlamydosporia* biotypes and information about the time, the amounts of enzymes secreted and the influence of different inducers was collected.

Enzymes are also required during saprophytic growth and high competitive saprophytic ability depends, together with rapid spore germination and high growth rate,

on high production of extracellular enzymes (Faull, 1988). Although *P. chlamydosporia* might not be considered a fungus with great saprophytic ability (Widden, 1997) as it is a weak competitor in soil (Bourne & Kerry, 2000), it must produce enzymes which may help it to survive as a saprophyte in soil. Therefore, to determine the relation between enzyme production and performance, the data collected in chapters three, four and five were put together and were analysed using Kendall's coefficient of concordance and the Spearman rank correlation coefficient. Kendall's coefficient of concordance was used to rank nine biotypes of *P. chlamydosporia*, from 1 (smallest in the rank) to 9 (greatest in the rank), according to their individual abilities in colonising the rhizosphere, parasitise nematode eggs and produce different enzymes *in vitro* in order to determine if biotypes with best parasitic performance were also good saprophytes or good enzyme producers. Spearman rank correlation coefficients were calculated for the relationship between different enzymes produced by the biotypes and their rhizosphere colonisation and egg parasitism ability. The outputs given by these two tests are presented in Tables 7.1 and 7.2. The results show that there is not a significant relation between parasitic and saprophytic growth. Biotype 16 was the highest in the rank (Table 7.1), among the nine biotypes analysed, and although it was the best rhizosphere coloniser and the best producer of proteases and chitinases, it was only average in parasitizing eggs. In contrast, the second in the rank was biotype 280, a poor saprophyte in the rhizosphere of maize, but the best egg parasite in the *in vitro* test. Biotype 400 was last in the ranking table, and although it was average in its performance as parasite and rhizosphere coloniser, it produced very small amounts of enzymes, with the exception of lipases. In previous studies, no relation was found between egg parasitism ability and fungal growth rate or the ability to produce chlamydospores by *P. chlamydosporia* (Kerry *et al.*, 1986). Furthermore, the enzymes studied in Chapter 5 did not correlate with the results in performance investigated in Chapter 3 and Chapter 4 (Table 7.2). Only a strong correlation (> 0.8) was found between protease and lipase production but given the low amounts of lipases secreted by the majority of the biotypes, this relationship may not be of importance and can be an artefact of the method of analysis.

Some of the enzymes studied and included in the analysis, may have no contribution for either saprophytic or parasitic growth. Esterases are known to be important in fungal metabolic processes and in substrate degradation but their role in

virulence was not investigated, at least in nematophagous fungi. Chitinases and proteases, in particular the serine protease VCP1, are known to play a role in degrading the nematode eggshell (Segers, 1996; Tikhonov *et al.*, 2002) and for this reason might play a role in the parasitic phase of the fungus but the importance of these enzymes in the saprophytic phase of the fungus is less clear. Segers, (1996) considered that VCP1 was not a useful indicator of the change of trophic stage *in vitro*, as it was produced when the fungus was grown saprophytically, as well as in the presence of nematodes. The results found in this thesis confirmed such findings; VCP1 activity was even higher in minimal basal medium than in medium amended with nematode eggs (Figure 5.5). Cellulase activity has been detected in one biotype of *P. chlamydosporia* (Segers, 1996) and might be important for the saprotrophic phase of the fungus and other enzymes might be produced according to the presence and type of substrates available in soil and rhizosphere. However, the saprophytic and parasitic stages of the fungus are probably influenced by a complex combination of factors, which are coordinated and interact in the switch from one stage to the other. Although enzyme production is important and particular enzymes might be important in specific pathways of growth or pathogenicity, they might not be critical. Therefore, other factors affecting performance may have been overlooked when virulence or saprophytic growth was correlated with enzyme production: fungal pathogenicity is not determined by one single factor.

Table 7.1. Rank of *Pochonia chlamydosporia* biotypes based on their saprophytic growth and parasitic growth and ability to produce selected enzymes *in vitro*. Values ranging from 1 (smallest in the rank) to 9 (greatest in the rank) were attributed to each biotype according their activity. The ranking was originated from means of root colonisation ability (Chapter 3), egg parasitism on *Meloidogyne* and *G. pallida* eggs (Chapter 4) and specific enzymatic activity produced by individual enzymes (Chapter 5). Kendall's coefficient of concordance was calculated in Genstat®.

Kendall's coefficient of concordance

Variables: Rhizosphere colonisation, the production of proteases, chitinases, lipases, esterases, and egg parasitism (*Meloidogyne* and *G. pallida*),

Coefficient: 0.110
Adjusted for ties: 0.113
Sample size: 9
Number of samples: 7
Sum of squares: 324.00
Chi-square: 6.32
Degrees of freedom: 8
Probability: 0.611

Ranks

Biotype	**Rhizosphere colonisation**	**Proteases**	**Chitinases**	**Lipases**	**Esterases**	**Parasitism (*Meloidogyne*)**	**Parasitism (G. *pallida*)**	**Mean**
16	9	9	9	3	8	6	3	6.7
280	2	6	8	3	4	8	9	5.7
399	7	2	4	8	2	7	8	5.4
10	5	3	5	6	3	9	6	5.3
132	8	8	7	3	5	4	1	5.1
104	1	5	6	3	9	3	4	4.4
392	3	4	2	7	7	5	2	4.3
60	4	7	3	3	6	1	5	4.1
400	6	1	1	9	1	2	7	3.9

Table 7.2. Spearman's rank correlation coefficient of nine Pochonia *chlamydosporia* biotypes (10, 16, 60, 104, 132, 280, 392, 399 and 400) based on their saprophytic growth (Chapter 3), parasitic growth (Chapter 4) and ability to produce selected enzymes *in vitro* (Chapter 5). Spearman's rank correlation coefficient was calculated in Genstat®.

Spearman's rank correlation coefficient

Sample size = 9
Degrees of freedom = 7

Exact critical values for two-sided test:

probability	0.100	0.050	0.020	0.010	0.005	0.002	0.001
critical value	0.600	0.700	0.783	0.833	0.867	0.917	0.933

Correlation matrix (adjusted for ties)

Chitinases	1	1.000						
Egg parasitism (*Meloidogyne*)	2	0.450	1.000					
Egg parasitism (*G. pallida*)	3	-0.117	0.333	1.000				
Esterases	4	0.400	-0.233	-0.650	1.000			
Lipases	5	-0.749	0.073	0.329	-0.676	1.000		
Proteases	6	0.717	-0.100	-0.533	0.667	-0.895	1.000	
Rhizosphere colonisation	7	0.183	0.083	-0.233	-0.267	0.146	0.200	1.000
		1	2	3	4	5	6	7

P-values

Chitinases	1	*						
Egg parasitism (*Meloidogyne*)	2	0.224	*					
Egg parasitism (*G. pallida*)	3	0.765	0.381	*				
Esterases	4	0.286	0.546	0.058	*			
Lipases	5	0.020	0.852	0.388	0.046	*		
Proteases	6	0.030	0.798	0.139	0.050	0.001	*	
Rhizosphere colonisation	7	0.637	0.831	0.546	0.488	0.708	0.606	*
		1	2	3	4	5	6	7

7.1.3. **Possible role of nutrition in fungal growth an infection of nematode eggs**

Nutrition is among the factors that may influence the trophic stage of the fungus. The facultative parasite and soil borne fungus *Pochonia chlamydosporia* is able to produce complex combinations of extracellular enzymes to degrade more intractable substrates such as chitin or nematode eggs. However, this requires considerable energy costs and therefore, the utilization of nematodes as an additional food source must confer some advantage to the fungus. Such 'investment' might be induced by the depletion of nutrients in the soil/rhizosphere environment caused by competition (Morton, 2002), or may reflect the 'need' to search for particular nutrients existing in the nematodes which are not present/available in the soil/rhizosphere. The choice for a pathogenic lifestyle may therefore be optional and the nutrient availability in the environment may play an important role in determining the trophic mode of the fungus.

Nutrient deprivation has been associated with an increase of disease in plant and entomopathogenic fungi. Nutritional studies on *Colletotrichum acutatum*, a pathogen responsible for causing anthracnose in a wide range of horticultural plants, showed that nutrient depletion was required for appressorium formation (Blakeman & Parbery, 1977). Similarly, the formation of appressoria in *M. anisopliae* is thought to be a nutritional response, with the most conducive conditions being provided by low levels of complex nitrogen sources such as low concentrations of yeast (St. Leger *et al.*, 1989). A similar response might occur in *P. chlamydosporia*. When grown in high-nutrient conditions *P. chlamydosporia* became a saprotroph, ignoring the presence of nematode eggs (Segers, 1996). However, simple direct relationship between nutrient scarcity and virulence might not exist, as a minimum of nutrients seem to be required for growth and infection. The number of appressoria formed *in vitro* was greater in medium containing a minimal quantity of YEM than in water (Figure 4.5). Similarly, eggs were infected more quickly in 0.0125% YEM than in water (Table 4.1 and Figure 4.2), suggesting that yeast extract might stimulate or speed up the formation of infection structures in *P. chlamydosporia*. After 48 hours of growth in YEM, high rates of egg infection could be detected for all the biotypes tested (Table 4.1). Microscopy studies confirmed the presence of the fungus colonising the eggs after three days of growth (Plates 5.2, 5.3 and 5.4), the time when VCP1 secretion was maximal (Figure 5.5). Complete destruction of the egg contents was confirmed after seven days of growth (Plates 4.3 to

4.5). Therefore, infection of nematode eggs by this fungus in medium containing yeast extract occurs quickly and possibly as soon as the nematode is in contact with the egg. Additionally, VCP1 activity is greater in these early stages of infection. Low levels of yeast seem to favour *in vitro* egg parasitism and appressorial formation. The C:N ratio of yeast extract (approximately 1:1) may be similar to the nutritional levels that can be found in the rhizosphere. Interestingly, these results seem to agree with Segers (1996), who found the greatest levels of egg pathogenicity by a biotype of *P. chlamydosporia* on an agar media with a C:N ratio of 1:1 rather than in medium with higher C:N ratios. In his work, a correlation between the decline of virulence with C:N ratios was found. The importance of the C:N ratio of media on pathogenesis has also been demonstrated in the fungus *Colletotrichum truncatum* (Jackson & Schisler, 1992). Spores of this fungus which were produced in a medium with a low C:N ratio were more effective in causing disease in *Sesbania exaltata* than spores produced in a medium with a higher C:N ratio. Therefore, the cultivation of *P. chlamydosporia* in culture media with particular combinations and amounts of nutrients may influence the activity of the fungus in the soil/rhizosphere and may influence the trophic switch.

7.1.4. Stability of *Pochonia chlamydosporia* cultures after repeated culture

An important requirement for the success of a fungal BCA is the maintenance of the characteristics by which the organism was selected. This aspect is particularly important when mass producing the fungus and must be carefully controlled and monitored to ensure the fungus retains pathogenicity, together with other characteristics important for biocontrol performance (Ryan *et al.*, 2002). Sub-culturing on agar is a common method traditionally used to maintain and grow organisms under laboratory conditions (Smith & Onions, 1994). However, changes in fungal physiology and morphology have been associated with this technique (Hajek *et al.*, 1990; Ryan *et al.*, 2002; Quesada-Moraga & Vey, 2003). In some cases, loss of virulence/pathogenicity has been reported and associated with continuous *in vitro* manipulation of organisms (Schaerffenberg, 1964; Nagaich, 1973; Hajek *et al.*, 1990) but have not been found in others (Hall, 1980; Ignoffo *et al.*, 1982; Brownbridge *et al.*, 2001; Vandenberg & Cantone, 2004; Scully & Bidochka, 2005). No changes in virulence were detected in biotypes of *Lecanicillium lecanii* against *Macrosiphoniella sanborni*, when this fungus

was cultivated *in vitro* up to 98 times, although changes in colony morphology and colony growth rate were detected (Hall, 1980). Similarly, *in vitro* passage of *Beauveria bassiana* in Sabouraud dextrose agar supplemented with 0.1 % yeast extract, did not affect virulence to *Bemisia argentifolii* (Brownbridge *et al.*, 2001). The quality of inoculum produced by mass culture was investigated in *P. chlamydosporia* during the course of this thesis.

