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BIOLOGICAL CONTROL OF CLOVER CYST NEMATODE *HETERODERA TRIFOLII*

A thesis presented in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
in Plant Science
(Plant Pathology)
at

Massey
University

Palmerston North
New Zealand

Pyone Pyone Kyi

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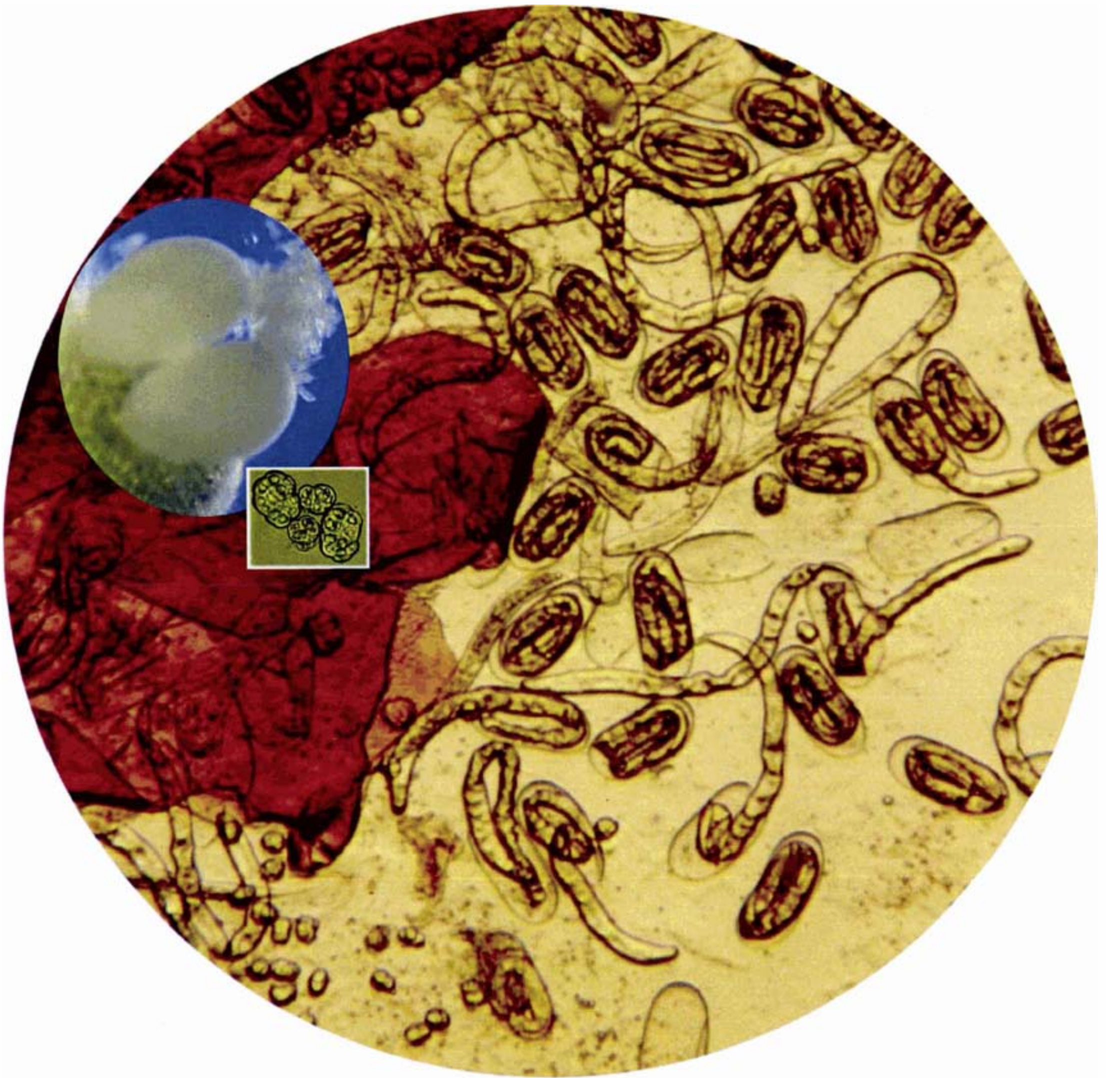

ABSTRACT

Heterodera trifolii is one of the most damaging pests in New Zealand pastures and the aim of this work was to study the potential use of 'biological control' as an alternative strategy to the use of chemicals. Natural enemies, especially fungi, isolated from *H. trifolii* were the main organisms investigated for control of *H. trifolii* in this study. As a first step, more information was required on population dynamics of this nematode species in pasture soil and the possible causes of its population peaks and troughs. Soil cores were taken at fortnightly intervals from a permanent white clover/ryegrass pasture at AgResearch, Palmerston North, New Zealand, from March 1999 to March 2001 and numbers of second stage juveniles (J2), adult females and cyst stages of *H. trifolii* were recorded together with those of other soil nematodes. Emergence of J2 peaked in winter in both 1999 and 2000, and appeared to follow root growth, which in turn reflected soil moisture levels.

Young cysts and mature females were assessed for parasitism by fungi and such fungi were isolated into pure culture as a first stage in assessing their biological control potential. Fungal genera such as *Verticillium, Fusarium*, *Gliocladium*, *Paecilomyces*, and *Trichoderma* were assessed for their pathogenicity to white clover seedlings *Trifolium repens* on 1.0% water agar *in vitro* then in sand in pots. As these fungal isolates were not pathogenic to the *T. repens* plants in pots, some, such as species of *Fusarium*, *Gliocladium*, and *Verticillium* were tested for their potential parasitism on *H. trifolii* in pots of sand with a view to assessing their use as biological control agents. Oatmeal was one substrate on which fungi were grown but it caused poor growth of *T. repens*.

On the basis of these experiments, only isolate Vc6 (a *Verticillium chlamydosporium* isolate) consistently reduced the numbers of *H. trifolii* and was selected for further experiment. Vc6 was grown on a range of media such as alginate beads, bran culture alginate beads, potato dextrose broth culture alginate beads, dry soil inoculum and wheat flour/sand inoculum and it was assessed for its potential pathogenicity to *H. trifolii* females and cysts. Vc6 grown on alginate beads containing wheat bran significantly reduced the numbers of females and of cysts and it increased plant growth of *T. repens.*

There were more than 100 *V. chlamydosporium* isolates from the two-year study so there was a need to screen the isolates for biocontrol activity as they varied in the production of chlamydospores from which eggs of adult females are parasitised. For screening experiments, clover cyst nematode was successfully cultured on 0.5% Hoagland & Knop's agar monoxenically using J2 surface-sterilised with 0.5% Hibitane in a watch glass. A number of *V. chlamydosporium* isolates obtained from young cysts and females of *H. trifolii* in the two-year field study were screened for pathogenicity to *H. trifolii* in monoxenic culture using *T. repens* seedlings on 0.5% Hoagland & Knop's agar, and in sand in pipette tips *in vitro*. Females of *H. trifolii* developed in some of the *V. chlamydosporium* isolate treatments and it was concluded that there were some variations in their pathogenicity to *H. trifolii* in the *in vitro* tests.

In addition to screening the isolates for their parasitism to *H. trifolii in vitro*, variation among the isolates was investigated at the molecular level using the RAPD PCR-based technique. Cluster analysis of 10 *V. chlamydosporium* isolates using RAPD PCR data showed that isolate Vc6 consistently differed from other *V. chlamydosporium* isolates tested.

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To May May Gyi, Phae Phae and May May I dedicate this thesis.

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1 GENERAL INTRODUCTION

1.1 White clover

The genus *Trifolium* (Leguminosae, Papilionaceae) includes approximately 250 annual and perennial species, commonly called clovers, which are native to the humid, temperate regions of the world (Evans 1976). About ten species are agriculturally important including *Trifolium repens* L. (white clover) and *Trifolium pratense* L. (red clover).

White clover is cultivated worldwide and is the major forage legume in the genus *Trifolium* (Frame et al. 1998). Growth of white clover in New Zealand pastures has four major benefits. It: (1) fixes nitrogen by rhizobial nodules, (2) improves nutritive value of sward, (3) complements seasonal growth patterns of commonly used grass species, and (4) improves forage intake and utilization rates of animals (Caradus et al. 1995). White clover contributes directly to animal production through its high digestibility (Thomson 1984). Compared with pasture grasses, white clover herbage is less fibrous and has a higher ratio of soluble to insoluble carbohydrate; these attributes result in rapid growth of stock on clover-dominant pasture. *T. repens* phenotypic plasticity allows it to adapt to a wide range of environments in continental, Mediterranean, oceanic and subalpine regions; this adaptability extends to the micro-environments found across a single pasture (Watson & Barker 1993). The estimated total contribution of white clover to the New Zealand economy is more than $3 billion (Caradus et al. 1995).

The principal aim of the work described in this thesis was to assess the possibility of biological control of *H. trifolii* on white clover in New Zealand. Monitoring of population dynamics of *H. trifolii* was undertaken to determine population peaks, activity, its possible causes and relation to environmental factors.

1.1.1 Pests and diseases

In New Zealand pastures, pests cost hundreds of millions of dollars per annum in lost production. Several pests on white clover in New Zealand have been listed (Gaynor & Skipp 1987). Of those, caterpillars of *Wiseana* spp. (Lepidoptera: Hepialidae), the larvae of Tasmanian grass grub *Aphodius tasmaniae* (Coleoptera:

Scarabaeidae), the larvae of the native grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae), slugs *Deroceras reticulatum* (Mollusca: Agriolimacidae), and lucerne flea *Sminthurus viridis* (Collembola: Sminthuridae) all feed on white clover and are well known to farmers in New Zealand (Kain et al. 1979a, b, c). Larvae of the clover root weevil *Amnemus quadrituberculatus* (Coleoptera: Curculionidae) also significantly damage nodules and reduce foliar nitrogen content in both pure and mixed swards (Gerard 2002).

White clover is a host for numerous pathogenic microorganisms, and many aspects of its growth, and thus its role in livestock production can be affected by disease. Fungal diseases recorded in New Zealand are clover rot caused by *Sclerotinia trifoliorum* (Latch & Skipp 1987), stolon rots caused by *Fusarium*, *Rhizoctonia*, *Phoma*, *Phomopsis* and *Cylindrocarpon* (Latch & Skipp 1987, R. A. Skipp & M. J. Christensen unpublished data), root rots caused by several *Fusarium* species (including *F. oxysporum*), *Codinaea fertilis*, *Ceratobasidium cornigerum*, *Thielaviopsis basicola*, dark, sterile mycelium of an unknown fungus, species of *Cylindrocarpon*, *Phoma*, *Chrysosporium*, and *Pythium* (Skipp & Christensen 1981, Skipp et al. 1982). Furthermore, *Verticillium* spp., *Phytophthora* spp., and *Macrophomina* spp. are also common clover pathogens in New Zealand (Skipp et al. 1986).

Waipara et al. (1996) found that mean root and shoot dry weights were lower in white clover plants inoculated with conidia of *C. fertili* from roots of white clover in New Zealand pasture, in pot experiments. Total dry matter yield difference of white clover was 2-fold in high vigour plants than low vigour for the two months and more fungi were isolated from low vigour white clover roots than high vigour in a white clover/ryegrass pasture in Bay of Plenty (Sarathchandra et al. 2000).

Dry matter weights of white clover were reduced by the soil-borne pathogenic fungi *Rhizoctonia solani* and *F. oxysporum* (Choi et al. 1999). Pathogens of the genera *Fusarium*, *Rhizotonia*, and *Phoma* are frequently reported in white clover in Sweden (Lager & Gerhardson 2002, Zahid et al. 2001a). *Sclerotinia trifoliorum*, *Pseudopeziza trifolii*, *Leptosphaerulina trifolii*, *Uromyces* spp., *Peronospora trifoliorum*, *Cymadothea trifolii* and *Erysiphe trifolii* caused damage to white clover in the UK (Clements & Cook 1998). In Australia, Zahid et al. (2001b) found species of *Fusarium*, *Gliocladium*, *Codinaea, Alternaria*, *Colletotrichum*, *Drechslera*, *Rhizoctonia*, *Phoma*, *Pythium*, *Phytophthora*, *Penicillium*, *Rhizopus* and *Trichoderma* on roots and stolons of white

clover. Fungal rots of roots and stolons were most severe in white clover-based dairy pastures.

Latch & Skipp (1987) listed 23 virus species on white clover internationally. In New Zealand, alfalfa mosaic virus (AMV) and white clover mosaic virus (WCMV) are among the more important. Both of these reduced herbage yield by over 25% in the glasshouse. Important clover viruses that have spread around the world include bean yellow mosaic virus (BYMV), pea common mosaic virus (PCMV), red clover vein mosaic virus (RCVMV), red clover mottle virus (RCMV), red clover necrotic mosaic virus (RCNMV), and (WCMV) (Gerhardson & Lindsten 1973, Smith et al. 1985).

1.1.2 Nematodes

Plant-parasitic nematodes utilize a wide range of plants, and white clover, with its high nitrogen content, is no exception. Plant feeding by nematodes can be associated with pathology, yield reduction and economic loss, particularly when the host plant is under some additional form of stress (e.g. nutrient, water, defoliation, other pests and diseases).

Nematodes from New Zealand pastures identified by Yeates (1984) included plant-parasitic nematode genera belonging to Tylenchida (*Aphelenchoides*, *Aphelenchus*, *Ditylenchus*, *Heterodera*, *Paratylenchus*, *Pratylenchus*, *Tylenchorhynchus*, *Helicotylenchus*) and soil nematodes belonging to Rhabditida, and Dorylaimida. *Heterodera trifolii* occurred in 75% of white clover-based pastures in New Zealand, *Meloidogyne* spp. have been detected on white clover in 58% and *Pratylenchus* spp. in 48% of New Zealand pastures (Skipp & Christensen 1983).

Plant-parasitic nematodes detected on white clover worldwide include *H. trifolii*, *Ditylenchus dipsaci*, *Meloidogyne* spp., *Pratylenchus* spp., and *Heterodera daverti* (Cook & Yeates 1993). Clements & Cook (1998) (in a UK survey) reported that major nematode species were *D. dipsaci*, *H. trifolii*, *Meloidogyne* spp. and *Pratylenchus* spp. The sedentary endoparasitic nematodes *Meloidogyne trifoliophila*, *H. trifolii* and the ectoparasitic nematode *Helicotylenchus dihystera* were the numerically dominant nematodes on white clover in Australia (Zahid et al. 2001b). Mcleish et al. (1997) detected *Meloidogyne* spp. on white clover in 77% of Australian pastures and *Pratylenchus* spp. in 94%.

The endoparasitic nematodes *H. trifolii*, *Meloidogyne* spp. and *Pratylenchus* spp. are known to be pathogenic to white clover (Colman 1964) and have been reported to depress clover growth in New Zealand pasture (Yeates 1976, 1977). Plant-feeding nematodes reduce pasture production in New Zealand by around 15% annually, mainly through their effect on white clover (Watson & Mercer 2000). Watson et al. (1985) treated pasture with pesticide and there was an increase of nitrogen fixation by 57% and herbage yield by 13%. The impact of clover nematodes in reducing nitrogen inputs and forage quality in New Zealand is estimated to exceed $1 billion annually in lost production potential in New Zealand (Watson & Mercer 2000). In the U.S., estimated yield loss is 6% in clover pasture, resulting in a loss of US $33 million (Hague 1980).

H. trifolii is one of the most damaging plant-parasitic nematode species in New Zealand pastures (Skipp & Christensen 1983, Watson et al. 1985, Mercer & Woodfield 1986). *H. trifolii* is therefore, an important nematode species to study. It causes reductions in yield, nitrogen fixation and persistence of white clover in pasture (Skipp & Watson 1987, Yeates et al. 1977, Mercer 1994) through general root malfunction (Widdowson et al. 1973, Yeates et al. 1975). The populations of *H. trifolii* second stage juveniles (J2) in 250 ml of soil in areas of low vigour and in high vigour 2 years after establishment of a white clover/ryegrass pasture in the coastal Bay of Plenty region were 42.7 and 9.7, respectively (Sarathchandra et al. 2000).

H. trifolii has been identified on clovers in New Zealand (Grandison 1963), England (Franklin 1939, 1940), Utah (Mcbeth 1938), California (Raski & Hart 1953), Illinois (Gerdeman & Linford 1953), Kentucky (Chapman 1962), Canada (Singh & Norton 1970), British Columbia (Hastings & Bosher 1952), Hawaii (Mulvey 1972), the Netherlands (Oostenbrink 1951), the Estonia (Riispere & Krall 1967) and Japan (Inagaki 1985).

Methods used to control plant pathogenic nematodes include nematicides, resistant varieties, and cultural practices such as crop rotation. Control of pasture nematodes by chemicals is environmentally unacceptable (Skipp & Gaynor 1987). Chemical control is expensive, and there is increasing concern over the safety of nematicides and the threat of groundwater contamination (Thomason 1987).

The research programme on pasture nematodes conducted within AgResearch has included evaluation of management practices that may reduce nematode impacts, selection of white clover seedlines for resistance or tolerance to nematodes, and

identification of agents for biological control of nematodes within New Zealand pastures (Watson & Mercer 2000). Breeding to improve resistance to *H. trifolii* has succeeded in reducing the numbers and fecundity of nematode females on white clover selections (Mercer et al. 1999).

1.2 Life cycle of clover cyst nematode *H. trifolii* (Tylenchida: Heteroderidae)

H. trifolii is a monosexual parthenogenetic species (Triantaphyllou & Hirschmann 1978). It is closely related to *H. schachtii*, *H. galeopsidis*, *H. glycines*, *H. lespedezae*, *H. limonii* and *H. rosii*.

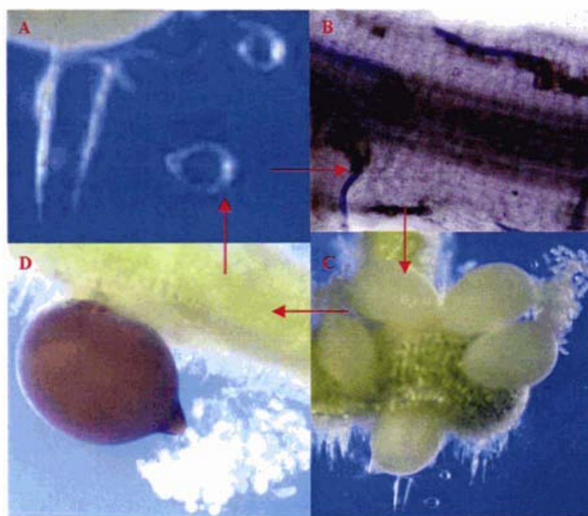

Figure 1-1 Life cycle of *H. trifolii* on sterile *T. repens* roots
(A) Second stage of juvenile (J2), (B) Stained J2 in root, (C) Females, (D) Cyst.

The soil phase of the life cycle starts when the J2 (Fig. 1.1 A) hatches from the egg. *H. trifolii* has four juvenile stages and one adult stage. The J2 hatches from egg, moves through the soil, and penetrates the root cortex (Fig. 1.1 B) and establishes a syncytial feeding site in the stele; the sedentary nematode undergoes three more moults to the adult. The white female enlarges as its gonads increase in size, and it eventually ruptures through the root cortex and epidermis so that its spheroid body lies exposed, with only the head and neck embedded in the root (Fig. 1.1C). Eggs are contained

within the uterus, which extends to fill the body cavity prior to encystment. In *H. trifolii*, up to 200 eggs are extruded from the vulva in a gelatinous matrix and cysts remain dormant in the soil (Thorne 1961). When the female dies, the cuticle tans to form a tough, protective cyst (Fig. 1.1D) containing up to 200 eggs. Mercer (1990) investigated the development of *H. trifolii* on white clover and showed that the number of degree days above 10°C for development to egg-producing females was 244 and for development to the appearance of distinct juveniles inside eggs (taken as completion of one generation) 310 degree days. Following the death of the female, eggs within the cyst may remain viable for at least 180 weeks (Yeates & Visser 1979).

1.3 Population dynamics of *H. trifolii* in New Zealand

Understanding dynamics of plant-parasitic nematode populations is one of the key factors to their control and information on the distribution and population dynamics of *H. trifolii* is essential to allow an assessment of the population status of the nematode.

Many nematode genera showed distinct annual population cycles and the mean annual temperature and rainfall appeared to be a greater influence on the distribution of nematodes than did soil texture (Yeates 1975). There were two reproductive cycles of *H. trifolii* and spring mortality of juveniles in roots in the studies of Yeates (1973) in Wairarapa and Yeates & Risk (1976) in Southland. Occurrence of juveniles in the soil generally preceded root invasion and times of invasion corresponded with the autumn and spring periods of pasture growth, but emergence of juveniles from cysts did not correspond with these periods.

The microorganisms that parasitise or prey on nematodes or reduce nematode populations by their antagonistic behaviour include some fungi that have shown great potential as biological control agents because of their constant association with nematodes in the rhizosphere (Stirling 1991).

To assess the possibility of biological control of *H. trifolii* on white clover under New Zealand conditions, examination of fungi that associate with this nematode is a first step.

1.4 Fungi associated with cyst nematodes

Hay & Skipp (1993) identified microorganisms colonising surface-sterilised *H trifolii* from the North Island of New Zealand, on water agar. Predominant species were *Fusarium oxysporum*, *F. culmorum*, *F. solani*, *Verticillium chlamydosporium*, *Gliocladium roseum*, *Mortierella alpina*, *Paecilomycetes lilacinus*, unidentified *Paecilomyces* spp., *Exophiala pisciphila* and *Trichocladium opacum*.

Fungal parasites are known to attack a number of cyst nematodes, including beet cyst nematode *Heterodera schachtii* (Tribe 1979) and cereal cyst nematode *Heterodera avenae* (Kerry & Crump 1977). An unidentified fungus has been associated with poor multiplication of the potato cyst nematodes *Globodera rostochiensis* in Germany (Goswami & Rumpenhorst 1978) and *V. chlamydosporium* isolated from *G. rostochiensis* released toxic metabolites into the medium and killed males of *G. pallida* and *G. rostochiensis* on agar plates (Saifullah 1996). Eggs of all cyst nematode species were susceptible to *V. chlamydosporium*, parasitised females contain few eggs and those were infected and thus this fungus may be an important potential weapon against such nematodes (Kerry 1981).

Costa et al. (1997) identified *F. solani*, *F. oxysporum*, *Gliocladium viride*, *Scytalidium* spp., *Dactylaria* spp., *Penicillium* spp., *Eurotium repens*, *P. lilacinus* and *P. variotti* from cysts of *H. glycines* in soya bean fields at several sites in Brazil. Chen & Chen (2002) investigated mycofloras in cysts, females and eggs of *H. glycines* from southern Minnesota; cysts were commonly colonized by *Cylindrocarpon destructans*, *C. olidum, F. oxysporum, F. solani*, *Pyrenochaeta terrestris*, females by *F. solani*, *F. oxysporum*, *C. destructans*, *Oidiodendron cerealis*, *P. terrestris*, and eggs by *C. destructans*, *F. oxysporum, F. solani*, *P. terrestris*, *Exophiala pisciphila*.

Alternaria alternata, *Curvularia lunata*, *Paecilomyces lilacinus*, *Syncephalastrum racemosum*, *Trichoderma viride*, *Drechslera tetramera*, *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Trichophyton* spp. and *Monilia* spp. were identified infecting the cysts of *H. oryzicola* from banana crops in Kerala, India (Charles et al. 2000).

Although many genera of fungi colonise eggs, females or cysts of *Heterodera* and *Globodera*, it is necessary to know whether the fungi are pathogenic to the host plants before testing their potential for biological control of nematodes. Therefore,

testing the pathogenicity to white clover of any fungi obtained from *H. trifolii* is essential.

1.5 Biological control of cyst nematodes

As described in Section 1.1.2, there is a need for alternative means of nematode control and biologically-based systems are one way to reduce chemical inputs in agriculture. A range of systems exhibiting nematode suppression involving naturally suppressive soils are known, including control with *V. chlamydosporium*. This fungus was first recognised as a parasite of cyst nematodes after it had been isolated from the eggs of *H. schachtii* (Willcox & Tribe 1974) and *H. avenae* (Kerry 1975).

Cyst nematodes are most effectively controlled by *V. chlamydosporium* when females are parasitised early in their development. *V. chlamydosporium* readily colonises females of cyst nematodes and reduces their fecundity, giving rise to small cysts containing few healthy eggs (Kerry 1990) and is now known to be a common parasite of cyst nematode females and eggs (Kerry & Crump 1977, Vinduska 1982) and one of the fungi responsible for the decline of *H. avenae* populations under intensive cereal cropping in the UK (Kerry et al. 1982).

No evidence showing *V. chlamydosporium* is pathogenic to plants or higher animals has been reported, e.g., *V. chlamydosporium* has been found to colonise the rhizosphere but not invade the root cortex, cause lesions or affect the growth of wheat (Kerry et al. 1984).

Many of the fungal species isolated from *H. trifolii* cysts are known to be colonists of the roots of white clover in New Zealand (Hay & Skipp 1993). In this study, *V. chlamydosporium* was one of the most frequently found fungal species from young cysts of *H. trifolii* and is therefore, more of interest as a potential biocontrol agent of *H. trifolii* than are species of *Fusarium* and *Gliocladium*.

1.6 *V. chlamydosporium (= V. chlamydosporium* var. *chlamydosporium* Goddard = *Pochonia chlamydosporia* var. *chlamydosporia* Zare et al.*)*

In addition to the records from nematodes given above, *V. chlamydosporium* has been isolated from roots of clover and ryegrass in New Zealand (Thornton 1965).

Elsewhere it has also been isolated from snail eggs (Barron & Onions 1966) and from oospores of some fungi (Sneh et al. 1977), so the fungus does not depend solely on nematodes for its nutrition.

V. chlamydosporium is characterized by the remarkable hyaline to yellowish multicellular, resting and propagative chlamydospores (dictyochlamydospores), which are borne on pedicels, and occur together with verticillate conidiophores when the fungus is grown on agar media. The chlamydospores are approximately globose, rectangular box-shaped or irregular, and typically 15-30 x 10-25 µm. Their component cells are initially thin-walled but later become very thick-walled with only a small central lumen remaining inside each cell. In culture, the verticillate conidiophores bear false heads of small hyaline conidia generally subglobose in shape and 2-3 µm in diameter (Bursnall & Tribe 1974, Gams 1971, Domsch et al. 1980).

The resting structures of chlamydospores persist in the soil and act as a selective substratum for fungal colonisation by egg parasites. Certain clavicipitaceous anamorphs, now comprised in the genus *Pochonia* (Zare et al. 2001), are specialised to parasitise such resting structures (Morgan-Jones & Rodriguez-Kabana 1988).

According to Zare et al. (2001), if dictyochlamydospores occurr in a species of the genus *Pochonia*, it is considered to be the species *Pochonia chlamydosporia*. Within this species, if conidia are only in heads, it is described as *P. chlamydosporia* var *chlamydosporia* (*Diheterodspora chlamydosporia* Barron & Onions 1966 or *Verticillium chlamydosporium* var. *chlamydosporium* Goddard 1913), a variety that mainly parasitises cyst nematodes. If the conidia are in chains, it is *P. chlamydosporia* var. *catenulata* (*Diheterospora catenulata* Barron & Onions 1966 or *Verticillium chlamydosporium* var. *catenulata* Gams 1988).

V. chlamydosporium can be cultured readily on a range of standard laboratory media but is relatively slow growing with a mean growth rate on corn meal agar of 1.6 mm/day at the optimum temperature of 25°C. Isolates differ in their temperature optima and in their ability to produce chlamydospores and conidia (Kerry et al. 1986). As repeated subculturing may lead to a decline in chlamydospore production, original isolates need to be freeze dried and stored (Dourhourt et al. 1993). Production of large numbers of chlamydospores of *V. chlamydosporium* has proved difficult (Sykes 1994) and the method of mass culturing of *V. chlamydosporium* for experiments markedly affected the subsequent, survival and proliferation of the fungus in soil (Kerry 1995).

Isolates of *V. chlamydosporium* differred markedly in their growth and sporulation *in vitro* (Irving & Kerry 1986), and in their virulence, saprophytic competitiveness and rhizosphere competence (Kerry & de Leij 1992). Thus, there is a need for careful selection of isolates and laboratory-based screening methods to select the most promising isolates for further testing.

In this study, monoxenic culture of *H. trifolii* was improved to allow screening of a range of *V. chlamydosporium* isolates from *H. trifolii* over a 2-year period.

1.7 Monoxenic culture of nematodes

In vitro culture provides the opportunity to observe nematode behaviour and interaction with the host more closely (Chen et al. 1961, Jones 1980, Lauritus et al. 1983, Aumann 1997) and to observe fungal parasitism of nematode eggs *in situ* (Hay 1993, Meyer et al. 1999, Meyer & Wergin 1998).

A difficulty in gnotobiotic culture is selecting a sterilisation method rigorous enough to kill contaminant bacteria and fungi on the surface of the nematodes without affecting nematode development. Hibitane diacetate has been used successfully to decontaminate various nematode species (Koenning & Baker 1985, Hooper 1986). Hay (1994) and Hay & Renault (1995) used a combination of Hibitane, Penicillin + streptomycin sulphate, and Tween 80 as a disinfectant for surface-sterilisation of *H. trifolii* J2.

Besides screening the isolates for their parasitism to *H. trifolii in vitro*, it would be useful to distinguish variation among the isolates at the molecular level. The PCR technique could be used for this purpose.

1.8 Observation of variation among nematophagous fungi by PCR-based techniques

Three major techniques: randomly amplified polymorphic DNA (RAPD) (Williams et al. 1991), arbitrarily primed PCR (AP-PCR) (Welsh & McClelland 1990), and DNA amplification fingerprinting (DAF) (Caetono-Anolles et al. 1991) have been applied to the study of genomic variation of fungal species. Of those, RAPD has been the most widely used because of its simplicity and wide applicability.

Zare et al. (2000) differentiated the anamorph genus *Verticillium* morphologically and phylogenetically, based on the sequences of the ITS (internal transcribed spacers) regions of nrDNA; one of the clades comprises mainly parasites of nematode eggs or cysts. The PCR-based procedures ERIC (enterobacterial repetitive intergenic consensus) and REP-PCR (repetitive extragenic palindromic) were found to be rapid tools for detection of different isolates of *V. chlamydosporium* within IGS (intergenic spacers) regions of the DNA (Arora et al. 1996). Hirsch et al. (2000) developed a method utilizing specific primers designed from an amplified and cloned fragment of the *V. chlamydosporium* ß-tubulin gene; one of the primer sets could identify the fungus on tomato roots infested with *Meloidogyne* spp. PCR-fingerprinting differentiated *V. chlamydosporium* isolates of Europe from the tropical ones (Sosnowska et al. 2001). Mauchline et al. (2002) first observed growth of *V. chlamydosporium* obtained from potato cyst nematode (PCN), in the rhizosphere of PCN-infested plants by using a PCR-based quantification method.

Although several PCR-based techniques have been used for the detection of *V. chlamydosporium*, it was important to confirm first that the isolates from *H. trifolii* were *V. chlamydosporium*. Differences at the molecular level could then be evaluated. The molecular variation among the *V. chlamydosporium* isolates using ITS and RAPD PCR techniques will be discussed in this thesis.

1.9 Aims of this study

The goal of the work described in this thesis was to assess the possibility of biological control of *H. trifolii* on white clover in New Zealand. Experiments were done to achieve the following aims as a way to reach the goal of this study.

1. Monitor population dynamics of *H. trifolii* to determine population peaks and possible causes of population decline, such as parasititic fungi and environmental factors such as soil moisture and temperature.
2. To investigate whether fungi commonly isolated from cysts of *H. trifolii* can be pathogenic to white clover in agar plates or in pots.
3. To develop the method of Hay's monoxenic culture that could be useful for screening of potential biological control fungal isolates *in vitro* in order to save the time of conducting pot trials in a glasshouse.

4. To determine which of the fungi isolated from *H. trifolii* might be the most effective for control of *H. trifolii* using a glasshouse screening test.

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2 SEASONAL POPULATION DYNAMICS OF THE CLOVER CYST NEMATODE (*HETERODERA TRIFOLII*) AND ASSOCIATED FUNGI

2.1 Introduction

2.1.1 Clover cyst nematode (*Heterodera trifolii*)

Heterodera trifolii Goffart, 1932 (Nematoda: Tylenchida: Heteroderidae) was first described parasitising red clover in Germany (Goffart 1932). *H. trifolii* occurs throughout New Zealand (Grandison 1963, Skipp & Christensen 1983, Mercer & Woodfield 1986). It has been identified principally on clovers and causes reductions in yield, nitrogen fixation and persistence of white clover in pasture (Mercer 1994) through general root malfunction and the accompanying depression of foliage growth (Widdowson et al. 1973, Yeates et al. 1975).

H. trifolii is one of the plant-feeding nematodes, which, together, reduce pasture production in New Zealand by around 15% annually, mainly through their effect on white clover (Watson & Mercer 2000). Nematicide application may increase clover yields in pasture by an average 40% and N-fixation levels by over 50% (Watson et al. 1985). The economic impact of nematodes on clover in reducing nitrogen inputs and forage quality has been estimated to exceed $1 billion annually in lost production potential (Watson & Mercer 2000).