Sub-cultures of three *P. chlamydosporia* biotypes were not different from their initial cultures in their root colonisation ability both, *in vitro,* and in soil (Figures 3.9 and 3.12). Similar results were found in the ability to parasitise eggs *in vitro*, which was maintained after 60 weeks of sub-culturing (Figure 4.6). In soil, virulence against eggs was even increased by some of the biotypes that have been sub-cultured for this length of time. Parasitism of biotypes 132 against *M. incognita* eggs was significantly greater in soil after 60 weeks of sub-culture (Figure 4.7). After 60 weeks of continuous culturing on agar, biotypes 132 and 392 were also stable in the production of chlamydospores, retained spore viability and kept similar enzyme profiles to their parent cultures (Figures 3.7, 3.8, 3.9, 3.10, 3.11 and 5.7). Therefore, no loss of performance was associated with the continuous *in vitro* manipulation in these two biotypes. In previous work, reduction in chlamydospore production with no loss of virulence was associated with frequent sub-culturing of *P. chlamydosporia* (Coosemans, 1990). Conversely, other studies demonstrated no differences in morphology and ability to produce spores. A biotype of *P. chlamydosporia*, was maintained on agar for 80 weeks without changes in morphology, spore production or physiology (Montes de Oca *et al.*, 2005). However, in this research, changes were detected in one biotype of *P. chlamydosporia*. Sub-cultures from biotype 280 showed a decrease on the ability to produce chlamydospores and a decrease in spore viability (Figures 3.10 and 3.11). Additionally, changes in enzyme profiles were also detected in this biotype - the ability to produce chitinases and esterases decreased with sub-culturing (Figure 5.7- B and C). Similarly, changes in the enzyme profiles due to repeated culturing were reported in sub-cultures of *M. anisopliae* (Ryan *et al.*, 2002). Four out of ten sub-cultures retained an enzyme profile similar to the control but six others failed to produce one or more enzymes. Examples of enzymes that failed to be produced were ß-glucosidase, α-fucosidase, ß-glucuronidase, ß-glucosamidase. The enzymes α- fucosidase and ß-

glucuronidase were the most commonly lost, suggesting that this character was not stable. Esterases and ß- chitobiosidase were produced by both parents and sub-cultures. It was not known if such reductions affected virulence but they were correlated with a lack of sporulation and decreases in the ability to produce secondary metabolites (Ryan *et al.*, 2002).

Although sub-cultures of biotype 280 in this study, decreased some activities (spore production, spore viability and enzyme activity), these did not affect rhizosphere or *in vitro* parasitic growth, parameters considered essential in a potential BCA. However, this biotype had a very poor performance when both initial and sub-culture were tested in soil. This biotype may be less genetically stable and more susceptible to changes than other biotypes of *P. chlamydosporia.* To explain fungal culture instability at the molecular level, a few studies have been done. Fungal morphological instability in *Cryphonectria parasitica* was linked to dsRNA virus infection in chestnut blight (Dawe & Nuss, 2001). Recently, it was demonstrated that degenerative sectors in cultures of *M. anisopliae* are under strong oxidative stress and show signs of ageing/senescence (Wang *et al.*, 2005). Most of the differentially expressed genes were involved in catabolic or anabolic pathways, the latter including genes for sporulation (Wang *et al.*, 2005). Karyotypic changes were reported in an isolate of *Saccharomyces cerevisiae* and in a pathotype of the rice blast fungus, *Magnaporthe grisea*, caused by prolong serial transfers. Nevertheless, if it is true that long-term serial transfer can result in changes in karyotype of some organisms, it may not occur in others or may not always result in alterations in morphology and physiology.

The effect of sub-culturing on virulence and other phenotypic traits was shown to vary by biotype in entomopathogenic fungi (Vandenberg & Cantone, 2004; Shah & Butt, 2005). Virulence of some biotypes of *Paecilomyces fumosoroseus* were affected by serial passage *in vitro* and *in vivo* whereas others remain stable (Vandenberg & Cantone, 2004). Consequently, different biotypes of the same fungus can change or adapt more or less to certain culture conditions than others. The type of mycological media may also have an influence on the consequences of *in vitro* sub-culturing. Two biotypes of *Metarhizium anisopliae* differed in their stability when grown in different nutrient media (Shah & Butt, 2005) .Similarly, the virulence of a biotype of *B. bassiana* on *Locusta migratoria* was significantly reduced after two passages through Sabouraud

dextrose agar, whereas two passages through malt agar increased its virulence and toxiphore activity (Quesada-Moraga & Vey, 2003). Therefore, aspects related to conditions of growth, such as culture media, pH, and temperature can influence the genetic stability of the organism being studied and must be taken into account when comparing fungal cultures (Ibrahim *et al.*, 2002).

The effects of culture history on virulence can pose a special problem if the precise history of the culture is unknown (Butt & Goettel, 2000). However, this was not a problem in this research. Information about the history of all the biotypes studied was recorded from the moment of isolation and the information has been kept since then (Table 2.1). Interestingly, biotype 280, in which changes occurred, was the most recent biotype to be isolated (year 2000). Therefore, such changes are not related with the length of preservation. Overall, biotype 280 showed stability when parameters consider essential in a potential BCA were tested, and attenuation cannot, consequentially, be attributed to a decrease in the performance.

The results confirmed that the performance of the fungus as a saprophyte/parasite was not affected by continuous culturing on agar, with stability observed and confirmed using different approaches. However, the results found in this study reinforce the need for careful selection of the organisms and biotypes with high genetic stability for mass production.

7.2 RESUMÉ OF MAIN ACHIEVMENTS

- **Optimisation and development of *in vitro* bioassays to study saprophytic and parasitic activity**: a method to quantify spores in suspension was successfully developed; a bioassay to study saprophytic growth was reduced to 8 eight days using maize as the plant host; a new bioassay to estimate *in vitro* egg parasitism was developed and an LD50 was calculated. The importance of the use of *in vitro* bioassays to measure performance and to compare biotypes was discussed.
- **The formation of appressoria was measured *in vitro* for a range of *P. chlamydosporia* biotypes.** The numbers of appressoria formed *in vitro* was higher in a medium containing a minimal quantity of YEM than in water (Figure

4.5). Similarly, eggs were infected more quickly in 0.0125% YEM than in water (Table 4.1 and Figure 4.2), suggesting that yeast extract might stimulate or speed up the formation of infection structures in *P. chlamydosporia*.

- **Microscopy observations on fungal colonisation inside RKN nematode eggs:** Fungal colonisation was observed inside single eggs of *Meloidogyne* spp. eggs after 7 days of growth. Internal colonisation by *P. chlamydosporia* was observed in individual whole RKN eggs, for the first time. It was possible to visualise and confirm destruction of the egg contents in eggs, which had been infected with the fungus for seven days, using the method developed in Section 4.2.1.
- **Enzyme activities were studied for a range of *P. chlamydosporia* biotypes.** Information about the time, amounts of enzymes secreted and the influence of different medium inducers was collected. *P. chlamydosporia* biotypes produced enzymes in different amounts and times and responded differently when different inducers were added to the medium. Enzymatic activity varied with the biotype and the type of enzyme assayed and biotypes could be ranked according to their similarities in the production of proteases, chitinases, esterases and lipases.
- Information on saprophytic and parasitic growth and enzyme production was collected for a range of *P. chlamydosporia* biotypes. No significant relationships were found between saprophytic or parasitic growth and enzyme production. It was concluded that factors affecting performance are complex and are multifactor dependent.
- **The effects of *in vitro* repeated culturing were studied for three biotypes of *P. chlamydosporia.*** Results showed that the majority of the biotypes of the fungus tested were stable in their performance. Although one biotype decreased the ability to produce chlamydospores, spore viability and enzyme activity after 60 weeks of growth, other parameters such as rhizosphere colonisation and egg parasitism remained stable. It was concluded that the stability of the fungus varied with the biotype but overall the performance of the fungus as a parasite/saprophyte was not affected by continuous culturing in agar, with stability observed and confirmed using different approaches.

- **The effects of osmotic and matric stress on growth and accumulation of endogenous reserves were studied for three biotypes of *P. chlamydosporia*.** Results revealed that *P. chlamydosporia* growth was affected by both osmotic and matric stress, although tolerance to matric stress was observed. Under water stress, the fungus accumulated a combination of different sugar alcohols, important in osmoregulation, which were dependent on the solute used.

7.3 RECOMMENDATIONS FOR FUTURE WORK

Future work should be directed towards:

a) Studying aspects related to the basic biology of *P. chlamydosporia* and infection

More research is needed to study mechanisms of germination, spore attachment and infection by the fungus. Hydrophobicity, appressorium formation and protease production are factors known to be involved in early parasitism of nematode eggs by *P. chlamydosporia* (Lopez-Llorca *et al.*, 2002) but further research is needed to understand which stimuli are involved in adhesion, attachment, formation of appressoria and penetration into the nematode eggshell. Most of such studies can be done *in vitro* using artificial solid surfaces (Apoga *et al.*, 2004). Adhesion to host surfaces is thought to be an important step in fungal infection of plants (Epstein & Nicholson, 1997), insects (Zacharuk, 1970) and is likely to be important for nematophagous fungi such as *P. chlamydosporia*. The production of mucilage has been associated with adhesion of conidia and appressoria to the host (Lopez-Llorca & Claugher, 1990; Jones, 1994; Epstein & Nicholson, 1997). Such extracellular material is thought to contain glycoproteins and carbohydrates (Sela-Buurlage *et al.*, 1991; Jones, 1994; Epstein & Nicholson, 1997; Jansson & Friman, 1999; Apoga *et al.*, 2004). Therefore, the existence and identification of these proteins responsible for adhesion of *P. chlamydosporia* to the nematode egg might be important in pathogenicity. Biotypes with greater saprophytic competitiveness may lack these or produce different protein compared to biotypes that are more virulent.

b) Optimising conditions for mass production of the fungus and for the improvement of the quality of the inocula

Studies in mass production of the fungus should focus not only on the search for cheaper substrates to grow the fungus and to obtain high yields of spores, but substrates that can possibly produce spores with higher quality. Conditions of *in vitro* growth can influence spore quality in fungi (Jackson & Schisler, 1992; Ibrahim *et al.*, 2002; Ypsilos & Magan, 2004; Ypsilos & Magan, 2005) and even enhance pathogenicity (Chandler *et al.*, 2005; Shah *et al.*, 2005). As it was mentioned in the general discussion the importance of nutrition in pathogenicity deserves to be further studied for this fungus. Therefore, future studies on the production of the fungus should focus on this important area of research.

c) Understanding mechanisms responsible for the parasitic switch and pathogenicity using molecular techniques

The development of a transformation system for the fungus would help to understand differences between biotypes under field conditions and the possible interaction between different biotypes coexisting in the same root or soil environment. Preliminary attempts have aimed to transform a biotype of *P. chlamydosporia* with the green fluorescent protein marker gene (*gfp*) and the hygromycin resistance gene (*hph*) but the poor sensitivity to fungal inhibitors has impeded the development of stable transformants (Atkins *et al.*, 2004). The biotype was also resistant to a wide range of potential markers such as the antibiotics ampicillin, hygromycin, kanamycin, tetracycline and phleomycin (Segers, 1996). New attempts are being carried out in Rothamsted Research in order to create transformants using *Agrobacterium* (Helen Ward, unpublished results).

Another important advance for the study of the fungus would be the identification of key genes related with pathogenicity and saprophytic growth. The construction of mutants lacking these genes would help to understand mechanisms of parasitism. Also, the tritrophic interactions between plant, nematode and fungus need further understanding. It would be interesting to investigate if signals transmitted from the plant when under attack by nematodes can influence fungal growth including the

switch from saprophytic to parasitic growth. Differential gene expression in *P. chlamydosporia* would help to examine genes that are expressed during infection.

To conclude, the successful use of *P. chlamydosporia* as a BCA is dependent on several biological, ecological and commercial factors. Because of the complexity involved, it is important to study several factors that affect the performance of the fungus and try to improve them. The work in this thesis has increased basic understanding of the biology of the fungus and provided information about the variability and stability of the fungus, important for the implementation of efficient quality control systems in production.

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APPENDIX I – Media composition

All media described in this appendix were autoclaved at 121°C for 15 minutes before use. In media containing antibiotics, these were introduced when media was cool to touch.

Medium	Used in	Ingredients	g l^{-1}
Corn meal agar	*P. chlamydosporia* growth (2.3; 2.4; 2.5; 4.2.1)	CMA (Oxoid)	17
Czapek Dox Broth	Production of extracellular enzymes (5.2.1)	Sucrose	10
		$NaNO_3$	3
		KCl	0.5
		$MgSO_4$	0.5
		K_2HPO_4	1
		Yeast extract (Merck)	0.5
		$FeSO_4$	0.006
Germination seed media	Studies on saprophytic ability (3.2.2)	Technical agar	12
		Glucose	10
		Peptone	0.1
		Yeast extract (Merck)	0.1
Minimal Czapek Dox Broth	Production of extracellular enzymes (5.2.1)	Sucrose	0.001
		$NaNO_3$	0.014
		$MgSO_4$	0.25
		KCl	0.25
		K_2HPO_4	0.5
		$FeSO_4$	0.0006
Minimal medium composition	Production of extracellular enzymes using *p*-nitrophenyl substrates (5.2.2)	NaCl	0.3
		$MgSO_4.7\ H_2O$	0.3
		K_2HPO_4	0.3
		Yeast extract (Merck)	0.2
Nutrient broth	Suspend chlamydospores and fungal material for freeze drying (2.5)	Nutrient broth n° 2 (Oxoid)	25
		Glucose	75
Potato Dextrose Agar	*P. chlamydosporia* growth (2.3; 4.2.1; 4.2.2; 6.2)	PDA (Oxoid)	39
Semi selective medium (SM)	Numbers of CFU in roots and soil (2.7.1; 2.7.2; 3.23)	CMA	17
		NaCl	17.5
		Rose Bengal	0.075
		After autoclaving add:	
		Triton X-100	3 ml
		Streptomycin sulphate	0.05
		Chloramphenicol	0.05
		Chlortetracycline	0.05
		Thiabendazole	0.0375
		Carbendazim	0.0375

Medium	Used in	Ingredients	$g\ l^{-1}$
Sorbose agar and antibiotics	Viability (% germination) of chlamydospores (2.4.2; 6.2.2)	Technical agar	12
		Sorbose	2
		After autoclaving add:	
		Streptomycin sulphate	0.05
		Chlortetracycline	0.05
		Chloramphenicol	0.05
Water-agar and antibiotics (WA)	Fungal parasitism of nematode eggs (2.7.3); Percentage of root colonisation (3.2.2)	Technical agar	8
		After autoclaving add:	
		Streptomycin sulphate	0.050
		Chloramphenicol	0.050
		Chlortetracycline	0.050

APPENDIX II to Chapter 3. - Outputs from statistical analysis

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Comparison between the average size of chlamydospores for five biotypes of *P. chlamydosporia* studied (biotypes 60, 104, 132, 280, 392)

Analysis of variance

Variate: chlamydospore size (μm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	4	1534.10	383.52	31.49	<.001
Residual	95	1156.96	12.18		
Total	99	2691.06			

Tables of means

Grand mean 19.34

Biotype	60.	104.	132.	280.	392.
	24.70	15.12	21.95	14.62	20.33

Standard errors of differences of means

Table	Biotype
rep.	20
d.f.	95
s.e.d.	1.104

Least significant differences of means (5% level)

Table	Biotype
rep.	20
d.f.	95
l.s.d.	2.191

Tests on rate of colonisation

Regression Analysis (GLM)

Response variate: Colonised
Binomial totals: total
Distribution: Binomial
Link function: Logit
Fitted terms: Constant + Days + Biotype + Days.Biotype

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance approx ratio	chi pr
+ Days	3	228.015	76.005	76.00	<.001
+ Biotype	2	293.711	146.856	146.86	<.001
+ Days.Biotype	6	45.330	7.555	7.56	<.001
Residual	23	61.060	2.655		
Total	34	628.116	18.474		

Predictions from regression model

Response variate: Colonised

Days	Prediction	s.e.
4	20.55	2.58
8	23.82	2.45
12	57.21	2.42
16	67.07	1.80

Biotype	Prediction	s.e.
10	50.20	2.07
280	13.45	1.81
400	67.19	2.05

Days	4		8	
Biotype	pred	s.e.	pred	s.e.
10	12.94	3.64	26.32	4.52
280	13.95	3.74	5.56	2.20
400	35.29	5.80	39.76	5.37

Days	12		16	
Biotype	pred	s.e.	pred	s.e.
10	67.37	4.81	85.05	3.45
280	15.15	4.41	18.69	3.77
400	89.22	3.07	97.22	1.58

Fitting lines to each biotype

Regression Analysis

Response variate: Colonised

Binomial totals: total
Distribution: Binomial
Link function: Logit
Fitted terms: Constant + ldays + biotype+ ldays.biotype

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
+ ldays	1	188.687	188.687	188.69	<.001
+ biotype	2	291.478	145.739	145.74	<.001
+ ldays.biotype	2	35.347	17.673	17.67	<.001
Residual	29	112.605	3.883		
Total	34	628.116	18.474		

__**Tests on root colonisation from different seed treatments**__

Analysis of variance

Variate: Logit (% rhizosphere colonisation – **BEAN**)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	8		20.3888	2.5486	2.85	0.033
Residual	17	(1)	15.1980	0.8940		
Total	25	(1)	35.4036			

Tables of means

Grand mean 1.21

Biotype	10.	16.	60.	69.	132.	280.	309.
	0.79	1.99	0.82	1.18	0.18	2.07	0.16

Biotype	392.	399.
	2.87	0.79

Standard errors of differences of means

Table	Biotype
rep.	3
d.f.	17
s.e.d.	0.772

Least significant differences of means (5% level)

Table	Biotype
rep.	3
d.f.	17
l.s.d.	1.629

Analysis of variance

Variate: Logit (% rhizosphere colonisation – **MAIZE**)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	8		7.07069	0.88384	11.07	<.001
Residual	17	(1)	1.35749	0.07985		
Total	25	(1)	7.94080			

Tables of means

Grand mean 0.098

Biotype	10.	16.	60.	69.	132.	280.	309.
	0.083	1.087	-0.249	0.731	0.284	-0.372	-0.587

Biotype	392.	399.
	-0.259	0.162

Standard errors of differences of means

Table	Biotype
rep.	3
d.f.	17
s.e.d.	0.2307

Least significant differences of means (5% level)

Table	Biotype
rep.	3
d.f.	17
l.s.d.	0.4868

Analysis of variance

Variate: Logit (% rhizosphere colonisation – **PEA**)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	8		27.1241	3.3905	4.04	0.010
Residual	15	(3)	12.5756	0.8384		
Total	23	(3)	38.8815			

Tables of means

Grand mean 3.31

Biotype	10.	16.	60.	69.	132.	280.	309.
	3.80	3.80	3.80	0.76	3.80	3.80	2.39

Biotype	392.	399.
	3.80	3.80

Standard errors of differences of means

Table	Biotype
rep.	3
d.f.	15
s.e.d.	0.748

Least significant differences of means (5% level)

Table	Biotype
rep.	3
d.f.	15
l.s.d.	1.593

Comparison between numbers of CFU per seed (biotype 400) adhering to seeds of pea, bean and maize in water-agar and semi selective medium.

Analysis of variance

Variate: CFU/seed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Media	1	$5.091 x 10^8$	$5.091 x 10^8$	10.10	0.003
Seed	2	$2.090 x 10^9$	$1.045 x 10^9$	20.73	<.001
Media.Seed	2	$2.050 x 10^8$	$1.025 x 10^8$	2.03	0.149
Residual	30	$1.512 x 10^9$	$5.042 x 10^7$		
Total	35	$4.316 x 10^9$			

Tables of means

Grand mean 21948.

Media	Semi selective	Water agar
	18188.	25708.

Seed	Bean	Maize	Pea
	11938.	30406.	23500.

Media	Seed	Bean	Maize	Pea
Semi selective		11063.	23688.	19813.
Water agar		12812.	37125.	27188.

Standard errors of differences of means

Table	Media	Seed	Media Seed
rep.	18	12	6
d.f.	30	30	30
s.e.d.	2366.8	2898.7	4099.4

Effect of repeated culturing on the ability to produce chlamydospores and colonise the rhizosphere using an *in vitro* bioassay, by three biotypes of *P. chlamydosporia* (biotypes 132, 280 and 392)

Analysis of variance

Variate: Log (number of chlamydospores)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	2.6334	1.3167	7.77	0.001
Type_subculture	1	0.0033	0.0033	0.02	0.890
Biotype.Type_subculture	2	0.3991	0.1995	1.18	0.318
Residual	42	7.1140	0.1694		
Total	47	10.1497			

Tables of means

Grand mean 2.027

Biotype	132.	280.	392.
	2.129	1.703	2.248

Type of culture	Initial	Subculture
	2.035	2.018

Biotype	Type of culture	Initial	Subculture
132.		2.257	2.001
280.		1.692	1.713
392.		2.155	2.341

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	16	24	8
d.f.	42	42	42
s.e.d.	0.1455	0.1188	0.2058

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	16	24	8
d.f.	42	42	42
l.s.d.	0.2936	0.2398	0.4153

Analysis of variance

Variate: Logit (% chlamydospore viability)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	2.11474	1.05737	31.55	<.001
Type of culture	1	0.00221	0.00221	0.07	0.800
Biotype. Type of culture	2	0.08838	0.04419	1.32	0.292
Residual	18	0.60328	0.03352		
Total	23	2.80860			

Tables of means

Grand mean 0.475

Biotype	132.	280.	392.
	0.440	0.130	0.855

Type of culture	Initial	Subculture
	0.466	0.485

Biotype	Type of culture	Initial	Subculture
132.		0.514	0.366
280.		0.096	0.165
392.		0.787	0.923

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	8	12	4
d.f.	18	18	18
s.e.d.	0.0915	0.0747	0.1295

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	8	12	4
d.f.	18	18	18
l.s.d.	0.1923	0.1570	0.2720

Analysis of variance

Variate: Logit (% rhizosphere colonisation)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		31.4340	15.7170	16.35	<.001
Type of culture	1		0.0314	0.0314	0.03	0.860
Biotype. Type of culture	2		1.5893	0.7947	0.83	0.465
Residual	10	(2)	9.6126	0.9613		
Total	15	(2)	39.5118			

Tables of means

Grand mean 0.97

Biotype	132.	280.	392.
	1.05	-0.69	2.55

Type of culture	Initial	Subculture
	1.01	0.93

Biotype	Type of culture	Initial	Subculture
132.		0.78	1.31
280.		-0.74	-0.63
392.		2.99	2.10

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	10	10	10
s.e.d.	0.566	0.462	0.801

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	10	10	10
l.s.d.	1.261	1.030	1.784

Effect of repeated culturing on the ability to produce chlamydospores and colonise roots in a pot experiment, by three biotypes of *P. chlamydosporia* (biotypes 132, 280 and 392)

Analysis of variance

Variate: Log (number of chlamydospores)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		2.01937	1.00969	11.16	<.001
Type of culture	1		0.06293	0.06293	0.70	0.407
Biotype. Type of culture	2		1.67180	0.83590	9.24	<.001
Residual	86	(4)	7.77750	0.09044		
Total	91	(4)	11.51941			

Tables of means

Grand mean 4.090

Biotype	132.	280.	392.
	4.031	3.950	4.290

Type of culture	Initial	Subculture
	4.116	4.065

Biotype	Type of culture	Initial	Subculture
132.		3.904	4.158
280.		4.145	3.755
392.		4.299	4.281

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	32	48	16
d.f.	86	86	86
s.e.d.	0.0752	0.0614	0.1063

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	32	48	16
d.f.	86	86	86
l.s.d.	0.1495	0.1220	0.2114

Analysis of variance

Variate: Logit (% chlamydospore viability)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	1.16274	0.58137	20.19	<.001
Type of culture	1	0.26059	0.26059	9.05	0.011
Biotype. Type of culture	2	0.50714	0.25357	8.81	0.004
Residual	12	0.34547	0.02879		
Total	17	2.27594			

Tables of means

Grand mean 0.718

Biotype	132.	280.	392.
	1.058	0.446	0.651

Type of culture	Initial	Subculture
	0.839	0.598

Biotype	Type of culture	Initial	Subculture
132.		1.109	1.006
280.		0.798	0.095
392.		0.609	0.693

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	6	9	3
d.f.	12	12	12
s.e.d.	0.0980	0.0800	0.1385

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
l.s.d.	0.2134	0.1743	0.3018