Adequate documentation of the density of plant-parasitic nematode populations is a key factor to assess effectiveness of any control programme. Seasonal variation in plant and soil nematode populations has frequently been correlated with variation in environmental factors such as soil temperature and soil moisture. Sequential sampling of soil nematode populations is necessary to establish temporal patterns of development, but the frequency of such sampling varies with the purpose. For economically important species in crops (e. g. cyst nematodes of the genera *Heterodera* and *Globodera*) pre-and post-cropping samples may be adequate (Southey et al. 1978); more frequent samples may be necessary to follow the life history of such genera (Franklin et al. 1971, Mikhailyukov 1980, Yeates 1978).

A factor apparently limiting a particular nematode population, such as moisture, temperature, soil texture, vegetation, soil chemistry or pathogens, can usually be found in any situation (Evans 1974, Jones 1975). Soil nematode populations are large and diverse. Population dynamics reflect the way in which factors [soil texture, climatic (moisture, temperature), plant (species, productivity) and management factors] interact with the food, temperature and moisture requirements of the various nematodes. Understanding the population dynamics of nematodes in soil may provide a better basis for managing economically important species (Yeates 1981).

The life cycle of *H. trifolii* in New Zealand was described by Yeates (1973). Yeates & Risk (1976) demonstrated two reproductive cycles per year in the field. Invasion by second stage juveniles (J2) occurred in early spring and autumn. Numbers of J2 in roots followed a similar pattern to the numbers in soil and the number of females followed the same trend as the number of J2 in roots. The mortality of larvae in the roots was found to be very high in springtime.

In the Netherlands, *H. trifolii* J2 hatched readily from February to November, and Oostenbrink (1967) presumed they maintained the potential to invade available host tissue. Eggs in immature females and egg matrices both hatched readily providing a rapid means of re-infestation and overlapping generations (Oostenbrink 1967, Gerdeman & Linford 1953). Gerdeman & Linford (1953) found all stages present at all times of the year in Illinois. Similarly, Mulvey (1959a) observed multiple generations of *H. trifolii* and reported that generation time varied from 31 days at 20°C to 45 days at 15.5°C under glasshouse conditions. In another development study, Mulvey (1959b) reported that at 15.5°C females were produced after 26 days and J2 after 43 days. In more detailed studies, Jones (1975) has shown the rate of development of *H. schachtii* is primarily dependent on accumulated effective temperature (i.e. degree-days above 4.4°C at 10-cm soil depth). The peak number of juvenile *Heterodera* in spring in grasslands in Netherlands indicated a distinct annual population cycle which could largely be related to seasonal changes in the temperature and moisture contents of the soil for this genus (Verschoor et al. 2001).

Plant-parasitic nematodes reported as infecting white clover in pastures at Fitzherbert West, Palmerston North, New Zealand were *H. trifolii* (Mercer 1990),

Meloidogyne trifoliophila, *Pratylenchus* sp. (Mercer et al. 1997), and *Ditylenchus dipsaci* (Williams & Barclay 1972).

2.1.2 The role of fungal parasitism of *H. trifolii*

There have been several reports of the natural control of nematodes in intensive agricultural systems in which microbial parasites have increased to densities that have significantly reduced nematode populations. Surveys have found a range of fungi to be associated with cysts of several heteroderid nematode species (Rodriguez-Kabana & Morgan Jones 1988, Stirling 1991). Kerry (1982) reported that populations of the cereal cyst nematode (*H. avenae*) were maintained below an economically damaging threshold by parasitic fungi in some fields in which cereals had been continuously cropped for a number of years.

Costa et al. (1997) found *Dactylaria* spp., *Eurotium repens*, *Fusarium oxysporum*, *F. solani*, *Gliocladium viride*, *Paecilomyces lilacinus* and *P. variotti*, *Penicillium* spp. and *Scytalidium* spp. associated with cyst of *H. glycines* in Brazil. Chen & Chen (2002) observed that *Cylindrocarpon destructans*, *C. olidum*, *F. oxysporum*, *F. solani* and *Pyrenochaeta terrestris* were the most common fungi colonising cyst of *H. glycines* in Minnesota. *Verticillium chlamydosporium* was the most common, but *Verticillium lecanii* and *P. lilacinus* were also found associated with the cyst nematodes *H. avenae* and *H. schachtii* in Spanish soils (Olivares-Bernabeu & Lopez-Llorca 2000).

The soils and plant roots in pastures in New Zealand contain a diverse range of fungi and actinomycetes, many of which are intimately associated with cysts of *H. trifolii*. Some of these fungi may play roles in the population dynamics of *H. trifolii*. Hay & Skipp (1993) identified the predominant species colonising *H trifolii* in New Zealand as *Exophiala pisciphila*, *F. oxysporum*, *F. culmorum*, *F. solani*, *Gliocladium roseum*, *Mortierella alpina*, *P. lilacinus*, unidentified *Paecilomyces* spp., *Trichocladium opacum* and *V. chlamydosporium*. Waipara et al. (2001) found that *Verticillium* infestations of *H. trifolii* was greater in autumn and *Fusarium* was abundant in summer samplings and these species were influenced by seasonal variations of temperature and moisture. In contrast *Gliocladium* spp. were not influenced by seasonal variations as no significant population fluctuations were observed.

Environmental concerns over the use of chemicals in general are increasing and clearly, there is a requirement for alternative means of nematode control. Biologically-based control systems could provide a partial answer to the problem. *H. trifolii* is one of the most damaging plant-parasitic nematode species in New Zealand pastures and using fungal biocontrol agents to control *H. trifolii* is environmentally and economically desirable. It is, thus, important to study the role of biological agents which are naturally associated with *H. trifolii* for biomanagement of nematodes in New Zealand pastures.

2.2 Objectives

(1) To characterise clover cyst nematode populations over 2 years determining the abundance of life stages and correlating these with soil, climate, and plant conditions.

(2) To assess the fungi associated with cyst of *H. trifolii* at each sampling.

2.3 Materials and Methods

2.3.1 The site and sampling

Soil samples were taken with a soil corer (85 mm in depth, 45 mm in diameter) (Fig. 2.1B) at fortnightly intervals from March 1999 to March 2001. One sample (Fig. 2.1 C, D) was collected from each quadrant of a permanent pasture paddock (12. 1 m x 11.7 m) (Fig. 2.1A), at AgResearch Grasslands, Fitzherbert West, Palmerston North, New Zealand (latitude 40.20S and longitude 175.37E, New Zealand Meteorological Service 1983).

The area is free draining Manawatu fine sandy loam (Dystric Fluventic Eutrochrept) overlying deep sands and gravels (Cowie 1978). The site had been cropped with Grasslands Tama annual ryegrass (*Lolium multiflorum* L.) and white clover was the main legume for 5 years (1990-1994) but others have appeared since principally *Bromus mollis*, *Poa* spp., and a little brown top (Brock et al. 2000). It was low in available soil nitrogen and grazed by sheep. The seedbed was prepared over summer, fertilised with superphosphate (250 kg / ha, 9% P, 12% S) and sown by hand on 18-19 April 1995 (mid autumn) (Brock et al. 2000). Average annual rainfall was 991 mm (New Zealand Meteorological Service 1984) and mean annual temperature at 10 cm depth was 12.7°C (New Zealand Meteorological Service 1983).

On each sampling occasion, a further three soil samples (75 mm in depth and 25 mm in diameter), were taken from random points in the same paddock for determination of soil moisture content (method is described in Section 2.3.6).

Figure 2-1 Soil sampling process

(A) White clover/ryegrass pasture, (B) Soil corers, (C) Soil core, (D) Soil cores in plastic bags.

2.3.2 Extraction of second stage juveniles (J2) of *H. trifolii*

Approximately 134 ml soil per core from each of the 4 cores was extracted by a variant of the method of Whitehead and Hemming (1965). One coarse (60 mm x 50 mm) mesh, one fine (1.4 mm x 1.4 mm) mesh and one single layer of a paper towel were used in the tray (Fig. 2.2A). Each sample was spread on a paper towel and water was carefully added at the side of the tray (650-700 ml/tray) until there was sufficient to wet the paper towel (Fig. 2.2B). The tray was left for 24 h to allow time for nematodes to pass through the towel and accumulate in the water underneath. Water was collected

from the tray, poured into a separation funnel (Fig. 2C) and left at room temperature for nematodes to accumulate at the bottom. After 24 h, the nematode suspension was collected in a beaker and left to stand at 5°C for 24 h until nematodes concentrated at the bottom. The supernatant water was removed and 10 ml of nematode suspension collected. An aliquot of 0.5 ml was taken by pipette and transferred to a Doncaster dish. Numbers of *H. trifolii* J2, total nematodes, free living nematodes belonging to orders Rhabditida and Dorylaimida and plant-parasitic nematodes belonging to Tylenchida (*Helicotylenchus*, *Meloidogyne*, *Paratylenchus*, *Pratylenchus*, *Tylenchus*, *Cephalenchus*), and *Aphelenchus* (fungal feeder) were identified to genus using temporary water mounts, and examined at x100 magnification and counted at x60 magnification. The counts were expressed as nematodes per 100 ml soil. Systemic positions of these nematodes are presented in Appendix II.

It should be noted that nematode numbers are not calibrated for extraction efficiency. The Whitehead and Hemming tray technique extracts only 50-70% of vermiform nematodes from the soil (Whitehead & Hemming 1965). The vermiform nematodes could be lost by concentrating the 700 ml suspension to 10 ml then to 0.5 ml. Also, this method depends on the activity of nematodes. Some nematodes just do not have time to leave the soil, some do not move downwards to go through the paper, some do not find a hole in the paper, and some get trapped.

Figure 2-2 Extraction process for vermiform nematodes from soil

(A) Preparation of tray, (B) Soil sample spread on paper towel in tray, (C) Setting funnel.

2.3.3 Extraction of clover cysts

The soil sample from the paper towel in the Whitehead and Hemming tray was transferred to an elutriation tower (Wood & Foot 1977) (Fig. 2.3A), stirred with a stick and elutriated for 2 min. The overflow was passed through stacked 1-mm and 180-µm-aperture sieves (Fig. 2.3B). Cysts retained on the 180-µm sieve were collected in a plastic cup and left for the cysts and soil to sediment. The cysts were poured into a Doncaster dish (Fig. 2.3C) and sorted into black, brown and white cysts, corresponding to old cysts, new cysts, and females respectively, using a dissecting microscope at x40 magnification with incident light (Fig. 2.3D). Counts were converted to numbers per 100 ml of soil. New brown cysts were picked up with fine forceps and stored in distilled water at 5°C for not more than 4 days and then assayed for the detection of fungi (Section 2.3.5).

Soil from the 1-mm sieve was centrifuged using 760.24 relative centrifugal force (RCF) with sugar solution (1.2 kg sugar in 1 litre water) (Southey 1986) to check for any remaining cysts. Because few black or brown cysts (average 50) and females (average 1) were found, this step was used in the first sampling only (March 1999) and was considered unnecessary in subsequent samplings.

Figure 2-3 The extraction of *H. trifolii* cysts and their observation

(A) Extraction of *H. trifolii* cysts using elutriation tower, (B) Sieves through which outflow from tower flowed, (C) Observing *H. trifolii* cysts in a Doncaster dish on a microscope, (D) Using a cold light source for incident illumination.

2.3.4 Staining of clover roots and pasture dry matter yields

Clover roots (Fig. 2.4A) from the 1-mm sieve were stained by boiling for 1 min in 0.05% Aniline Blue solution (300 ml water + 10 ml of stain solution containing 15 g / L of Aniline Blue) (Fig. 2.4B) followed by destaining in acidified glycerol (14 drops of 5N HCl in 500 ml glycerol) (Byrd et al. 1983), then microscopic examination using transmitted light and x60 magnification. Numbers of juvenile and adult *H. trifolii* in the roots were counted together with other plant-parasitic nematodes. *H. trifolii* J2 in roots with no distinct body content were recorded as dead J2. The roots were blot dried with filter paper before weighing. Numbers were expressed as numbers per g of wet weight (glycerol) of roots.

The dry matter yield was taken from sheep-grazed pasture. It was cut in 500 x 80 mm areas about 20 m off the ground, dissected into components (grass, clover, etc.) and dried overnight at 80°C and weighed. Cuts were taken before grazing about every two months.

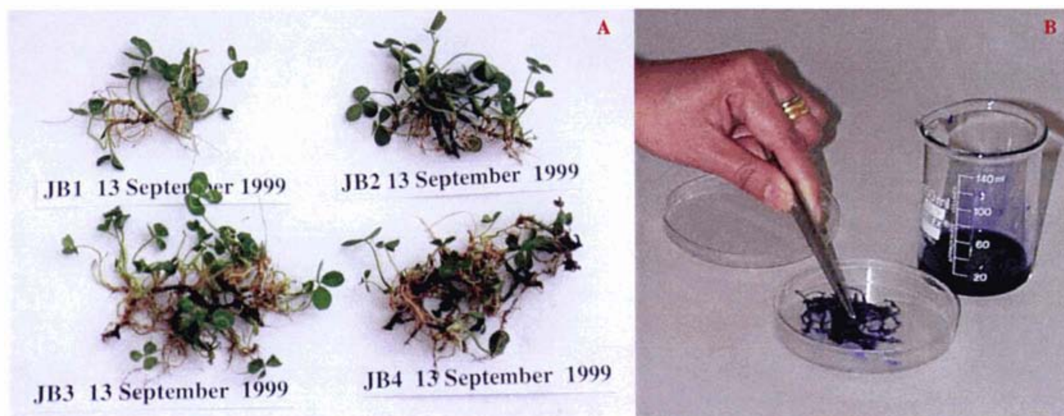


Figure 2-4 Staining of white clover roots
(A) White clover plant parts, (B) Staining and destaining white clover roots.

2.3.5 Culturing fungi from *H. trifolii*

2.3.5.1 Surface-sterilisation of cysts

The suspension of new brown cysts was poured into a Gelman Sciences filter flask apparatus fitted with a Whatman No. 1 filter paper (42 mm diameter). The filter paper had been oven sterilised at 160°C for 4 h. Water was removed by vacuum aspiration and was replaced with 15 ml of 0.3% NaOCl for 2 min. The solution was drawn through the filter and cysts were then rinsed with three changes (aliquots) of 10 ml each of sterilised water (Hay & Skipp 1993).

2.3.5.2 Isolation of the surface-sterilised cysts

A total of 30 surfaced-sterilised new cysts from each sampling was placed on 1.5% tap water agar (containing 10 µg / ml of oxytetracycline) in Petri plates. The plates were incubated at 20°C for up to 2 weeks, being examined every 2 to 3 days. Fungal hyphae or spores growing from cysts were transferred onto potato dextrose agar (PDA) in Petri plates and incubated at 20°C until cultures could be identified (Hay &

Skipp 1993). The fungi grown on PDA were kept at 5°C or in sterile distilled water in McCartney bottles at room temperature.

2.3.5.3 Grouping and identification of fungi from **H. trifolii** *cysts*

Isolates of fungi were grouped on the basis of their morphological characteristics for identification and to determine which were the most common. They were kept at 5°C for subsequent screening for biological control activity.

2.3.6 Soil moisture, temperature and rainfall

The soil from three 25 mm diameter cores was mixed together, crushed, and all plant parts removed. Three 50-ml glass beakers were weighed individually, their weights noted and 10 g of moist soil was added to each beaker. Soil samples were dried at 75°C for 24 h. Dried soil samples were weighed individually and the soil moisture on a dry weight basis at each sampling calculated from the following formula

$$\text{Soil moisture \%} = \frac{100\ (\text{Beaker} + \text{wet soil}) - (\text{Beaker} + \text{dried soil})}{(\text{Beaker} + \text{dry soil} - \text{Beaker}).}$$

Soil moisture content at each sampling was averaged for the three cores.

2.3.7 Soil temperature

Soil temperatures (°C, 10 cm depth at 0900 h) and daily rainfall were obtained from the meteorological station, AgResearch, Fitzherbert West, Palmerston North, New Zealand, and fortnightly values were calculated. This station was 30 m from the population study site.

2.3.8 Statistics

Means and standard errors were calculated using Microsoft Excel.

2.4 Results

As variability in the fortnightly population estimates may obscure longer trends, four-fortnight running averages (Four, individual fortnightly data measurements

averaged) were used to follow the *H. trifolii* population changes over the 2-year period. Individual data are presented in Appendix I.

2.4.1 Second stage *H. trifolii* and other plant-parasitic nematodes in the soil

Low numbers of second stage juveniles in soil in May 1999 were followed by distinct peaks (1176 / 100 ml soil) in August - September 1999 (Fig. 2.5a). After low numbers from October 1999 to February 2000, there was a small, distinct peak in February - March 2000. The numbers decreased in April but gradually increased from May 2000, with high numbers (843 / 100 ml soil) until August 2000. There was a low population from mid-September to March 2001(Fig. 2.5a).

These distinct peaks in numbers of J2 in soil in August to September 1999 and in July to September 2000 were followed by a corresponding increase in J2 numbers in roots. The J2 matured and the population returned to the soil as white females, with the peak numbers in October-November 1999 and in November-December 2000.

The numbers of J2 in soil started to increase in winter 1999 and in January 2000, when the wet root weights started to increase and in general, the numbers of J2 in soil followed the pattern of root growth. The significant increases in numbers of J2 in soil also preceded significant increases in J2 in roots in winter 1999 and in January 2000.

Although the numbers of J2 in soil started to increase in May 2000 when the wet root weight increased, the winter peak of J2 in soil in 2000 did not reflect the wet root weight, which declined in winter 2000. Again, the winter peak of J2 in soil preceded the J2 in roots but the numbers of J2 in roots did not follow the numbers of J2 in soil.

Plant-parasitic nematodes such as *Pratylenchus* sp., *Helicotylenchus* sp., *Paratylenchus* sp. and *Meloidogyne* sp., free-living nematodes belonging to the Orders Dorylaimida, and Rhabditida and other nematodes (without stylets) were found throughout the sampling period (Table 2.1).

2.4.2 Second stage juveniles in clover roots

A small, but noticeable increase in J2 numbers in clover roots was found in May - July 1999 (Fig. 2.5b) followed by a distinct peak (750 / g of root) in August – September 1999. The numbers decreased to November 1999 and remained low until

January 2000. A distinct peak (343 / g of root) in March – May 2000 was followed by low numbers until July 2000 when they again increased with moderate numbers (181 / g of root) in August and November 2000 (Fig. 2.5b). The numbers then slowly decreased until March 2001. Most (50-77%) of second stage juveniles in early October 2000 sampling were dead.

Small peaks in numbers of J2 in roots in late June 1999 and in early May 2000 probably resulted from J2 emergence in May 1999 and March 2000. Although the numbers of J2 in roots were higher in autumn 2000 than those in 1999 because of the May J2 emergence, the average of the numbers in both years was higher in spring than in autumn, possibly reflecting the higher levels of J2 emergence in winter.

Other plant-parasitic nematode, such as, *Pratylenchus* spp., and *Meloidogyne* spp. were commonly found in clover roots throughout the sampling period.

2.4.3 White females in soil

Small peaks in numbers (up to 15 / 100 ml) were observed in April and May 1999, with low numbers in June to July 1999 (Fig. 2.5c). There was a distinct peak (reaching 59 / 100 ml soil) in October to November 1999. The numbers slowly decreased until January 2000 and small increases in numbers were then recorded in February to May 2000. Numbers dropped in June to September 2000 followed by a distinct peak (28 / 100 ml soil) in November to December 2000 and gradual increase to March 2001 (Fig. 2.5c).

2.4.4 New cysts in soil

The high numbers of new cysts in April to July 1999 (40 / 100 ml soil) were followed by a slow decline in numbers until October 1999. The numbers in October 1999 lasted until April 2000 when small increases in numbers (20 / 100 ml soil) occurred in May to July 2000. The numbers then declined slowly and dropped in September to October 2000 followed by a slow increase in numbers in November to February 2001. No distinct peak was observed in this two-year study (Fig. 2.5d).

2.4.5 Old cysts in soil

The numbers of old cysts were high (580 / 100 ml soil) in April and May 1999 and then decreased slowly until February 2000 when a small increase in numbers (197 / 100 ml soil) in March to May 2000 was seen. The numbers decreased again in June 2000 and these were lasting until March 2001. No distinct peak was observed in this two-year study (Fig. 2.5e).

2.4.6 The wet root weight and white clover dry matter yield

A peak (0.3 g / 100 ml soil) of the weight of wet clover roots was seen in May 1999. A high amount of weights (0.2 g / 100 ml soil) in July 1999 was followed by a small amount in September to December 1999. The amount then decreased in January 2000 and increased (up to 0.2 g / 100 ml soil) in February to May 2000 followed by a slow decline until March 2001 (Fig. 2.6b).

White clover dry matter yield (g/m^2) was obtained from AgResearch Grassland Research Centre, Palmerston North). The white clover dry matter yields (g/m^2) were high in spring and summer both in 1999 and 2000 (Fig 2.8).

2.4.7 Soil moisture

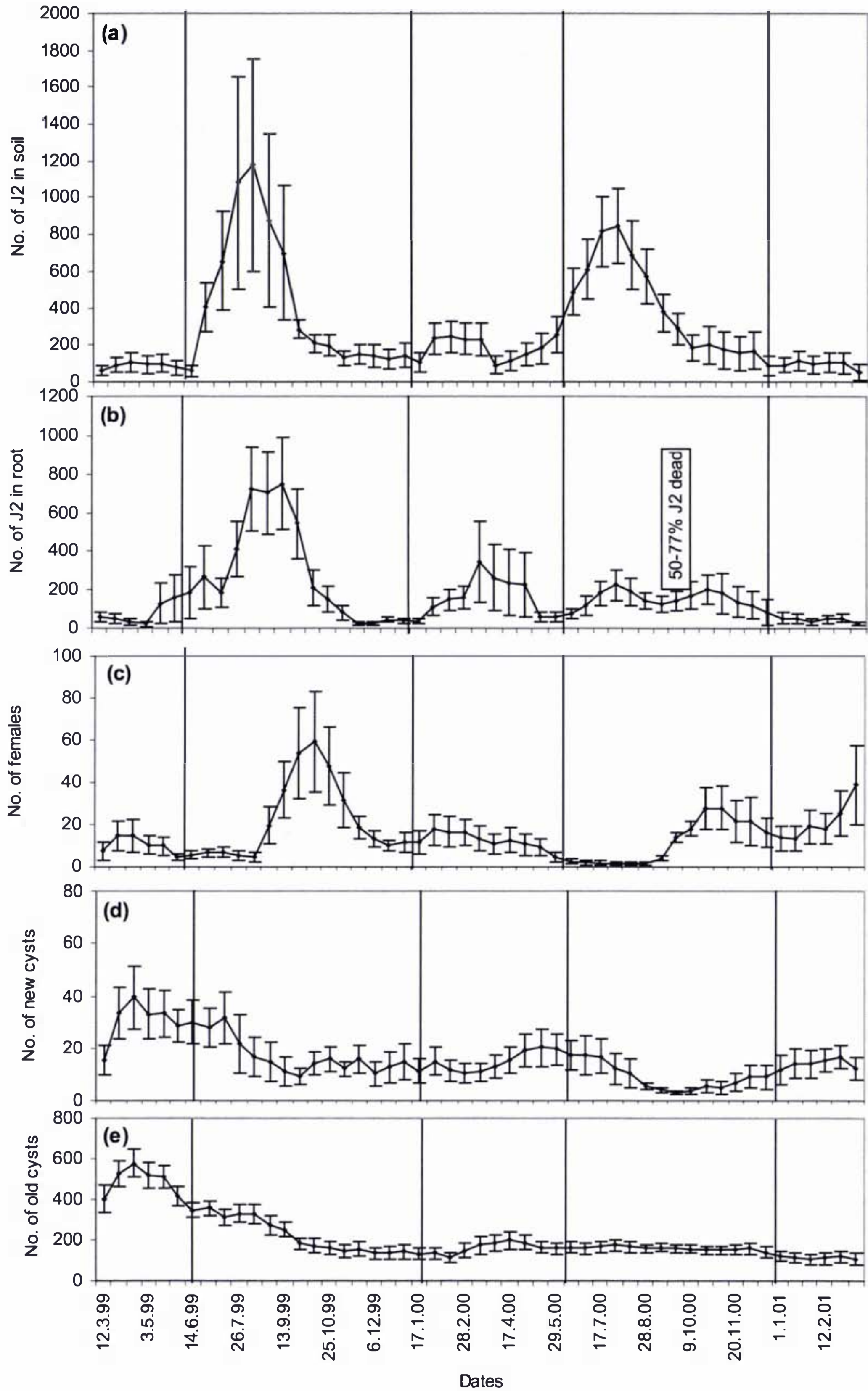
The soil moisture level was high (up to average 31%) in May to August and low (average 13%) in February both in 1999 and 2000. There were small peaks in December 1999 and January 2000 (Fig. 2.6a). The soil moisture started to fall in December 1999 and it started to increase in February 2000 but it fell in August 2000 through to March 2001.

Soil moisture content rose each autumn, was high in winter and fell in late spring/early summer. In both winters, the maximum soil moisture content (gravimetric) was up to 34.5% for individual readings but the averaging reduced these peaks to the low 30s. The biggest difference between a peak and average was on 9 October 2000 when the peak was 34.2% but the averaging reduced this to 25.9%. The consistent readings of 32-34% in winter would indicate that this was field capacity for this soil. Averaging had less effect on low soil moisture content readings with the biggest difference being on 29 January 2001 when the low of 10% was converted to 13.75% by averaging.

Rainfall (mm) was high in winter both in 1999 and 2000, and there was a small peak in January-May 2000 (Fig. 2.7).

2.4.8 Soil temperature

Soil temperature levels were opposite to soil moisture levels, they were high (up to 19°C) in January to February and low (7°C) in June to July both in 1999 and 2000 (Fig. 2.6a).


(a)
No. of J2 in soil
0
200
400
600
800
1000
1200
1400
1600
1800
2000
(b)
No. of J2 in root
0
200
400
600
800
1000
1200
50-77% J2 dead
(c)
No. of females
0
20
40
60
80
100
(d)
No. of new cysts
0
20
40
60
80
(e)
No. of old cysts
0
200
400
600
800
12.3.99
3.5.99
14.6.99
26.7.99
13.9.99
25.10.99
6.12.99
17.1.00
28.2.00
17.4.00
29.5.00
17.7.00
28.8.00
9.10.00
20.11.00
1.1.01
12.2.01
Dates

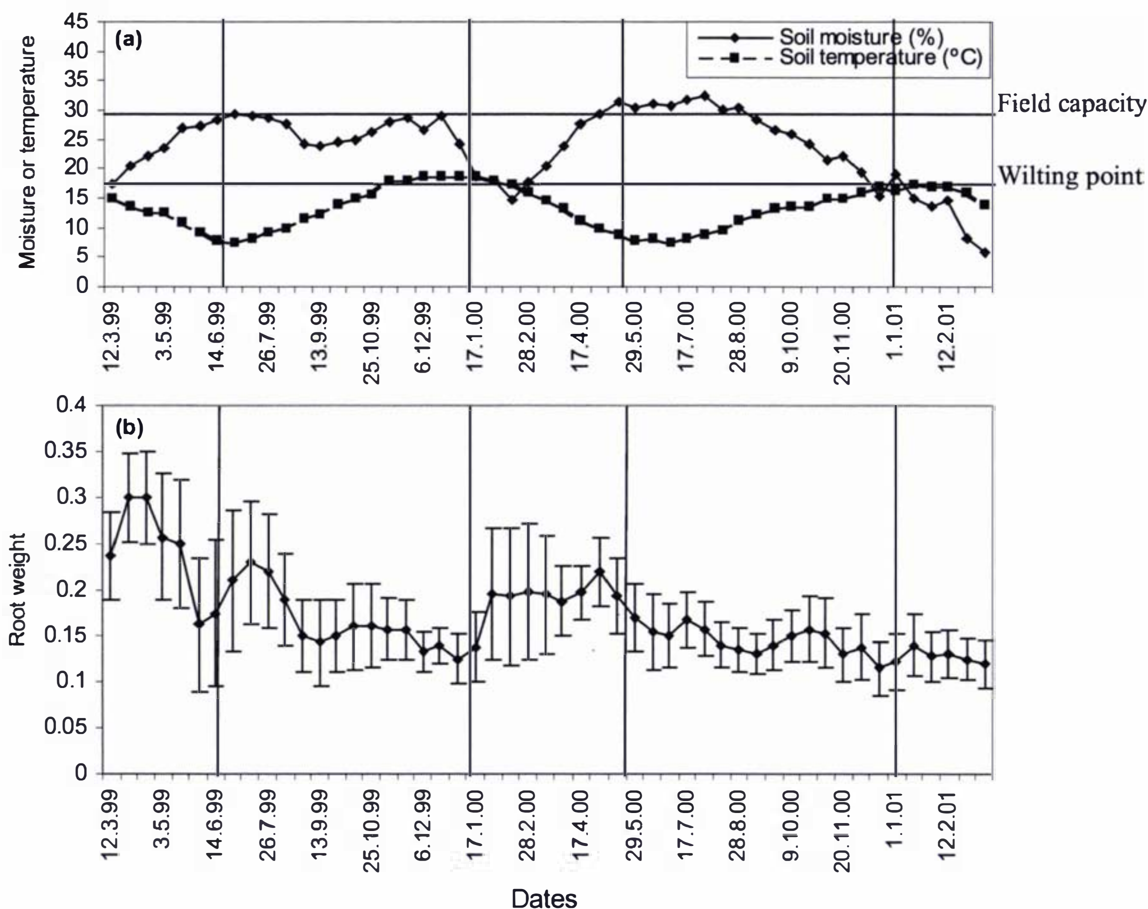


Figure 2-6 (a) Soil temperature (°C, 10 cm soil depth) from the Meteorological records at AgResearch Palmerston North and mean soil moisture levels (% dry weight), (b) Mean wet root weights (g) / 100 ml soil in fortnightly samples from a permanent white clover/ryegrass pasture at Palmerston North from March 1999-March 2001 expressed as a four-fortnight running averages. Bars represent ± SE. (n=4)

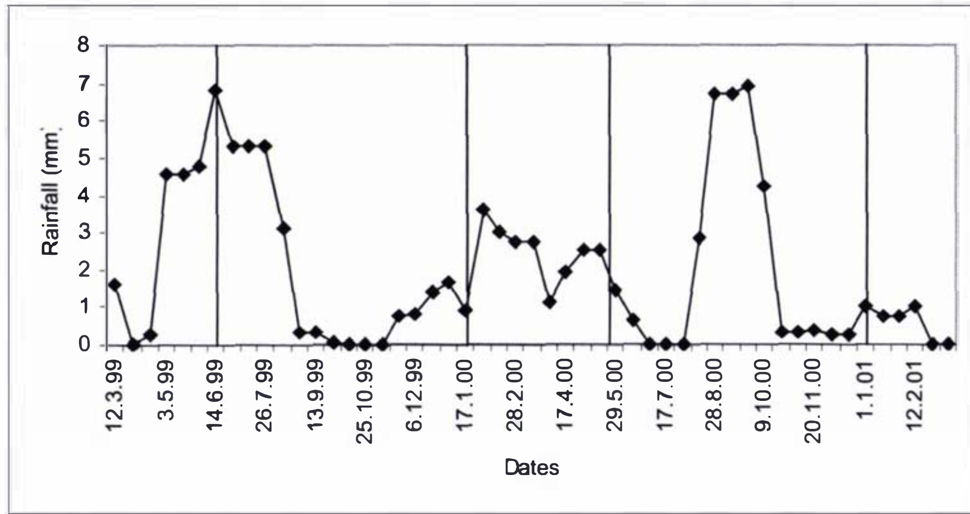


Figure 2-7 Rainfall (mm) from the Meteorological records at AgResearch Palmerston North expressed as a four-fortnight running averages

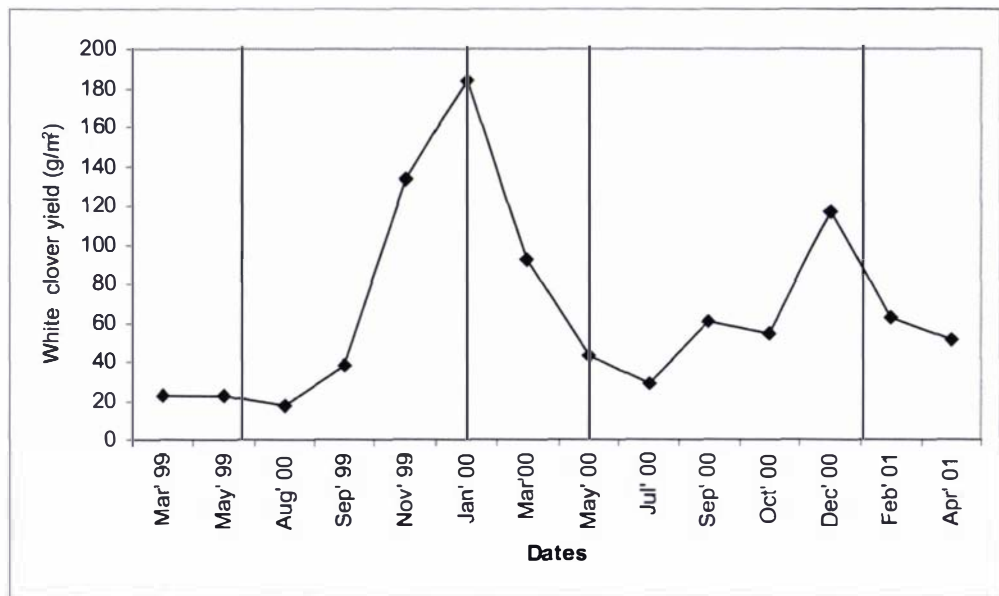


Figure 2-8 White clover yield (dry matter g/m^2)

2.4.9 Fungi from clover cysts

Fungi most frequently isolated from young cysts/females of *H. trifolii* were *Fusarium* spp. (25%), *Verticillium chlamydosporium* (22%) and *Gliocladium roseum* (14%) (Table 2.2).