Analysis of variance

Variate: Log (CFU_g+0.5) **CFU/ g root**

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
BLOCK stratum	4	135.399	33.850	5.07	
BLOCK.*Units* stratum					
Biotype	2	498.757	249.379	37.35	<.001
Type of culture	1	4.066	4.066	0.61	0.437
Biotype. Type of culture	2	110.062	55.031	8.24	<.001
Residual	80	534.120	6.677		
Total	89	1282.404			

Tables of means

Grand mean 6.14

Biotype	132	280	392	control
	7.35	2.85	8.22	

Type of culture	Control	Initial	Subculture
		6.35	5.93

Biotype	Type of culture	Control	Initial	Subculture
132			6.17	8.52
280			4.38	1.31
392			8.50	7.95

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	30	45	15
d.f.	80	80	80
s.e.d.	0.667	0.545	0.944

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype. Type of culture
rep.	30	45	15
d.f.	80	80	80
l.s.d.	1.328	1.084	1.878

APPENDIX III to Chapter 4. - Outputs from statistical analysis

GenStat Eighth Edition (Service Pack 1)
GenStat Procedure Library Release PL16.1

Comparison between biotypes of *P. chlamydosporia* in their ability to parasitise *Meloidogyne* spp and *G. pallida* eggs using a novel test (in liquid)

Analysis of variance

Variate: Logit (% egg parasitism – *Meloidogyne incognita*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	8	44.1078	5.5135	16.54	<.001
Residual	27	9.0013	0.3334		
Total	35	53.1091			

Tables of means

Grand mean 0.996

Biotype	10	104	132	16	280	392
	0.778	0.748	1.183	-0.661	-0.100	1.371

Biotype	399	400	60	*B. cinerea*	Control
	1.711	3.450	0.483		

Standard errors of differences of means

Table	Biotype
rep.	4
d.f.	27
s.e.d.	0.4083

Least significant differences of means (5% level)

Table	Biotype
rep.	4
d.f.	27
l.s.d.	0.8377

Analysis of variance

Variate: Logit (% egg parasitism – *Globodera pallida*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	8	32.2148	4.0268	6.43	<.001
Residual	27	16.8975	0.6258		
Total	35	49.1123			

Tables of means

Grand mean 1.90

Biotype	10	104	132	16	280	392
	1.74	1.40	1.47	0.68	3.26	1.19

Biotype	399	400	60	*B. cinerea*	Control
	1.53	3.80	2.01		

Standard errors of differences of means

Table	Biotype
rep.	4
d.f.	27
s.e.d.	0.559

Least significant differences of means (5% level)

Table	Biotype
rep.	4
d.f.	27
l.s.d.	1.148

Comparison between the numbers of juveniles hatched in water and in 0.0125% YEM medium

Analysis of variance

Variate: Logit (% egg hatch)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	5	87.02584	17.40517	499.73	<.001
Media	1	0.46140	0.46140	13.25	0.001
Days.Media	5	0.16891	0.03378	0.97	0.456
Residual	24	0.83590	0.03483		
Total	35	88.49205			

Tables of means

Grand mean -0.347

Days	0.	6.	9.	12.	15.	20.
	-3.801	0.013	0.243	0.403	0.488	0.568

Media	Water	Yeast extract (0.0125%)
	-0.461	-0.234

Days	Media	Water	Yeast extract (0.0125%)
0.		-3.801	-3.801
6.		-0.170	0.197
9.		0.033	0.453
12.		0.319	0.487
15.		0.389	0.587
20.		0.465	0.672

Standard errors of differences of means

Table	Days	Media	Days Media
rep.	6	18	3
d.f.	24	24	24
s.e.d.	0.1077	0.0622	0.1524

Least significant differences of means (5% level)

Table	Days	Media	Days Media
rep.	6	18	3
d.f.	24	24	24
l.s.d.	0.2224	0.1284	0.3145

Effect of conidial concentration on the proportion of *Meloidogyne* spp. and *G. pallida* eggs by two biotypes of *P. chlamydosporia* (400 and 392).

Regression analysis – PROBIT (considering 3 groups)

Response variate: Parasitised eggs
Binomial totals: Total
Distribution: Binomial
Link function: Probit
Fitted terms: C1 + Concentration

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	3	2312.2	770.74	770.74
Residual	32	322.2	10.07	
Total	35	2634.4	75.27	

Estimates of parameters

Parameter	estimate	s.e.	t(*)
C1 *Globodera pallida* 392	-3.632	0.105	-34.44
C1 *Globodera pallida* 400	-3.482	0.123	-28.38
C1 *Meloidogyne* spp. 400	-3.201	0.103	-31.19
Concentration	1.2509	0.0344	36.36

Effective doses

Log10 scale

Group	LD	estimate	s.e.	lower 95%	upper 95%
Globodera pallida 392	50.00	2.904	0.02986	2.845	2.962
Globodera pallida 400	50.00	2.783	0.06327	2.659	2.907
Meloidogyne spp. 400	50.00	2.559	0.04265	2.476	2.643

Natural scale

Group	LD	estimate	lower 95%	upper 95%
Globodera pallida 392	50.00	800.8	700.2	916.4
Globodera pallida 400	50.00	606.9	456.5	807.5
Meloidogyne spp. 400	50.00	362.4	299.0	439.2

Regression analysis – PROBIT (considering 3 groups)

Response variate: Parasitised eggs
Binomial totals: Total
Distribution: Binomial
Link function: Probit
Fitted terms: Constant, Concentration

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	1	2269.4	2269.39	2269.39
Residual	34	365.0	10.74	
Total	35	2634.4	75.27	

Estimates of parameters

Parameter	estimate	s.e.	t(*)
Constant	-3.4287	0.0976	-35.13
Concentration	1.2275	0.0338	36.37

Effective doses

Log10 scale

LD	estimate	s.e.	lower 95%	upper 95%
50.00	2.793	0.02306	2.748	2.838

Natural scale

LD	estimate	lower 95%	upper 95%
50.00	621.0	559.8	689.1

Regression analysis – PROBIT (considering 2 groups – compatible and non compatible interaction)

Response variate: Parasitised_eggs
Binomial totals: Total
Distribution: Binomial
Link function: Probit
Fitted terms: C2 + Concentration

Summary of analysis

Source	d.f.	mean deviance	deviance deviance	ratio
Regression	2	2309.3	1154.661	1154.66
Residual	33	325.1	9.851	
Total	35	2634.4	75.269	

Estimates of parameters

Parameter	estimate	s.e.	t(*)
C2 *Globodera pallida* 392	-3.599	0.103	-34.84
C2 *Meloidogyne* spp. 400	-3.195	0.102	-31.18
Concentration	1.2486	0.0344	36.35

Effective doses

Log10 scale

Group	LD	estimate	s.e.	lower 95%	upper 95%
Globodera pallida 392	50.00	2.883	0.02705	2.830	2.936
Meloidogyne spp. 400	50.00	2.559	0.04269	2.476	2.643

Natural scale

Group	LD	estimate	lower 95%	upper 95%
Globodera pallida 392	50.00	763.4	676.0	862.6
Meloidogyne spp. 400	50.00	362.4	299.0	439.3

Regression analysis – PROBIT (considering 2 groups – compatible and non compatible interaction)

Response variate: Parasitised eggs
Binomial totals: Total
Distribution: Binomial
Link function: Probit
Fitted terms: Constant, Concentration

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	1	2269.4	2269.39	2269.39
Residual	34	365.0	10.74	
Total	35	2634.4	75.27	

Estimates of parameters

Parameter	estimate	s.e.	t(*)
Constant	-3.4287	0.0976	-35.13
Concentration	1.2275	0.0338	36.37

Effective doses

Log10 scale

LD	estimate	s.e.	lower 95%	upper 95%
50.00	2.793	0.02306	2.748	2.838

Natural scale

LD	estimate	lower 95%	upper 95%
50.00	621.0	559.8	689.1

Comparison between biotypes of *P. chlamydosporia* in their ability to parasitise *Meloidogyne* spp and G. *pallida* eggs using a standard method (agar)

Analysis of variance

Variate: Logit (% egg parasitism – *Meloidogyne incognita*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	8	5.95645	0.74456	23.59	<.001
Residual	18	0.56803	0.03156		
Total	26	6.52449			

Tables of means

Grand mean -0.760

Biotype	10	104	132	16	280	392	399
	-0.078	-1.131	-1.113	-0.949	-0.082	-0.966	-0.157

Biotype	400	60	CONTROL
	-1.138	-1.223	

Standard errors of differences of means

Table	Biotype
rep.	3
d.f.	18
s.e.d.	0.1450

Least significant differences of means (5% level)

Table	Biotype
rep.	3
d.f.	18
l.s.d.	0.3047

Analysis of variance

Variate: Logit (% egg parasitism – *Globodera pallida*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	8	13.95625	1.74453	18.11	<.001
Residual	18	1.73439	0.09635		
Total	26	15.69064			

Tables of means

Grand mean -0.632

Biotype	10	104	132	16	280	392	399
	-0.714	-1.006	-1.204	-1.016	1.280	-1.143	-0.370

Biotype	400	60	Control
	-0.667	-0.847	

Standard errors of differences of means

Table	Biotype
rep.	3
d.f.	18
s.e.d.	0.2534

Least significant differences of means (5% level)

Table	Biotype
rep.	3
d.f.	18
l.s.d.	0.5325

Effect of type (chlamydospore and conidia) and concentration of fungal inoculum on the proportion (%) of *Meloidogyne* spp. eggs infected using a standard method (agar)

Analysis of variance

Variate: Logit (% egg parasitism – biotype 392)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Spore	1	0.09138	0.09138	3.56	0.077
Concentration	3	0.80795	0.26932	10.49	<.001
Spore.Concentration	3	0.48284	0.16095	6.27	0.005
Residual	16	0.41062	0.02566		
Total	23	1.79279			

Tables of means

Grand mean -1.018

Spore	Chlamydospores	Conidia
	-1.080	-0.957

Conc.	100.	1000.	10000.	100000.
	-1.319	-1.003	-0.913	-0.838

Spore	Conc.	100.	1000.	10000.	100000.
Chlamydospores		-1.625	-1.008	-0.892	-0.794
Conidia		-1.014	-0.998	-0.933	-0.881

Standard errors of differences of means

Table	Spore	Conc.	Spore Conc.
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.0654	0.0925	0.1308

Least significant differences of means (5% level)

Table	Spore	Conc	Spore. Conc
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.1386	0.1961	0.2773

Analysis of variance

Variate: Logit (% egg parasitism – biotype 400)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	3	2.16150	0.72050	15.98	<.001
Spore	1	0.25563	0.25563	5.67	0.030
Concentration.Spore	3	0.28020	0.09340	2.07	0.144

Residual	16	0.72132	0.04508
Total	23	3.41865	

Tables of means

Grand mean -0.819

Conc.	100.	1000.	10000.	100000.
	-1.332	-0.661	-0.574	-0.708

Spore	Chlamydospores	Conidia
	-0.716	-0.922

Conc.	Chlamydospores	Conidia
100.	-1.375	-1.289
1000.	-0.544	-0.778
10000.	-0.496	-0.653
100000.	-0.448	-0.968

Standard errors of differences of means

Table	Conc.	Spore	Conc. Spore
rep.	6	12	3
d.f.	16	16	16
s.e.d.	0.1226	0.0867	0.1734

Least significant differences of means (5% level)

Table	Conc.	Spore	Conc. Spore
rep.	6	12	3
d.f.	16	16	16
l.s.d.	0.2599	0.1838	0.3675

<u>Effects of different solutions on the formation of appressoria *in vitro* using a biotype of *P. chlamydosporia* (biotype 392)</u>

Analysis of variance

Variate: Logit (% appressoria)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Solution	3	34.1072	11.3691	15.48	<.001
Residual	28	20.5627	0.7344		
Total	31	54.6699			

Tables of means

Grand mean -1.96

Solution	0.1 %Triton	Ringers solution	Water	0.0125%YEM
	-3.41	-1.47	-2.32	-0.63

Standard errors of differences of means

Table	Solution
rep.	8
d.f.	28
s.e.d.	0.428

Least significant differences of means (5% level)

Table	Solution
rep.	8
d.f.	28
l.s.d.	0.878

Comparison between biotypes of *P. chlamydosporia* on the formation of appressoria *in vitro*

Analysis of variance

Variate: Logit (% appressoria)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	10	55.598	5.560	2.31	0.020
Residual	77	185.299	2.406		
Total	87	240.897			

Tables of means

Grand mean -1.51

Biotype	10.	16.	60.	69.	104.	132.	280.
	-0.98	-2.20	-1.44	-1.56	-2.15	-1.08	-3.44

Biotype	309.	392.	399.	400.
	-0.72	-0.63	-0.83	-1.63

Standard errors of differences of means

Table	Biotype
rep.	8
d.f.	77
s.e.d.	0.776

Least significant differences of means (5% level)

Table	Biotype
rep.	8
d.f.	77
l.s.d.	1.545

Comparison between appressoria formation and egg parasitism ability

Spearman's rank correlation coefficient (Appressoria/ egg parasitism – *Meloidogyne* spp.)