Percentage isolations of *Fusarium* spp. and *G. roseum* were high in autumn/winter and low in spring/summer but those of *V. chlamydosporium* were high in spring/summer and low in autumn/winter (Fig. 2.9).

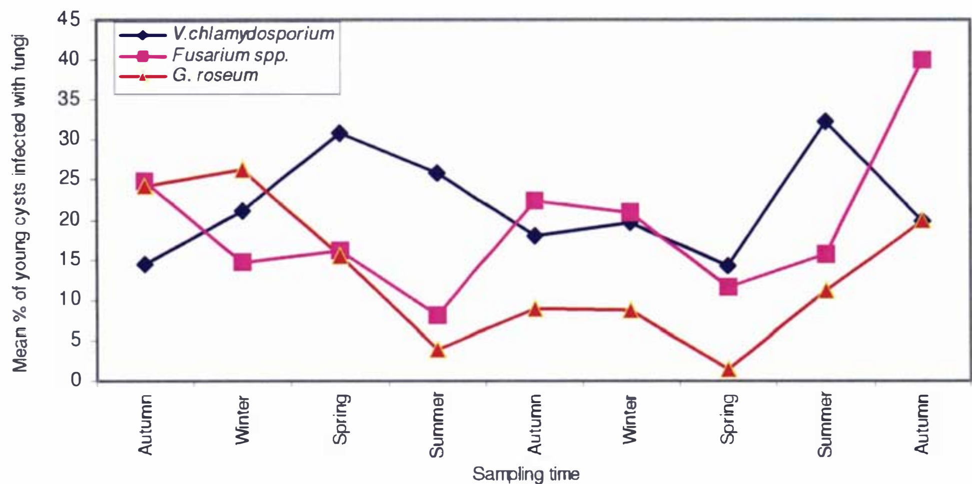


Figure 2-9 Mean percentage of young cysts *H. trifolii* infected with *Fusarium* spp., *Gliocladium roseum*, and *Verticillium chlamydosporium* in a permanent white clover/ryegrass pasture at AgResearch, Palmerston North, New Zealand, over a 2-year period (March 1999-March 2001)

Table 2-1 Nematode counts from fortnightly sampling in a white clover/ryegrass pasture at AgResearch, Palmerston North, over a 2-year period (March 1999 – March 2001)

	Sampling date	Dorylaimida	Rhabditida	Tylenchida						Other nematodes (without stylets)
				1^	2	3	4	5	6	
1	12.3.99	30*	59	0	96	0	0	63	44	52
2	1.4.99	74	936	44	59	0	4	44	19	466
3	19.4.99	422	870	89	185	0	0	248	311	999
4	3.5.99	96	1080	152	93	0	0	104	429	1606
5	17.5.99	167	1558	30	529	0	163	263	289	3108
6	31.5.99	130	1354	41	215	0	89	296	426	1902
7	14.6.99	63	651	41	104	163	74	67	263	707
8	28.6.99	44	562	15	296	56	56	59	237	638
9	12.7.99	93	1080	96	126	4	11	511	178	1458
10	26.7.99	96	1613	144	63	0	59	141	122	992
11	16.8.99	141	1543	74	248	0	59	263	118	2375
12	30.8.99	211	1066	11	159	22	30	189	141	1894
13	13.9.99	252	1669	44	111	70	44	259	155	1798
14	27.9.99	266	1395	89	152	522	107	178	189	2597
15	11.10.99	96	1332	15	167	15	67	215	437	1843
16	25.10.99	337	958	85	74	0	107	366	266	1347
17	8.11.99	385	807	104	63	0	37	244	241	2153
18	22.11.99	122	781	0	18.5	0	44	137	322	818
19	6.12.99	352	1132	163	303	0	26	266	270	2035
20	20.12.99	562	870	44	111	0	329	429	167	1968
21	3.1.00	622	840	126	237	15	26	333	133	2105
22	17.1.00	400	422	22	96.2	185	41	318	126	699
23	31.1.00	1269	940	167	107	0	41	307	178	1621
24	14.2.00	688	625	70	96	41	22.2	444	725	1191
25	28.2.00	1369	525	107	48	33	107	518	126	2146
26	13.3.00	1117	485	137	155	4	37	322	329	2446
27	27.3.00	618	577	19	52	163	15	144	118	640
28	17.4.00	596	629	392	229	0	67	204	118	1421
29	1.5.00	566	814	104	96	26	59	1021	226	1898
30	15.5.00	1143	1154	181	26	0	141	163	163	1817
31	29.5.00	603	1920	133	89	11	44	518	426	2797
32	12.6.00	884	847	141	126	0	1195	588	366	1354
33	26.6.00	829	1343	155	19	0	78	100	159	1602
34	17.7.00	966	1288	130	81	0	74	514	289	2509
35	31.7.00	3271	1029	315	78	19	33	433	237	2375
36	14.8.00	2435	973	56	30	0	553	544	244	2287
37	28.8.00	1536	540	155	37	0	22	459	862	1717
38	11.9.00	1580	544	222	41	41	44	252	625	1761
39	25.9.00	1265	1536	130	33	259	33	215	426	1998
40	9.10.00	2176	899	96	37	440	37	192	518	2250
41	23.10.00	677	1006	37	11	30	70	315	355	781
42	6.11.00	466	581	11	33	44	126	300	322	577
43	20.11.00	955	751	70	74	15	74	440	178	1006
44	4.12.00	685	755	37	33	4	85	488	381	688
45	18.12.00	377	370	22	100	100	11	1302	100	315
46	1.1.01	755	322	26	11	4	63	374	204	463
47	15.1.01	529	311	26	7	4	78	59	52	170
48	29.1.01	259	163	11	0	7	104	0	26	93
49	12.2.01	511	185	19	7	19	26	174	67	126
50	26.2.01	292	155	22	11	44	4	26	26	67
51	13.3.01	229	152	7	19	7	22	15	189	78
	Mean	659	863	87	102	46	91	302	253	1407

^ 1 = *Aphelenchus*, 2 = *Helicotylenchus*, 3 = *Meloidogyne*, 4 = *Paratylenchus*, 5 = *Pratylenchus*, 6 = *Tylenchus* + *Cephalenchus*.

* = Values are numbers per 100 ml soil from Whitehead and Hemming tray extractions.

Table 2-2 Percentage of microbial taxa isolated from young cysts of *H. trifolii* on each of 51 samples over the study period (March 1999 – March 2001)

	Sampling date	1^	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	12.3.99	46.6*	7	43	7	22	0	0	7	7	7	0	0	0	0	0	0	0	0	0	0
2	1.4.99	63.3	47.2	0	42	5.4	0	0	0	5.4	0	0	0	0	0	0	0	0	0	0	0
3	19.4.99	76.6	52	0	39	0	0	0	4.5	4.5	0	0	0	0	0	0	0	0	0	0	0
4	3.5.99	66.6	20	20	55	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
5	17.5.99	87.5	15.3	11.5	50	0	0	11.5	4	0	7.7	0	0	0	0	0	0	0	0	0	0
6	31.5.99	80	37	13	42	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0
7	14.6.99	90	55	0	33	3	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0
8	28.6.99	70	42.8	0	38	0	0	5	4.7	0	0	0	9.5	0	0	0	0	0	0	0	0
9	12.7.99	76.6	21.7	47.8	4.3	4.3	0	4.3	4.3	0	0	0	13	0	0	0	0	0	0	0	0
10	26.7.99	80	4.2	16.7	41.7	0	0	0	12.5	4.2	0	0	0	0	4.2	17	0	0	0	0	0
11	16.8.99	60	11	22.2	22.2	0	0	11.4	0	22.2	5.5	0	5.5	0	0	0	0	0	0	0	0
12	30.8.99	66.7	25	40	20	10	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
13	13.9.99	73.3	36.4	9	22.7	13.6	0	0	0	4.7	0	0	14	0	0	0	0	0	0	0	0
14	27.9.99	36.7	18.2	27.3	27.3	27.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	11.10.99	73.3	9	72.8	4.6	0	0	0	0	0	4.6	0	0	0	0	0	0	9	0	0	0
16	25.10.99	83.3	8	28	12	4	0	28	4	0	4	8	0	0	4	0	0	0	0	0	0
17	8.11.99	63.3	26.4	31.6	5.3	0	5.3	15.5	0	5.3	5.3	0	5.3	0	0	0	0	0	0	0	0
18	22.11.99	76.7	13	17.4	21.7	8.7	8.7	8.7	0	8.7	8.7	0	0	0	0	0	0	0	0	0	4.4
19	6.12.99	83.3	16	24	0	20	0	0	12	4	4	0	12	4	0	0	0	0	4	0	0
20	20.12.99	56.7	16.6	29.4	12	24	0	6	0	0	12	0	0	0	0	0	0	0	0	0	0
21	3.1.00	50	33.5	26.7	0	20	0	0	0	0	6.6	6.6	0	6.6	0	0	0	0	0	0	0
22	17.1.00	53.3	37.5	18.8	6.3	25	0	0	0	0	0	0	0	0	12.4	0	0	0	0	0	0
23	31.1.00	63.3	10.5	31.6	0	15.8	0	0	5.3	10.5	0	21	5.3	0	0	0	0	0	0	0	0
24	14.2.00	56.7	41.2	23.5	0	23.5	0	0	5.9	0	0	5.9	0	0	0	0	0	0	0	0	0
25	28.2.00	66.7	20	35	10	5	10	10	0	0	5	0	0	5	0	0	0	0	0	0	0
26	13.3.00	46.7	7.1	14.3	35.7	21.4	7.2	14.3	0	0	0	0	0	0	0	0	0	0	0	0	0
27	27.3.00	46.7	35.7	14.3	0	7.1	21.4	14.4	0	0	0	0	0	0	0	0	0	0	0	7.1	0
28	17.4.00	53.3	12.5	18.8	12.5	12.5	12.5	12.5	0	0	0	0	0	0	0	0	12.5	0	6.2	0	0
29	1.5.00	60	22.2	11.1	5.6	33.3	0	0	11.1	0	0	5.6	0	0	11.1	0	0	0	0	0	0
30	15.5.00	40	58.4	16.7	0	8.3	0	8.3	0	0	0	0	0	0	8.3	0	0	0	0	0	0
31	29.5.00	20	33.3	33.3	0	16.7	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0
32	12.6.00	43.3	15.3	23.1	23.1	15.4	0	0	7.7	7.7	0	7.7	0	0	0	0	0	0	0	0	0
33	26.6.00	13.3	50	25	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0
34	17.7.00	60	22.2	0	16.7	5.6	27.6	16.7	5.6	0	5.6	0	0	0	0	0	0	0	0	0	0
35	31.7.00	40	25	16.7	8.3	41.7	0	0	8.3	0	0	0	0	0	0	0	0	0	0	0	0
36	14.8.00	60	0	44.4	0	11.1	22.2	5.6	5.6	0	0	0	0	11.1	0	0	0	0	0	0	0
37	28.8.00	76.7	13	8.7	4.3	0	69.7	0	4.3	0	0	0	0	0	0	0	0	0	0	0	0
38	11.9.00	36.7	9.1	9.1	18.2	36.3	0	9.1	18.2	0	0	0	0	0	0	0	0	0	0	0	0
39	25.9.00	54.5	16.7	0	0	33.3	0	0	33.3	0	17	0	0	0	0	0	0	0	0	0	0
40	9.10.00	60	11.1	11.1	0	0	0	72.3	0	0	0	5.5	0	0	0	0	0	0	0	0	0
41	23.10.00	56.7	11.8	11.8	0	29.4	0	23.5	17.6	0	5.9	0	0	0	0	0	0	0	0	0	0
42	6.11.00	46.7	21.2	35.6	0	7.2	0	0	7.2	0	0	7.2	14	7.2	0	0	0	0	0	0	0
43	20.11.00	83.3	0	4	0	4	92	0	0	0	0	0	0	0	0	0	0	0	0	0	0
44	4.12.00	16.5	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45	18.12.00	100	40	40	10	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0
46	1.1.01	60	5.6	55.7	5.5	0	22.2	0	0	0	0	5.5	0	5.5	0	0	0	0	0	0	0
47	15.1.01	40	58.4	33.3	0	0	0	0	0	8.3	0	0	0	0	0	0	0	0	0	0	0
48	29.1.01	60	50	16.7	27.8	0	0	0	5.5	0	0	0	0	0	0	0	0	0	0	0	0
49	12.2.01	70	38.1	47.6	9.5	0	0	0	0	4.8	0	0	0	0	0	0	0	0	0	0	0
50	26.2.01	80	20.8	33.2	20.8	4.2	0	0	0	0	0	4.2	0	16.8	0	0	0	0	0	0	0
51	12.3.01	33.3	50	20	20	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0
	Mean	60	24.6	22.2	14.4	12.2	5.9	5.6	4.2	2.3	2.1	1.8	1.5	1.3	0.8	0.3	0.2	0.2	0.2	0.1	0.1

* = Numbers are percentages of fungal infected cysts, ^=(1) Fungal infected *H. trifolii* cysts, (2) *Fusarium* spp., (3) *V. chlamydosporium,* (4) *Gliocladium* spp., (5) Sterile mycelium, (6) Bacteria, (7) Zygomycetes, (8) *Cylindrocarpon* spp., (9) *Paecilomyces* spp., (10) *Verticillium* spp., (11) *Gliomastix* spp., (12) *Trichoderma* spp., (13) *Penicillium* spp., (14) *Pythium* spp., (15) *Humicola* spp., (16) *Aureobasidium* spp., (17) *Cercosporella* spp., (18) *Menisporella* spp., (19) *Aspergillus* spp., (20) *Chaetomium* spp.

2.5 Discussion

Small peaks in numbers of J2 in the soil in May 1999 and March 2000 occurred when soil moisture levels rose and high soil temperature levels started to fall. Both distinct peaks of J2 in soil occurred 12-14 weeks after soil moisture levels had risen in the autumn and when soil temperatures were low and starting to rise. Distinct peaks in numbers of J2 in soil were found in the winter of both 1999 and 2000 and the distinct peak of J2 in soil in 1999 was higher than that in 2000. A smaller peak occurred in the summer/autumn of 2000 but not in 2001. Yeates and Risk (1976) reported that the autumn peak was as large as the spring peak in 1975, while it was small in 1974 in Southland. Yeates (1973) found spring and autumn peaks were approximately of equal size in Wairarapa.

The average numbers of J2 in soil in winter were higher than those in summer possibly reflecting the high numbers of new cysts in autumn but more likely the high soil moisture levels in winter. Yeates (1973) reported that the higher level of J2 invasion in spring reflected the higher spring moisture levels. Soil moisture levels in Southland (Yeates & Risk 1976) were considerable higher than in the other studies and all peaks of J2 in soil were at soil moisture levels higher than 30%. In all three studies the spring peaks of J2 in soil commenced when the winter temperature was below 10°C and the summer/autumn temperature was above 15°C.

The highest previously recorded population densities of *H. trifolii* in New Zealand were 228 cysts / 100 ml of soil in autumn and spring (Yeates 1973) and 86 cysts / 100 ml soil (Yeates & Risk 1976). Maximum numbers of J2 in roots were 83-300 / 100 ml in autumn-spring and females were 73-55 / 100 ml in autumn-spring (Yeates 1973). In the present study, highest J2 numbers in soil in summer-winter in 1999 were 85-1816 / 100 ml and 633-1025 / 100 ml in 2000. The maximum numbers of J2 in roots in autumn-spring in 1999 were 91-1377 / 100 ml and 725-215 in 2000. The highest numbers of females in soil were 31-73 / 100 ml in autumn-spring in 1999 and 30-44 / 100 ml in 2000. Although the numbers and times of peaks of all stages of *H. trifolii* differed between their studies (Yeates 1973, Yeates & Risk 1976) and the current study, there appears to be good overall agreement between the three studies and the pattern of J2 populations in soil appears to be similar in the three geographically distinct areas of the country.

The trend of soil moisture was similar to that of rainfall, but it did not follow a small peak of rainfall in January - February 2000. It was likely that the soil could not maintain the water content (although there was rainfall) because of the dry period during the summer in 1999, and this was reflected in the soil moisture.

Dry matter yield of white clover in summer 1999 was higher (maximum 184 g/ m^2) than that of 2000 (maximum 117g/m^2). The yields increased in September 2000, coinciding with the rainfall in that period.

The trend of root weights paralleled that of the rainfall; root weights increased when the rainfall increased in March 1999, June 1999, January – April 2000, and September – October 2000. The amount of roots in cores was generally higher in autumn and in spring. A small peak in August in both years followed the return to normal soil moistures after rain in the previous 2 weeks. The clover root weights and white clover dry matter yields tended to be lower in 2000 than in 1999. One explanation would be that rainfall was high in May - August 1999 but it was high in August-September in 2000 and soil could not maintain the moisture because field capacity (30s) and available water for plants was low at the permanent wilting point for plants (16%), consequently the soil dried quickly and there was poor root growth. Manawatu sandy loams are quite variable and a permanent wilting point of 16% has been recorded in the area of the field plots (D. Horne Pers. Comm.) and this point was exceeded in a dry spell at the beginning of 2001.

In general, the numbers of J2 in soil reflected the rainfall and soil moisture but a small peak of J2 numbers in soil in January-February 2000 did not follow the soil moisture, which was lower than normal for that time. J2 emergence coincided with increase of root weights in June 1999. J2 emerged in soil when root weight and white clover dry matter yield started to increase in January - March 2000 and July 2000 – January 2001.

The year 2000 was relatively drier than that in 1999 and the low root weights probably reflected the dry period in 2000. J2 in soil could die because host plant roots were not available for winter emerged J2 and movement of emerged J2 in winter 2000 was probably restricted by the drier conditions in 2000. Consequently, numbers of J2 in roots and all cyst stages were also lower in 2000 than in 1999. There was marked mortality of J2 with a lack of distinct body contents both in the 1973 and 1976 studies followed by reinvasion of J2 to plants, but there was no discussion on the reason for the

mortality in the studies of Yeates (1973) and Yeates & Risk (1976). A similar mortality occurred in 2000 spring (9.10.00) in this study and subsequent reinvasion was observed 2-4 weeks later. J2 mortality was probably because of high rainfall which could have caused anaerobic conditions and hence the O_2 concentration in the flooded soil and in the roots fell to a level below that at which the J2 could survive. Alternatively, the numbers of J2 in roots were a feature of the relationship between *H. trifolii* and white clover.

There were clear sequences of J2 in soil followed by J2 in roots then females in all three studies with J2 in roots appearing after a 1- to 2-week delay in the current study and that of Yeates (1973). Yeates and Risk (1976) found that numbers of females followed the same trend as that of J2 in roots in Southland with a 1- to 2-week delay only, but there was a 3-4 week delay in the current study and that of Yeates (1973) in Wairarapa.

In the studies of Yeates (1973), Yeates and Risk (1976) there was a clear relationship between the number of females and the subsequent number of cysts. In the current study, there were few new cysts in August-November 2000 following a low number of females in April-October but the large numbers of females in September-December 2000 were not followed by an increase in new cysts. The reason for this is not known but one explanation could be large scale mortality of the high numbers of females.

The percentages of infected cysts with *Fusarium* spp. and with *G. roseum* were high in autumn but low in spring and summer. *V. chlamydosporium* frequencies were high in spring/summer but low in autumn/winter. Its peak coincided with that of white females in spring so this could be the key time for *V. chlamydosporium* parasitisation of eggs of white female *H. trifolii*. According to Leij et al. (1992), *V. chlamydosporium* was most abundant in soil at 22 °C, which was also about the optimal temperature for sporulation and infection of egg-masses of *Meloidogyne* spp. exposed on tomato root surfaces.

The numbers of new cysts were higher in autumn than those in spring and seemed to be corresspond to the high soil temperature (15-17°C) in autumn as opposed to spring (13°C). The numbers of old cysts were higher in autumn than those in spring reflecting the autumn high numbers of new cysts. A low, stable numbers of old cysts in 2000 were possibly due to parasitism by fungi in the soil especially *V.*

chlamydosporium. Kerry and Crump (1977) found the population decline of *H. avenae* was due to failure of many cysts to develop and fecundity was reduced because eggs and females were parasitised frequently by *V. chlamydosporium.*

Nematodes belonging to the Orders Rhabditida, Dorylaimida and nematodes genera belonging to Tylenchida (*Aphelenchus, Helicotylenchus*, *Heterodera*, *Paratylenchus, Pratylenchus*, *Tylenchus*,) were found in this study and Yeates (1975) also recorded these nematodes from Manawatu soil. Plant-parasitic nematodes; *H. trifolii*, *Meloidogyne* spp. and *Pratylenchus* spp. were found in this study. Plant-parasitic nematodes infected white clover in pastures at Fitzherbert West, Palmerston North, New Zealand were *H. trifolii* (Mercer 1990), *Meloidogyne trifoliophila,* (Mercer et al 1997), and *Pratylenchus* sp. (Mercer et al 1997).

Fungi commonly isolated from young *H. trifolii* cysts in this study were similar to those listed by Hay & Skipp (1993) who reported that predominant fungal species isolated from *H. trifolii* collected from 10 pastures sites in North Island of New Zealand included *Fusarium* spp., *V. chlamydosporium*, and *G. roseum*.

Waipara et al. (2001) found that *Verticillium* and *Fusarium* were abundant in autumn and summer samplings infested with *H. trifolii*, respectively, and they were influenced by seasonal variations of temperature and moisture but *Gliocladium* spp. was not. In the present study, *V. chlamydosporium* was high in spring and summer but *Fusarium* spp. and *Gliocladium roseum* were high in autumn/winter. Clearly the numbers of these three fungal species differed among the seasons and also the peak time of these fungal species differed between those two studies. Possible factors were a) different agar medium and b) different soil samples but more likely because different cyst stages were incubated on agar. Other fungal genera from this study could be commonly found in soil and roots of plants in pastures. Hay & Skipp (1993) reported fungi in soil and roots of plants in New Zealand pastures, which are intimately associated with cysts of *H. trifolii*.

2.6 Conclusion

There were two reproductive cycles of *H. trifolii* each year. The seasonal population changes of new and old *H. trifolii* cysts were not as large as those of the juveniles and females. Nematode population changes followed basically the same

pattern each year, but levels differed. Changes of numbers of J2 both in soil and in roots and numbers of females generally reflected root growth which in turn reflected changes of soil moisture and soil temperature. Numbers of all stages of *H. trifolii* tended to be lower in 2000 than in 1999. One possible reason is mortality caused by fungi such as *V. chlamydosporium, Fusarium* spp. and *Gliocladium roseum*. Because of this reason, fungal isolates collected at this time warrant further testing for their pathogenicity to white clover plants and clover cyst nematode *H. trifolii*.

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3 PATHOGENICITY TO WHITE CLOVER PLANTS OF SOME FUNGI ISOLATED FROM CLOVER CYST NEMATODE

3.1 Introduction

The clover cyst nematode *Heterodera trifolii* is widespread in pasture soils throughout New Zealand and many other countries (Skipp & Gaynor 1987). It is an important pest of white clover (*Trifolium repens*) causing reduced plant establishment and persistence, yield and nitrogen fixation. Many pastures in New Zealand have been established for a number of years, affording the opportunity for populations of fungal parasites to have reached equilibrium with nematode populations at some sites.

Fungal parasites of eggs of *H. trifolii* could be candidates for biological control of this pest. In evaluating such fungi it is as important to determine their lack of pathogenicity to man, plants, or other valuable non-target entities as well as their efficacy in parasitising females and cysts, and in killing eggs (See Chapter 4). Fungi found in and around cysts on roots are often common soil fungi that invade and colonise living, moribund or dead root tissue. Many of the fungi isolated from cysts and females in the rhizosphere belong to species that are known plant pathogens (Kerry 2000). Some promising nematode biocontrol fungi, such as, *Fusarium solani*, *F. oxysporum* and *Nectria vasinfecta* have biotypes that are plant pathogenic (Domsch et al. 1980) and *Paecilomyces lilacinus* has been reported pathogenic to man (Agrawal et al. 1979, Malbran et al. 1973, Takayasu et al. 1977).

Root-invading fungi known to be pathogenic to white clover include several *Fusarium* species, particularly *F. oxysporum* which can cause root rot (Leath et al. 1971; Menzies 1973a,b, Campbell 1980, 1981). *Gliocladium* spp. have also been found pathogenic to white clover in pot trials using pure culture inoculum (Thornton 1965, Skipp & Christensen 1982). Root lesions were formed on seedling white clover roots by most fungi, which were commonly isolated from surface-sterilised roots of clover grown in pasture soils: *Codinaea fertilis*, *Cylindrocarpon destructans, Fusarium culmorum*, *F. oxysporum*, *Phoma chrysanthemicola*, *Phoma exigua*, *Phoma fimeti*, *Thielaviopsis basicola*, species of *Acremonium, Chrysoporium, Coniothyrium,*

Gliocladium and *Phoma,* unidentified basidiomycetes, and fungi having sterile dark mycelium (Skipp & Christensen 1981, Skipp & Christensen 1982, Skipp et al. 1982). In these studies *V. chlamydosporium* was also isolated from clover roots but was referred to by its older synonym, *Diheterospora chlamydosporia* (Skipp & Christensen 1981). As with other *Verticillium* spp. found, it did not penetrate the root tissues or form necrotic root lesions on roots of white clover seedlings (Skipp & Christensen 1982). Isolates of *Acremonium* spp., *Cylindrocarpon* spp., *Microdochium bolleyi*, and *Trichoderma* spp. caused necrosis in both invaded and non-invaded cells suggesting that these fungi produced diffusible toxins in an agar plate test (Skipp & Christensen 1989).

In similar Australian studies, Zahid et al. (2001a,b) also reported isolation of a wide range of fungi from white clover roots. Species of *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Phoma*, *Codinaea*, *Gliocladium*, *Microsphaeropsis*, *Trichoderma*, *Nectria* and *Macrophomina* were pathogenic to white clover roots *in vitro*. Lager & Gerhardson (2002) also found that isolates of the genera *Fusarium*, *Cylindrocarpon*, *Phoma*, and *Pythium*, were pathogenic to the clovers in greenhouse tests using four different types of inoculum (agar cultures, sand mixtures, wheat-kernel culture, or a spore suspension root dip).

The choice of an appropriate pathogenicity test is important for effective screening of isolates. Christensen et al. (1988) used white clover seedlings grown on water agar in Petri plates to test pathogenicity of fungi to seedling roots and induction of fungal sporulation. Waipara et al. (1996) used this method for rapid screening for pathogenicity to a range of pasture grasses and legumes.

Hay and Skipp (1993) recovered a total of 707 isolates of fungi and actinomycetes from 488 surface-sterilised clover cysts from ten pasture sites in the North Island of New Zealand, many of the species isolated are known colonists of the roots of white clover. The biocontrol potential of some of those fungal isolates has been assessed in tests for pathogenicity to clover cysts in that study but they have not been tested for pathogenicity to white clover seedlings. That is an important area for study.

3.2 Objective

To investigate whether fungi commonly isolated from cysts of *H. trifolii* can be pathogenic or non-pathogenic to white clover in agar plates or in pots.

3.3 Materials and Methods

3.3.1 Agar plate test

Seeds of white clover *Trifolium repens* cv. Huia were surface-sterilised in H_2SO_4 (50% for 15 min) followed by NaOCl (0.3% for 15 min) with three rinses in 10 ml of sterile distilled water (SDW) after each treatment. Seeds were placed on half strength Potato Dextrose Agar (PDA) in Petri plates and germinated at 21° C for 4 days after which they were transferred to 1.0% water agar (WA). Each plate was taped with clear plastic food wrap and placed on edge with the rows of seeds aligned horizontally to ensure that seedling roots grew across the surface of the agar. Plates were incubated under a mixture of lights (SYLVANIA, GRO - LUX, F18W / GRO - T8 and PHILIPS, TLD 18W / 33, COOL WHITE) on a 12 h/12 h day/night regime, at 20°C.

Small discs (3 mm diameter) cut from 3 week old PDA cultures of fungi isolated from cysts of *H. trifolii* (Table 3.1) were inoculated onto the agar surface of each plate close to the white clover seedlings (Christensen et al. 1988). All inoculated plates were oriented as described above and returned to the cabinet. There were eight treatments and 3 replicates for each treatment in a complete randomised design.

Seedlings were observed at intervals and effects of test fungi assessed. Seedlings were harvested 6 weeks after inoculation of fungi and oven-dried at 85°C overnight. The dried weights of seedlings were recorded. Data was analysed by ANOVA, using SAS.

3.3.2 Pot test

The white clover seeds (cv. Huia) were surface-sterilised as described in Section 3.3.1. The seeds were germinated on three layers of sterile moist filter paper in a 10 cm Petri dish. The Petri dishes were incubated at 20°C for 48 h. A seedling was then transplanted into a 6 cm diameter pot containing pasteurised sand. Pots were assigned at random to steel trays (80 x 44 cm), to give 40 pots per tray. Trays were placed on a heated wet mat in a glasshouse (soil temperature range 19-22°C). The pots were re-arranged in the same tray and between the trays on alternate weeks. Each tray received 1 L of soluble fertiliser (THRIVE, 4.0 g / 4.5 L) dissolved in water. Immediately after application, a 200 x 20 mm synthetic sponge (Wettex, Sweden) wick was introduced to slowly remove surplus solution.

Fungal suspensions in distilled water were prepared from cultures of 3 wk-old fungal isolates grown on PDA, filtered through a 70 μm Falcon cell strainer. The spores were counted with a haemocytometer and were adjusted to 1 x 10^4, 1 x 10^6 and 1 x 10^8 conidia per ml.

Two weeks after planting, pots were inoculated with fungal spore suspensions. There were 7 treatments and 3 replicates in a randomised complete block design for both wounded and unwounded plants. Six plants were used for each isolate, three intact and three wounded by cutting off one third of the root. The plant roots were dipped in the inoculum suspension for 1 h (Skipp et al. 1986). Roots of control plants, both wounded and unwounded, were soaked in sterilised-distilled water. All plants were then transferred singly to plastic pots in the glasshouse. Plants were harvested 8 weeks after inoculation and shoots were oven-dried at 80°C for 24 h, and their dry weight determined. Effects of fungi on seedling growth were assessed from the dry weights of seedlings.

Immediately after harvesting, roots were washed free of soil with tap water and they were surface-sterilised in 0.3% NaOCl solution for 1 min, rinsed in sterilised-distilled water and the primary taproot cut into 1-2 mm long segments. Five segments from each treatment were placed on water agar containing oxytetracycline (10 μg/ml) in a Petri dish and were incubated at 20°C in an incubator room until fungi could be identified (Skipp & Christensen 1981). Data were analysed by SAS using ANOVA with factorial arrangement.

3.4 Results

3.4.1 Agar plate test

Plant weights from the *F. oxysporum* and *G. roseum* treatments were significantly lower than from the remaining treatments (Table 3.1). The seedlings died 4 weeks after inoculation with *F. oxysporum*.

Seedling weights from the *V. chlamydosporium* treatments were significantly higher than those of the remaining treatments but did not differ from those on uninoculated agar. Although seedling weights were significantly higher in *Verticillium* sp., *P. lilacinus*, and *Trichoderma* sp. compared with *F. oxysporum* and *G. roseum* treatments, they were not significantly different from the control checks (Table 3.1).

Necrotic symptoms were found on the roots of *F. oxysporum*, *G. roseum*, and *Trichoderma*-inoculated seedlings (Table 3.2).

Table 3-1 Mean dry weights of white clover seedlings on agar harvested 6 weeks after inoculation with a range of fungi

No.	Treatments	Mean seedling weight (g)
1	*Gliocladium roseum*	0.0004c
2	*Fusarium oxysporum*	0.0001c
3	*Trichoderma* sp.	0.0015b
4	*Paecilomyces lilacinus*	0.0016b
5	*Verticillium* sp.	0.0016b
6	*V. chlamydosporium*	0.0024a
7	Potato dextrose agar	0.0022a
8	Control	0.0016b

Means followed by the same letter are not significantly different ($P<0.05$).

Table 3-2 Lesion formation on white clover seedlings on agar 6 weeks after inoculation with fungi

No.	Treatments	Lesions on clover
1	*Gliocladium roseum*	+
2	*Fusarium oxysporum*	+
3	*Trichoderma* sp.	+
4	*Paecilomyces lilacinus*	-
5	*Verticillium* sp.	-
6	*V. chlamydosporium*	-
7	Potato dextrose agar	-
8	Control	-

+ = Lesions present

- = Lesions absent

3.4.2 Pot test

Root weights were significantly higher in the *V. chlamydosporium* treatment than those from the *G. roseum, F. oxysporum*, or *Trichoderma* sp. treatments (P< 0.05), but not from the weights of *P. lilacinus*, *Verticillium* sp., and the control (Table 3.3).