Sample size = 9
Correlation = -0.250
t Approximation = -0.68
Degrees of freedom = 7
P-value = 0.516

Exact critical values for two-sided test:

probability	0.100	0.050	0.020	0.010	0.005	0.002	0.001
critical value	0.600	0.700	0.783	0.833	0.867	0.917	0.933

Spearman's rank correlation coefficient (Appressoria/ egg parasitism – *G. pallida* spp.)

Sample size = 9
Correlation = -0.467
t Approximation = -1.40
Degrees of freedom = 7
P-value = 0.205

Exact critical values for two-sided test:

probability	0.100	0.050	0.020	0.010	0.005	0.002	0.001
critical value	0.600	0.700	0.783	0.833	0.867	0.917	0.933

Effect of repeated culturing on the ability to parasitise *Meloidogyne* spp. eggs using an *in vitro* bioassay by three biotypes of *P. chlamydosporia* (biotypes 132, 280 and 392)

Analysis of variance

Variate: Logit (% egg parasitism)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Type of culture	1	0.01654	0.01654	0.18	0.687
Biotype	2	0.35718	0.17859	1.93	0.225
Type of culture.Biotype	2	0.41272	0.20636	2.23	0.188
Residual	6	0.55449	0.09242		
Total	11	1.34093			

Tables of means

Grand mean -0.748

Type of culture	Initial	Subculture
	-0.785	-0.711

Biotype	132.	280.	392.
	-0.612	-0.640	-0.991

Type of culture	Biotype	132.	280.	392.
Initial		-0.506	-0.558	-1.290
Subculture		-0.718	-0.721	-0.692

Standard errors of differences of means

Table	Type of culture	Biotype	Type of culture Biotype
rep.	6	4	2
d.f.	6	6	6
s.e.d.	0.1755	0.2150	0.3040

Least significant differences of means (5% level)

Table	Type of culture	Biotype	Type of culture

		Biotype	
rep.	6	4	2
d.f.	6	6	6
l.s.d.	0.4295	0.5260	0.7439

Effect of repeated culturing on the ability to parasitise *Meloidogyne* spp. eggs by three biotypes of *P. chlamydosporia* (biotypes 132, 280 and 392) (pot experiment)

Analysis of variance

Variate: Logit (% egg parasitism)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Type of culture	1	0.01654	0.01654	0.18	0.687
Biotype	2	0.35718	0.17859	1.93	0.225
Type of culture.Biotype	2	0.41272	0.20636	2.23	0.188
Residual	6	0.55449	0.09242		
Total	11	1.34093			

Tables of means

Grand mean -0.748

Type of culture	Initial	Subculture
	-0.785	-0.711

Biotype	132.	280.	392.
	-0.612	-0.640	-0.991

Type of culture	Biotype	132.	280.	392.
Initial		-0.506	-0.558	-1.290
Subculture		-0.718	-0.721	-0.692

Standard errors of differences of means

Table	Type of culture	Biotype	Type of culture. Biotype
rep.	6	4	2
d.f.	6	6	6
s.e.d.	0.1755	0.2150	0.3040

Least significant differences of means (5% level)

Table	Type of culture	Biotype	Type of culture. Biotype
rep.	6	4	2
d.f.	6	6	6
l.s.d.	0.4295	0.5260	0.7439

Analysis of variance

Variate: Log (CFU/g soil) + 0.5))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	82.515	20.629	2.48	
Block.*Units* stratum					
Type of culture	1	40.878	40.878	4.91	0.030
Biotype	2	1183.380	591.690	71.04	<.001
Type of culture.Biotype	2	71.857	35.928	4.31	0.017
Residual	80	666.292	8.329		
Total	89	2044.920			

Tables of means

Grand mean 5.52

Type of culture	Control	Initial	Subculture
		4.84	6.19

Biotype	132	280	392	Control
	9.88	5.67	1.00	

Type of culture	Biotype	132	280	392	Control
Initial		9.07	6.15	-0.69	
Subculture		10.69	5.18	2.70	

Standard errors of differences of means

Table	Type of culture	Biotype	Type of culture Biotype
rep.	45	30	15
d.f.	80	80	80
s.e.d.	0.608	0.745	1.054

Least significant differences of means (5% level)

Table	Type of culture	Biotype	Type of culture Biotype
rep.	45	30	15
d.f.	80	80	80
l.s.d.	1.211	1.483	2.097

Number of egg masses per root/number of eggs per egg mass; number of infected eggs per root system

Analysis of variance

Variate: Number of egg masses per g of root

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	334.90	83.73	1.52	
Block.*Units* stratum					
Subdiv	1	138.43	138.43	2.51	0.126
Subdiv.Type of culture	1	12.03	12.03	0.22	0.644
Subdiv.Biotype	2	96.03	48.01	0.87	0.431
Subdiv.Type of culture.Biotype	2	164.43	82.21	1.49	0.245
Residual	24	1322.47	55.10		
Total	34	2068.30			

Tables of means

Grand mean 13.1

Subdiv	Control	factorial
	18.0	12.3
rep.	5	30

Subdiv	Type of culture	Control	Initial	Subculture
Control		18.0		
	rep.	5		
factorial			12.9	11.7
	rep.		15	15

Subdiv	Biotype	132	280	392	Control
Control					18.0
	rep.				5
factorial		12.7	14.3	9.9	
	rep.	10	10	10	

Subdiv	Type of culture. biotype	132	280	392	Control
Control	Control				18.0
factorial	Initial	11.0	18.1	9.7	
	Subculture	14.4	10.4	10.1	

Standard errors of differences of means

Table	Subdiv	Subdiv. Type of culture	Subdiv. biotype	Subdiv. Type of culture. biotype	
rep.	unequal	unequal	unequal	5	
d.f.	24	24	24	24	
s.e.d.		4.69X	4.69X		min.rep
	3.59	3.83	4.07	4.69	max-min

		2.71	3.32		max.rep

Least significant differences of means (5% level)

Table	Subdiv	Subdiv. Type of culture	Subdiv. Biotype	Subdiv. Type of culture. Biotype	
rep.	unequal	unequal	unequal	5	
d.f.	24	24	24	24	
l.s.d.		9.69X	9.69X		min.rep
	7.40	7.91	8.39	9.69	max-min
		5.59	6.85		max.rep

Analysis of variance

Variate: Number of eggs per egg mass

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	98226.	24557.	1.67	
Block.*Units* stratum					
Subdiv	1	5376.	5376.	0.37	0.551
Subdiv.Type of culture	1	5810.	5810.	0.40	0.535
Subdiv.Biotype	2	62667.	31334.	2.14	0.140
Subdiv.Type of culture.Biotype	2	114033.	57016.	3.89	0.035
Residual	24	352139.	14672.		
Total	34	638252.			

Tables of means

Grand mean 380.

Subdiv	Control	factorial
	411.	375.
rep.	5	30

Subdiv	Type of culture	Control	Initial	Subculture
Control		411.		
	rep.	5		
factorial			362.	389.
	rep.		15	15

Subdiv	Biotype	132	280	392	Control
Control					411.
	rep.				5
factorial		369.	323.	434.	
	rep.	10	10	10	

Subdiv	Type of culture	biotype	132	280	392	Control
Control	Control					411.
Factorial	Initial		420.	226.	438.	
	Subculture		318.	420.	431.	

APPENDIX IV to Chapter 5 - Outputs from statistical analysis

GenStat Eighth Edition (Service Pack 1)
GenStat Procedure Library Release PL16.1

Quantitative studies on the production of extracellular enzymes

Enzyme activity in response to medium amendments

(a) Proteases

Analysis of variance

Variate: LOG10 [(specific+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		1.5460	0.1546	0.98	0.470
Media	3		7.3899	2.4633	15.56	<.001
Biotype.Media	30		3.2245	0.1075	0.68	0.884
Residual	85	(3)	13.4554	0.1583		
Total	128	(3)	25.2392			

Tables of means

Grand mean 0.615

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.537	0.694	0.812	0.683	0.678	0.742	0.548

Biotype	309.	392.	399.	400.
	0.442	0.498	0.574	0.556

Media	Minimal	Chitin	Gelatin	Lipid
	1.018	0.419	0.543	0.480

Biotype	Media	Minimal	Chitin	Gelatin	Lipid
10.		0.929	0.177	0.304	0.738
16.		1.355	0.573	0.449	0.400
60.		1.179	0.463	0.943	0.661
69.		0.939	0.574	0.641	0.578
104.		1.068	0.602	0.609	0.431
132.		1.184	0.460	0.881	0.442
280.		1.042	0.274	0.536	0.339
309.		0.914	0.361	0.494	0.000
392.		0.987	0.422	0.114	0.470
399.		0.847	0.410	0.269	0.772
400.		0.750	0.291	0.736	0.448

Standard errors of differences of means

Table	Biotype	Media	Biotype Media
rep.	12	33	3
d.f.	85	85	85
s.e.d.	0.1624	0.0979	0.3249

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	12	33	3
d.f.	85	85	85
l.s.d.	0.3230	0.1947	0.6459

(a) Chitinases

Analysis of variance

Variate: LOG10 [(specific+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		6.78296	0.67830	16.26	<.001
Media	2		36.07933	18.03967	432.48	<.001
Biotype.Media	20		7.82672	0.39134	9.38	<.001
Residual	63	(3)	2.62787	0.04171		
Total	95	(3)	52.59511			

Tables of means

Grand mean 0.554

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.461	0.845	0.363	1.235	0.480	0.599	0.599

Biotype	309.	392.	399.	400.
	0.510	0.340	0.370	0.291

Media	Minimal	chitin	gelatin
	0.260	0.007	1.395

Biotype	Media	Minimal	chitin	gelatin
10.		0.000	0.000	1.383
16.		0.686	0.016	1.835
60.		0.000	0.000	1.090
69.		1.845	0.000	1.859
104.		0.000	0.000	1.439
132.		0.000	0.054	1.743
280.		0.000	0.000	1.798
309.		0.011	0.007	1.513
392.		0.318	0.000	0.703
399.		0.000	0.000	1.111
400.		0.000	0.000	0.872

Standard errors of differences of means

Table	Biotype	Media	Biotype Media
rep.	9	33	3
d.f.	63	63	63
s.e.d.	0.0963	0.0503	0.1668

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	9	33	3
d.f.	63	63	63
l.s.d.	0.1924	0.1005	0.3332

(c) Lipases

Analysis of variance

Variate: LOG10 [(Lipase activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Media	2		0.67468	0.33734	4.87	0.011
Biotype	10		0.34979	0.03498	0.50	0.881
Media.Biotype	20		1.09719	0.05486	0.79	0.714
Residual	64	(2)	4.43648	0.06932		
Total	96	(2)	6.54278			

Tables of means

Grand mean 0.120

Media	Minimal	gelatin	lipid
	0.040	0.087	0.234

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.095	0.126	0.191	0.021	0.084	0.125	0.025

Biotype	309.	392.	399.	400.
	0.174	0.211	0.112	0.160

Media	Biotype	10.	16.	60.	69.	104.	132.
Minimal		0.000	0.000	0.000	0.000	0.104	0.000
gelatin		0.000	0.144	0.445	0.000	0.065	0.000
lipid		0.284	0.233	0.130	0.062	0.083	0.376

Media	Biotype	280.	309.	392.	399.	400.
Minimal		0.000	0.338	0.000	0.000	0.000
gelatin		0.000	0.000	0.207	0.000	0.094
lipid		0.075	0.184	0.427	0.335	0.384

Standard errors of differences of means

Table	Media	Biotype	Media Biotype
rep.	33	9	3
d.f.	64	64	64
s.e.d.	0.0648	0.1241	0.2150

Least significant differences of means (5% level)

Table	Media	Biotype	Media Biotype
rep.	33	9	3
d.f.	64	64	64
l.s.d.	0.1295	0.2479	0.4295