There was a significant difference between wounded and unwounded in overall mean root weights (P< 0.05), mean unwounded-root dry weights were significantly higher than wounded-root weights. None of the spore suspensions of any fungal treatments caused differences in root dry weights (Table 3.3).

There was a significant effect of fungal treatment on weights of shoots (P< 0.001) and a significant interaction between fungal treatment and wounding (P< 0.01) (Table 3.4). Shoot dry weight was significantly higher in the *V. chlamydosporium* treatment than in all other treatments except *P. lilacinus* (Table 3.4).

The treatments of *Trichoderma* and *Paecilomyces* did not differ significantly from the control in shoot dry weight, but all three were significantly greater than the *Gliocladium* or *Fusarium* treatments. No significant difference in shoot dry weight was found between wounded and un-wounded treatments, or among the fungal inoculation rates (Table 3.4). Only *F. oxysporum* was recovered from root segments (more than 65%) 5-10 days after re-isolation.

Table 3-3 Mean dry weights of roots (g/plant) harvested from white clover plants 8 weeks after inoculation by dipping the roots in a suspension of fungal conidia (1 x 10^4, 1 x 10^6 or 1 x 10^8/ml) with or without wounding

		Type						
		Unwounded			Wounded			
		Spores level			Spores level			
No.	Treatments	10^4	10^6	10^8	10^4	10^6	10^8	**Treatment mean**
1	*Gliocladium roseum*	0.18	0.13	0.17	0.11	0.12	0.16	**0.14b**
2	*Fusarium oxysporum*	0.13	0.13	0.10	0.14	0.11	0.15	**0.13b**
3	*Trichoderma* sp.	0.20	0.16	0.11	0.11	0.12	0.12	**0.14b**
4	*Paecilomyces lilacinus*	0.17	0.15	0.17	0.14	0.14	0.12	**0.15ab**
5	*Verticillium* sp.	0.20	0.12	0.19	0.16	0.11	0.17	**0.16ab**
6	*V. chlamydosporium*	0.16	0.18	0.20	0.17	0.18	0.20	**0.18a**
7	Control	0.20	0.16	0.18	0.15	0.14	0.13	**0.16ab**
	Type mean		**0.16a**			**0.14b**		
	Type	*						
	Treatment	*						
	Spore	ns						
	Type*treatment	ns						
	Type*spore	ns						
	Treatment*spore	ns						
	Type*treatment*spore	ns						

Means within columns followed by the same letter are not significantly different (P<0.05), NS, *: non significant or significant at p=0.05.

Table 3-4 Mean dry weights of shoots (g/plant) harvested from white clover plants 8 weeks after inoculation by dipping the roots in a suspension of fungal conidia (1 x 10^4, 1 x 10^6 or 1 x 10^8/ml) with or without wounding

		Type						
		Unwounded			Wounded			
		Spores level			Spores level			
No.	Treatments	10^4	10^6	10^8	10^4	10^6	10^8	**Treatment mean**
1	*Gliocladium roseum*	0.50	0.44	0.38	0.43	0.40	0.42	**0.43c**
2	*Fusarium oxysoprum*	0.34	0.31	0.29	0.42	0.39	0.36	**0.35d**
3	*Trichoderma* sp.	0.62	0.54	0.41	0.40	0.53	0.53	**0.50b**
4	*Paecilomyces lilacinus*	0.52	0.50	0.68	0.56	0.50	0.45	**0.54ab**
5	*Verticillium* sp.	0.57	0.32	0.43	0.49	0.61	0.53	**0.49bc**
6	*V. chlamydosporium*	0.56	0.69	0.67	0.53	0.55	0.62	**0.60a**
7	Control	0.60	0.61	0.61	0.49	0.42	0.43	**0.53b**
	Type mean		**0.50a**			**0.48a**		
	Type	ns						
	Treatment	***						
	Spore	ns						
	Type*treatment	**						
	Type*spore	ns						
	Treatment*spore	ns						
	Type*treatment*spore	ns						

Means within columns followed by the same letter are not significantly different ($P<0.05$), NS, **, ***: non significant or significant at $p=0.01$ or 0.001 respectively.

3.5 Discussion

3.5.1 Agar plate test

F. oxysporum and *G. roseum* caused necrotic lesions on roots and reduced seedling growth, with *F. oxysporum* causing the most severe damage and eventually the death of seedlings. Although *Trichoderma* sp. caused necrotic lesions, the seedling weights did not differ from the control check, and probably the fungus was weakly pathogenic to white clover seedlings. These findings were consistent with results of Leath et al. (1971), Menzies (1973a, b), Campbell (1980, 1981), Skipp & Christensen (1981, 1982), Waipara et al. (1996), and Lager & Gerhardson (2002) who all found that most *Fusarium* spp. were pathogenic to white clover seedlings. Zahid et al. (2001b) and Skipp & Christensen (1981, 1982) found that some species of *Gliocladium*, and *Trichoderma* were pathogenic to roots of white clover seedlings.

Although *V. chlamydosporium* treatment increased seedling growth compared with any of the fungal treatments and the control check, the increase was not significantly greater than on PDA alone. Thus it is not certain whether the fungus stimulated seedling growth. The unidentified *Verticillium* sp., and *P. lilacinus* were not pathogenic to white clover seedlings as the weights from those treatments were not significantly different from the control check and no lesions were found on those seedlings. Waipara et al. (1996) had previously found that *Paecilomyces* spp., and *Verticillium* spp., were not pathogenic to white clover seedlings.

The pathogenicity of all these fungal species was then tested in sand in pots in a glasshouse test to confirm the results of the agar test.

3.5.2 Pot test

In the agar plate test, using young seedlings, high levels of inoculum, and pure cultures, the isolates of *Fusarium* and *Gliocladium* spp. obtained from *H. trifolii* cysts were pathogenic to white clover seedlings. However, in the pot tests, these fungal species did not reduce the root dry weights below that of the control. The possible reasons were: 1) they might be weak pathogens that could not produce adverse effects on the root dry weights, 2) the environmental conditions in soil were not conducive to severe pathogenic effects of those fungal species, and 3) the fungal inoculation method did not favour severe root disease that could affect root weight.

Many clover root pathogens are considered weak pathogens (Chi & Hanson 1961, Siddiqui et al. 1968, Stutz et al. 1985, Rufelt 1986). For instance, *F. oxysporum* was regarded as a weak pathogen of white clover in pasteurised soil in pot tests (Garrett 1950, Talboys 1978). Skipp & Christensen (1982) reported that although *F. oxysporum* was one of the primary root-invaders and potential pathogens of white clover roots, roots usually appeared healthy and free from root lesions, therefore, it may not express its potential pathogenicity in pasture soils. Furthermore, in pathogenicity tests on agar (Skipp & Christensen 1989) most fungi invade root tissues regardless of any field specificity, and the test may have created conditions in which roots were unnaturally susceptible to the isolates because of the non-competitive environment. This view may be supported by other work with pot experiments using sterile and unsterile soil (Shipton 1967, Wong et al. 1986). In autoclaved soil, *F oxysporum* was a virulent

pathogen, causing severe root rot and seriously affecting plant growth of subterranean clover in pots, whereas in pasteurised soil it was only a weak or non-pathogen (Pung et al. 1992).

Only plants inoculated with *V. chlamydosporium* had produced significant more shoot dry weight than the control. This probably indicates that the fungus or some fungal product had an effect on seedling growth that increased shoot dry weight. The *Trichoderma, Paecilomyces*, *Verticillium* sp. isolates did not reduce shoot dry weights compared with the control. Some *Trichoderma* spp. are known to be pathogenic fungi in plant roots (Abada 1994) while others have no effect or may be associated with higher vigour (Sarathchandra et al. 2000).

There was an adverse effect of *G. roseum* and *F. oxysporum* on shoot dry weights, which were significantly lower than the control. These isolates appeared to be weak pathogens and effects on shoot dry weight was not affected by inoculum rate.

There was no significant effect of wounding which suggested that these fungal species could enter the uninjured root tissues.

3.6 Conclusion

In the agar plate test, *G. roseum* and *F. oxysporum* isolates were virulent to white clover seedlings and the *Fusarium* seriously affected plant growth. In pasteurised soil in the pot test, these fungal species affected shoot weight but they did not cause root disease.

V. chlamydosporium, *P. lilacinus*, and *Verticillium* sp. were not pathogenic to white clover plants in either the agar plate or pot tests. Although, the *Trichoderma* sp. produced necrosis on white clover seedling roots, it did not affect growth of the plants in either test and is regarded as non-pathogenic. *V. chlamydosporium* appeared to stimulate plant growth.

The results of the agar plate test were generally consistent with those of the pot test and the Petri plate method appears suitable for screening a large numbers of fungal isolates within a short time to distinguish probable pathogens from non-pathogens.

V. chlamydosporium, F. oxysporum, and *G. roseum* were commonly found from the Manawatu pasture within March 1999-March 2001. As these fungal species were

not virulent in the pot experiment, they were chosen for further testing as biocontrol agents to control clover cyst nematode *H. trifolii*.

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4 PATHOGENICITY TO CLOVER CYST NEMATODE OF SOME FUNGI ISOLATED FROM CLOVER CYST NEMATODE: GLASSHOUSE TESTS

4.1 Introduction

Interest in developing fungi as biological control agents for plant-parasitic cyst nematodes was stimulated by Kerry's (1981) report that long-term suppression of the cereal cyst nematode *(Heterodera avenae*) in England was caused by *Verticillium chlamydosporium* and *Nematophthora gynophila* (Kerry & Crump 1977). Studies on soybean cyst nematode in the United States (Morgan-Jones et al. 1981, Carris et al. 1986) have identified numerous fungi occurring in cysts of *H. glycines.* Some of these fungi were potentially pathogenic to soybean, and some to *H. glycines.*

Verticillium species of the section *Prostrata* constitute a group of fungi that can parasitise and destroy nematode eggs and juveniles. *Verticillium chlamydosporium*, *V. lamellicola*, *V. lecanii*, *V. leptobactrum*, and *V. psalliotae* occur in cysts and (or) eggs of *Globodera*, *Heterodera*, and *Meloidogyne* species (Kerry et al. 1982, Morgan-Jones et al. 1981, Morgan-Jones & Rodriguez-Kabana 1988, Stirling & Kerry 1983, Tribe 1977). It has been reported that the most common fungal egg parasites of the plant endoparasitic nematodes *Heterodera avenae* or *Heterodera schachtii* in Spanish soils was *V. chlamydosporium* var. *chlamydosporium*, with *Verticillium lecanii* and *Paecilomyces lilacinus* also found (Olivares-Bernabeu & Lopez-Llorca 2000). Pathogenicity of isolates of *V. chlamydosporium* was demonstrated in the laboratory in tests with the root knot nematode, *M. javanica*, where 70-100% egg infection and 35-40 penetrating hyphae/egg were observed.

Some species of genera other than *Verticillium* isolated from cysts have been shown to be parasites of nematode eggs. These include actinomycetes (Sayre & Starr 1988, Walter & Kaplan 1990), *P. lilacinus*, *Fusarium oxysporum, F. solani*, species of *Gliocladium, Phialophora, Exophiala, Acremonium* and *Cylindrocarpon* (Jatala 1986, Morgan-Jones & Rodriguez-Kabana 1988). There is also evidence that some cyst-colonising fungi may affect development by causing reduced egg hatch and egg abortion even though they do not parasitise nematode eggs directly (Jatala 1986). Walia

& Vats (2000) distinguished three groups of fungi with different modes of parasitism or predation that may have potential as biological control agents of phytonematodes. These were: (1) parasitic or opportunistic fungi, including *P. lilacinus*, *V. chlamydosporium*, *Hirsutella rhossiliensis*, *Dactylella oviparasitica* and *Cylindrocarpon destructans* (*Nectria radicicola*), (2) endozoic fungi including *Nematophthora gynophila* and *Catenaria* spp., and, (3) fungi that trap free-living nematode juveniles, such as *Arthrobotrys* spp., *Monacrosporium* spp. and *Dactylaria* spp.

Most of the information on fungi in cysts of *H. trifolii* on white clover in pasture has been provided by Hay (Hay 1993, Hay & Skipp 1993) in New Zealand. Predominant fungal species isolated from surface-sterilised clover cysts from 10 pastures sites in North Island of New Zealand were *Exophiala pisciphila, Gliocladium roseum*, *Fusarium oxysporum*, *F. culmorum*, *F. solani*, *Mortierella alpina*, unidentified *Paecilomyces* spp., *P. lilacinus*, *Trichocladium opacum* and *V. chlamydosporium.* Actinomycetes were also commonly present. Many of the fungal species isolated from *H. trifolii* cysts are known to be colonists of the roots of white clover (*Trifolium repens*) and some will parasitise eggs of *H. trifolii* and *M. hapla* (Hay & Skipp 1993, Mercer 1994).

In a pathogenicity test, three fungal parasites isolated from diseased eggs of *H. trifolii* and a parasite of *H. trifolii* J2 isolated from soil (*Hirsutella rhossiliensis*) were added to soil in the form of alginate beads (Hay & Bateson 1996). The mean number of cysts that developed per pot eight weeks after sowing was reduced by *Scolecobasidium constrictum*, one of the two sterile fungi, and *H. rhossiliensis.*

Various substrates and inoculation methods have been used to apply candidate fungi to soil or roots to test pathogenicity experimentally or to attempt biological control in the field. *Verticillium chlamydosporium* has been widely studied because of its perceived potential and because its thick-walled chlamydospores allow survival under unfavourable conditions and can prolong shelf life. Chlamydospores of *V. chlamydosporium* have been produced on a maize medium and applied by physically mixing the inoculum with soil, drenching a suspension into soil, or dipping bare roots in a spore suspension then transplanting colonized seedlings. The former two methods resulted in sufficient colonization of the soil and root surface to reduce significantly numbers of *Meloidogyne incognita* found in the roots of tomato and lettuce in a glasshouse trial (Bourne & Kerry 1999).

Granular formulations of *Arthrobotrys oligospora*, *P. lilacinus*, *V. chlamydosporium*, *Gliocladium virens* and *Trichoderma harzianum*, prepared using a medium of wheat bran, maize, and vegetable oil, reduced nematode multiplication and resulted in improved growth of tomato plants (Sankaranarayanan et al. 2000).

In New Zealand, biocontrol potential of some of the fungal isolates from *H. trifolii* have been tested for their parasitism against to *H. trifolii* but *Fusarium oxysporum*, *Gliocladium roseum* and *Verticillium chlamydosporium* have not been tested for pathogenicity to *H. trifolii*. In attempting such a study of the pathogenicity of these fungi it is important to use a medium suitable for both host plant and biocontrol agents.

4.2 Objective

4.2.1 (Trial 1) Screening a range of fungal species

To determine, using a glasshouse screening test, which of the fungi isolated from clover cyst nematode *H. trifolii*, from the study area (Section 2.3.1), might be the most effective for control of clover cyst nematode.

4.2.2 (Trial 2) Pathogenicity of *V. chlamydosporium* isolate 6 (Vc6) to *H. trifolii* and to clover seedlings.

In the previous experiment, only *V. chlamydosporium* (Vc6) gave substantial reductions in *H. trifolii* parasitism and improvements plant growth. This experiment was carried out to confirm these findings.

4.2.3 (Trial 3) Influence of fungal inoculum medium on pathogenicity tests

In Trial 2, white clover plants grew well in the presence of *V. chlamydosporium* inoculum grown on an oatmeal/sand medium. However, addition of the oatmeal/sand medium without fungus resulted in poor plant growth and reduced numbers of *H. trifolii* cysts. Results from *V. chlamydosporium*-inoculated treatment could not be compared with oatmeal alone treatment statistically because of this apparent effect of medium on plant growth. The aim of Trial 3 was to find other types of media and inocula that would be suitable for both *V. chlamydosporium* (Vc6) and white clover plants. This has

included the use of inoculum encapsulated in alginate beads (Stirling and Mani, 1995) and inoculum mixed in with pasteurised soil and dried (Hancock 1985).

4.3 Materials and methods

Unless, otherwise stated, the following methods were standard in all trials.

Germinating white clover seed

White clover seed (cv. Huia) was surface-sterilised as described in Section 3.3.1 then germinated on sterilised-moistened filter paper (3 layers) in a 10 cm Petri dish for 48 h in an incubator room at 20°C.

Preparing fungal inoculum

Fungal inocula of *F. oxysporum*, *G. roseum* and *V. chlamydosporium* were prepared by adding small pieces of PDA fungal culture to a sterilised medium consisting of 5 g coarse oatmeal, 95 g sand, and 15 ml distilled water in a 250 ml Erlenmeyer flask which was then incubated for 3 weeks at 20°C (Skipp & Christensen 1983).

Sowing white clover seed in soil inoculated with a fungal isolate

Manawatu silt loam collected from No 1 dairy Unit, Massey University, Palmerston North, was sieved through a 6 mm sieve and defaunated by freezing at -20°C for 48 h, stored at 4°C until required. The frozen soil was mixed (1:1) with sand (Yeates et al. 1999). Fifty grams of fungal inoculum (4.3), was mixed with 150 g of frozen soil/sand in a 6 cm diameter pot. Two germinated white clover seeds were sown in each pot. Pots were assigned at random to a steel tray (80 x 44 cm), to give 40 pots per tray. Trays were placed on a heated wet mat in a glasshouse (soil temperature range 19-22°C). The pots were re-arranged in the same tray and between trays on alternative weeks. Each tray received 1 L of soluble fertiliser (THRIVE, 4.0 g / 4.5 L) dissolved in water. Immediately after application, a 200 x 20 mm synthetic sponge (Wettex, Sweden) wick was introduced to slowly remove surplus solution.

***Inoculating white clover seedlings with* H. trifolii**

Two weeks after sowing, each clover seedling was inoculated with 2000 eggs suspended in 3 ml of water. To inoculate a seedling, a pencil was used to make a hole in the soil directly under the plant close to the roots of the seedling. The 3 ml of egg suspension was injected into the hole, which was refilled with soil to prevent drying (van den Bosch & Mercer 1996).

Measurements

Six weeks after inoculation, plants were harvested and oven dried at 80°C for 24 h. The dried shoot and root weights were recorded. Cysts and females were extracted from the soil with an elutriation tower and numbers per pot were counted under a dissecting microscope. Data were transformed to square-root as required and analysed by ANOVA, using SAS.

4.3.1 (Trial 1)

There were 14 treatments (Table 4.1) and 10 replicates in a randomised complete block design for each treatment.

4.3.2 (Trial 2)

4.3.2.1 Preparing Vc6 spore suspension and inoculating white clover seedlings

Fungal chlamydospore suspensions were prepared by flooding 3 wk-old cultures grown on PDA with distilled water, scraping the surface, and filtering through a 70 μm Falcon cell strainer. The spores were counted with a haemocytometer and adjusted to 10^6 chlamydospores per ml. The chlamydospore suspension of Vc6 was pipetted (10^6 chlamydospores/ml/plant) in the soil next to the roots.

There were 8 treatments (Table 4.2) and 5 replicates in a randomised complete block design for each treatment. Data were transformed to square-root as required and analysed by ANOVA, using SAS.

4.3.3 (Trial 3)

4.3.3.1 Alginate/kaolin beads (AB)

Inoculum encapsulated in alginate beads containing kaolin as a filler was prepared by a modification of the method of Stirling and Mani (1995) using post-encapsulation incubation in a liquid nutrient medium to increase fungal biomass in the beads. Twenty-five plugs 11.5 mm diameter of 2-week-old CMA culture of *V. chlamydosporium* were macerated in 50 ml sterile 1% Na alginate in a 100 ml Schott bottle containing 30 g of 4 mm diameter glass beads. Macerates were tipped aseptically into plugged 1 litre flasks containing 350 ml of autoclaved alginate/kaolin (Na alginate 10 g/l: 100 g/l kaolin were mixed together in a blender to ensure they are well mixed with no lumps before autoclaving). The macerates were suspended by manual shaking and the suspension was then removed with a sterile Pasteur pipette and dripped into a 2.0 l flask containing 1.0 litre sterile 0.05M Ca gluconate solution (22 g/l = 0.05M).

After 30 min, when beads had set, gluconate was aseptically poured off and beads were washed in three changes of sterile water. Then, they were dispensed aseptically as three equal portions into 500 ml flasks containing 200 ml of GPY broth (Glucose 15 g; Peptone 2 g; Yeast extract 5 g; Asparagine 1 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.25 g; Thiamine HCL 0.001 g; water 1litre), and the flasks were incubated for 2 days at 21°C on a shaker. Beads were rinsed in three changes of sterile water and they were then collected on an alcohol-sterilised, wire-covered white tray. The beads were allowed to dry for 3 days in a laminar flow cabinet without airflow and then collected and stored at 5°C. Beads without fungi were prepared for medium control check.

4.3.3.2 Bran culture alginate beads (BB)

Ground bran (10 g) was placed in a plugged 100 ml conical flask and autoclaved on two successive days. Potato dextrose broth (PDB) (10 ml) [1 L juice of boiled potatoes (200 g) + 10 g dextrose] was added aseptically to moisten the bran, and then 3 plugs (11.5 mm diameter) cut from the edge of a 2-week-old CMA culture of *V. chlamydosporium* were added to the mix, and incubated at 21°C for 2 weeks. The culture was washed by adding sterile water, shaking gently, and then filtering through muslin and draining off the excess water. The inoculum (3 g) was placed in a

McCartney bottle together with 10 ml of sterile 1% alginate / 5% bran mix (mix solutions 75 ml water containing 1 g of Na alginate and 25 ml water containing 5 g ground bran) and they were macerated by manual shaking with glass beads (4 mm). The macerates were added drop wise to a solution of 0.1M Calcium gluconate. After 20 min, beads were collected by filtration onto muslin. They were washed, collected then dried and stored as described in Section 4.3.3.1.

4.3.3.3 Potato dextrose broth culture alginate beads (PB)

Three plugs from the edge of CMA cultures of *V. chlamydosporium* were added to Petri dishes each containing 10 ml PDB, and grown for 2 weeks at 21°C. Mycelium was rinsed and excess water was drained off. The contents of 2 dishes were tipped into a McCartney bottle and 10 ml sterile 1% alginate / 5% bran mix (75 ml water containing 1 g of Na alginate mixed with 25 ml water containing 5 g ground bran) was added. The mycelium was macerated by manual shaking with glass beads (4 mm). The macerates were added drop wise to a solution of 0.1M Calcium gluconate. After 20 min, beads were collected by filtration onto muslin. Then they were washed, dried and stored as described in Section 4.3.3.1.

4.3.3.4 Dry soil inoculum (DS)

The method used was adapted from Hancock (1985). Field soil was adjusted to a soil moisture of 40% water holding capacity then disinfested by subjecting it to treatment in a microwave oven (Phillips 5210 V, 650 watts) for 4 minutes at full power. Fungal inoculum was prepared by adding 5 plugs, taken from the edge of CMA cultures of *V. chlamydosporium,* to McCartney bottles containing 10 ml PDB, and incubating for 2 weeks at 21°C. The resulting mycelium was macerated by adding glass beads and manual shaking. Then 1 ml of macerate was pipetted into Petri plates each containing 10 g microwave-treated soil and incubated for 1 week at 21°C in dark. The soil inoculum was dried and stored as described in Section 4.3.3.1.

4.3.3.5 Wheat flour/Sand inoculum (WS)

Wheat flour (approximately 30 ml) was washed on a 63 µm mesh sieve. The underside of the sieve was blotted dry and the flour was mixed with an equal volume of coarse sand. The mixture was left to air dry until slightly moist and then put into a 250

ml flask and autoclaved. When cool, the mixture was shaken and 5 plugs of *V. chlamydosporium* culture grown on CMA were added and the flasks incubated at 21°C for 3 weeks (Kerry et al. 1993).

4.3.3.6 Preparing Vc spore suspension

Vc spore suspension was prepared by tipping the Vc colonised WS medium onto a 250 µm sieve over 63 µm sieve over a sieve tray. The medium was washed with water and the suspension was collected from the tray into 10 µm sieve. A sponge was used on the base of the sieve to help draw the water through. The base of the sieve was blotted with tissue and chlamydospores were scraped off as they were collected on the mesh (Kerry et al. 1993). Spores were counted using a Haemocytometer under a stereomicroscope.

4.3.3.7 Fungal inoculation

V. chlamydosporium chlamydospores in the individual media were quantified using a Haemocytometer under a stereomicroscope and then mixed with microwaved soil (microwave oven, Phillips 5210 V, 650 watts for 2 min) to give 10^6 spores/g of soil. Pre-germinated white clover seeds were sown in the pots (5 seedlings/pot).

4.3.3.8 Inoculation of clover cyst nematode

One week after sowing, the seedlings were thinned to 2 seedlings/pot and two weeks after sowing, each seedling was inoculated with 4000 eggs.

4.3.3.9 Measurements

Eight weeks after inoculation, plants were harvested, dried, and weighed. Numbers of females, new and old cysts of *H. trifolii*, shoot and root weights were recorded. At 19-22°C it was anticipated that after 5 weeks of its life cycle *H. trifolii* could have developed first generation females (mean 380 µm in length x 220 µm in width) and after 8 weeks second generation females (260 x 120 µm). They were, thus, recorded as 1st generation females and 2nd generation females based primarily on time and secondarily on size. Eggs from mature females were isolated on water agar containing oxytetracycline (10µg/ml), (100 eggs/treatment/plate for 4 plates/treatment)

and the plates were incubated at 21°C in an incubator room for 2 weeks to check fungal parasitism on clover cyst nematode eggs. There were 24 treatments (Table 4.3) and 5 replicates for each treatment in a randomised complete block design for each treatment. Data were transformed to square-root as required and analysed by ANOVA, using SAS.

4.4 Results

4.4.1 (Trial 1) Screening a range of fungal species

Low levels of clover cyst nematode contamination, seen as white females, new cysts and old cysts (Table 4.1), were present in the control and all other treatments to which *H. trifolii* had not been intentionally added. However, the numbers of all these cyst stages in the untreated control and most of the other –*H. trifolii* treatments were significantly less than in the control+*H. trifolii.* The numbers of white females did not differ significantly among any of the –*H. trifolii* treatments.

Addition of oatmeal with *H. trifolii* significantly reduced the numbers of all three stages of the nematode compared with the control+*H. trifolii* (Table 4.1).

There were significantly more white females in the *F. oxysporum*+*H. trifolii* treatment than in the remaining +*H. trifolii* treatments. Numbers of females in the Vc1+ *H. trifolii,* Vc6+ *H. trifolii,* and oatmeal+*H. trifolii* treatment were significantly lower than in the control+*H. trifolii.* Only the Vc6+*H. trifolii* reduced the numbers of white females below that of oatmeal+*H. trifolii.*

Numbers of new cysts mainly followed trends seen with white females. The +*H. trifolii* treatments all had significantly fewer new cysts than the control+*H. trifolii* (Table 4.1). Of the –*H. trifolii* treatments, the *G. roseum*, Vc6, and oatmeal treatments had fewer new cysts than the control.

There were no significant differences in the numbers of old cysts among the –*H. trifolii* treatments (Table 4.1). However, Vc6 alone had fewer old cysts than the *F. oxysporum* only treatment. There were significant treatment effects on the numbers of old cysts in pots inoculated with *H. trifolii.* Fewer old cysts were found in all the Vc+*H. trifolii* treatments and the oatmeal+*H. trifolii* treatment than in the control+*H. trifolii.*

Addition of oatmeal significantly affected the root weight as it had less root weight than the control. Shoot dry weight in the –*H. trifolii* treatments was greatest with Vc6, while Vc1 was significantly less than the control (Table 4.1). Root dry weights

were significantly higher in the Vc6 and *F. oxysporum* treatments, and less in the *G. roseum* and Vc1 treatments, than in the control. For the +*H. trifolii* treatments, the *F. oxysporum* +*H. trifolii* and the Vc6+*H. trifolii* had greater shoot dry weight than the control and the remaining treatments. *G. roseum*+*H. trifolii* treatments had significantly less shoot or root weight than in the control+*H. trifolii*.

Table 4-1 The effect of a range of fungal isolates on reproduction of *H. trifolii* and on yield of white clover in the presence or absence of *H. trifolii* (no. of nematodes/pot)

Treatment	No. of white females/pot	No. of new cysts/pot	No. of old cysts/pot	Shoot dry weight (g/pot)	Root dry weight (g/pot)
Control	0.2f	0.8b	5.9cde	0.60cde	0.18b
Control+*H. trifolii*	14.5b	2.3a	10.8a	0.50def	0.14cde
O+*Fusarium oxysporum*	1.2f	0.3bcde	7.9abcd	0.67bc	0.23a
O+*F. oxysporum* +*H. trifolii*	26.1a	0.3bcde	9.1abc	0.76ab	0.25a
O+*Gliocladium roseum*	0.1f	0.2cde	7.0bcde	0.45efg	0.11cdef
O+*G. roseum* +*H. trifolii*	13.9b	0.7bc	9.7ab	0.33g	0.08f
O+Vc1	0.1f	0.2bcd	5.4cde	0.42fg	0.12cdef
O+Vc1+*H. trifolii*	6.2de	0.5bcde	6.1bcde	0.37fg	0.09ef
O+Vc2	0.2f	0.3bcde	6.0bcde	0.63bcd	0.16bc
O+Vc2+*H. trifolii*	12.5bc	0.5bcde	6.5bcde	0.42efg	0.10def
O+Vc6	0.0f	0.0e	3.9e	0.84a	0.27a
O+Vc6+*H. trifolii*	3.4ef	0.1de	4.8de	0.77ab	0.24a
O	0.5f	0.0e	4.4de	0.47defg	0.14cde
O+*H. trifolii*	8.4cd	0.6bcd	7.3bcd	0.36fg	0.12cdef

O = Oatmeal

Means within columns followed by the same letter are not significantly different ($P<0.05$).

The effect of a range of fungal isolates on reproduction of *H. trifolii* was also analysed based on the numbers of *H. trifolii* females and cyst stages/g of root weight (Table 4.1a).

Table 4-1a The effect of a range of fungal isolates on reproduction of *H. trifolii* (no. of nematodes/g of root weight)

Treatment	No. of white females/g of root wt	No. of new cysts/g of root wt	No. of old cysts/g of root wt
Control	1.1d	4.2c	35.6cde
Control+*H. trifolii*	111.8b	18.8a	90.5b
O+*Fusarium oxysporum*	7.2cd	1.5c	36.1cde
O+*F. oxysporum* +*H. trifolii*	108.0b	1.5c	37.0cde
O+*Gliocladium roseum*	1.7d	2.0c	65.3bcd
O+*G. roseum* +*H. trifolii*	233.5a	11.3b	142.3a
O+Vc1	0.6d	1.7c	50.4bcde
O+Vc1+*H. trifolii*	71.3bc	5.9bc	76.0bc
O+Vc2	2.2cd	2.3c	50.3bcde
O+Vc2+*H. trifolii*	123.9b	5.2bc	64.0bcd
O+Vc6	0.0d	0.0c	18.0e
O+Vc6+*H. trifolii*	14.0cd	0.4c	19.8e
O	3.5cd	0.0c	33.2de
O+*H. trifolii*	88.6b	5.9bc	69.8bcd

4.4.2 (Trial 2) Pathogenicity of *V. chlamydosporium* isolate 6 (Vc6) to *H. trifolii* and to clover seedlings

As in the previous experiment, all the clover cyst nematode stages were present in the control and almost all the treatments to which *H. trifolii* had not intentionally been added (Table 4.2). There were no significant differences among –*H. trifolii* treatments. None of the treatments reduced any stages of *H. trifolii* below oatmeal alone. Oatmeal significantly reduced numbers of white females and new cysts but did not affect numbers of old cysts.

The control +*H. trifolii* treatment had significantly more white females, new cysts, and old cysts than any of the remaining treatments (Table 4.2), indicating that all, including the oatmeal medium, reduced parasitism of white clover by *H. trifolii*. There were significantly fewer white females or new cysts in Vc6+*H. trifolii* than in oatmeal+*H. trifolii* treatment. The numbers of old cysts did not differ between those two treatments, however. No significant differences in white females and cyst stages were found between Vc6+*H. trifolii* and Vc6 spores +*H. trifolii* treatments.

The shoot and root dry weights were significantly reduced in control+*H. trifolii* compared with the control (Table 4.2), indicating that parasitism by *H. trifolii* in the experimental system affected growth of white clover seedlings. In the Vc6 and Vc6+*H. trifolii* treatments, the shoot dry weights were significantly higher than in all the remaining treatments except the control (–*H. trifolii*). The oatmeal and the oatmeal+*H. trifolii* treatments gave the least shoot weight with the former being lower than the –*H.*

trifolii control, and the latter lower than the +*H. trifolii* control. The Vc6 spores treatment did not increase shoot dry weight in the presence or absence of *H. trifolii.*

The root dry weights of Vc6 treatment was significantly higher than the remaining treatments, but not the Vc6+*H trifolii* treatment (Table 4.2).

Table 4-2 The effect of selected fungal isolate (Vc6) on reproduction of *H. trifolii* and on yield of white clover in the presence or absence of *H. trifolii* (no. of nematodes/pot)

Treatment	No. of white females/pot	No. of new cysts/pot	No. of old cysts/pot	Shoot dry weight (g/pot)	Root dry weight (g/pot)
Control	20.8bc	0.0d	1.4bc	1.6ab	0.8b
Control+*H. trifolii*	197.0a	5.6a	4.4a	0.9d	0.2cd
O+Vc6	1.6c	0.0d	0.6c	1.9a	1.1a
O+Vc6+*H. trifolii*	11.0c	0.2cd	0.8bc	1.8a	0.9ab
O	1.4c	0.0d	0.4c	0.7de	0.2cd
O+*H. trifolii*	51.2b	1.8b	3.0ab	0.4e	0.1d
Vc6 spores	2.2c	0.0d	1.8bc	1.3bc	0.3c
Vc6 spores+*H. trifolii*	24.6bc	1.2bc	2.0bc	1.0cd	0.2cd

O = Oatmeal

Means within columns followed by the same letter are not significantly different (P<0.05).

The effect of selected fungal isolate (Vc6) on reproduction of *H. trifolii* was also analysed based on the numbers of *H. trifolii* females and cyst stages/g of root weight (Table 4.2a).

Table 4-2a The effect of selected fungal isolate (Vc6) on reproduction of *H. trifolii* (no. of nematodes/g of root weight)

Treatment	No. of white females/g of root wt	No. of new cysts/g of root wt	No. of old cysts/g of root wt
Control	29.0b	0.0c	1.9b
Control+*H. trifolii*	1858.6a	37.1a	15.2b
O+Vc6	1.7b	0.0c	0.7b
O+Vc6+*H. trifolii*	11.9b	0.2c	0.8b
O	10.0b	0.0c	3.3b
O+*H. trifolii*	669.2ab	20.6ab	43.7a
Vc6 spores	6.1b	0.0c	4.4b
Vc6 spores+*H. trifolii*	85.9b	4.4bc	7.2b

4.4.3 (Trial 3) Influence of fungal inoculum medium on pathogenicity tests

4.4.3.1 Numbers of 1st generation females

No females were found in any –*H. trifolii* treatments (Table 4.3). In the +*H. trifolii* treatments the medium used almost always caused a depression in numbers of females counted compared with the Control+*H. trifolii*. Exceptions were: the AB and PB media for the 1st females, BB for the 2nd females and AB for new cysts. WS depressed production of 1st females by 83%. For the pots receiving *H. trifolii* but not *V. chlamydosporium,* the greatest numbers of females were found in those receiving *H. trifolii* alone, alginate/kaolin beads (AB+*H. trifolii*), and potato dextrose broth alginate beads (PB+*H. trifolii*), with significantly fewer than for these treatments being found in pots with wheat flour/sand (WS+*H. trifolii*), dry soil inoculum (DS+*H. trifolii*), and bran alginate beads (BB+*H.trifolii*). As an indication of the efficacy of *V. chlamydosporium*, there were significantly fewer females in the +Vc, than the –Vc, treatments for all inoculum media except bran (BB) and wheat flour/sand (WS).

4.4.3.2 Numbers of 2nd generation females

No females were found in any –*H. trifolii* treatments (Table 4.3). For the pots receiving *H. trifolii* but not *V. chlamydosporium,* the greatest numbers of females were found in those receiving *H. trifolii* alone or BB+*H. trifolii*, with significantly fewer being present in all remaining treatments. Only VAB+*H. trifolii* and VBB+*H. trifolii* had significantly fewer 2nd females than their respective medium+*H. trifolii* controls.

4.4.3.3 Numbers of new cysts

No new cysts were found in any –*H. trifolii* treatments (Table 4.3). For the pots receiving *H. trifolii* but not *V. chlamydosporium*, the greatest numbers of new cysts were found in *H. trifolii* and AB+*H. trifolii*, with significantly fewer being present in all remaining treatments. There were significantly fewer new cysts in the +Vc, than the –Vc, treatments for all inoculum media except WS. There were no significant differences among the different media in numbers of new cysts in pots receiving different types *V. chlamydosporium* inoculum.

4.4.3.4 Numbers of old cysts

There were old cysts in all +*H. trifolii* and a few in most of the –*H. trifolii* treatments except BB and VAB (Table 4.3). For the pots receiving *H. trifolii* but not *V. chlamydosporium* the greatest numbers were found in AB+*H. trifolii* and BB+*H. trifolii* treatments while none had significantly fewer than *H. trifolii* alone. There were significantly fewer old cysts in the +Vc, than the –Vc, treatments for all inoculum media except Vc and DS.

4.4.3.5 Dry shoot and root weights

AB and BB significantly increased clover shoot weights compared with the control, while WS reduced it (Table 4.3). None of the media affected root dry weight. Adding *H. trifolii* to media did not affect plant dry weight except with BB where addition of *H. trifolii* significantly increased root dry weight. Addition of Vc6 to media did not affect plant growth except with BB where both shoot and root weights were increased. Addition of Vc6 to BB+*H. trifolii* increased shoot dry weight.

4.4.3.6 Recovery of **V. chlamydosporium**

V. chlamydosporium (65-85%) was recovered from eggs in the females from all +Vc treatments. A maximum (85%) of the fungus was recovered from Vc spores+*H. trifolii* treatment.

Table 4-3 The effect of *V. chlamydosporium* (Vc6) grown on a range of media on reproduction of *H. trifolii* and on yield of white clover in the presence or absence of *H. trifolii* (no. of nematodes/pot)

Treatment	No. of 1st females/pot	No. of 2nd females/pot	No. of new cysts/pot	No. of old cysts/pot	Shoot wt (g/pot)	Root wt (g/pot)
Control	0.0i	0.0e	0.0d	1.6def	0.41ef	0.16ef
H. trifolii	100.8a	87.0a	21.0a	5.6bc	0.05h	0.02h
AB	0.0i	0.0e	0.0d	0.6f	0.98cd	0.32bc
VAB	0.0i	0.0e	0.0d	0.0f	1.02cd	0.33b
AB+*H. trifolii*	100.6a	56.4b	22.0a	9.8a	0.81d	0.23bcdef
VAB+*H. trifolii*	44.6de	13.2cde	0.8d	4.8bcde	0.90cd	0.32bc
BB	0.0i	0.0e	0.0d	0.0f	0.82d	0.25bcde
VBB	0.0i	0.0e	0.0d	1.4def	1.40a	0.54a
BB+*H. trifolii*	73.4b	86.2a	13.4b	11.0a	1.10bc	0.25bcde
VBB+*H. trifolii*	61.0bc	9.0cde	3.8cd	5.2bcd	1.30ab	0.55a
PB	0.0i	0.0e	0.0d	0.4f	0.55e	0.22cdef
VPB	0.0i	0.0e	0.0d	0.4f	0.53e	0.19def
PB+*H. trifolii*	92.6a	29.6c	6.2c	8.0ab	0.54e	0.23bcdef
VPB+*H. trifolii*	35.4ef	6.4cde	1.2d	2.6cdef	0.54e	0.26bcd
DS	0.0i	0.0e	0.0d	1.0ef	0.42ef	0.20def
VDS	0.0i	0.0e	0.0d	0.8f	0.42ef	0.17def
DS+*H. trifolii*	52.6cd	25.0cd	6.6c	5.6bc	0.40ef	0.14fg
VDS+*H. trifolii*	24.2fgh	4.2de	0.8d	1.8cdef	0.43ef	0.17def
WS	0.0i	0.0e	0.0d	0.8f	0.20fgh	0.05gh
VWS	0.0i	0.0e	0.0d	0.4f	0.10gh	0.04gh
WS+*H. trifolii*	17.8gh	9.0cde	1.0d	8.0ab	0.05h	0.02h
VWS+*H. trifolii*	8.2hi	0.4de	0.6d	2.2cdef	0.04h	0.01h
Vcspores	0.0i	0.0e	0.0d	0.6f	0.38ef	0.19def
Vcspores+*H. trifolii*	33.4efg	2.8de	1.2d	7.2ab	0.32efg	0.19def

Means within columns followed by the same letter are not significantly different (P<0.05).

AB =Alginate beads

BB = Alginate beads with bran

PB = Alginate beads from potato dextrose broth culture medium

DS =dry soil inoculum

WS =wheat flour/sand

Vc =*V. chlamydosporium* spores

VAB =Vc in alginate beads

VBB = Vc in alginate beads with bran

VPB=Vc grown on potato dextrose broth and made into alginate beads

VDS = Vc in dry soil

VWS = Vc grown on wheat flour/sand

The effect of *V. chlamydosporium* (Vc6) grown on a range of media on reproduction of *H. trifolii* was also analysed based on the numbers of *H. trifolii* females and cyst stages/g of root weight (Table 4.3a).

Table 4-3a The effect of *V. chlamydosporium* (Vc6) grown on a range of media on reproduction of *H. trifolii* (no. of nematodes/g of root weight)

Treatment	No. of 1st females/g of root wt	No. of 2nd females/g of root wt	No. of new cysts/g of root wt	No. of old cysts/g of root wt
Control	0.0b	0.0b	0.0b	11.2b
H. trifolii	21740.0a	20377.0a	3986.7a	1626.7a
AB	0.0b	0.0b	0.0b	4.0b
VAB	0.0b	0.0b	0.0b	0.0b
AB+*H. trifolii*	460.0b	276.0b	104.4b	49.1b
VAB+*H. trifolii*	144.0b	42.0b	2.6b	15.2b
BB	0.0b	0.0b	0.0b	0.0b
VBB	0.0b	0.0b	0.0b	2.7b
BB+*H. trifolii*	337.0b	453.0b	71.8b	55.9b
VBB+*H. trifolii*	112.0b	17.0b	6.7b	9.6b
PB	0.0b	0.0b	0.0b	3.4b
VPB	0.0b	0.0b	0.0b	1.6b
PB+*H. trifolii*	463.0b	173.0b	35.6b	44.8b
VPB+*H. trifolii*	161.0b	32.0b	5.1b	8.6b
DS	0.0b	0.0b	0.0b	7.7b
VDS	0.0b	0.0b	0.0b	6.0b
DS+*H. trifolii*	413.0b	194.0b	52.9b	43.2b
VDS+*H. trifolii*	141.0b	23.0b	6.0b	10.6b
WS	0.0b	0.0b	0.0b	28.7b
VWS	0.0b	0.0b	0.0b	12.2b
WS+*H. trifolii*	800.0b	364.0b	48.0b	412.0b
VWS+*H. trifolii*	770.0b	40.0b	60.0b	160.0b
Vcspores	0.0b	0.0b	0.0b	3.2b
Vcspores+*H. trifolii*	186.0b	18.0b	7.7b	45.3b

4.5 Discussion

4.5.1 Trial 1 & trial 2

The presence of white females, new cysts or old cysts in the control and –*H. trifolii* treatments suggested that the soil used for this experiment was contaminated with *H. trifolii* to some extent, probably because some indigenous *H. trifolii* nematodes had survived the freezing treatment at –20°C. In the –*H. trifolii* control and treatments most of the cyst stages found were old whereas in the +*H. trifolii* control and treatments there were many white females and some young cysts. These had presumably developed from larvae released from the egg inoculum which was added to the soil. Thus data for white females and young cysts would provide the best indication of fungal treatment effects.

The growth of white clover plants from oatmeal and oatmeal+*H. trifolii* treatments had problems. The soil treated with oatmeal alone smelled very strongly five days after planting. It is assumed that microbial growth on the oatmeal substrate created an unfavourable environment for plant growth through reduced aeration and perhaps production of toxic substances. Even though oatmeal was a control check for the effect of fungal growth medium, the numbers of white females were lower than in the control

check (without medium). Poor plant growth and production of new rootlets probably reduced egg hatching and J2 development.

Only isolate Vc6 significantly reduced the numbers of females to less than the oatmeal+*H. trifolii* treatment, suggesting that Vc6 had some affect on *H. trifolii* cyst production. There were fewer white females and new cysts in Vc6+*H. trifolii* than in the +*H. trifolii* control and the oatmeal+*H. trifolii* treatment which suggested that this formulation of the fungus reduced infection and reproduction of *H. trifolii*. However, the numbers of old cysts did not differ between those treatments, possibly because Vc6 caused premature tanning of the female cuticle, and for young females showing this effect to be regarded as old cysts, as reported for *H. avenae* and *H. schachtii* affected by *V. chlamydosporium* (Crump 1987). Both Vc6 grown on oatmeal/sand medium and Vc6 spores appeared to affect *H. trifolii* reproduction but Vc6 spore alone did not have as much of an effect on plant dry matters as Vc6 grown on oatmeal/sand medium. A possible reason was that Vc6 may have received additional nutrient from oatmeal to support the fungal growth in soil, which in turn may have allowed it to improve plant shoot and root weights significantly.

Fusarium oxysporum inoculum appeared to enhance the development of J2s to females because the numbers of females were significantly higher than the remaining +*H. trifolii* treatments. This result was similar to those of Zahid et al. (2002) who reported that *F. avenaceum* infection of the roots allowed not only increased nematode penetration, but also greater nematode development than in control plants, an effect that could be reproduced by pre-treating plants with *F. avenaceum* culture filtrate. In the present study, although, more females developed, the numbers of new cysts were lower, and old cysts were similar in *F. oxysporum*+*H. trifolii* and in control+*H. trifolii*. *Fusarium oxysporum* may have affected the development of white females into further cyst stages possibly by parasitising eggs of females that could be a source of new cysts. Sosnowska (1996) found that *F. sambucinum* parasitised *H. schachtii* and Mizobutsi et al. (2000) reported that, *F. oxysporum* and *F. solani* parasitised *H. glycines*.

None of fungi+*H. trifolii* treatments reduced the numbers of new and old cysts more than the oatmeal+*H. trifolii* treatment because poor plant growth in oatmeal appeared to affect *H. trifolii* reproduction. Another possibility was that microbes that developed to high populations in the oatmeal alone treatment, and/or their toxic products, could have affected reproduction of *H. trifolii*. Furthermore, the improved

plant growth in the Vc+oatmeal treatment could have been caused by the growth of the fungus in the Vc+oatmeal inoculum exhausting free nutrients and thus encouraging less growth of these microbes in soil.

The root dry weights were lower in control+*H. trifolii* than in control-*H. trifolii* as might be expected if nematode parasitism was having a substantial effect on plant growth. Shoot dry weights, however, did not differ between those two treatments, possibly because the effects on root physiology were not sufficient to influence shoot weight in an experimental system where water and nutrient supply were abundant. Although the numbers of all cyst stages were higher in control+*H. trifolii* than the remaining treatments, the shoot or root dry weights only differed from the control in the *F. oxysporum*+*H. trifolii* and the Vc6+*H. trifolii* treatment, again probably because in this system *H. trifolii* did not have a severe effect on plant growth. *F. oxysporum* and Vc6 appeared to enhance formation of new rootlets and consequently those isolates caused weight increases in roots and shoots. *G. roseum*, Vc1 and Vc2 had little effect on growth of *H. trifolii*-infected clover plants.

H. trifolii reduced the shoot or root dry weights as the dry weights were lower in control+*H. trifolii* compared with control check. The increased shoot and root dry weights of plants treated with Vc6 grown in the oatmeal/sand medium may reflect a direct effect on seedling growth, or be the indirect result of reduced numbers of viable J2 released from parasitised cysts. Vc6 spore treatment did not affect root growth.

4.5.2 Trial 3

In contrast to the previous experiments, in which freezing failed to kill all *H. trifolii* in the experimental soil, no *H. trifolii* females or new cysts were found in any of the -*H. trifolii* treatments. This suggests that treatment in the microwave oven for 4 min was sufficient to kill the existing *H. trifolii* in the soil.

This experiment provided strong evidence that inoculation with *V. chlamydosporium* reduced the numbers of *H. trifolii* females. Even the *V. chlamydosporium* spore inoculum without medium (Vc) reduced the numbers of 1st generation females, possibly through an effect of *V. chlamydosporium* itself on *H. trifolii* reproduction. Siddiqui et al. (2000) also found that the soil application of spore suspension of *V. chlamydosporium* or *P. lilacinus* significantly reduced the galling of

M. javanica on aubergine. The numbers of 1st or 2nd generation females were significantly lower in the +Vc+*H. trifolii* treatments than the -Vc+ *H. trifolii* treatments with the same medium/inoculum except for the bran bead inoculum. In this treatment (VBB), showed reduced numbers of 2nd generation, but not of 1st generation, white females compared with the medium alone (BB). Possibly, the presence of large numbers of 1st generation females may have increased release of nutrients into the rhizosphere which stimulated the growth and parasitism of the fungus so that its effect was evident in the 2nd generation. Such an effect was reported by Dourhourt et al. (1993), who found that the presence of the nematodes increased release of nutrients into the rhizosphere, which the fungus was able to exploit. Leij & Kerry (1991) found that an isolate of *V. chlamydosporium* reduced the population by more than 80% after the first nematode generation of *M. arenaria* on tomato plants and that growth of *V. chlamydosporium* was greater on galled root tissue than on unaffected roots.

The DS and WS inocula were different from others as they were not bead-based. DS only had nutrients present in soil or as released during the microwave process, whereas WS had the wheat flour. Of the bead-based treatments the BB and PB would have had residual nutrients in the bran to support fungal growth and colonisation in soil; the AB treatments had no such nutrient base. In general, AB, BB or PB media themselves did not reduce the numbers of females, but Vc grown on those media did reduce the numbers of females. In a field experiment, wheat bran inocula of *V. chlamydosporium* or *P. lilacinus* significantly reduced galling of *M. javanica* on aubergine (Siddiqui et al 2000). *V. chlamydosporium* multiplied on wheat bran significantly reduced cyst number of *H. avenae* on wheat and improved plant growth (Bharadwaj & Trivedi 1996). Granules of *V. chlamydosporium* multiplied on wheat bran reduced multiplication of *M. incognita* on tomato, and it also improved plant growth (Sankaranarayanan et al 2000).

The numbers of females present in all treatments were reflected in the numbers of new cysts, and to some extent old cysts in relevant treatments. For instance, there were high numbers of old cysts in AB+*H. trifolii*, BB+*H. trifolii* or Vc+*H. trifolii* treatments which also had high numbers of females and new cysts.

Shoot and root mean dry matters in the *H. trifolii* alone treatment were reduced to about 12% of those in the control (Table 4.3), indicating that inoculation with clover cyst nematode in these experimental conditions did adversely affect plant growth. The

weights of shoots or roots were not different among PB, DS, WS media -*H. trifolii* or those media+*H. trifolii* treatments, possibly because these media caused poor plant vigour that might affect the *H. trifolii* reproduction so that the reduced numbers of nematodes were not sufficient to affect plant growth.

Adding *V. chlamydosporium* in the BB or AB treatments (VAB or VBB) resulted in a greater effect on shoot or root weight than when used in the treatments Vc alone, VPB, VDS, or VWS. Sankaranarayanan et al. (2001) reported that considerable reduction of *H. cajani* cyst population on pigeon pea and a significant increase in plant height and root weight were observed following addition of *V. chlamydosporium* cultured on different substrates such as sorghum grain, broken maize grain, broken wheat grain and wheat bran.

Rates of fungal parasitism may have been underestimated as parasitised females are fragile and difficult to handle. The maximum recovery of the fungus was from the eggs of Vc spores+*H. trifolii* treatment, probably because: 1) Vc alone had some effect on parasitism of eggs, 2) establishment of Vc spores could be greater in soil by adding Vc alone than by adding Vc+medium. Establishment of *V. chlamydosporium* in soil was significantly greater if the fungus was introduced without a foodbase, i.e. as hyphal fragments and chlamydospores rather than colonised sand-bran (Leij & Kerry 1991). The fungus did not have any effect on improving plant growth in this pot trial.

4.6 Conclusion

Oatmeal is not a suitable medium for addition to soil used for growth of white clover seedlings. *G. roseum* did not reduce the numbers of *H. trifolii,* but it seemed to reduce the dry weights of shoots and roots of clover. *F. oxysporum* had some positive effect on shoot and root dry weight of clover but it also increased the numbers of *H. trifolii* females. Vc1 and Vc2 did not reduce the numbers of *H. trifolii* and did not affect dry weight.

V. chlamydosporium (Isolate Vc6) appeared to have had some effect in reducing female populations, possibly through egg parasitism. Consequently, there were few white females and cyst stages in Vc inoculated treatments. Vc6 may have better potential parasitism, and ultimately biological control, of *H. trifolii* than *F. oxysporum* or *G. roseum*. Vc treatment, using chlamydospores without any nutrient medium or

alginate encapsulation, reduced the numbers of females and new cyst stages. The fungus was re-isolated from 85% of eggs examined but no significant effect on plant growth could be detected. The VBB treatment (*V. chlamydosporium* in alginate beads containing bran) reduced the numbers of females and new cysts and increased both dry shoot and root weights. Therefore, the bran alginate bead inoculum seemed to be the most suitable of those tested for multiplication of *V. chlamydosporium*, control of clover cyst nematode *H. trifolii* and improving clover plant growth in this pot trial.

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5 MONOXENIC CULTURE OF CLOVER CYST NEMATODE *HETERODERA TRIFOLII* ON WHITE CLOVER *TRIFOLIUM REPENS*

5.1 Introduction

Gnotobiotic techniques, whereby nematodes are cultured *in vitro* in the presence of a known number of other organisms, have proved useful in many aspects of nematology (Jones 1980), including studying the effect of nematodes on plants (Chen et al. 1961), culturing obligate parasites of nematodes (Verdejo & Jaffee 1988), feeding behaviour of nematodes, their effects on host metabolism (Jones 1980), nematode development (Lauritus et al. 1983), and observation of fungal parasitism of nematode eggs *in situ* (Hay 1993). Such techniques may overcome some of the limitations of traditional methods of screening fungi against nematodes.

A major difficulty in achieving gnotobiotic culture is the development of a sterilisation method rigorous enough to kill contaminant bacteria and fungi, while being harmless to nematodes. One example is the use of Hibitane diacetate, which has been used successfully to decontaminate various nematode species (Koenning & Barker 1985, Hooper 1986).

Several species of *Heterodera*, *Globodera*, and *Meloidogyne* have been raised on roots using a variety of media, for example, White's and Tiner's media (Hopper 1986), Gamborg's B-5 media (Gamborg et al. 1976) without cytokinins or auxins (Huettel, 1990), and Skoog, Tsui and White's media (Orion et al. 1980, Lauritus et al. 1983).

Monoxenic culture of several species of plant-parasitic nematodes on callused host tissues has been described in the nematological literature (Riedel & Foster 1970). Christie and Crossman (1936) successfully cultivated *Aphelenchoides fragariae* in agar plates inoculated with a fungus. Todd and Atkins (1958) described a method for culturing mass populations of *Aphelenchoides besseyi* from infested rice seeds.

Krusberg (1961) aseptically cultured *Ditylencus dipsaci* and *Aphelenchoides ritzemabosi* by passing them singly with a needle through five or six baths of a solution of 20 ppm malachite green plus 1000 ppm streptomycin sulphate, contained in sterile

Syracuse watch glasses for 3 to 4 hours before transfer to agar. Miller (1963) cultured *Meloidogyne* spp. aseptically by rinsing eggs of *M. hapla* and *M. incognita* for 2 min in 0.1% formaldehyde followed by two separate rinses in SDW after which the eggs were transferred to water agar containing 5% neomycin. Paracer and Zuckerman (1967) surface-sterilised adult *Dolichodorus heterocephalus* by immersion in 0.5% Hibitane diacetate for 5 to 7 min and then rinsing in SDW before transfer to corn root callus tissue.

Aumann (1997) described a method for monoxenic culture of *Xiphinema index* on the root tips of *Ficus carica* in a sterile nutrient agar medium, maintained for up to a year with 2 nematode generations in a single Petri dish. Chavarria-Hernanadez & Torre (2001) cultured *Steinernema feltiae* monoxenically on liquid media, such as soyabean flour/egg yolk/yeast extract and egg yolk/yeast extract.

Chen et al. (1961) used a solution of streptomycin sulphate (0.1%) and malachite green (30 ppm) as disinfectant for surface-sterilisation of J2 of *H. trifolii*. Lauritis et al. (1983) used yellow females with egg masses from stock cultures to cultivate *H. glycines* on soybean gnotobiotically. *H. schachtii* were monoxenically cultured from J2 which were surface-sterilised by immersion for 72 h at 25°C in a solution of 10 p.p.m. ethoxyethyl mercury chloride with 0.01% dioctyl sodium sulphosuccinate and Crystamycin (1000 units sodium penicillin G + 1 mg. Streptomycin/ml) (Moriarty 1964).

Monoxenic cultures were reported for the following species: *H. avenae*, *G. rostochiensis*, *H. glycines*, *H. oryzae*, and *H. schachtii* (Lauritis 1983). Hay & Regnault (1995) surfaced sterilised J2 of *H. trifolii* in a syringe filter holder with 20% Hibitane, a solution of Penicillin G, Streptomycin sulphate, and 0.05% Tween 80. *H. trifolii* was monoxenically reared by inoculation of the surfaced-sterilised J2 on white clover (*Trifolium repens),* growing in small volumes of sand in pipette tips for several weeks (Hay & Regnault 1995), growing on a modified Hoagland & Knop's agar (Hay 1993) and on excised root tips of white clover on White's medium (Hay 1994).

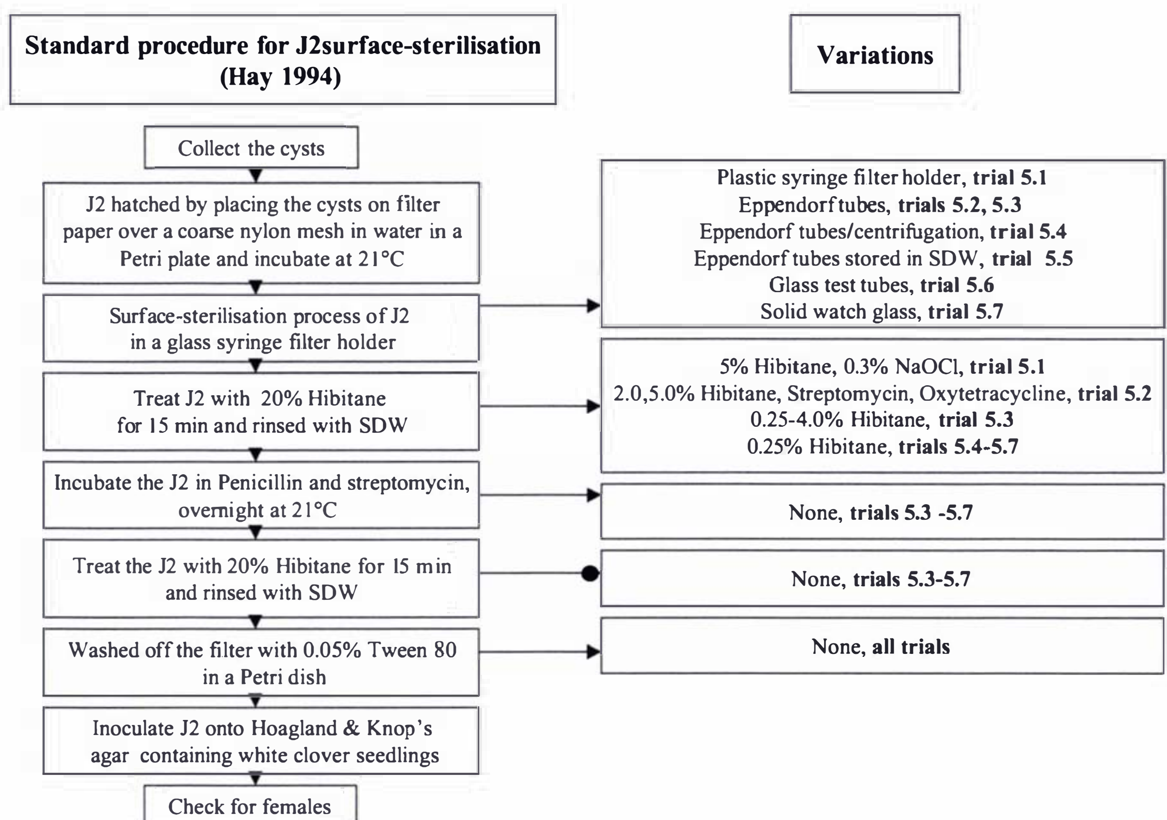
Monoxenic culture could be useful for screening of *V. chlamydosporium* isolates *in vitro* in order to save the time of conducting pot trials in a glasshouse.

The main aim of this work was to study the potential of fungal parasitism for biocontrol of *H. trifolii*. To reach this aim, a pre-requisite was to grow the *H. trifolii* in monoxenic culture. The J2 of *H. trifolii* are very small and difficult to handle during the

process of surface sterilisation for monoxenic culture, with the result that most are lost during the sterilisation process. The aim of this section of work was to improve the efficiency of the sterilisation process.

The general procedure for disinfecting nematodes is to treat them with disinfectant (e.g. Hibitane) and antibiotics (e.g. Penicillin and Streptomycin) then add them to medium with white clover seedlings. Problems can arise with chemical toxicity, adherence to containers and physical damage to nematodes during the transfer in surface-sterilisation process.

A series of experiments was carried out to evaluate losses and consider ways to improve the efficiency of each of these factors (Fig. 5.1).



SDW = Sterilised, distilled water

Figure 5-1 Variations in the procedure for surface-sterilisation of *H. trifolii* J2

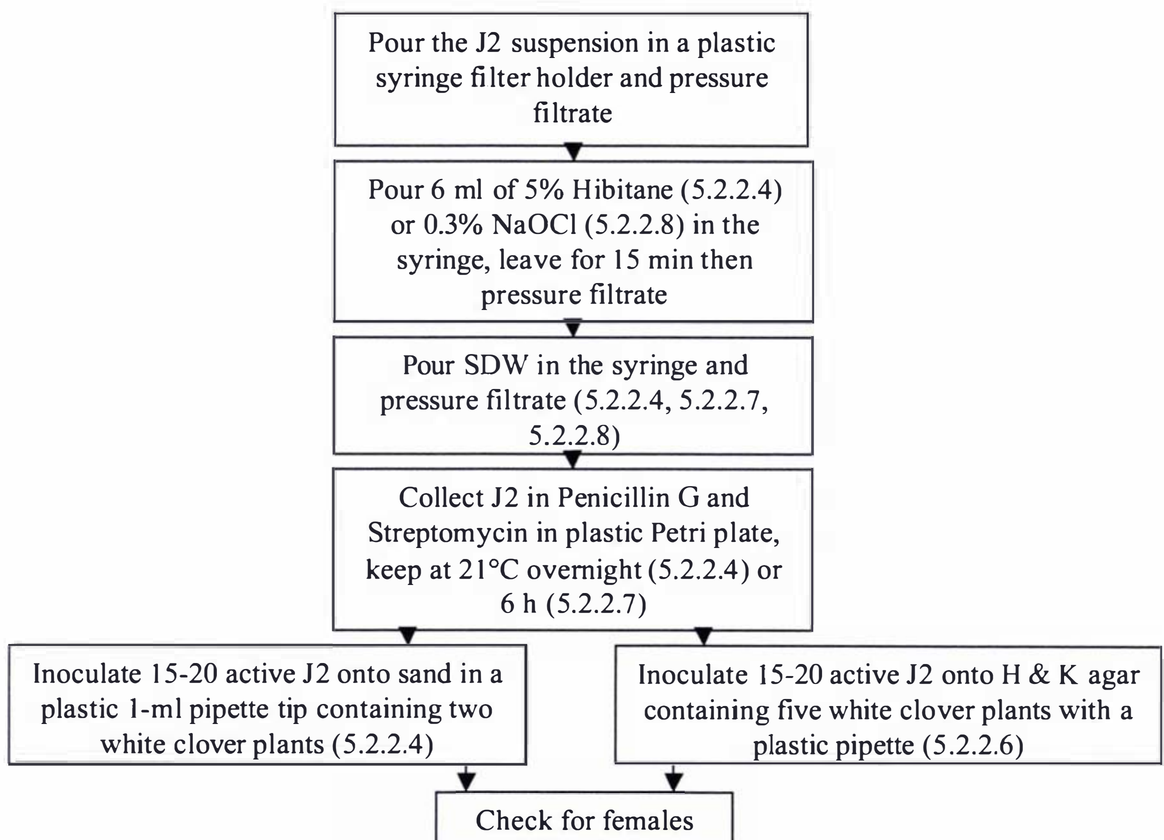
5.2 Trial (5.1) Surface-sterilisation of *H. trifolii* J2 with Hibitane and culture of *H. trifolii* in sand or on agar

5.2.1 Objectives

(1) To assess the surface-sterilisation of J2 with Hibitane using a plastic syringe filter holder and inoculation of clover plants growing in sand or in agar plates.

(2) To assess the surface-sterilisation of J2 with Hibitane followed by 6 h in antibiotics or in NaOCl using a syringe filter holder and inoculation of clover plants growing in agar plates.

5.2.2 Materials and Methods



H & K = Hoagland & Knop's

Figure 5-2 Procedure for surface-sterilisation of *H. trifolii* J2 in trial (5.1)

5.2.2.1 Germination of white clover seeds

Seeds of white clover *T. repens* cv. Huia were surface sterilised in $H_2 SO_4$ (50% for 15 min) followed by NaOCl (0.3% for 15 min) with three rinses in 10 ml of sterilised distilled water (SDW) after each treatment. Seeds were placed on half strength potato-dextrose agar (PDA) in Petri plates under aseptic condition and germinated at 21° C for 4 days.