(d) Esterases

Analysis of variance

Variate: LOG10 [(Esterase activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
media	2		17.67661	8.83831	129.81	<.001
Biotype	10		4.96251	0.49625	7.29	<.001
media.Biotype	20		5.97221	0.29861	4.39	<.001
Residual	65	(1)	4.42553	0.06809		
Total	97	(1)	32.76530			

Tables of means

Grand mean 0.518

media	gelatin	Lipid	Minimal
	1.078	0.056	0.421

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.535	0.806	0.561	0.631	0.611	0.820	0.479

Biotype	309.	392.	399.	400.
	0.278	0.677	0.197	0.110

media	Biotype	10.	16.	60.	69.	104.	132.
gelatin		1.090	1.453	1.301	1.571	1.606	1.221
Lipid		0.000	0.078	0.000	0.069	0.000	0.155
Minimal		0.517	0.887	0.381	0.253	0.226	1.083

media	Biotype	280.	309.	392.	399.	400.
gelatin		1.229	0.633	1.360	0.391	0.000
Lipid		0.000	0.000	0.225	0.064	0.030
Minimal		0.208	0.201	0.447	0.136	0.299

Standard errors of differences of means

Table	media	Biotype	media Biotype
rep.	33	9	3
d.f.	65	65	65
s.e.d.	0.0642	0.1230	0.2130

Least significant differences of means (5% level)

Table	media	Biotype	media Biotype
rep.	33	9	3
d.f.	65	65	65
l.s.d.	0.1283	0.2457	0.4255

VCP1 activity

Analysis of variance

Variate: LOG10 [(VCP1 activity+1)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		1.36883	0.13688	5.31	<.001
Media	2		2.34148	1.17074	45.38	<.001
Biotype.Media	20		2.46696	0.12335	4.78	<.001
Residual	57	(9)	1.47050	0.02580		
Total	89	(9)	6.94656			

Tables of means

Grand mean 0.365

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.265	0.354	0.403	0.270	0.487	0.619	0.403

Biotype	309.	392.	399.	400.
	0.368	0.185	0.418	0.241

Media	Minimal	chitin	gelatin
	0.307	0.575	0.212

Biotype	Media	Minimal	chitin	gelatin
10.		0.166	0.498	0.130
16.		0.255	0.685	0.122
60.		0.541	0.660	0.006
69.		0.018	0.791	0.001
104.		0.335	0.587	0.540
132.		0.784	0.576	0.497
280.		0.640	0.442	0.127
309.		0.470	0.476	0.159
392.		0.039	0.513	0.004
399.		0.087	0.695	0.471
400.		0.041	0.405	0.276

Standard errors of differences of means

Table	Biotype	Media	Biotype Media
rep.	9	33	3
d.f.	57	57	57
s.e.d.	0.0757	0.0395	0.1311

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	9	33	3
d.f.	57	57	57
l.s.d.	0.1516	0.0792	0.2626

(b) Enzyme activity and time of secretion

Proteases and time of secretion

Analysis of variance

Variate: LOG10 [(Protease activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		2.54607	0.25461	3.09	0.003
Day	2		0.85078	0.42539	5.17	0.008
Biotype.Day	20		1.52817	0.07641	0.93	0.556
Residual	62	(4)	5.10378	0.08232		
Total	94	(4)	9.66288			

Tables of means

Grand mean 0.919

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.916	1.087	1.144	0.956	1.023	1.126	0.860

Biotype	309.	392.	399.	400.
	0.865	0.795	0.708	0.631

Day	3.	5.	7.
	0.945	1.018	0.795

Biotype	Day	3.	5.	7.
10.		0.938	0.929	0.880
16.		1.149	1.355	0.756
60.		1.147	1.179	1.105
69.		1.198	0.939	0.731
104.		1.336	1.068	0.665
132.		1.240	1.184	0.954
280.		0.874	1.042	0.666
309.		0.824	0.914	0.856
392.		0.665	0.987	0.732
399.		0.553	0.847	0.724
400.		0.468	0.750	0.675

Standard errors of differences of means

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	62	62	62
s.e.d.	0.1353	0.0706	0.2343

Least significant differences of means (5% level)

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	62	62	62
l.s.d.	0.2704	0.1412	0.4683

Chitinases and time of secretion

Analysis of variance

Variate: LOG10 [(Chitinase activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		9.19694	0.91969	10.41	<.001
Day	2		11.36197	5.68099	64.30	<.001
Biotype.Day	20		5.44253	0.27213	3.08	<.001
Residual	63	(3)	5.56625	0.08835		
Total	95	(3)	31.48202			

Tables of means

Grand mean 1.064

Biotype	10.	16.	60.	69.	104.	132.	280.
	1.089	1.492	0.806	1.515	1.066	1.414	1.140

Biotype	309.	392.	399.	400.
	1.031	0.532	0.692	0.934

Day	3.	5.	7.
	0.599	1.395	1.199

Biotype	Day	3.	5.	7.
10.		0.923	1.383	0.960
16.		1.201	1.835	1.439
60.		0.603	1.090	0.725
69.		1.235	1.859	1.450
104.		0.708	1.439	1.051
132.		1.040	1.743	1.459
280.		0.000	1.798	1.620
309.		0.132	1.513	1.449
392.		0.043	0.703	0.848
399.		0.000	1.111	0.964
400.		0.702	0.872	1.226

Standard errors of differences of means

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	63	63	63
s.e.d.	0.1401	0.0732	0.2427

Least significant differences of means (5% level)

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	63	63	63
l.s.d.	0.2800	0.1462	0.4850

Lipases and time of secretion

Analysis of variance

Variate: LOG10 [(Lipase activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		0.49551	0.04955	0.65	0.762
Day	2		0.68335	0.34168	4.51	0.015
Biotype.Day	20		1.25177	0.06259	0.83	0.675
Residual	65	(1)	4.92821	0.07582		
Total	97	(1)	7.35325			

Tables of means

Grand mean 0.214

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.214	0.196	0.109	0.198	0.199	0.241	0.148

Biotype	309.	392.	399.	400.
	0.157	0.206	0.378	0.304

Day	3.	5.	7.
	0.103	0.234	0.304

Biotype	Day	3.	5.	7.
10.		0.140	0.284	0.219
16.		0.109	0.233	0.247
60.		0.104	0.130	0.093
69.		0.157	0.062	0.375
104.		0.354	0.083	0.161
132.		0.041	0.376	0.305
280.		0.105	0.075	0.263

309.	0.000	0.184	0.288
392.	0.019	0.427	0.171
399.	0.108	0.335	0.691
400.	0.000	0.384	0.528

Standard errors of differences of means

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	65	65	65
s.e.d.	0.1298	0.0678	0.2248

Least significant differences of means (5% level)

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	65	65	65
l.s.d.	0.2592	0.1354	0.4490

<u>**Esterases and time of secretion**</u>

Analysis of variance

Variate: LOG10 [(Esterase activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		9.6073	0.9607	9.02	<.001
Day	2		0.2958	0.1479	1.39	0.257
Biotype.Day	20		8.2012	0.4101	3.85	<.001
Residual	63	(3)	6.7085	0.1065		
Total	95	(3)	23.4232			

Tables of means

Grand mean 1.000

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.759	1.251	1.058	1.257	1.388	1.031	1.078

Biotype	309.	392.	399.	400.
	0.497	1.364	0.892	0.432

Day	3.	5.	7.
	0.966	1.078	0.958

Biotype	Day	3.	5.	7.
10.		0.539	1.090	0.649
16.		0.979	1.453	1.320
60.		0.672	1.301	1.201

69.	1.604	1.571	0.595
104.	1.357	1.606	1.200
132.	0.541	1.221	1.331
280.	1.008	1.229	0.997
309.	0.594	0.633	0.264
392.	1.141	1.360	1.591
399.	1.135	0.391	1.149
400.	1.057	0.000	0.239

Standard errors of differences of means

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	63	63	63
s.e.d.	0.1538	0.0803	0.2664

Least significant differences of means (5% level)

Table	Biotype	Day	Biotype Day
rep.	9	33	3
d.f.	63	63	63
l.s.d.	0.3074	0.1605	0.5324

VCP1 activity in minimal medium

Analysis of variance

Variate: LOG10 [(VCP1 activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Day	2		0.092106	0.046053	4.73	0.024
Biotype	2		0.835783	0.417891	42.91	<.001
Day.Biotype	4		0.228671	0.057168	5.87	0.004
Residual	16	(2)	0.155837	0.009740		
Total	24	(2)	1.197375			

Tables of means

Grand mean 0.312

Day	3.	5.	7.
	0.361	0.230	0.345

Biotype	280.	309.	392.
	0.560	0.213	0.165

Day	Biotype	280.	309.	392.
3.		0.520	0.208	0.356
5.		0.519	0.072	0.100
7.		0.640	0.357	0.039

Standard errors of differences of means

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	16	16	16
s.e.d.	0.0465	0.0465	0.0806

Least significant differences of means (5% level)

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	16	16	16
l.s.d.	0.0986	0.0986	0.1708

VCP1 activity in medium amended with *Meloidogyne* spp. eggs

Analysis of variance

Variate: LOG10 [(VCP1 activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Day	2		0.021665	0.010832	1.57	0.239
Biotype	2		0.090578	0.045289	6.55	0.008
Day.Biotype	4		0.040347	0.010087	1.46	0.261
Residual	16	(2)	0.110654	0.006916		
Total	24	(2)	0.255279			

Tables of means

Grand mean 0.105

Day	3.	5.	7.
	0.128	0.121	0.065

Biotype	280.	309.	392.
	0.187	0.064	0.063

Day	Biotype	280.	309.	392.
3.		0.189	0.071	0.124
5.		0.268	0.043	0.052
7.		0.102	0.078	0.014

Standard errors of differences of means

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	16	16	16
s.e.d.	0.0392	0.0392	0.0679

Least significant differences of means (5% level)

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	16	16	16
l.s.d.	0.0831	0.0831	0.1439

<u>**VCP1 activity in medium amended with *Globodera pallida* spp. eggs**</u>

Analysis of variance

Variate: LOG10 [(VCP1 activity+1)]

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	2	0.0271050	0.0135525	17.48	<.001
Biotype	2	0.0249259	0.0124630	16.07	<.001
Day.Biotype	4	0.0264788	0.0066197	8.54	<.001
Residual	18	0.0139575	0.0007754		
Total	26	0.0924672			

Tables of means

Grand mean 0.0353

Day	3.	5.	7.
	0.0799	0.0094	0.0166

Biotype	280.	309.	392.
	0.0781	0.0106	0.0173

Day	Biotype	280.	309.	392.
3.		0.1835	0.0129	0.0435
5.		0.0161	0.0077	0.0044
7.		0.0348	0.0111	0.0040

Standard errors of differences of means

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	18	18	18
s.e.d.	0.01313	0.01313	0.02274

Least significant differences of means (5% level)

Table	Day	Biotype	Day Biotype
rep.	9	9	3
d.f.	18	18	18
l.s.d.	0.02758	0.02758	0.04777

(c) Enzyme activity in different biotypes of *P. chlamydosporia* (5 days of growth)

Analysis of variance

Variate: LOG10 [(Activity+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	10		5.39109	0.53911	7.71	<.001
Enzyme	3		24.10461	8.03487	114.86	<.001
Biotype.Enzyme	30		8.95403	0.29847	4.27	<.001
Residual	85	(3)	5.94608	0.06995		
Total	128	(3)	43.45967			

Tables of means

Grand mean 0.931

Biotype	10.	16.	60.	69.	104.	132.	280.
	0.921	1.219	0.925	1.108	1.049	1.131	1.036

Biotype	309.	392.	399.	400.
	0.811	0.869	0.671	0.502

Enzyme	chitinases	esterases	lipases	proteases
	1.395	1.078	0.234	1.018

Biotype	Enzyme	chitinases	esterases	lipases	proteases
10.		1.383	1.090	0.284	0.929
16.		1.835	1.453	0.233	1.355
60.		1.090	1.301	0.130	1.179
69.		1.859	1.571	0.062	0.939
104.		1.439	1.606	0.083	1.068
132.		1.743	1.221	0.376	1.184
280.		1.798	1.229	0.075	1.042
309.		1.513	0.633	0.184	0.914
392.		0.703	1.360	0.427	0.987
399.		1.111	0.391	0.335	0.847
400.		0.872	0.000	0.384	0.750

Standard errors of differences of means

Table	Biotype	Enzyme	Biotype Enzyme
rep.	12	33	3
d.f.	85	85	85
s.e.d.	0.1080	0.0651	0.2160

Least significant differences of means (5% level)

Table	Biotype	Enzyme	Biotype Enzyme

rep.	12	33	3
d.f.	85	85	85
l.s.d.	0.2147	0.1295	0.4294

Kendall's coefficient of concordance

Variates: Proteases_S, Chitinases_S, Lipases_S, Esterases_S (Specific activity)

Coefficient: 0.209
Adjusted for ties: 0.223
Sample size: 11
Number of samples: 4
Sum of squares: 368.00
Chi-square: 8.93
Degrees of freedom: 10
Probability: 0.539

Ranks

Proteases	Chitinases	Lipases	Esterases	mean
4.0	5.0	8.0	4.0	5.2
11.0	10.0	4.0	9.0	8.5
9.0	3.0	4.0	7.0	5.8
5.0	11.0	4.0	10.0	7.5
7.0	6.0	4.0	11.0	7.0
10.0	8.0	4.0	6.0	7.0
8.0	9.0	4.0	5.0	6.5
3.0	7.0	4.0	3.0	4.2
6.0	2.0	9.0	8.0	6.2
2.0	4.0	10.0	2.0	4.5
1.0	1.0	11.0	1.0	3.5

Kendall's coefficient of concordance

Variates: Proteases_T, Chitinases_T, Lipases_T, Esterases_T.