5.2.2.2 Planting of white clover seedlings in sand-filled pipette tips

Plastic pipette tips (1000 µL) were filled with sieved sand (125-250 µm) and tips were placed individually into test-tubes (15 x 2 cm) containing 700 µL of modified Hoagland & Knop's solution, capped with translucent polypropylene closures before autoclaving. Two seedlings free of contaminants were placed on the inside edge of each plastic pipette tip and the tips were placed under lights (a mixture of two of SYLVANIA, GRO-LUX, F18W/GRO-T8 and two of PHILIPS, TLD 18W/33, COOL WHITE) under a 12 h / 12 h day/night regime, at 20°C.

5.2.2.3 Preparation of Hoagland & Knop's solution

The modified Hoagland & Knop's solution was prepared as follows and adjusted to a pH of 6.0: macroelements, $Ca (NO_3)_2 . 4H_2O$, 0.95 g, KNO_3, 0.61 g, $MgSO_4.7H_2O$, 0.49 g, $NH_4H_2PO_4$, 0.12 g; microelements (stock solution, 1,000 ml in vol), $MnSO_4.4H_2O$, 3.0 g, $ZnSO_4.7H_2O$, 0.5 g, H_3BO_3, 0.5 g, $CUSO_4.5H_2O$, 0.025 g, $Na_2MoO_4.2H_2O$, 0.025 g, H_2SO_4 (sp gr 1.83), 0.5 ml; ferric citrate (stock solution, 1,000 ml in vol), $FeC_6O_5H_7.5H_2O$, 10 g, distilled water to 1,000 ml. One ml of the microelement-stock solution and 2 ml of the ferric citrate stock solution were used for each liter of solution.

5.2.2.4 Surface-sterilisation and inoculation of J2 (Second stage juveniles)

J2 were pre-cleaned by placing 50 young cysts recently collected from a white clover/ryegrass permanent pasture trial, AgResearch, Grasslands, Palmerston North, New Zealand, on a paper towel nested in water in a plastic Petri plate (Fig. 5.1). Active J2 were collected 2 days after hatching. J2 were surface sterilised in a plastic syringe filter holder fitted with a cellulose acetate filter (8 µm) by adding 6 ml 5% Hibitane [1%

active ingredient (a.i.), 100 Chlorhexidine Gluconate 20% Solution, City Health Pharmacy LTD] for 15 min. They were rinsed with 20 ml SDW, then washed from the filter and incubated overnight at 21°C in a solution of antibiotics Penicillin-G and Streptomycin sulphate BP (both at 0.1% w/v) (Fig. 5.2). They were treated again with 5% Hibitane (1% a.i.) for a further 15 min, rinsed in SDW and washed off the filter with SDW into a plastic Petri plate. J2 were counted under a dissecting microscope in a laminar flow cabinet and 15-20 active J2 were drawn up with a 50 μL plastic pipette and inoculated onto one of the sand-filled tips with two 4-day-old white clover plants. Each tip was replaced under the lights. Thirty to 45 days after inoculation, plants were checked under a dissecting microscope for mature females and cysts. Non-surface sterilised J2 were inoculated onto sand-cultured plants as control checks (Fig. 5.2) and there were 7 replications for each treatment.

5.2.2.5 Hoagland & Knop's agar

The medium was prepared by adding agar (5.0 g/1000 ml) into the Hoagland and Knop's solution as described in Section 5.2.2.3.

5.2.2.6 Inoculation of J2 in agar plates

Using a stereomicroscope, 20-25 surface-sterilised active 2-day-old J2 were drawn up with a 50 μL plastic pipette and inoculated around the roots of white clover seedlings in plates containing modified 0.5% Hoagland & Knop's agar (5 seedlings/plate). Each plate was sealed with plastic tape and placed under lights as in Section 5.2.2.2. From 2 weeks after inoculation, plants were checked microscopically for mature females and cysts.

5.2.2.7 Surface-sterilisation of J2 with antibiotics

J2 were surface-sterilised as in Section 5.2.2.4 except they were incubated at 20°C in antibiotics for 6 h (Fig. 5.2) and then inoculated (20-25 J2/plate) into plants in Hoagland & Knop's agar plates as in Section 5.2.2.6.

5.2.2.8 Surface-sterilisation of J2 with NaOCl

J2 were surface-sterilised for 2 minutes by placing them in 15 ml of 0.3% NaOCl and drawing through a plastic filter holder on a syringe (Fig. 5.2). After three rinses of 10 ml SDW, J2 were washed off the filter into a sterile plastic Petri plate with 10 ml SDW. J2 were inoculated (20-25 J2/plate) around the roots of seedlings in the Hoagland & Knop's agar plates.

5.2.3 Results

Forty-five days after inoculation of J2 onto sand-filled tips, each J2-inoculated seedling had an average of one or two females from each of sterilised and non-sterilised J2-inoculated plant respectively. One plant with a mature female was found from one of ten Hoagland & Knop's agar plates. Two plants with one mature female each were found from one of two plates in the 6 h antibiotics treatment. Only one plant with a mature female was found from one of two plates in the NaOCl treatment.

5.2.4 Discussion

The numbers of replicates for each experiment were not the same because J2 were lost after the surface-sterilisation treatments. More females were found from the non-sterilised J2-inoculated plants than from sterilised ones from sand culture. The combination of Hibitane with 6 h in antibiotics might be a better disinfectant than Hibitane with antibiotics in overnight or NaOCl. Although females developed into cysts, they had not hatched by 45 days as had cysts in agar plates either J2 surface-sterilised with Hibitane or with NaOCl. The cysts were crushed and the eggs examined microscopically. J2 within the eggs were not fully developed. The principal causes of lost J2 during the surface-sterilisation process and undeveloped J2 within the eggs could be due to 1) adhesion to the filter holder, 2) possible toxicity of potential Hibitane, 3) possible toxicity of the antibiotics Penicillin G and Streptomycin, or NaOCl.

In Hay & Regnaults' report (1995), J2 were surfaced sterilised with 20% Hibitane (1 % a.i) for 15 min, followed by 1 rinse in SDW and they were then incubated in Penicillin-G / Streptomycin Sulphate BP (both at 0.1% w/v) overnight. The Hibitane treatment was repeated and J2 were washed with 0.05% Tween 80 before inoculation. They found that on average, 10-24% of the J2 developed into egg-containing females in

sand in tips. In the current study, only (5-10%) of the J2 which were surfaced sterilised with 5% Hibitane (100 Chlorhexidine Gluconate 20% Solution), developed into females in sand in tips. The differences between their investigation and this one were 1) a different type of Hibitane, 2) glass syringe (Hay) and plastic syringe (current study).

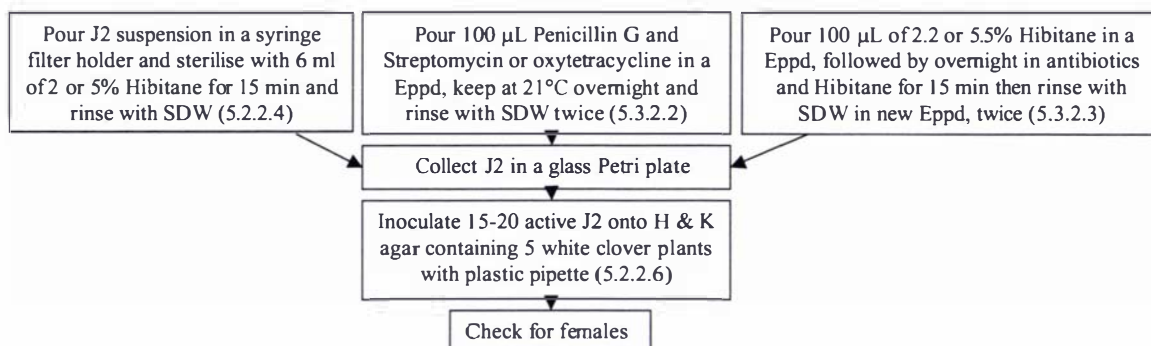
5.3 Trial (5.2) Effect of Hibitane and antibiotics as surface sterilants

5.3.1 Objectives

(1) To assess the surface-sterilisation of J2 with 2 or 5% Hibitane using a syringe filter holder.

(2) To assess the surface-sterilisation of J2 with antibiotics Penicillin-G and Streptomycin or Oxytetracycline alone, or a combination of Hibitane and antibiotics using an Eppendorf tube.

5.3.2 Materials and Methods



Eppd = Eppendorf tube

Figure 5-3 Procedure for surface-sterilisation of *H. trifolii* J2 in trial (5.2)

5.3.2.1 Surface-sterilisation of J2 with Hibitane

J2 were surface-sterilised with either 2% or 5% Hibitane (1% a.i.) for 15 min and rinsed with 20 ml SDW in a plastic syringe filter holder (Fig. 5.3) as described in Section 5.2.2.4. SDW was used instead of Hibitane for the control check.

5.3.2.2 Surface-sterilisation of J2 with antibiotics in Eppendorf tubes

J2 were placed in 10 μL of distilled water in an Eppendorf and 100 μL of the antibiotics Penicillin-G and Streptomycin sulphate BP (both at 0.1% w/v) were pipetted into the Eppendorf and the J2 suspension was incubated overnight at 21°C, followed by two changes of SDW. J2 in SDW in an Eppendorf and in a glass Petri plate were used as control checks (Fig. 5.3). Plants in agar plates were inoculated as described in Section 5.2.2.6.

J2 were surface-sterilised with Oxytetracycline (10 μg/ml) instead of Penicillin-G and Streptomycin (Fig. 5.3).

5.3.2.3 Surface-sterilisation of J2 with combination of Hibitane and antibiotics in Eppendorf tubes

J2 were surface-sterilised with either 2.5 or 5.5 % Hibitane for 15 min followed by antibiotics Penicillin-G and Streptomycin sulphate using Eppendorfs (Fig. 5.3) as described in Section 5.3.2.2. J2 were then treated with either 2.2 or 5.5% Hibitane (1% a.i.) for a further 15 min, and rinsed in SDW twice (Fig. 5.3). J2 placed in an Eppendorf and in a glass Petri plate containing SDW were used as check controls. J2 were counted under a dissecting microscope in a laminar flow cabinet. Plants in agar plates were inoculated (10-20 J2/plate) as described in Section 5.2.2.6.

5.3.3 Results

J2 (9-14%) were found 15-22 h after sterilisation with Hibitane. Survival of J2 was 3% in the untreated filtered control treatment and 95% in untreated without filter control treatment (Table 5.1).

Table 5-1 Percentage survival of J2 after treatment with Hibitane, using filter holder

	2% Hibitane		5% Hibitane		Sterilised, distilled water			
					Filter		Without filter	
	No.	%	No.	%	No.	%	No.	%
Initial count	47	100	105	100	26	100	77	100
15 - 22 h aftertreatment	7	14	10	9	1	3	73	95

Sixteen hours after sterilisation with Streptomycin, 65% of J2 were still alive, but the numbers of J2 from Oxytetracycline were not counted because they were mixed with crystals from Oxytetracycline making it difficult to draw them up into the pipette for inoculation after a 16 h incubation. J2 (92-95%) had survived from the control checks (Table 5.2). Two days after inoculation, bacteria were found in the agar plates inoculated with Streptomycin treated J2 and with J2 from control checks.

Table 5-2 Percentage survival of J2 after treatment with antibiotics, using Eppendorf tubes

	Streptomycin		Oxytetracycline		Eppd, control		Petri plate, control	
	No.	%	No.	%	No.	%	No.	%
Initial count	20	100	25	100	50	100	55	100
16 h after treatment	13	65	?		46	92	52	95

Eppd = Eppendorf, ? = no. of J2 were not counted

Sixteen hours after sterilisation with Hibitane, 67-78% of J2 survived and 65 % of J2 survived from Streptomycin treatment. Survival of J2 was (95- 100%) from the Petri plate and Eppendorf control checks (Table 5.3). Agar plates inoculated with J2 from the Streptomycin treatment and control checks were contaminated with bacteria.

Table 5-3 Percentage survival of J2 after treatment with Hibitane and Streptomycin, using Eppendorf tubes

	2% Hibitane		5% Hibitane		Streptomycin		SDW, Eppd		SDW, Petri plate	
	No.	%	No.	%	No.	%	No.	%	No.	%
Initial count	70	100	15	100	20	100	15	100	55	100
16 h after treatment	55	78	10	67	13	65	15	100	52	95

SDW = sterilised, distilled water, Eppd = Eppendorf

Forty-five days after inoculation, 1 – 2 females/plate were found on plants which were inoculated with J2 that had been treated with either 2.2 or 5.5% Hibitane followed by Streptomycin and Penicillin G. Although those females became cysts, J2 were not seen to emerge from them 45 days after the J2 became females.

5.3.4 Discussion

It was concluded that the principal cause of the mortality of the J2 was filtering, not Hibitane treatment as more survival J2 were recovered in Hibitane/Eppendorf than in Hibitane/filter holder. Both Hibitane and antibiotics (Penicillin and Streptomycin) caused some mortality of J2. Contaminants did not grow in the two Hibitane combination with antibiotics treatments but did grow in the antibiotics and control treatments. The cysts from the Hibitane combination with antibiotics treatments were crushed and examined microscopically. J2 within the eggs were not fully developed suggesting that the Hibitane combination with antibiotics treatments may have affected the eggs of *H. trifolii.*

The investigation of the effects of surface sterilisation on J2 survival had to be done as a series of experiments, not as one experiment because, a) there were insufficient J2 available at any one time and, b) because it would not have been possible to accurately carry out treatments and assessments with more than three simultaneous treatments.

The hatching rate of eggs within the cysts was often low because the J2 could be not completely developed, and most were lost during the surface-sterilisation process so the initial numbers of living J2 used for surface sterilisation, differed between treatments in the same experiment.

5.4 Trial (5.3) Surface sterilisation of J2 with various concentrations of Hibitane

5.4.1 Objectives

(1) To assess surface-sterilisation of J2 with a range of concentrations of Hibitane using Eppendorf tubes.

(2) To assess the cause of J2 loss during surface-sterilisation with SDW using a filter holder or an Eppendorf tube.

5.4.2 Materials and Methods

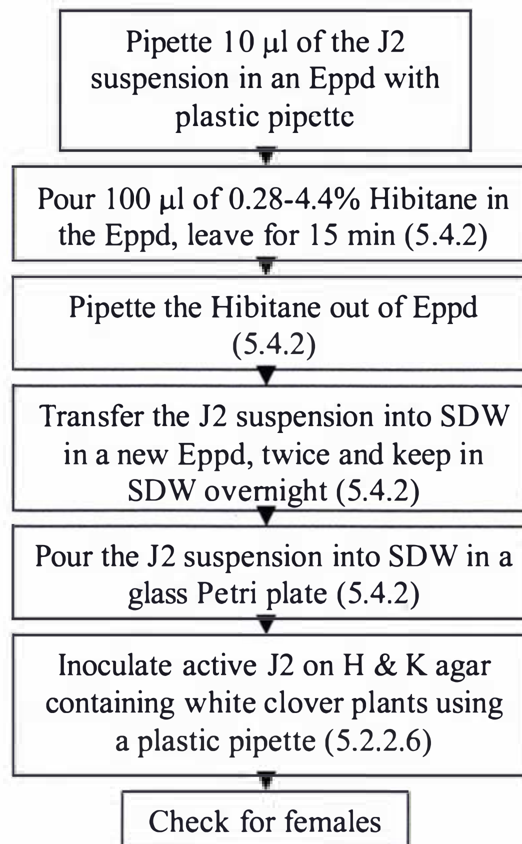


Figure 5-4 Procedure for surface-sterilisation of *H. trifolii* J2 with Hibitane in trial (5.3)

J2 were placed in 10 µL of SDW in an Eppendorf and 100 µL of Hibitane from each of 0.28%, 0.55%, 1.1%, 2.2%, and 4.4% were then added (Fig. 5.4). After 15 min, Hibitane was pipetted out and J2 were washed in 100 µL of SDW in an Eppendorf for 15 min twice. J2 in an Eppendorf of SDW were used as one control while J2 placed in SDW in a glass Petri plate (no transferring) were a control to check whether the process of transferring J2 affected survival. J2 surface-sterilised with two changes of SDW using a syringe filter holder was a check on whether the syringe filter holder affected J2 loss.

After sterilisation, J2 were counted under a stereomicroscope and then incubated in SDW overnight at 21°C before recounting the living J2 (Fig. 5.4). Surviving J2 were

inoculated in Hoagland and Knop's agar plates (eight J2 per plate, two seedlings per plate) as described in Section 5.2.2.6.

5.4.3 Results

There was a large loss (30-84%) of J2 during the sterilisation process with the Hibitane treatments and with the SDW/Eppendorf control but all J2 had survived in the SDW Petri plate control (Table 5.4). Sixteen hours after treatment, all J2 initially recovered in all treatments had survived (Table 5.4).

Table 5-4 Percentage survival of J2 after treatment with Hibitane in Eppendorf tubes

	Hibitane										SDW		Control	
	0.25%		0.50%		1.00%		2.00%		4.00%					
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Initial count	50	100	50	100	50	100	50	100	50	100	50	100	50	100
0 h after treatment	8	16	14	28	19	38	31	62	35	70	16	32	50	100
16 h after treatment	8	16	14	28	19	38	31	62	35	70	16	32	50	100

SDW = sterilised, distilled water

Two days after inoculation, bacteria were found in the agar plates inoculated with J2 from SDW and from the control check but there was no contamination in any of the Hibitane treatments 5 days after inoculation. Forty-five days after inoculation, one – two females were found from each Hibitane treatment, they had already hatched, and two – ten J2 were developing into females 60-70 days after inoculation.

Fifty-two to 68% of J2 were lost in both treatments during the sterilisation process. Sixteen hours after treatments, all J2 had initially recovered in the SDW/Eppendorf treatment but only 34% of the initial J2 count in SDW/filter holder treatment had survived. J2 (100%) were still active in the control check (Table 5.5). Two days after inoculation with J2, all of the agar plates were contaminated with bacteria.

Table 5-5 Percentage survival of J2 in SDW, using Eppendorf tubes or filter holders

	SDW,Eppd		SDW,Filter		Control	
	No.	%	No.	%	No.	%
Initial count	50	100	50	100	50	100
0 h after treatment	16	32	24	48	50	100
16 h after treatment	16	32	17	34	50	100

SDW = sterilised, distilled water; Eppd = Eppendorf; Filter = filter holder

5.4.4 Discussion

It was concluded from these experiments that most of the J2 were lost because (1) many failed to sink to the base of the Eppendorf within 15 min of treatment. They were therefore removed when the Hibitane or SDW were pipetted out of the Eppendorfs. The lower the concentration of Hibitane used, the fewer J2 descended to the base of the Eppendorf during the sterilisation period because most of the J2 were more active at lower concentrations of Hibitane than at the highest one. (2) They may have stuck to the Eppendorf wall. Hibitane controls contaminants at concentrations of 0.25% and above. The principal cause of the mortality of J2 was filtering. There was a loss of J2 in both treatments and some died in the SDW / filter treatment.

5.5 Trial (5.4) Surface sterilisation of J2 with Hibitane, using a centrifuge

5.5.1 Objective

Since most of the J2 appeared to be lost because many failed to sink to the base of the Eppendorf within 15 min of treatment with Hibitane, the objective of this experiment was to determine if J2 could survive centrifugation and Hibitane surface sterilisation of J2.

5.5.2 Materials and Methods

Forty active freshly-hatched J2 were used for each treatment. J2 were surface sterilised with 0.25% Hibitane in an Eppendorf. This was done by placing 10 µl of distilled water/nematode suspension in an Eppendorf and adding 100 µL of 0.28% Hibitane (Fig. 5.5). After 15 min, the Eppendorf was centrifuged at 1000 rpm for 1 min.

Hibitane was then removed with a 200 µL plastic pipette tip and the J2 were transferred to another Eppendorf containing 100 µL of SDW. They were gently shaken, re-centrifuged, re-washed, and centrifuged before pipetting into a glass Petri plate. J2 were surface sterilised with Hibitane but without centrifuging as a control to check whether centrifuging of J2 affected the loss or survival. J2 in an Eppendorf containing SDW only, with and without centrifuging were also used as controls. J2 placed in a glass Petri plate or an Eppendorf containing SDW (not using either pipetting or centrifugation) were used as a final control to check whether transfer of J2 affected loss or survival. After sterilisation, J2 were counted under a microscope and then incubated in SDW overnight at 21°C before recounting the living J2 (Fig. 5.5).

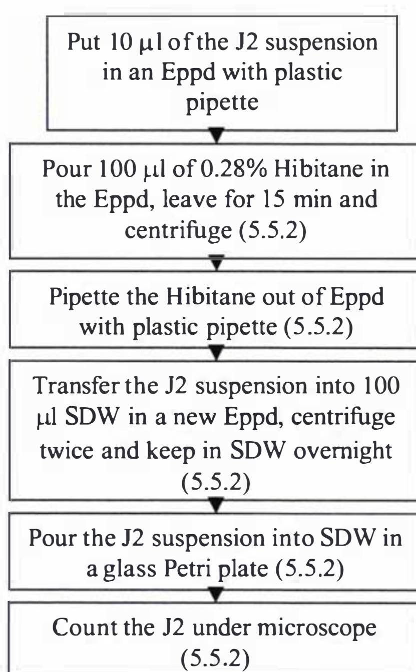


Figure 5-5 Procedure for surface-sterilisation of *H. trifolii* J2 with Hibitane using centrifuge in trial (5.4)

5.5.3 Results

All J2 initially recovered in all treatments were alive 16 hours after treatment (Table 5.6). A high proportion (47-87 %) of J2 were lost during the process of Hibitane treatments, especially when no centrifugation was used (Table 5.6). Losses were not as

great in the SDW controls (10 – 20%) and were less with no centrifugation. No loss was found in the glass Petri plate control check (Table 5.6).

Table 5-6 Percentage survival of J2 after treatment with Hibitane, using Eppendorf tubes with and without centrifugation

	0.25% Hibirtane				0.5% Hibitane				SDW				Glass Petri plate	
	Centrifuge		no centrifuge		Centrifuge		no centrifuge		Centrifuge		no centrifuge			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Initial count	40	100	40	100	40	100	40	100	40	100	40	100	40	100
0 h after treatment	10	25	5	13	21	53	17	43	32	80	36	90	40	100
16 h after treatment	10	25	5	13	21	53	17	43	32	80	36	90	40	100

5.5.4 Discussion

Hibitane did not reduce survival of J2. Most of the J2 were lost during the sterilisation process. Centrifugation had some effect on numbers of J2 recovered but this was still minor compared with the overall loss and in the SDW only treatment, fewer J2 were found after centrifugation. The most logical explanation is that J2 stuck to the walls of the Eppendorfs and pipette tips. Some J2 were observed on the walls of Eppendorfs and some also been observed sticking to plastic Petri plates. While it is possible that this is a chemical adhesion it is more likely to be as electrostatic adhesion since it appears to occur on plastics but not on glass.

5.6 Trial (5.5) Surface sterilisation of J2 with 0.25% Hibitane either in Eppendorf tubes/stored in SDW or in Eppendorf tubes

5.6.1 Objective

In the experiment above, J2 may have been lost because of electrostatic adhesion as occurs on plastics. The objective of this experiment was to reduce possible electrostatic adhesion by storing Eppendorf tubes in SDW prior to use.

5.6.2 Materials and Methods

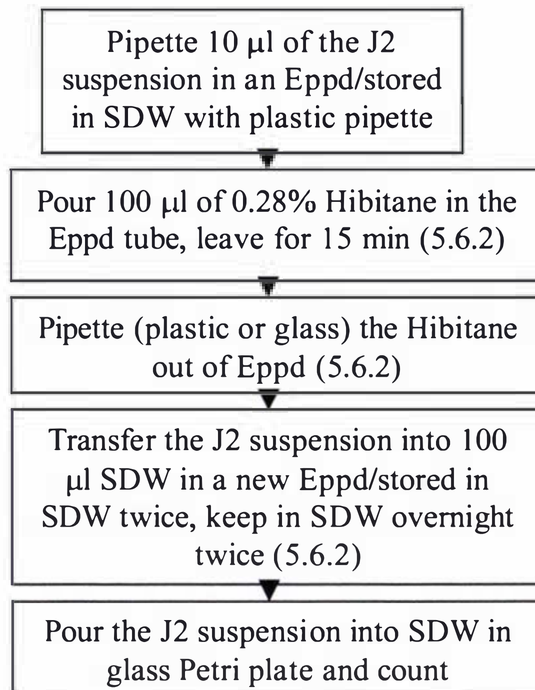


Figure 5-6 Procedure for surface-sterilisation of *H. trifolii* J2 with Hibitane using Eppendorf stored in SDW in trial (5.5)

Forty active freshly-hatched J2 were used for each treatment. J2 were surface sterilised with 0.25% Hibitane in either dry-stored Eppendorfs or Eppendorfs stored in SDW (ESDW) (Fig. 5.6). This was done by placing 10 µl of distilled water nematode suspension in an ESDW or an Eppendorf and adding 100 µL of 0.28% Hibitane. After 15 min, Hibitane was then removed using either a plastic or glass pipette and the J2 were transferred to another ESDW or Eppendorf containing 100 µL of SDW. They were re-washed before pipetting into a glass Petri plate. J2 sterilised with SDW using an ESDW or an Eppendorf were used as one set of controls. J2 placed in a glass Petri plate, ESDW and an Eppendorf containing SDW (not using pipettes) was used as a second control to check whether transfer of J2 affected survival. After sterilisation, J2 were counted under a microscope and then incubated in SDW overnight at 21°C before recounting as live or dead (Fig. 5.6).

5.6.3 Results

After treatment with Hibitane in an ESDW/glass pipette treatment, 17.5% of the J2 were found while only 10% were recovered from the Eppendorf/glass pipette (Table 5.7). All J2 were lost in treatments using a plastic pipette to transfer them. Sixty-five percentage of J2 were found from the ESDW/glass pipette control and 50% from the Eppendorf/glass pipette (Table 5.7). Only 5% of J2 were found from the control of ESDW/plastic pipette and all J2 were lost in Hibitane or control treatment (SDW), using plastic pipettes (Table 5.7). All J2 survived in a glass Petri plate and in the Eppendorf (no pipetting) control. All J2 initially recovered in all treatments were still active 16 h after treatments (Table 5.7).

Table 5-7 Percentage survival of J2 after treatment with Hibitane either in Eppendorf tubes/stored in SDW or in Eppendorf tubes

	0.25% Hibitane				Control, SDW			
	No.	%	No.	%	No.	%	No.	%
Initial count	40	100	40	100	40	100	40	100
Time after treatment	0 h		16 h		0 h		16 h	
	No.	%	No.	%	No.	%	No.	%
WG	7	18	7	18				
G	4	10	4	10				
WP	0	0	0	0				
P	0	0	0	0				
WG					26	65	26	65
G					20	50	20	50
WP					2	5	2	5
P					0	0	0	0
GlassPetri dish					40	100	40	100
Eppendorf / SDW					40	100	40	100
Eppendorf					40	100	40	100

WG = Eppendorf/stored in SDW / glass pipette

G = Eppendorf/glass pipette

WP = Eppendorf/stored in SDW / plastic pipette

P = Eppendorf/plastic pipette

Eppendorf/SDW = Eppendorf/stored in SDW

5.6.4 Discussion

Using Eppendorfs stored in SDW had some effect on the numbers of J2 recovered but this was still minor compared with the overall loss. It therefore appears

that J2 were lost because they stuck to the walls of Eppendorfs and plastic pipette tips when they were transferred during the sterilisation process. Hibitane had no effect on the J2 survival (Paracer & Zuckerman 1967). Hay (1994) and Hay & Regnault (1995) successfully cultured nematodes in monoxenic using 20% Hibitane for 15 min for surface sterilisation of nematodes. Koenning & Barker (1985) and Hooper (1986) also successfully surface sterilised various nematode species with Hibitane diacetate.

5.7 Trial (5.6) Surface sterilisation of J2 with 0.25% Hibitane using glass test tubes

5.7.1 Objective

To test whether glassware reduces the loss of J2 during the sterilisation process compared with use of plasticware.

5.7.2 Materials and Methods

5.7.2.1 J2 sterilisation with Hibitane in a glass test tube (25 x 5 mm)

Thirty freshly-hatched J2 were tested for each treatment. J2 were surface sterilised with 0.25% Hibitane in a glass test tube. This was done by pipetting about 50 μL of nematode suspension into a glass test tube (25 mm x 5 mm) and adding 50 μL of 0.5% Hibitane (Fig. 5.7). After 15 min, the J2 had settled and all the fluid in the tube was removed using a glass Pasteur pipette and the J2 were transferred to another glass test tube containing 100 μL of SDW. They were re-washed before pipetting into a glass Petri plate. J2 placed in SDW in a glass test tube or in a Petri plate were used as a set of controls to check whether transfer of J2 affected their survival and loss. After sterilisation, J2 were counted under a dissecting microscope and were then incubated in SDW overnight at 21°C before recounting the living J2 (Fig. 5.7). Twelve active surface-sterilised J2 were inoculated around the roots of seedlings in the Hoagland & Knop's agar plate and females were examined as described in Section 5.2.2.6.

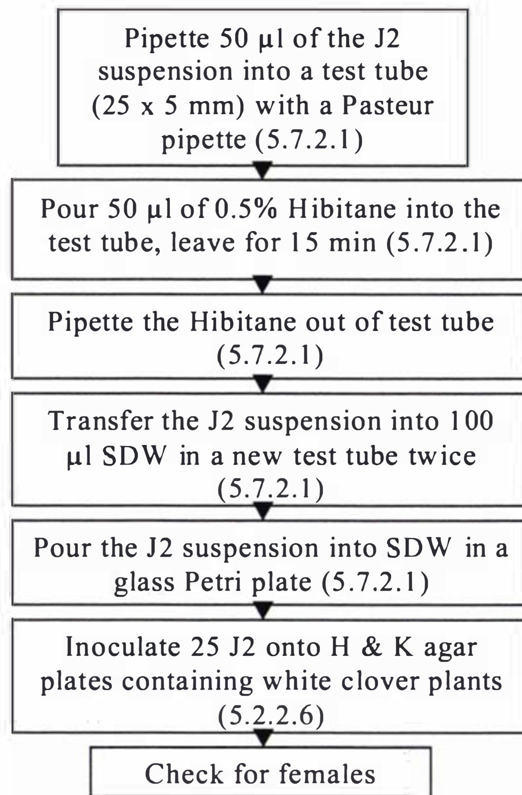


Figure 5-7 Procedure for surface-sterilisation of *H. trifolii* J2 with Hibitane using glass test tubes in trial (5.6)

5.7.2.2 J2 sterilisation with Hibitane in a glass test tube (53 x 5 mm)

Forty-five freshly-hatched J2 were used for each treatment. J2 were surface sterilised with 0.25% Hibitane in a glass test tube (53 mm x 5 mm). This was done as described in Section 5.7.2.1, except that two volumes of Hibitane and SDW were used. Twenty-five active surface-sterilised J2 were inoculated around seedling roots as described in Section 5.2.2.6.

5.7.3 Results

After sterilisation, 40% and 97-100% of J2 were recovered from the Hibitane treatment and the control checks, respectively. All J2 initially recovered in all treatments were still active 16 h after treatment (Table 5.8). One week after inoculation of seedlings, the agar plate was contaminated with bacteria.

Table 5-8 Percentage survival of J2 after treatment with Hibitane in glass test tubes (25 x 5 mm)

	0.25% Hibitane, glass test tube		SDW, glass test tube		SDW, glass Petri plate	
	No.	%	No.	%	No.	%
Initial count	30	100	30	100	30	100
0 h after treatment	12	40	29	97	30	100
16 h after treatment	12	40	29	97	30	100

After treatment, 56% and 100 % of J2 were found in Hibitane and in control checks, respectively. All J2 initially recovered in all treatments were still active 16 h after treatment (Table 5.9). One week after inoculation of J2, the agar plates were contaminated with bacteria.

Table 5-9 Percentage survival of J2 after treatment with Hibitane in glass test tubes (53 x 5 mm)

	0.25% Hibitane, glass test tube		SDW, glass test tube		SDW, glass Petri plate	
	No.	%	No.	%	No.	%
Initial count	45	100	45	100	45	100
0 h after treatment	25	56	45	100	45	100
16 h after treatment	25	56	45	100	45	100

5.7.4 Discussion

Forty-four to 60% of J2 were lost because they were more active after treatment with 0.25% Hibitane than at higher concentrations and glass tubes were so small that J2 were accidentally pipetted out during the sterilisation process. J2 did not stick to the glass test tube. However it was difficult to get the exact volume of J2 suspension because there were no gradation marks on the glass test tubes and consequently the volume of J2 suspension used in these tests could have diluted the concentration of Hibitane below the target of 0.25%. The concentration of Hibitane used in this test did not affect the survival of J2 but may not have given adequate decontamination.

5.8 Trial (5.7) Surface sterilisation of J2 with 0.25% Hibitane in watch glasses

5.8.1 Objective

To examine the J2 loss using a glass watch glass instead of a glass test tube for the surface-sterilisation process.