Coefficient: 0.274
Sample size: 11
Number of samples: 4
Sum of squares: 482.00
Chi-square: 10.95
Degrees of freedom: 10
Probability: 0.361

Ranks

Proteases	Chitinases	Lipases	Esterases	mean
3.0	6.0	8.0	4.0	5.2
10.0	11.0	7.0	8.0	9.0
5.0	2.0	1.0	7.0	3.8
1.0	10.0	2.0	9.0	5.5

11.0	5.0	5.0	10.0	7.8
7.0	9.0	6.0	6.0	7.0
9.0	8.0	3.0	5.0	6.2
2.0	7.0	4.0	3.0	4.0
8.0	3.0	9.0	11.0	7.8
6.0	4.0	11.0	2.0	5.8
4.0	1.0	10.0	1.0	4.0

(d) Effects of repeated culturing on the ability to produce enzymes (5 days of growth)

Proteases (minimal medium)

Analysis of variance

Variate: LOG10 [(protease_minimal+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		0.58300	0.29150	7.98	0.007
Type of culture	1		0.04592	0.04592	1.26	0.286
Biotype.Type of culture	2		0.33311	0.16656	4.56	0.036
Residual	11	(1)	0.40187	0.03653		
Total	16	(1)	1.23677			

Tables of means

Grand mean 1.121

Biotype	132.	280.	392.
	1.242	1.255	0.867

Type of culture	Initial	Sub-culture
	1.071	1.172

Biotype	Type of culture	Initial	Sub-culture
132.		1.184	1.300
280.		1.042	1.468
392.		0.987	0.747

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	11	11	11
s.e.d.	0.1104	0.0901	0.1561

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	11	11	11
l.s.d.	0.2429	0.1983	0.3435

Chitinases (medium amended with gelatin))

Analysis of variance

Variate: LOG10 [(Chitinases_gelatin+1)]

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	2.80042	1.40021	20.83	<.001
Type of culture	1	0.06465	0.06465	0.96	0.346
Biotype.Type of culture	2	0.54015	0.27007	4.02	0.046
Residual	12	0.80670	0.06722		
Total	17	4.21191			

Tables of means

Grand mean 1.355

Biotype	132.	280.	392.
	1.748	1.502	0.816

Type of culture	Initial	Sub-culture
	1.415	1.295

Biotype	Type of culture	Initial	Sub-culture
132.		1.743	1.753
280.		1.798	1.205
392.		0.703	0.928

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
s.e.d.	0.1497	0.1222	0.2117

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
l.s.d.	0.3262	0.2663	0.4613

Lipases (medium amended with olive oil)

Analysis of variance

Variate: LOG10 [(Lipase activity+1)]

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	0.03103	0.01551	0.30	0.747
Type of culture	1	0.24571	0.24571	4.74	0.050
Biotype.Type of culture	2	0.23212	0.11606	2.24	0.149
Residual	12	0.62189	0.05182		
Total	17	1.13075			

Tables of means

Biotype	132.	280.	392.
	0.196	0.118	0.213

Type of culture	Initial	Sub-culture
	0.293	0.059

Biotype	Type of culture	Initial	Sub-culture
132.		0.376	0.016
280.		0.075	0.161
392.		0.427	0.000

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
s.e.d.	0.1314	0.1073	0.1859

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
l.s.d.	0.2864	0.2338	0.4050

<u>**Esterases (medium amended with gelatin)**</u>

Analysis of variance

Variate: LOG10 [(Esterase activity+1)]

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Biotype	2	0.85960	0.42980	6.70	0.011
Type of culture	1	0.10299	0.10299	1.60	0.229
Biotype.Type of culture	2	0.67471	0.33736	5.26	0.023
Residual	12	0.77030	0.06419		
Total	17	2.40760			

Tables of means

Biotype	132.	280.	392.
	1.330	0.886	1.367

Type of culture	Initial	Sub-culture
	1.270	1.119

Biotype	Type of culture	Initial	Sub-culture
132.		1.221	1.438
280.		1.229	0.543
392.		1.360	1.374

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
s.e.d.	0.1463	0.1194	0.2069

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	12	12	12
l.s.d.	0.3187	0.2602	0.4507

<u>VCP1 activity (in minimal medium)</u>

Analysis of variance

Variate: LOG10 [VCP1 activity_Minimal+1)]

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		1.29084	0.64542	16.10	<.001
Type of culture	1		0.06496	0.06496	1.62	0.232
Biotype.Type of culture	2		0.05631	0.02816	0.70	0.518
Residual	10	(2)	0.40080	0.04008		
Total	15	(2)	1.62065			

Tables of means

Grand mean 0.428

Biotype	132.	280.	392.
	0.662	0.568	0.053

Type of culture	Initial	Sub-culture
	0.488	0.367

Biotype	Type of culture	Initial	Sub-culture
132.		0.784	0.541
280.		0.640	0.495
392.		0.039	0.067

Standard errors of differences of means

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	10	10	10
s.e.d.	0.1156	0.0944	0.1635

Least significant differences of means (5% level)

Table	Biotype	Type of culture	Biotype Type of culture
rep.	6	9	3
d.f.	10	10	10
l.s.d.	0.2575	0.2103	0.3642

APPENDIX V to Chapter 6 - Outputs from statistical analysis

GenStat Eighth Edition (Service Pack 1)
GenStat Procedure Library Release PL16.1

Effects of osmotic and matric potential on growth in three biotypes of *P. chlamydosporia* (biotypes 10, 280 and 392)

A) COMPARISON OF GROWTH PER SOLUTE

Analysis of variance

Variate: log(growth_rate) GLYCEROL – 25 days

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Water potential	3		47.908512	15.969504	11039.65	<.001
Biotype	2		0.491036	0.245518	169.73	<.001
Water potential.Biotype	6		0.430655	0.071776	49.62	<.001
Residual	46	(2)	0.066542	0.001447		
Total	57	(2)	47.489171			

Tables of means

Grand mean 0.2166

Water potential	-1.4MPa	-2.8MPa	-7.2 MPa	-0.7 MPa
	0.6225	0.4243	-1.2730	1.0925

Biotype	10.	280.	392.
	0.3177	0.0981	0.2339

Water potencial	Biotype	10.	280.	392.
-1.4 MPa		0.6990	0.4720	0.6964
-2.8 MPa		0.6497	0.1410	0.4821
-7.2 MPa		-1.2730	-1.2730	-1.2730
-0.7 MPa		1.1949	1.0524	1.0302

Standard errors of differences of means

Table	Water potential	Biotype	Water potential Biotype
rep.	15	20	5
d.f.	46	46	46
s.e.d.	0.01389	0.01203	0.02405

Least significant differences of means (5% level)

Table	Water potential	Biotype	Water potential Biotype
rep.	15	20	5
d.f.	46	46	46
l.s.d.	0.02795	0.02421	0.04842

Analysis of variance

Variate: log(Growth_rate) KCl – 25 days

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Water potential	3		9.166042	3.055347	381.96	<.001
Biotype	2		0.728217	0.364108	45.52	<.001
Water potential.Biotype	6		0.992162	0.165360	20.67	<.001
Residual	46	(2)	0.367964	0.007999		
Total	57	(2)	10.824361			

Tables of means

Grand mean 0.586

Water potential	-1.4 MPa	-2.8 MPa	-7.2 MPa	-0.7 MPa
	0.706	0.539	0.005	1.093

Biotype	10.	280.	392.
	0.431	0.682	0.643

Water potential	Biotype	10.	280.	392.
-1.4 MPa		0.521	0.878	0.718
-2.8 MPa		0.147	0.756	0.715
-7.2 MPa		-0.137	0.042	0.109
-0.7 MPa		1.195	1.052	1.030

Standard errors of differences of means

Table	Water potential	Biotype	Water potential Biotype
rep.	15	20	5
d.f.	46	46	46
s.e.d.	0.0327	0.0283	0.0566

Least significant differences of means (5% level)

Table	Water potential	Biotype	Water potential Biotype
rep.	15	20	5
d.f.	46	46	46
l.s.d.	0.0657	0.0569	0.1139

Analysis of variance

Variate: log (Growth_rate) PEG – 25 days

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Water potential	3		5.889728	1.963243	571.25	<.001
Biotype	2		0.012903	0.006452	1.88	0.171
Water potential.Biotype	6		0.076368	0.012728	3.70	0.007
Residual	29	(7)	0.099665	0.003437		
Total	40	(7)	5.811290			

Tables of means

Grand mean 0.6760

Water potential	-1.4 MPa	-2.8 MPa	-4.2 MPa	-0.7 MPa
	0.8751	0.5744	0.1603	1.0942

Biotype	10.	280.	392.
	0.6984	0.6597	0.6699

Water potential	Biotype	10.	280.	392.
-1.4 MPa		0.8958	0.9054	0.8241
-2.8 MPa		0.5706	0.5503	0.6022
-4.2 MPa		0.1396	0.1307	0.2106
-0.7MPa		1.1877	1.0524	1.0425

Standard errors of differences of means

Table	Water potential	Biotype	Water potential Biotype
rep.	12	16	4
d.f.	29	29	29
s.e.d.	0.02393	0.02073	0.04145

Least significant differences of means (5% level)

Table	Water potential	Biotype	Water potential Biotype
rep.	12	16	4
d.f.	29	29	29
l.s.d.	0.04895	0.04239	0.08478

B) EFFECT OF SOLUTE ON GROWTH OF INDIVIDUAL BIOTYPES

Analysis of variance –BIOTYPE 10

Variate: Growth rate

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Media	9		7.989297	0.887700	489.05	<.001
Residual	35	(2)	0.063530	0.001815		
Total	44	(2)	8.045631			

Tables of means

Grand mean 0.8262

Media	-1.4 MPa (Gly)	-1.4MPa (KCl)	-1.4MPa (PEG)	-2.8 MPa (Gly)	-2.8 MPa(KCl)
	1.0060	0.8420	1.2250	0.9580	0.5800

Media	-2.8 MPa (PEG)	-4.2 MPa (PEG)	-7.2 MPa(Gly	-7.2 MPa(KCl)	-0.7MPa
	0.8849	0.5750	0.1400	0.4380	1.6540

Least significant differences of means (5% level)

Table	Media
rep.	
d.f.	35
l.s.d.	0.06116

Analysis of variance -BIOTYPE 280

Variate: Growth rate

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Media	9		6.903256	0.767028	321.98	<.001
Residual	35	(2)	0.083377	0.002382		
Total	44	(2)	6.454724			

Tables of means

Grand mean 0.8391

Media	-1.4 MPa (Gly)	-1.4 MPa (KCl)	-1.4 MPa (PEG)	-2.8 MPa (Gly)	-2.8 MPa (KCl)
	0.8020	1.2040	1.2367	0.5760	1.0660

Media	-2.8 MPa (PEG)	-4.2 MPa (PEG)	-7.2 MPa (Gly)	-7.2 MPa (KCl)	-0.7 MPa
	0.8725	0.5700	0.1400	0.5240	1.4325

Least significant differences of means (5% level)