5.8.2 Materials and Methods

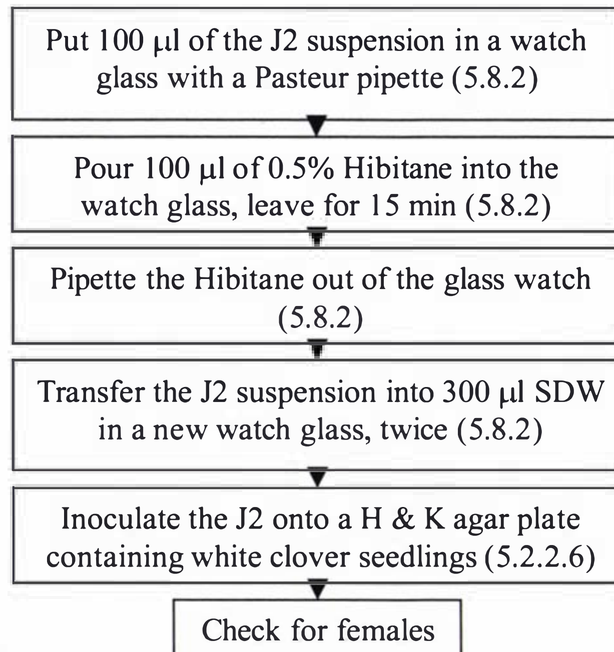


Figure 5-8 Procedure for surface-sterilisation of *H. trifolii* J2 with Hibitane in watch glasses in trial (5.7)

Forty freshly-hatched J2 were tested for each treatment. J2 were surface sterilised with 0.25% Hibitane in a solid watch glass. Hibitane (100 µL of 0.5%) was added to about 100 µl of nematode suspension in a sterilised watch glass (Fig. 5.8). After 15 min, the J2 had settled and all the fluid in the watch glass was removed using a glass Pasteur pipette and the J2 were transferred to another sterilised watch glass containing 300 µL of SDW. They were re-washed twice before inoculation. J2 placed in SDW in a sterilised watch glass were used as a control to check whether transfer of J2 affected survival and J2 loss. After sterilisation, J2 were counted under a microscope and were then incubated in SDW overnight at 21°C before recounting the living J2. Twenty-six active, surface-sterilised J2 were inoculated around the roots of seedlings in the Hoagland & Knop's agar plate using a Pasteur pipette (Fig. 5.8). The plate was taped and placed under lights and females were examined from two weeks after inoculation as described in Section 5.2.2.6.

5.8.3 Results

After treatment with Hibitane, 65% of J2 were found. All J2 survived in the control check. J2 initially recovered in all treatments were still active 16 h after treatment (Table 5.10). Twenty-five days after inoculation, 2-7 females were found.

Table 5-10 Percentage survival of J2 after treatment with Hibitane in watch glasses

	0.25% Hibitane, solid watch glass		SDW, solid watch glass	
	No.	%	No.	%
Initial count	40	100	40	100
0 h after treatment	26	65	40	100
16 h after treatment	26	65	40	100

Observations on reproduction of the *H. trifolii* on white clover roots in agar plates showed that the nematode completed its life cycle in about 1 month. J2 developed into females on the white clover roots at an average of five per plate after 2 to 3 weeks incubation in the first generation (Fig. 5.8A). Two weeks later, the first J2 generation hatched. They entered the roots and developed as a second generation of females on the rootlets after 2 months incubation (Fig. 5.8B). By 3 months of incubation, third generation females had been found (Fig. 5.8C) and the next generation was starting. *H. trifolii* cysts (Fig. 5.8D) can be produced while white clover plants are still green (up to 5 to 6 months old) in the agar plates.

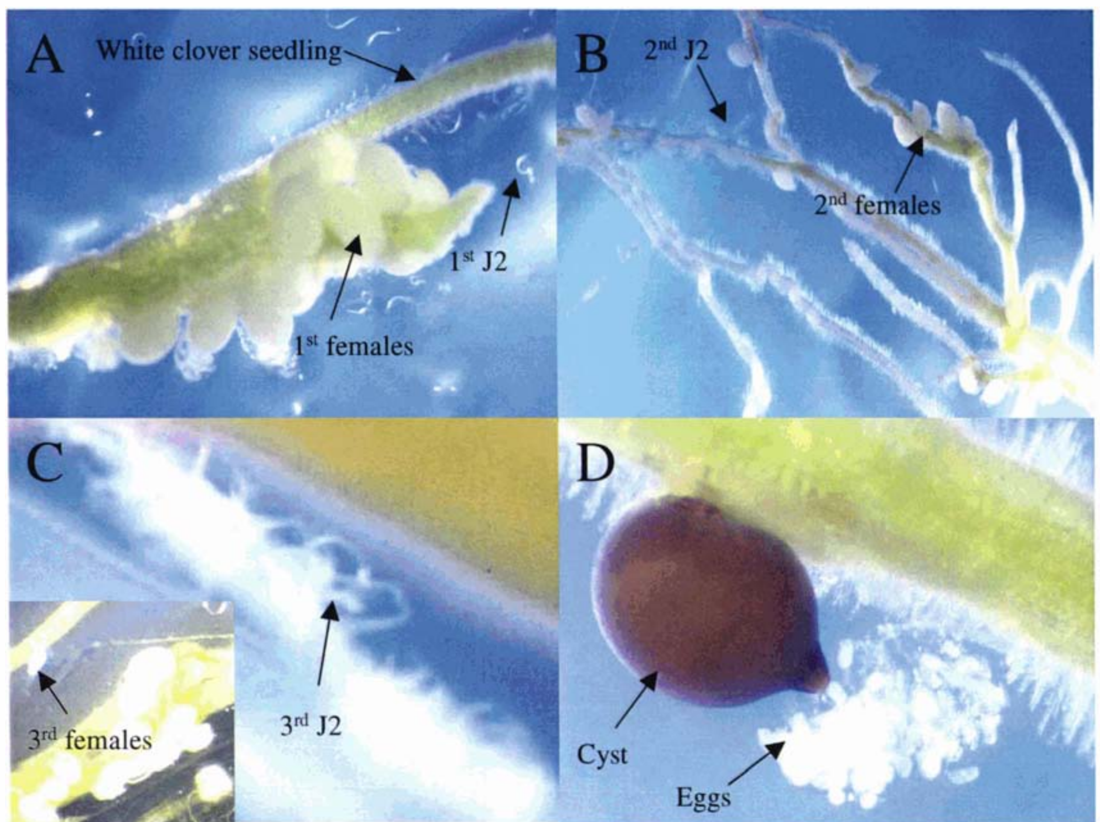


Figure 5-9 *H. trifolii* J2, females, and cyst monoxenically cultured from surface-sterilised J2 with 0.25% Hibitane in a watch glass on Hoagland & Knop's agar

(A) 1st generation *H. trifolii* females and J2, (B) 2nd generation *H. trifolii* females and J2, (C) 3rd generation *H. trifolii* females and J2, (D) *H. trifolii* cyst and eggs.

5.8.4 Discussion

Hibitane may have stimulated activity of J2. The lower the concentration of Hibitane, the fewer J2 descended to the bottom of the watch glass. Thirty-five percent of J2 were lost because they were more active after treatment with 0.25% Hibitane and were pipetted out with the Hibitane. J2 did not stick to the watch glass and the concentration of Hibitane used in this test did not affect the survival of J2 but it was strong enough to prevent contamination.

Hay (1993) reported that an average of 11.3% of inculated J2 developed into egg-containing females on white clover plants on Hoagland & Knop's agar. The J2

were surfaced sterilised with 10% Hibitane (0.5% Chlorhexidine Gluconate) for 4 min in a glass syringe, followed by 3 rinses in SDW and then incubated in 0.1% Penicillin-G / Streptomycin Sulphate BP for 4 h. This procedure was repeated before J2 inoculation.

In the current study, there was an average of (7-25%) J2 that developed into first generation, egg-containing females in Hoagland & Knop's agar by inculation J2 that had been surface-sterilised in 0.25% Hibitane (100 Chlorhexidine Gluconate 20% solution) for 15 min with 3 rinses in SDW followed by incubation in SDW overnight in a watch glass. There were at least 3 generations of females in the same Hoagland & Knop's agar plate. The differences between these two studies were 1) a different type of Hibitane, 2) syringe (Hay's study) and watch glass (this study).

5.9 Conclusion

Young cysts recently collected from the field and held not more than 5 days in 5°C or freshly-hatched J2 (not more than 2-day-old) should be used for monoxenic culture of *H. trifolii* because they are fresh and there is less contamination of cysts.

The plastic filter holder is the most important factor causing loss and mortality of J2 because J2 stick to it. The pressure used to wash the J2 using the filter holder may also damage them. J2 were lost during the process of sterilisation using a plastic Eppendorf, possibly because of adhesion to the plastic.

Streptomycin and NaOCl had no affect on J2 survival but did not control contaminants. Hibitane controlled contaminants at a concentration of 0.25% but it did appear to make J2 more active than at higher concentrations, so they were lost when the Hibitane was pipetted out.

Neither centrifugation nor use of Eppendorf tubes/ stored in SDW to reduce possible electrostatic adhesion, reduced J2 losses. Few J2 were lost when they were sterilised with Hibitane using glassware. The glass test tubes used in some experiments were too small and it was impossible to check the J2 under the microscope during the sterilisation process. The complete surface-sterilisation process could be done under a dissecting microscope using a watch glass and few J2 were lost during the sterilisation process.

The concentration of disinfectant is important. Although 0.25% Hibitane surface sterilised the J2, a lower concentration may be ineffective. A concentration of 0.5%

Hibitane allows a safety margin for surface-sterilisation of J2 for successful monoxenic culture of *H. trifolii*. It is therefore also possible *H. trifolii* can be produced monoxenically for experimental work by using *H trifolii* J2 which were surface sterilised by 0.5% Hibitane in a watch glass.

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6 SCREENING ISOLATES OF *VERTICILLIUM CHLAMYDOSPORIUM* FROM CLOVER CYST NEMATODE FOR PATHOGENICITY TO CLOVER CYST NEMATODE

6.1 Introduction

There is considerable variation between isolates of *Verticillium chlamydosporium* in their cultural characteristics, chlamydospore production and pathogenicity. The screening programmes used to identify isolates with biological control potential are therefore particularly important (Kerry et al. 1984, Irving & Kerry 1986). The inherent variation between different isolates of *V. chlamydosporium* means that isolates must be carefully selected for introduction into soil as potential biological control agents. It is expensive to screen large numbers of isolates in pot tests in nematode infested soil so development of appropriate *in vitro* tests would enable many isolates to be eliminated before more time-consuming screenings are conducted in pot trials (Kerry 1991).

Various soil fungi, mainly hyphomycetes, parasitise eggs of the cereal cyst nematode *Heterodera avenae* Woll. and other cyst nematodes (Tribe 1977, Kerry 1981, Morgan-Jones & Rodriguez-Kabana 1981). Assessing the extent of parasitism usually involves plating eggs onto a nutrient-poor medium and counting the numbers from which fungi develop (Kerry & Crump 1977, Nigh et al. 1980, Dackman & Nordbring-Hertz 1985). Irving & Kerry (1986) observed variations in pathogenicity of *V. chlamydosporium* to *H. avenae* or *H. schachtii* eggs on water agar. Wang et al. (1999) found that water agar is a good method for testing the parasitism of isolates of *Paecilomyces lilacinus*, *Verticillium chlamydosporium* and *V. lamellicola* from root-knot nematode (*Meloidogyne* spp.) eggs and adults towards eggs.

Meyer & Wergin (1998) observed penetration of *Verticillium lecanii* in females and cysts of *H. glycines* grown in monoxenic culture on soyabean roots three days after inoculation. Meyer et al (1990) reported that *V. chlamydosporium* isolated from cysts or eggs of *Heterodera glycines* caused a decrease in the number of viable eggs from *H. glycines* that grew monoxenically on excised root tips. *V. chlamydosporium* obtained

from cysts or eggs of clover cyst nematode *Heterodera trifolii* were able to infect eggs of root-knot nematode *Meloidogyne hapla* but not eggs of clover cyst nematode in gnotobiotic culture on Hoagland & Knop's agar (Hay 1993). Interactions between *Ditylenchus dipsaci*, white clover and nematophagous *Verticillium balanoides* have been studied using J2 surface-sterilised with Hibitane and cultured on white clover plants in sand-tips (Hay & Regnault 1995).

In New Zealand, biocontrol potential of a few fungal isolates from *H. trifolii* have been tested for their parasitism to *H. trifolii* but systematic screening of *V. chlamydosporium* isolates on their parasitism to *H. trifolii* has not been tested. This is therefore, an important area to develop for biocontrol potential by using suitable screening methods. Some isolates of *V. chlamydosporium* obtained from *H. trifolii* young cysts from each season of the 2-year study were taken at random and screened in the laboratory to assess their ability to parasitise *H. trifolii.*

6.2 Objective

To evaluate the pathogenicity to clover cyst nematode eggs or females, of *V. chlamydosporium* isolates, which have been found non-pathogenic to clover seedlings growing in 1) water agar plates, 2) sand-filled pipette tips (sand-tips), and 3) Hoagland & Knop's agar plates.

6.3 Materials and Methods

6.3.1 Screening of fungi for pathogenicity to *H. trifolii* eggs on agar

Three-week old *V. chlamydosporium* cultures grown on Corn Meal Agar (CMA) were washed with 2.5 ml of SDW and the surface rubbed with a glass rod to suspend the fungal material. A 0.5 ml aliquot of the fungal suspension was spread onto 1.0% water agar supplemented with oxytetracycline (10 μg/ml). They were sealed with plastic tape and incubated for 1 week at 21°C.

One week after incubation, 100 *H. trifolii* eggs were added to each fungus-inoculated agar plate. Adding *H. trifolii* eggs to non-inoculated CMA plates was the control check. There were 18 *V. chlamydosporium* isolates in this test (Table 6.1). These isolates were kept on PDA in 5°C room or in SDW at room temperature.The 1st code number of each isolate represents the number of sampling and the 2nd the number

of subcultures of *V. chlamydosporium* from single *H. trifolii* young cyst. There were 19 treatments with five replications and they were incubated for 1 week at 21°C before numbers of parasitised eggs were counted. Data were transformed to square-root as required and analysed by ANOVA using SAS.

6.3.2 Screening of *V. chlamydosporium* fungal isolates in sand-tips

Germination and planting of white clover *T. repens* (cv. Huia) seedlings in sand-tips were as described in Section 5.2.2.1 and 5.2.2.2. *V. chlamydosporium* spore suspensions (10^6/ml/tip) from 3-week old cultures in Corn Meal Broth (CMB) were inoculated around the roots of seedlings and incubated under lights as described in Section 5.2.2.2. Two weeks after inoculation of fungal spores, 25 *H. trifolii* J2, surface sterilised with 0.5% Hibitane in a solid watch glass as described in Section 5.8.2, were added to the sand in each pipette tip. Adding J2 or CMB only were control checks. There were 22 treatments (Table 6.2) with four replications. Five weeks after adding J2, plants were harvested and checked microscopically for mature females and cysts. Plants were ovendried at 85°C overnight for dry weight determination. Data were transformed to square-root as required and analysed by ANOVA using SAS.

6.3.3 Screening of *V. chlamydosporium* fungal isolates against *H. trifolii* on white clover grown on Hoagland & Knop's agar plates

Germination and planting white clover seedlings (three seedlings/plate) in Hoagland and Knops' agar were as described in Section 5.2.2.6. *V. chlamydosporium* spores (10^6/ml/plate) from 3-week-old cultures in CMB, were inoculated onto each agar plate and incubated under lights as described in Section 5.2.2.2. Two weeks after inoculation of fungal spores, 25 J2, which had been surface sterilised as described in Section 5.8.2, were added to each agar plate. Adding J2 or *V. chlamydosporium* suspension only were control checks. There were 22 treatments (Table 6.3) with four replications. From 2 weeks to 5 weeks after adding J2, plants were checked under a dissecting microscope for counting of mature females and cysts. Numbers of root tips and trifoliated leaves, and shoot lengths were recorded 5 weeks after adding J2. Data were transformed to square-root as required and analysed by ANOVA using SAS.

6.4 Results

6.4.1 Screening of *V. chlamydosporium* in water agar plates

Isolate 1.12 of *V. chlamydosporium* infected significantly more eggs of *V. chlamydosporium* infected eggs than isolates 15.301, or 49.843 treatment (P<0.0001) but it did not differ significantly from the remaining isolates (Table 6.1). The absence of *V. chlamydosporium* infections in the *H. trifolii* egg control shows that the eggs used in this experiment were free of natural *V. chlamydosporium* infections and that all the infections in the treatments were from the isolates being tested (Table 6.1).

Table 6-1 Percentages of *V. chlamydosporium* infected *H. trifolii* eggs on water agar after 1-week incubation at 21°C

No.	Treatments	% infected eggs
1	1.5+*H. trifolii*	39.4abc
2	1.12+*H. trifolii*	43.0a
3	9.188+*H. trifolii*	40.2abc
4	15.301+*H. trifolii*	25.4c
5	15.316+*H. trifolii*	36.0abc
6	19.388+*H. trifolii*	38.2abc
7	20.422+*H. trifolii*	35.6abc
8	25.509+*H. trifolii*	39.4abc
9	26.518+*H. trifolii*	35.4abc
10	30.563+*H. trifolii*	35.8abc
11	31.590+*H. trifolii*	26.6abc
12	32.606+*H. trifolii*	32.0abc
13	42.428+*H. trifolii*	31.4abc
14	47.740+*H. trifolii*	37.2abc
15	47.795+*H. trifolii*	27.4abc
16	49.843+*H. trifolii*	24.4c
17	50.866+*H. trifolii*	29.2abc
18	51.875+*H. trifolii*	30.4abc
19	*H. trifolii*	0.0d

Means followed by the same letter are not significantly different (P<0.05).

6.4.2 Screening of *V. chlamydosporium* in sand-tips

Although there were no significant differences in numbers of females in any *V. chlamydosporium* treatments, the numbers of females in isolate treatments 1.5, 1.12,

15.316, 19.388, 20.422, 30.563, 31.590, 47.740, 47.795, and 49.843 were significantly lower than those in *H. trifolii* alone or CMB+ *H. trifolii* treatment (P<0.05) (Table 6.2).

The numbers of new cysts were significantly lower in treatments of 1.5, 9.188, 15.301, 15.316, 19,388, 26.518, 30.563, 49.843, and 50.866 than those in *H. trifolii*, CMB+ *H. trifolii*, or 51.875 treatment (P<0.05) but those numbers did not significantly differ from remaining treatments (Table 6.2). No old cysts were found in any treatments.

Addition of *H. trifolii* alone did not depress plant growth and only isolate 1.12 increased plant growth compared with *H. trifolii* alone, CMB+ *H. trifolii*, control, CMB, and all *V. chlamydosporium* treatments (P<0.0001) except 9.188, or 15.301 treatment (Table 6.2).

Table 6-2 The effect of *V. chlamydosporium* isolates on mean numbers of females, new cysts and mean clover shoot dry weights in sand in pipette tips

No.	Treatments	Mean no. of females/tip	Mean no. of new cysts/tip	Mean shoot dry wt (g/tip)
1	1.5+*H. trifolii*	0.5bc	0.0c	0.0044bcd
2	1.12+*H. trifolii*	1.0bc	0.5bc	0.0058a
3	9.188+*H. trifolii*	1.5abc	0.0c	0.0052ab
4	15.301+*H. trifolii*	1.3abc	0.0c	0.0047abc
5	15.316+*H. trifolii*	0.8bc	0.0c	0.0030ef
6	19.388+*H. trifolii*	0.8bc	0.3c	0.0029ef
7	20.422+*H. trifolii*	0.8bc	1.5abc	0.0026ef
8	25.509+*H. trifolii*	1.3abc	0.5bc	0.0034def
9	26.518+*H. trifolii*	2.5ab	0.0c	0.0028ef
10	30.563+*H. trifolii*	0.3bc	0.0c	0.0029ef
11	31.590+*H. trifolii*	0.3bc	0.5bc	0.0034cdef
12	32.606+*H. trifolii*	1.3abc	1.0bc	0.0020f
13	42.428+*H. trifolii*	2.3abc	1.3abc	0.0033def
14	47.740+*H. trifolii*	1.0bc	2.0abc	0.0025ef
15	47.795+*H. trifolii*	0.8bc	1.3abc	0.0028ef
16	49.843+*H. trifolii*	0.8bc	0.0c	0.0038cde
17	50.866+*H. trifolii*	2.0abc	0.0c	0.0038cde
18	51.875+*H. trifolii*	1.5abc	2.8ab	0.0038cde
19	Control	0.0c	0.0c	0.0032bcde
20	*H. trifolii*	3.5a	2.8ab	0.0030def
21	CMB	0.0c	0.0c	0.0038cde
22	CMB+*H. trifolii*	3.5a	3.5a	0.0039cde

Means in the columns followed by the same letter are not significantly different (P<0.05).

6.4.3 Screening of *V. chlamydosporium* in Hoagland & Knop's agar plates

Although, the numbers of female *H. trifolii* were significantly higher in the *H. trifolii* and CMB+ *H. trifolii* treatments than those from all *V. chlamydosporium* treatments (P<0.0001), the numbers of new and old cysts were not significantly different in any treatments tested (Table 6.3). There was a significantly higher number of old cysts from the isolate 49.795+ *H. trifolii* treatment than in those from the remaining treatments (P<0.01) (Table 6.3). No new or old cysts were found in *H. trifolii* or CMB+ *H. trifolii* treatment.

Plants treated with isolates 15.301 and 50.866 had significantly higher numbers of root tips than isolate 9.188 or treatments *H. trifolii*, control, CMB, and CMB+ *H. trifolii* (P<0.05)(Table 6.4).

The mean number of trifoliated leaves in isolates 9.188, 15.301, 47.740, and 50.866 treatments were higher than those in *H. trifolii*, control, CMB, or CMB+ *H. trifolii* (P<0.01) (Table 6.4).

Shoot lengths from isolate 47.740 treatment only were significantly higher than the *H. trifolii*, control, CMB, or CMB+ *H. trifolii* (Table 6.4) (P<0.05). Addition of *H. trifolii* alone did not significantly depress plant growth compared with the controls (Table 6.4).

Females from the control checks contained eggs (Fig. 6.1A) but females from *V. chlamydosporium* treatments were parasitised by *V. chlamydosporium* (Fig. 6.1B) and they had become small, old cysts with no eggs (Fig. 6.1C) in Hoagland & Knop's agar plates.

Table 6-3 The effect of *V. chlamydosporium* isolates on mean numbers of females, new cysts, and old cysts in Hoagland & Knop's agar plates

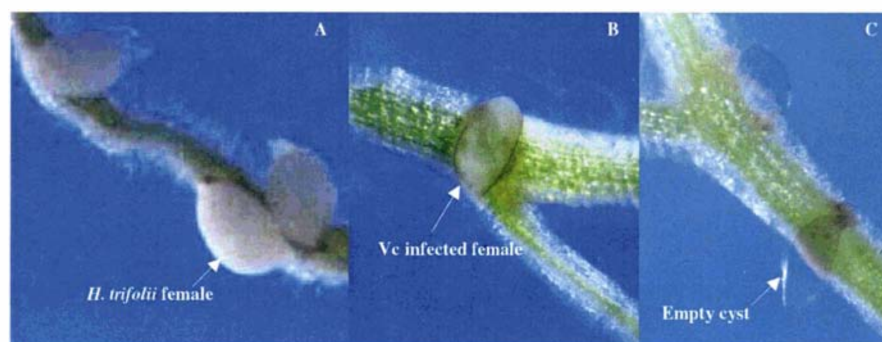
No.	Treatments	Mean no. of females/plate	Mean no. of new cysts/plate	Mean no. of old cysts/plate
1	1.5+*H. trifolii*	0.0b	0.0a	0.0b
2	1.12+*H. trifolii*	0.0b	0.0a	0.3b
3	9.188+*H. trifolii*	0.0b	0.0a	0.8b
4	15.301+*H. trifolii*	0.0b	0.0a	0.0b
5	15.316+*H. trifolii*	0.0b	0.0a	1.0b
6	19.388+*H. trifolii*	1.0b	0.3a	0.5b
7	20.422+*H. trifolii*	0.0b	0.0a	0.5b
8	25.509+*H. trifolii*	0.0b	0.0a	0.3b
9	26.518+*H. trifolii*	0.0b	0.0a	1.0b
10	30.563+*H. trifolii*	0.0b	0.0a	0.0b
11	31.590+*H. trifolii*	0.0b	0.0a	0.8b
12	32.606+*H. trifolii*	0.0b	0.0a	1.0b
13	42.428+*H. trifolii*	0.0b	0.0a	1.3b
14	47.740+*H. trifolii*	0.0b	0.0a	0.3b
15	47.795+*H. trifolii*	0.0b	0.3a	5.0a
16	49.843+*H. trifolii*	0.0b	0.0a	0.5b
17	50.866+*H. trifolii*	0.0b	0.0a	0.3b
18	51.875+*H. trifolii*	0.0b	0.0a	1.0b
19	Control	0.0b	0.0a	0.0b
20	*H. trifolii*	8.0a	0.0a	0.0b
21	CMB	0.0b	0.0a	0.0b
22	CMB+*H. trifolii*	7.3a	0.0a	0.0b

Means in the columns followed by the same letter are not significantly different at P<0.5.

Table 6-4 The effect of *V. chlamydosporium* isolates on mean numbers of root tips, trifoliated leaves, and clover shoot lengths in Hoagland & Knop's agar plates

No.	Treatments	Mean no. of root tips/plant	Mean.no. of trifoliated leaves/plant	Mean shoot length (mm)/plant
1	1.5+*H. trifolii*	3.08abc	2.92bcd	47.08abcd
2	1.12+*H. trifolii*	3.17abc	3.25bcd	44.33abcd
3	9.188+*H. trifolii*	2.33bc	4.42a	45.42abcd
4	15.301+*H. trifolii*	4.33a	3.83ab	48.33abc
5	15.316+*H. trifolii*	3.33abc	3.58abc	47.50abcd
6	19.388+*H. trifolii*	2.83abc	3.33bcd	50.00ab
7	20.422+*H. trifolii*	2.83abc	2.92bcd	38.75bcd
8	25.509+*H. trifolii*	3.33abc	3.25bcd	45.83abcd
9	26.518+*H. trifolii*	2.83abc	3.33bcd	43.75abcd
10	30.563+*H. trifolii*	2.75abc	3.50abc	45.42abcd
11	31.590+*H. trifolii*	2.83abc	3.25bcd	48.33abc
12	32.606+*H. trifolii*	2.67abc	3.08bcd	43.33abcd
13	42.428+*H. trifolii*	2.67abc	2.83bcd	42.50abcd
14	47.740+*H. trifolii*	3.42abc	3.83ab	52.08a
15	47.795+*H. trifolii*	3.25abc	3.50abc	46.25abcd
16	49.843+*H. trifolii*	2.67abc	3.42bcd	39.58bcd
17	50.866+*H. trifolii*	4.25a	3.75ab	47.08abcd
18	51.875+*H. trifolii*	3.58ab	3.33bcd	43.33abcd
19	Control	1.83bc	2.67cd	36.67cd
20	*H. trifolii*	2.25bc	2.42d	35.42d
21	CMB	2.17bc	2.67cd	35.42d
22	CMB+*H. trifolii*	1.67c	2.58cd	39.17bcd

Means in the columns followed by the same letter are not significantly different at $P<0.05$.



Vc=*Verticillium chlamydosporium*

Figure 6-1 A comparison of CCN female from control check with that from *V. chlamydosporium* treatment on Hoagland & Knop's agar

(A) *H. trifolii* healthy female from control check, (B) *H. trifolii* female infected with *V. chlamydosporium*, (C) Empty *H. trifolii* cyst after a virgin *H. trifolii* female was parasitised by *V. chlamydosporium*.

6.5 Discussion

No females or new cysts were found in control or CMB, indicating that sterilising the sand killed indigenous *H. trifolii* that could exist in the sand.

Although the percentage of *V. chlamydosporium* infected-eggs were significantly higher in isolate treatment 1.12 than in treatments with isolate 15.301 or 49.843, there were no significantly differences in numbers of females or new cysts among the *V. chlamydosporium* treatments in sand-tips or in Hoagland & Knop's agar plates. Possibly, eggs were more susceptible to isolate 1.12 than J2. Cayrol et al. (1982) reported that *H. avenae* eggs were not susceptible to parasitism by *V. chlamydosporium* after they had developed beyond the three-cell stage.

None of the *V. chlamydosporium* treatments differed in the numbers of females in sand-tips or Hoagland & Knop's agar plates, probably because all isolates tested might have been from the same genetic population or they had the same parasitic potential. The numbers of females that developed were not significantly different between treatments and consequently numbers of new cysts were not significantly different either.

Although the numbers of females were significantly higher in the *H. trifolii* treated control checks, the numbers of new cysts were not significantly different between treatments in Hoagland & Knop's agar plates, so the females probably failed to form new cysts or the eggs did not hatch in the experimental period on this medium.

Although none of females from *H. trifolii* or CMB+ *H. trifolii* treatment developed into old cysts in sand-tips or Hoagland & knop's agar plates, there were small, old cysts in all the *V. chlamydosporium* isolate treatments (except isolate 1.5, 15.301, 30.563, 19.388, 47.795) even though no females or new cysts were found in those treatments. Again, the numbers of females and new cysts were not significantly different between treatments, the numbers of old cysts from the isolate 47.795 treatment were significantly higher than those from the rest of the treatments. A possible reason was *H. trifolii* J2 developed into females but those females were possibly infected by *V. chlamydosporium* and became premature tanned females, which were then recorded as old cysts in this test. Infection by *V. chlamydosporium* caused premature tanning of the female cuticle of *H. avenae* and *H. schachtii* (Crump 1987).

No stages of *H. trifolii* were found in treatments of isolates 1.5, 15.301, and 30.563 on Hoagland & Knop's agar. These fungal isolates may produce some products which could affect the development of inoculated J2 in Hoagland & Knop's agar or the fungal isolates might affect J2 survival. Saifullah (1996) found that *V. chlamydosporium* isolated from *Globodera rostochiensis* released toxic metabolites into the medium and killed males of *G. pallida* and *G. rostochiensis* on agar plates. Khambay et al. (2000) detected nematicidal activity from *V. chlamydosporium* by bioassay-directed fractionation of the fungal cultures.

Old cysts from the *V. chlamydosporium* treatments had no eggs but there were eggs in females from the control checks, possibly *V. chlamydosporium* parasitised eggs in young females (virgin females). Small, empty cysts were produced when virgin females were infected by *V. chlamydosporium* and when egg-producing females were attacked, infected females produced fewer eggs than normal and many of the eggs were parasitised (Kerry 1975).

None of the *V. chlamydosporium* isolates increased plant growth compared with the control (except isolate 1.12), probably because; 1) the pipette tips in which plants were grown, were so small that the growth of plants was not sufficient to compare the effect of the treatments, 2) the duration (from adding *H. trifolii* J2 to harvesting) was too short for adequate plant growth, and 3) the numbers of females were too small to affect plant growth.

V. chlamydosporium isolates 15.301 and 50.866 or some of these fungal products had some effect on white clover seedling growth that increased numbers of root tips and trifoliated leaves in Hoagland & Knop's agar plates or in sand-tips.

Hay & Regnault (1995) observed that nematophagous *V. balanoides* could parasitise stem nematode *D. dipsaci* on white clover in sand-tips. The results from this study also showed that *V. chlamydosporium* affected the *H. trifolii* J2 development in sand-tips or Hoagland & Knop's agar but the results of using sand-tips or Hoagland & Knop's agar for screening fungal isolates against nematodes could not be compared with Hay & Regnault (1995) because they did not screen fungal isolates for potential biocontrol agent.

6.6 Conclusion

Although the measurements for pathogenicity of a range of *V. chlamydosporium* isolates on *H. trifolii* varied, no significant difference was found, indicating that all *V. chlamydosporium* isolates might be clonal or at least from a limited population. Alternatively, agar plates and sand-tips tests may not be sufficiently sensitive for screening fungi for biocontrol activity of *H. trifolii*.

Despite the lack of differing pathogenicities among *V. chlamydosporium* isolates found in these trials, they appeared to be simple techniques and suitable for studying the fungal parasitism of eggs or J2 of *H. trifolii*. Considerable time can be saved by using such screening procedures and further testing with a wider range of isolates to further validate the procedure is justified. Lopez-Laorca & Duncan (1986) reported that isolates, which failed in an *in vitro* selection process, were not effective if tested in soil hence isolates which have greater parasitism to nematodes then this selection could be effective for further studies as biocontrol agents.

6.7 References

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7 OBSERVATIONS ON MOLECULAR VARIATION OF *VERTICILLIUM CHLAMYDOSPORIUM* POPULATIONS ISOLATED FROM *HETERODREA TRIFOLII*

7.1 Introduction

Verticillium chlamydosporium Goddard is a common parasite of cyst nematode females and eggs (Bursnall & Tribe 1974, Kerry & Crump 1977) and is one of the fungi responsible for the decline of *Heterodera avenae* Woll. populations under intensive cereal cropping in the UK (Kerry et al. 1982). *V. chlamydosporium* has been isolated from roots of clover and ryegrass in New Zealand (Thornton 1965).

V. chlamydosporium infects females of cyst nematodes on roots, reducing their fecundity, and kills eggs at all stages of development. The fungus occurs as a number of strains (Bursnall & Tribe 1974, Kerry 1981), which vary in their pathogenicity, growth and chlamydospore production on laboratory media. Some authors regard the species as a complex, with continuous variation in morphology and physiology among different isolates (Bursnall & Tribe 1974, Irving & Kerry 1986, Kerry et al. 1986).