Table	Media
rep.	
d.f.	35
l.s.d.	0.07006

Analysis of variance- BIOTYPE 392

Variate: Growth rate

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Media	9		5.507943	0.611994	82.51	<.001
Residual	33	(4)	0.244762	0.007417		
Total	42	(4)	5.274665			

Tables of means

Grand mean 0.864

Media	-1.4 MPa (Gly)	-1.4 MPa (KCl)	-1.4 MPa(PEG)	-2.8 MPa (Gly)	-2.8 MPa (KCl)
	1.004	1.036	1.140	0.810	1.030

Media	-2.8 MPa (PEG)	-4.2 MPa (PEG)	-7.2 MPa (Gly)	-7.2 MPa (KCl)	-0.7MPa
	0.913	0.618	0.140	0.558	1.405

Least significant differences of means (5% level)

Table	Media
rep.	
d.f.	33
l.s.d.	0.1239

Effects of osmotic and matric potential on the accumulation of endogenous reserves in three biotypes of *P. chlamydosporia* (biotypes 10, 280 and 392)

(a) Total polyols in osmotically modified media (Glycerol and KCl) 25 days

Analysis of variance

Variate: LOG (total polyols)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Media	4	16.0460	4.0115	28.47	<.001
Biotype	2	0.1877	0.0939	0.67	0.521
Media. Biotype	8	1.9753	0.2469	1.75	0.127
Residual	30	4.2275	0.1409		
Total	44	22.4365			

Tables of means

Grand mean 9.837

Media	PDA+gly (-1.4)	PDA+gly (-2.8)	PDA+gly (-7.1)	PDA+KCl (-1.4)
	10.607			9.281

Media	PDA+KCl (-2.8)	PDA+KCl (-7.1)	unmodified
	9.786	10.413	9.098

Biotype	10.	280.	392.
	9.871	9.747	9.894

Media	Biotype	10.	280.	392.
PDA+gly (-1.4)		10.309	10.878	10.633
PDA+KCl (-1.4)		9.378	9.194	9.271
PDA+KCl (-2.8)		10.110	9.497	9.751
PDA+KCl (-7.1)		10.298	10.093	10.849
Unmodified		9.259	9.071	8.963

Standard errors of differences of means

Table	Media	Biotype	Media Biotype
rep.	9	15	3
d.f.	30	30	30
s.e.d.	0.1770	0.1371	0.3065

Least significant differences of means (5% level)

Table	Media	Biotype	Media Biotype
rep.	9	15	3
d.f.	30	30	30
l.s.d.	0.3614	0.2799	0.6260

(b) Total polyols in matrically modified media (PEG) 25 days

Analysis of variance

Variate: LOG(Total Polyols)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		1.59955	0.79977	12.88	<.001
Media	3		48.95991	16.31997	262.80	<.001
Biotype.Media	6		1.14701	0.19117	3.08	0.024
Residual	22	(2)	1.36621	0.06210		
Total	33	(2)	52.08613			

Tables of means

Grand mean 10.589

Biotype	10	280	392
	10.852	10.579	10.336

Media	Peg (-1.4)	Peg (-2.8)	Peg (-4.2)	Unmodified
	9.912	11.221	12.125	9.098

Biotype	Media	Peg (-1.4)	Peg (-2.8)	Peg (-4.2)	Unmodified
10		10.393	11.272	12.483	9.259
280		9.892	11.087	12.267	9.071
392		9.451	11.304	11.624	8.963

Standard errors of differences of means

Table	Biotype	Media	Biotype Media
rep.	12	9	3
d.f.	22	22	22
s.e.d.	0.1017	0.1175	0.2035

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	12	9	3
d.f.	22	22	22
l.s.d.	0.2110	0.2436	0.4220

(c) Osmotically modified media – Glucose. 25 days

Analysis of variance

Variate: LOG(glucose)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		4.3880	2.1940	6.04	0.006
Media	5		22.5288	4.5058	12.40	<.001

Biotype.Media	10		8.8379	0.8838	2.43	0.026
Residual	34	(2)	12.3515	0.3633		
Total	51	(2)	47.0049			

Tables of means

Grand mean 8.715

Biotype	10.	280.	392.
	9.109	8.444	8.593

Media	PDA+gly (-1.4)	PDA+gly (-2.8)	PDA+gly (-7.1)	PDA+KCl (-1.4)
	9.675	9.309		8.300

Media	PDA+KCl (-2.8)	PDA+KCl (-7.1)	unmodified
	8.223	8.944	7.841

Biotype	Media	PDA+gly (-1.4)	PDA+gly (-2.8)	PDA+gly (-7.1)	PDA+KCl (-1.4)
10.		9.856	10.380		9.216
280.		9.565	8.915		7.937
392.		9.602	8.631		7.746

Biotype	Media	PDA+KCl (-2.8)	PDA+KCl (-7.1)	unmodified
10.		8.571	8.497	8.136
280.		7.981	8.578	7.688
392.		8.118	9.759	7.701

Standard errors of differences of means

Table	Biotype	Media	Biotype Media
rep.	18	9	3
d.f.	34	34	34
s.e.d.	0.2009	0.2841	0.4921

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	18	9	3
d.f.	34	34	34
l.s.d.	0.4083	0.5774	1.0001

(d) Matrically modified media (PEG) - Glucose

Analysis of variance

Variate: LOG(glucose)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Biotype	2		1.6098	0.8049	3.68	0.044
Media	3		33.3854	11.1285	50.87	<.001
Biotype.Media	6		5.5677	0.9280	4.24	0.006
Residual	20	(4)	4.3752	0.2188		

Total	31	(4)	37.2269

Tables of means

Grand mean 9.314

Biotype	10	280	392
	9.552	9.353	9.038

Media	Peg (-1.4)	Peg (-2.8)	Peg (-4.2)	Unmodified
	9.109	10.364	9.943	7.841

Biotype	Media	Peg (-1.4)	Peg (-2.8)	Peg (-4.2)	Unmodified
10		9.771	10.814	9.487	8.136
280		8.565	10.354	10.807	7.688
392		8.990	9.925	9.536	7.701

Least significant differences of means (5% level)

Table	Biotype	Media	Biotype Media
rep.	12	9	3
d.f.	20	20	20
l.s.d.	0.3983	0.4599	0.7966

Analysis of endogenous reserves in *P. chlamydosporia* spores produced in standard and modified media (biotype 10)

(a) Total Polyols

Analysis of variance

Variate: Total Polyols

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	10003461.	5001731.	0.80	0.462
Residual	21	131032497.	6239643.		
Total	23	141035958.			

Tables of means

Variate: Total Polyols

Grand mean 9604.

Treatment	0%	10%	20%
	9284.	10505.	9024.

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	1249.0

Least significant differences of means (5% level)

Table		Treatment
rep.		8
d.f.		21
l.s.d.	2597.4	

(b) Glucose

Analysis of variance

Variate: glucose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Treatment	2		1792696.	896348.	0.04	0.958
Residual	20	(1)	418962869.	20948143.		
Total	22	(1)	420622337.			

Tables of means

Variate: glucose

Grand mean 6460.

Treatment	0%	10%	20%
	6767.	6512.	6103.

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	20
s.e.d.	2288.5

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	20
l.s.d.	4773.6

(c) Glycerol

Analysis of variance

Variate: glycerol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	7255924.	3627962.	1.03	0.373
Residual	21	73739103.	3511386.		
Total	23	80995027.			

Tables of means

Grand mean 2324.

Treatment	0%	10%	20%
	2252.	3030.	1689.

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	936.9

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	21
l.s.d.	1948.5

(d) Arabitol

Analysis of variance

Variate: Arabitol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	3457629.	1728815.	0.88	0.431
Residual	21	41443450.	1973498.		
Total	23	44901080.			

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	702.4

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	21
l.s.d. 1460.7	

(e) Erythritol

Analysis of variance

Variate: erythritol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	44298.	22149.	0.34	0.718
Residual	21	1379975.	65713.		
Total	23	1424273.			

Tables of means

Grand mean 2201.

Treatment	0%	10%	20%
	2261.	2173.	2167.

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	128.2

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	21
l.s.d.	266.5

(f) Mannitol

Analysis of variance

Variate: mannitol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	1033202.	516601.	0.32	0.732
Residual	21	34191265.	1628155.		
Total	23	35224467.			

Tables of means

Variate: mannitol

Grand mean 2804.

Treatment	0%	10%	20%
	2897.	2517.	2999.

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	638.0

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	21
l.s.d. 1326.8	

(g) Viability

Analysis of variance

Variate: Viability (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	1454.40	727.20	10.16	<.001
Residual	69	4939.36	71.58		
Total	71	6393.77			

Tables of means

Grand mean 77.20

Treatment	10% Glycerol	20 % Glycerol	Unmodified
	71.02	79.01	81.57

Standard errors of differences of means

Table	Treatment
rep.	24
d.f.	69
s.e.d.	2.442

Least significant differences of means (5% level)

Table	Treatment
rep.	24
d.f.	69
l.s.d.	4.872

(h) Quantity of chlamydospores per gram of rice extracted

Analysis of variance

Variate: Quantity of spores per g of rice

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	1507.	754.	0.66	0.525
Residual	21	23833.	1135.		
Total	23	25340.			

Tables of means

Grand mean 85.6

Treatment	10% Glycerol	20% Glycerol	Unmodified
	93.1	74.6	89.0

Standard errors of differences of means

Table	Treatment
rep.	8
d.f.	21
s.e.d.	16.84

Least significant differences of means (5% level)

Table	Treatment
rep.	8
d.f.	21
l.s.d.	35.03

APPENDIX VI to Chapter 7 - Outputs from statistical analysis

GenStat Eighth Edition (Service Pack 1)
GenStat Procedure Library Release PL16.1

Kendall's coefficient of concordance

Variates: Root colonisation, proteases, chitinases, lipases, esterases, egg parasitism (*Meloidogyne*), egg parasitism (*G. pallida*),

Coefficient: 0.110
Adjusted for ties: 0.113
Sample size: 9
Number of samples: 7
Sum of squares: 324.00
Chi-square: 6.32
Degrees of freedom: 8
Probability: 0.611

Ranks

Biotype / Root col.	Proteases	Chitinases	Lipases	Esterases	Egg parasitism (*Meloidogyne*)	Egg parasitism (*G. pallida*)	Mean
5.0	3.0	5.0	6.0	3.0	9.0	6.0	5.3
10							
9.0	9.0	9.0	3.0	8.0	6.0	3.0	6.7
16							
4.0	7.0	3.0	3.0	6.0	1.0	5.0	4.1
60							
1.0	5.0	6.0	3.0	9.0	3.0	4.0	4.4
104							
8.0	8.0	7.0	3.0	5.0	4.0	1.0	5.1
132							
2.0	6.0	8.0	3.0	4.0	8.0	9.0	5.7
280							
3.0	4.0	2.0	7.0	7.0	5.0	2.0	4.3
392							
7.0	2.0	4.0	8.0	2.0	7.0	8.0	5.4
399							
6.0	1.0	1.0	9.0	1.0	2.0	7.0	3.9
400							

Spearman's rank correlation coefficient

Sample size = 9
Degrees of freedom = 7

Exact critical values for two-sided test:

probability	0.100	0.050	0.020	0.010	0.005	0.002	0.001
critical value	0.600	0.700	0.783	0.833	0.867	0.917	0.933

Correlation matrix (adjusted for ties)

Chitinases	1	1.000						
Egg parasitism (*Meloidogyne*)	2	0.450	1.000					
Egg parasitism (*G. pallida*)	3	-0.117	0.333	1.000				
Esterases	4	0.400	-0.233	-0.650	1.000			
Lipases	5	-0.749	0.073	0.329	-0.676	1.000		
Proteases	6	0.717	-0.100	-0.533	0.667	-0.895	1.000	
Root colonisation	7	0.183	0.083	-0.233	-0.267	0.146	0.200	1.000
		1	2	3	4	5	6	7

P-values

Chitinases	1	*						
Egg parasitism (*Meloidogyne*)	2	0.224	*					
Egg parasitism (*G. pallida*)	3	0.765	0.381	*				
Esterases	4	0.286	0.546	0.058	*			
Lipases	5	0.020	0.852	0.388	0.046	*		
Proteases	6	0.030	0.798	0.139	0.050	0.001	*	
Root colonisation	7	0.637	0.831	0.546	0.488	0.708	0.606	*
		1	2	3	4	5	6	7