Molecular methods have been used to analyse the diversity of ribosomal DNA (rDNA) repeat units containing highly conserved DNA sequence regions such as the 28S and 18S genes, as well as more variable sequence regions such as Internal Transcribed Spacer (ITS) and have been used to detect genetic variation between related fungal species (Bruns & Palmer 1989, White et al. 1990). PCR, using universal DNA primers specific for the conserved 18S and 28S elements followed by direct sequencing, has been successfully used to detect fungal plant pathogens (White et al. 1990), and to produce characteristic DNA fragments from filamentous fungi (O'Donnell 1992, Yao et al. 1992).

PCR-based assays have been developed to differentiate ITS sequence diversity present in *V. dahilae*, *V. albo-atrum* and *V. tricorpus* (Nazar et al. 1991, Robb et al. 1993, Moukhamedov et al. 1994). Isolates of *V. chlamydosporium* were differentiated using primers designed to amplify ribosomal ITS and intergenic spacers (IGS), and using primers matching enterobacterial repetitive intergenic consensus (ERIC) sequences, and repetitive extragenic palindromic (REP) elements (Arora et al. 1996).

Carder et al. (1993) classified the nematophagous fungi *V. chlamydosporium* and *V. suchlasporium* by Restriction Fragment-Length Polymorphism analysis (RFLPs) and enzyme activities using a commercial test system (LRA ZYM kit, bio Merieux UK Ltd) that provides a simple, rapid and semiquantitative method for detecting enzymes secreted into the culture medium. Although there were subspecific groups of isolates, these methods could not support their division into two species.

Hirsch et al. (2000) developed a PCR-based method using specific primers designed from an amplified and cloned fragment of the *V. chlamydosporium* β-tubulin gene to detect the species on infected tomato plant roots. However, this method could not discriminate between different isolates of this fungal species.

Three major techniques: randomly amplified polymorphic DNA (RAPD) (Crowhurst et al. 1991, Guthrie et al. 1992, Mills et al. 1992, Smith et al. 1992, Williams et al. 1991a,b), arbitrarily primed PCR (AP-PCR) (Welsh & McClelland 1990), and DNA amplification fingerprinting (DAF) (Caetono-Anolles et al. 1992) have been applied to the study of genomic variation of various fungal species. These methods became very popular and RAPD has been the most widely used because of its simplicity and wide applicability. The number, reproducibility and intensity of bands in RAPD analysis have been found to be a function of multiple parameters, such as concentrations of salts, magnesium, deoxyribonucleoside triphosphates, primer, *Taq* DNA polymerase and DNA isolation methods, cycle number, annealing temperature and type of thermocycler. Despite the simplicity of this enzymatic procedure, because of considerable concerns on reproducibility of RAPD profiles (Penner et al. 1993), appropriate guidelines for optimisation should be followed and reproducibility of results confirmed.

Random amplified microsatellites (RAMS) technique originally described by Zietkiewicz et al. (1994) has also been shown to be applicable for investigations of fungi (Hantula etal. 1996). The technique combines most of the benefits of RAPD and microsatellite analyses, and is therefore promising for studies of genetic variation. In RAMS analysis the DNA between the distal ends of two closely located microsatellites is amplified and the resulting PCR products are separated electrophoretically in an agarose gel (Zietkiewicz et al. 1994, Hantula et al. 1996). Hantula & Muller (1997) reported that RAMS technique is useful for detection of genetic variation within *Gremmeniella abietina* isolates.

In the present work more than 100 *V. chlamydosporium* isolates were obtained from (*H. trifolii* Goffart) during a 2-year study of this nematode in a white clover/ryegrass pasture. There were variations in colony morphology of the isolates on both Potato Dextrose Agar (PDA) and Corn Meal Agar (CMA), hence attempts were made to determine whether there were variations in pathogenicity to *H. trifolii in vitro*. Isolates which varied in morphology, some from each season, were taken at random and their pathogenicity to *H. trifolii* was examined in (1) water agar plates, (2) sand-filled pipette tips, and (3) Hoagland & Knop's agar plates as described in Sections 6.3.1, 6.3.2, and 6.3.3, respectively. Although, all isolates of *V. chlamydosporium* tested parasitised eggs and young females, their pathogenicity to females was not significantly different in *in vitro* tests (Sections 6.4.1, 6.4.2, and 6.4.3). This raised the question of whether the field population was clonal.

7.2 Objectives

(1) To use ITS sequences for confirmation that the isolates obtained in this work are *V. chlamydosporium.*

(2) To examine whether there are variations in ITS and 5.8S regions among the *V. chlamydosporium* isolates by amplifying those regions with PCR universal primers.

(3) To investigate whether there are molecular variations among the *V. chlamydosporium* isolates tested using RAPD analysis.

(4) To investigate the relationship among 10 *V. chlamydosporium* isolates tested based on (Semi-Partial R-Squared 'minimum variance') generated by cluster analysis of RAPD profiles.

7.3 Materials and Methods

One aim of this study was to investigate whether there were molecular variations among *V. chlamydosporium* isolates nation wide. Clover cyst nematode was extracted from soil samples from Lincoln University, Otago University, Kaikohe Research Centre, Keri Keri, and Ruakura. The fungi growing from the females and young cysts on water agar were identified microscopically. Because of the time limitation and the unavailability of *V. chlamydosporium* isolates from elsewhere in New Zealand (except

small numbers of isolates from Kaikohe), the study of molecular variation of *V. chlamydosporium* isolates nationwide could not be done. The present study was conducted on a small number of isolates that were collected from one pasture field in Palmerston North.

7.3.1 Culturing *Verticillium chlamydosporium*

Two discs of *V. chlamydosporium* grown on CMA (Appendix III) were placed on sterile cellophane discs on PDA (Appendix III) and the plates incubated at 21°C for 4 days. Because of the slow growth of *V. chlamydosporium*, the mycelium grown on the cellophane was macerated and resuspended in 0.5 ml sterile distilled water before spreading onto new sterile cellophane discs on PDA and incubating for a further 5 days. The mycelium from all cellophane discs was removed and placed in Eppendorf tubes. Two pin-holes were made in the lid of each Eppendorf tube and the holes were sealed with parafilm and tubes were kept in a freezer (-18°C) until required, then the parafilm was removed and the samples freeze-dried overnight.

7.3.2 Extraction of DNA

The method of Al-Samarrai & Schmid (2000) was used because it is very good for polysaccharides-free DNA. About 30 mg of freeze-dried *V. chlamydosporium* mycelium was ground in liquid nitrogen using a sterile pestle and mortar and placed in an Eppendorf tube. Lysis buffer (500 µL, Appendix III) was added and the suspension was pipetted vigorously until the viscosity increased and froth formed. RNAse A (20 µL of 1 mg/ml, Appendix III) was then added and the Eppendorf tube kept at 37°C for 5 min. A concentration of 5 M NaCl (165 µL) was added to the tube before centrifugation at 13,000 rpm at 4°C for 20 min. The supernatant was transferred into a fresh Eppendorf tube. Chloroform and phenol (400 µL each) were added to the Eppendorf tube and mixed gently by inverting the tube until the suspension appeared milky. The tube was centrifuged at 13,000 rpm at 4°C for 20 min and the aqueous (upper) phase was transferred to a new Eppendorf tube with an equal volume of chloroform. The tube was shaken, then centrifuged at 13,000 rpm for 5 min (MSE, Micro Centaur, SANYO) and the upper phase was transferred to a new Eppendorf tube. DNA was precipitated by adding two volumes of 95% ethanol and keeping it at 4°C for at least 1 h.

The tube was centrifuged at 13,000 rpm for 5 min and the ethanol poured off. The pellet that remained was resuspended in 500 µL lysis buffer and mixed using a vortex mixer. 5M NaCl (165 µL) was added and the tube inverted to achieve a complete mixture. An equal volume of chloroform (665 µL) was added to the pellet mixture before centrifugation at 13,000 rpm at 4°C for 20 min. Supernatant was transferred to a new Eppendorf tube and two volumes of 95% ethanol added to the new tube to precipitate the DNA. The tube was centrifuged at 13,000 rpm for 5 min and the ethanol tipped off. The pellet in the tube was washed with 70% cold ethanol three times, with a 3-min spin and ethanol removal between each spin. The tube with pellet was air dried for 30 min to remove all ethanol. Finally, the pellet was resuspended in 50 µL TE buffer and left at 4°C overnight for the DNA to resuspend. The extracted DNA was kept at – 20°C for long-term use.

7.3.3 Measuring DNA concentration by the fluorometer method

DNA measurements were determined by fluorescence in the presence of bisbenzimide (bisBenzimide Hoechst 33258, Sigma Chemical Co., St. Louis, MQ) using the 'DyNA Quant 200, fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Working buffer was a mixture of 90 ml water + 10 ml 10 x TNE (Appendix III) + 10 µL Hoechst dye (1 mg/ml, Appendix II). Two ml of buffer in a glass cuvette (Pharmacia Biotech, Hoefer ref. DQ105) was used as a blank and calf thymus DNA (2 µL, 1 mg/ml) as a standard for calibration. The DNA sample (2 µL) was added to the 2 ml buffer in the cuvette and the sample concentration read in ng/µL.

7.3.4 Agarose gel electrophoresis

The concentration of DNA samples was also determined by electrophoresing DNA samples on an agarose gel (usually 0.7-1.2% with 1 x TBE buffer, Appendix III) alongside a series of Lambda (λ) DNA (Invitrogen, Appendix III) concentration standards of 10 ng, 25 ng, and 50 ng/5 µL. The concentration of the fragment of interest was estimated by comparing the intensity of the ethidium bromide (EtBr) (Appendix III) fluorescence to that of the known DNA concentration standards.

Gel loading buffer (Appendix III) was diluted with milli-Q water or TE buffer and DNA sample to a final 1x concentration before loading the sample. A mixture (5 μL) of DNA, TE buffer, and gel loading buffer (1 μL + 3 μL + 1 μL) was made on a piece of parafilm and pipetted in each well, alongside wells of the standard λ DNA. Five microlitres (40 ng/μL) of 1 kb ladder (Invitrogen) was loaded. The molecular weight of the unknown fragments was determined by measuring the distance migrated (from the wells) by the ladder fragments and comparing their relative mobility to that of the unknown DNA sample. The gel was run for about 1 h at 80 volt and placed in the EtBr for 20 min before washing in milli Q water for 30 min to destain. Separated DNA was visualised and photographed under UV light using the IS-1000 Digital Imaging System. Negative image (black on white) was taken instead of positive image (white on black) because of its clarity especially for faint bands.

7.3.5 PCR amplification and sequencing of ribosomal ITS regions

7.3.5.1 Universal PCR ITS amplification

PCR reactions were set up on ice using a cocktail which contained all common reagents used in the PCR in a ratio of n + 1 PCR reactions (where n = the number of PCR reactions to be amplified, including positive and negative controls). Uncommon reagents were pipetted separately. Components were added in order and kept on ice at all times. Amplifications were performed in a Techne Genius air cooled thermal cycler. Following the amplification, the reactions were stored at 4°C and the products viewed using agarose gel electrophoresis.

The reactions contained final concentrations of 1 x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 100 μM dNTPs (Roche), 2 μM each of ITS4 and ITS5 primers, 1.0 U *Taq* DNA polymerase (Invitrogen) and 20-50 ng of template DNA. These reactions were performed in a total reaction volume of 50 μL.

The cycling conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 5 min. Following a final elongation of 72°C for 2 min, the reaction mix was held at 4 °C. Amplification products were analysed by electrophoresis of 2 μL of the reaction products as described in Section 7.3.4.

7.3.5.2 Purification of PCR products

PCR products were purified using a QIAquick PCR purification kit (Qiagen®) according to the manufacturer's instructions. When necessary, a gel extraction kit (GIBCOBRL, Concert ™, Rapid Gel Extraction System) was used to purify PCR products from a mixture.

7.3.5.3 Sequencing

The *V. chlamydosporium* ITS region was sequenced using the universal primer ITS5 (Table 7.1) by directly sequencing the Qiagen® (QIAquick) purified PCR products at the Massey University Sequencing Unit (MUseq), Palmerston North.

7.3.5.4 Sequence alignment

DNA sequences were aligned using the CLUSTAL W algorithm (Thompson et al. 1994), which is available in the Gene-Jockey II Sequence handling program. The 18S and 28S ribosomal DNA sequences which flank the ITS regions were deleted prior to alignment. Sequence for ITS1, 5.8S, and ITS2 regions of published *V. chlamydosporium* sequence was obtained from the GenBank database and was added to the sequences resulted from this study in order to anchor the regions. Matching of the central 5.8S rDNA region in each sequence was checked to ensure accuracy of the alignment.

7.3.6 Optimisation of Randomly Amplified Polymorphic DNA, (RAPD) PCR

Standard conditions used for RAPD amplification were carried out with PCR reactions using random 10-mer oligonucleotides obtained from Gensys (Australia). Each reaction was in a total volume of 25 µL and contained 1 x PCR buffer (Invitrogen), 3.0 mM $MgCl_2$, 0.2 mM dNTPs (Roche), 0.4 µM primer, 0.8 U of *Taq* DNA polymerase (Invitrogen), Q solution (20% of total volume), and 20 ng of template DNA.

The PCR cycling conditions were an initial 4 min at 94°C, followed by 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min. A final 72°C elongation step for 6 min was followed by holding at 4°C. The amplification products were analysed by

agarose gel electrophoresis as described in Section 7.3.4. These conditions were chosen after optimisation of the parameters listed below. Unless otherwise stated, all other conditions were standard.

Concentration of DNA template, magnesium chloride, annealing temperature, PCR amplification conditions, buffer for gel electrophoresis, adding Q (Qiagen) solution to PCR reaction were optimised for reproducible RAPD products.

Parameters for PCR optimisation were:

Concentration of DNA template: (10 or 20 ng)

Concentration of $MgCl_2$: 1.5, 2.0, 2.5, or 3.0 mM

Annealing temperature: 36, 37, 38, 39, or 40°C

Electrophoresis buffer: 1 x TBE or 1 x TAE

PCR reaction mix: with or without Q solution

Q solution is a PCR enhancer marketed by Qiagen. Generally, Q-solution facilitates amplification of G-C rich templates by modifying the melting behaviour of DNA. It can be used for increasing PCR specificity in certain primer-template systems that do not perform well using standard conditions.

7.3.7 RAPD PCR with Anchored primers

The diversity within the 10 *V. chlamydosporium* isolates was also investigated using anchored primers (Anchored AAG, Anchored TG and Anchored CT, Table 7.1).

Optimum conditions for amplification and gel electrophoresis were as described for RAPD primers in Section 7.3.6.

Table 7-1 PCR and sequencing primers

Primer	Size (nt)	Sequence (5'-3')	Source
rDNA			
ITS4	20	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
ITS5	22	GAA AGT AAA AGT CGT AAC AAG G	White et al. (1990)
Microsatellite			
Anchored CT	19	KKV RVR VCT CTC TCT CTC T	Fisher et al. (1996), Ganley (2000)
Anchored TG	19	KKV RVR VTG TGT GTG TGT G	Ganley (2000)
Anchored AAG	22	KKY NSS HAA GAA GAA GAA GAA G	Ganley (2000)
RAPD			
Ngen 3-60-1	10	GAGTGTCTCG	Péros et al. (1996)
Ngen 3-60-2	10	CACATAGCGC	Péros et al. (1996)
Ngen 3-60-3	10	CGAAGCGATC	Péros et al. (1996)
Ngen 3-60-4	10	CCCTCATCAC	Péros et al. (1996)
Ngen 3-60-5	10	CCTGTTAGCC	Péros et al. (1996)
Ngen 3-60-6	10	GCAGCTCATG	Péros et al. (1996)
Ngen 3-60-7	10	CGCTTGCTAG	Péros et al. (1996)
Ngen 3-60-8	10	GAACCTACGG	Péros et al. (1996)
Ngen 3-60-9	10	CTAGCTGAGC	Péros et al. (1996)
Ngen 3-60-10	10	GAGCAGGCTG	Péros et al. (1996)
P-A10	10	GTGATCGCAG	Péros et al. (1996)
P-A15	10	TTCCGAACCC	Péros et al. (1996)
P-B02	10	TGATCCCTGG	Péros et al. (1996)
P-C13	10	AAGCCTCGTC	Péros et al. (1996)
P-C20	10	ACTTCGCCAC	Péros et al. (1996)
P-D18	10	GAGAGCCAAC	Péros et al. (1996)
P-E03	10	CCAGATGCAC	Péros et al. (1996)
P-E08	10	TCACCACGGT	Péros et al. (1996)
P-E15	10	ACGCACAACC	Péros et al. (1996)
P-P05	10	CCCCGGTAAC	Péros et al. (1996)
P-P06	10	GTGGGCTGAC	Péros et al. (1996)
P-P14	10	CCAGCCGAAC	Péros et al. (1996)
OP-F11	10	TTGGTACCCC	Bielikova et al. (2002)
OP-B 6	10	TGCTCTGCCC	Hirst (1997)

R=A+G, S=C+G, Y=C+T, K=G+T, V=A+C+G, H=A+G+T, N=A+C+G+T

7.3.8 Overnight large gel

A large overnight gel was used to analyse PCR products from those RAPD primers that produced reproducible polymorphic amplification patterns. Agarose (3.6 g/ 300 ml) was dissolved in 1 x TAE buffer (Appendix III) to give a 1.2% (w/v) gel. This was cooled to 50°C and then poured into a larger gel carrier (20 x 25 x 0.6 cm). Once set, the gel was kept at 4° C for 30 min.

There are two lines in the gel carrier and two different PCR reactions can be run at the same time. Loading dye (10 x) was added to the PCR products to give a final concentration of 1 x and this mixture was loaded into wells of the second line and run at 80 volts for 10 min. Then, the wells of first line were filled with PCR products of different PCR reaction and the products were run at 80 volts. After 30 min at 80 volts, the products were separated slowly at 18 volts for 14 h (overnight at 4° C). Then, the products were run quickly at 80 volts until the loading dye reached two thirds of the way along the edge of the gel. The gel was divided using a scalpel blade at the middle across the gel and stained in EtBr for 20 min and destained in milli-Q water for 20 min before the DNA was visualized on a short wave UV transilluminator and photographed using an IS-1000 Imaging System.

7.3.9 Analysis of RAPD data

Although amplification patterns were extremely reproducible it was occasionally difficult to score faint bands. Two independent DNA preparations from separate cultures of each isolate were used to check reproducibility and consistency for RAPD analysis. Molecular weights from reproducible RAPD products of the 10 *V. chlamydosporium* isolates with six RAPD primers were analysed. Banding patterns from RAPD markers were compiled by scoring '1' for the presence of major bands and '0' for their absence. Markers which produced faint or inconsistent bands, were discarded from the analysis. The isolates were grouped by cluster analysis (Ward linkage, SAS version 8.2) of 10 *V. chlamydosporium* isolates (duplicate culture of each isolate) using molecular weights of reproducible RAPD fragments.

7.4 Results and Discussion

7.4.1 Concentration of genomic DNA

DNA was measured from separate cultures of each isolate. That DNA concentration differed among isolates and between replicates, probably because of the dry weights of mycelium or separate DNA extractions. For example, DNA extracted from 50 mg dry weight of mycelium gave more than 50 ng/μL but 30 mg gave less than 25 ng/μL. Negative images were used in these results because it showed clear and distinct bands compared with positive images.

Gel electrophoretic analyses were carried out to check that high molecular weight DNA had been obtained (Fig. 7.1). Only lane 4 & 5 did not show clear high molecular weight bands, probably because too much DNA was present. Alternatively, the cause could be degradation or impurity of DNA.

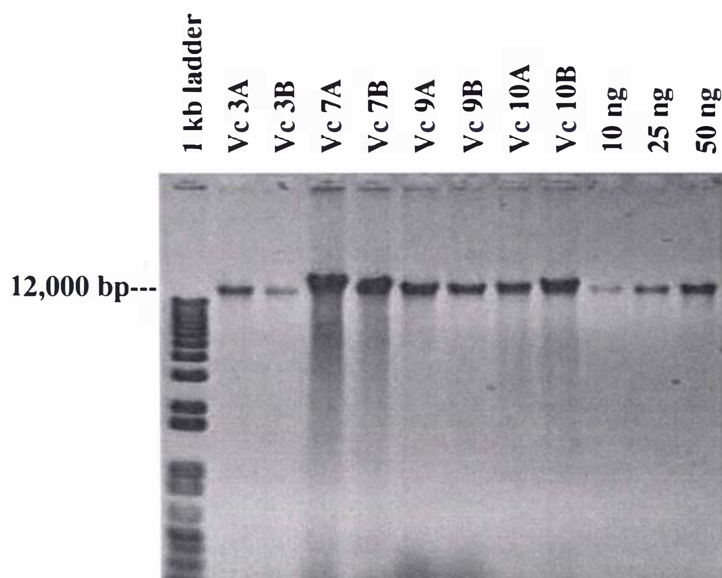


Figure 7-1 Gel electrophoretic analyses of genomic DNA isolated from three *V. chlamydosporium* isolates (duplicates A and B of each isolate)

1 kb ladder (lane 1), Vc isolates 3A, 3B, 7A, 7B, 9A, 9B, 10A, 10B, respectively (lane 2-9), λ standard DNA 10 ng, 25 ng, and 50 ng, respectively (lane 10-12). Number at the sides of the figure indicates the fragment size of the 1 kb+ ladder (Invitrogen).

7.4.2 Concentration of PCR-amplified DNA

Gel electrophoresis of PCR products from *V. chlamydosporium* with the ITS5 and ITS4 primers always yielded a single band approximately 650 bp in length (Fig. 7.2). The size of this fragment was approximately the same in all *V. chlamydosporium* isolates examined when estimated on agarose gels.

A measurement of the concentration of purified DNA was required for sequencing. For example, concentration of the PCR-purified product of *V. chlamydosporium* isolate Vc 6B was determined by gel electrophoresis by comparing

three different λ standard DNA, showing that Vc 6B (Fig. 7.2, lane 2) had about 40 ng/μL.

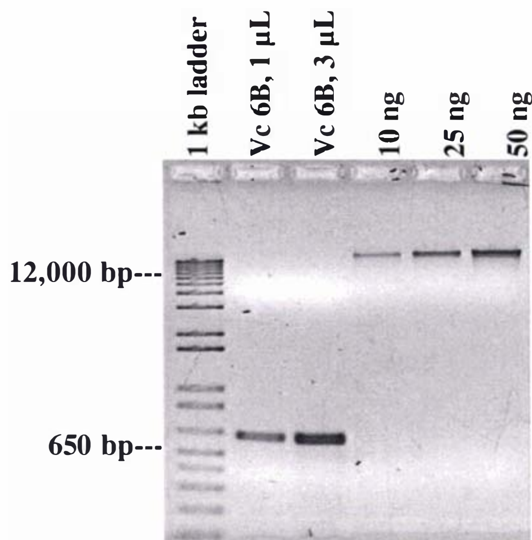


Figure 7-2 Agarose gel (1.2%) with purified-PCR amplification products of a *V. chlamydosporium* isolate 6B, using universal primers ITS4 and ITS5

1 kb+ ladder (lane 1), PCR-purified products of *V. chlamydosporium* isolate Vc 6B, 1 μL (lane 2), 3 μL (lane 3), λ standard DNA 10 ng, 25 ng, and 50 ng, respectively (lane 4-6). Numbers at the sides of the figure indicate the fragment sizes of the 1 kb+ ladder (Invitrogen).

7.4.3 ITS region sequences

ITS primers ITS4 and ITS5 specifically amplified the entire length of the ITS region including the 5.8S rDNA, plus small portions of the 3' end of 18S and the 5' end of 28S rDNA (Fig. 7.3).

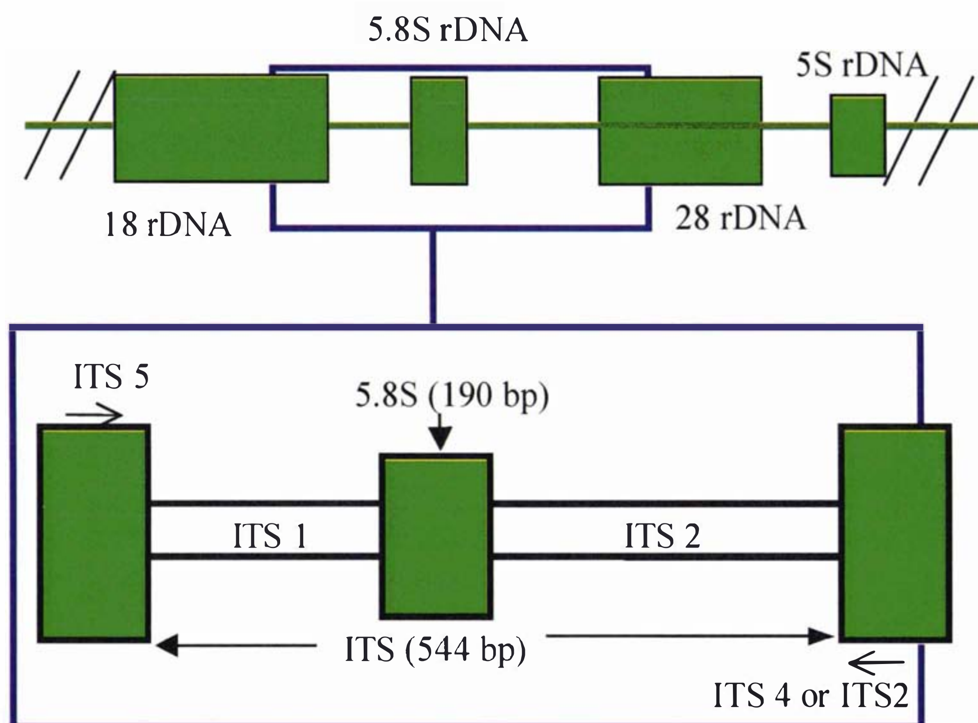


Figure 7-3 Location on nuclear rDNA of PCR primers designed to PCR-amplify the ITS regions. The arrowheads represent the 3' end of each primer

Only sequences from 5' to 3' primed by ITS5 were obtained in this study. Although, attempts were made for reverse sequencing using primers ITS2 and ITS4, none of the results were reliable with many N residues and possibly a mixed sequence. One possible explanation is contamination of the primers ITS2 and ITS4 used in this study.

The ITS5-primed sequences of four isolates (Vc 1A, 6B, 7B, 12B) were in agreement with the ITS sequence obtained for a *V. chlamydosporium* isolate from the UK (Genbank accession number AJ292397) and no polymorphic sites were seen. The length of sequences was 544 bp (for the regions of ITS1+5.8S+ITS2) and the conserved

5.8S was found at the centre of the alignment (169-360 bp) from all isolates sequenced (Fig. 7.4).

There were NNNN sequences (304 - 307 bp) in Vc 7B and 12B (Fig. 7.4). Possibly this was because only single-stranded sequences were obtained and there was some ambiguous sequence that could not be resolved.

7.4.4 Optimisation for Randomly amplified polymorphic DNA, (RAPD) PCR

7.4.4.1 Optimisation of DNA template concentration

The amount of DNA template (10 ng or 20 ng) did not affect the numbers and concentrations of PCR products, however, sharper bands were produced as the amount of genomic template increased (data not shown).

7.4.4.2 Optimisation of magnesium concentration

Although the amount of magnesium (1.5, 2.0, 2.5, or 3.0 mM) did not affect the numbers and concentrations of PCR products, the higher the concentrations used, the sharper bands were produced. The concentration of 3.0 mM gave intense, sharper bands of amplified products (data not shown).

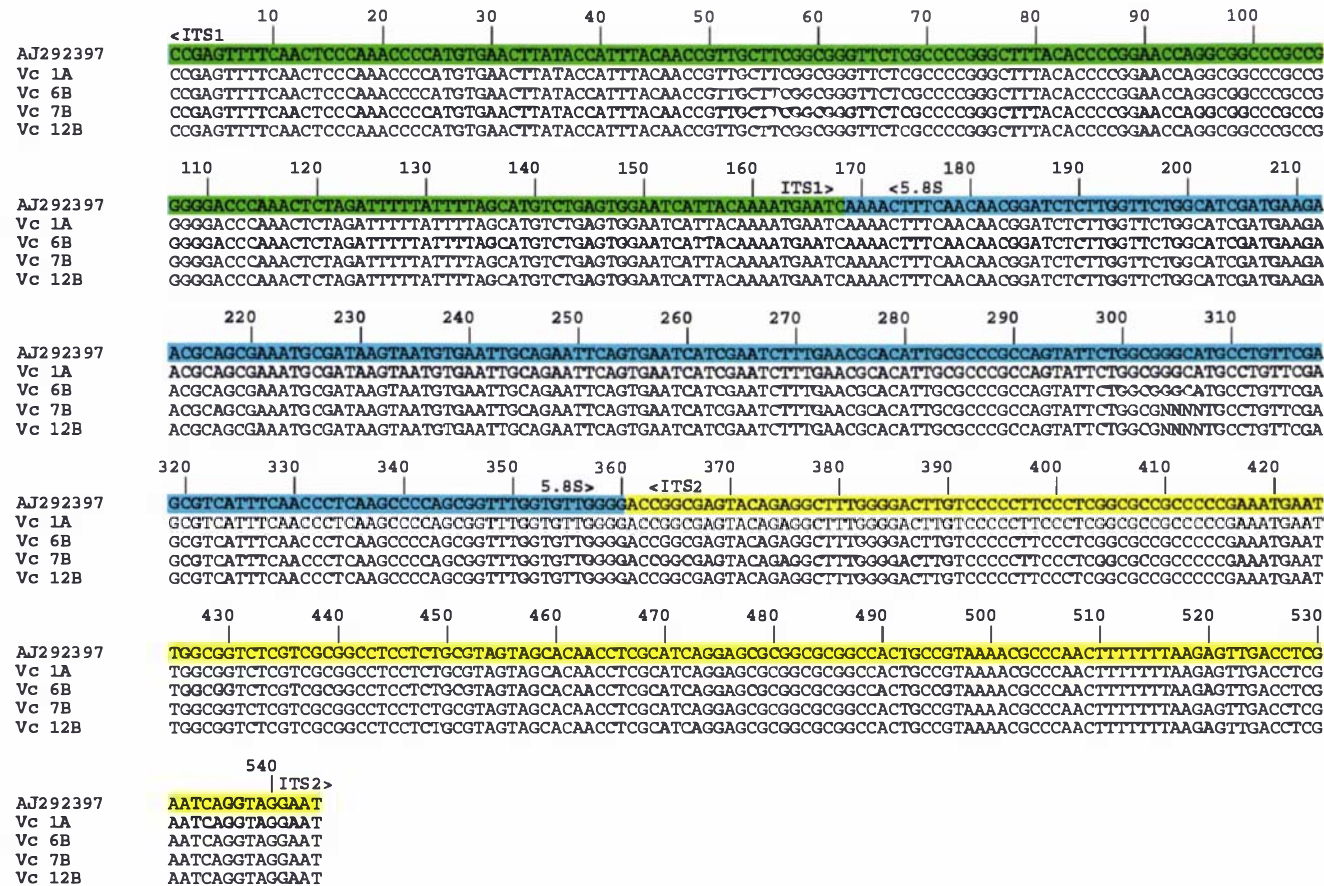


Figure 7-4 Sequence alignments of ITS1+5.8S+ITS2 regions of AJ292397 (*Verticillium chlamydosporium* var. *chlamydosporium* (Zare et al. 2000) and four *V. chlamydosporium* isolates (Vc 1A, Vc 6B, Vc 7B, Vc 12B)

7.4.4.3 Optimisation of annealing temperature

Annealing temperatures (36°C - 40°C) were tested for reproducible products. At an annealing temperature at 36°C, both the number and amount of the PCR products and the clarity of gel bands were less with primer Anchored CT and *V. chlamydosporium* isolate 5B (Fig. 7.5, lane 1) than at 37°C and above (Fig. 7.5, lane 2-5).

There were faint bands using RAPD primer PA 10 with *V. chlamydosporium* isolate 5B at temperature of 39°C (Fig. 7.5, lane 9) and no band was produced at 40°C (Fig. 7.5, lane 10). Temperatures of 36°C - 38°C produced clear and distinct bands (Fig. 7.5, lane 6-8). An annealing temperature of 37°C was chosen as being appropriate for both Anchored primers and RAPD primers.

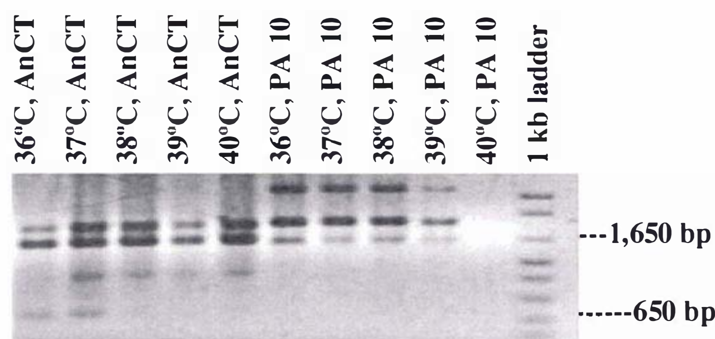


Figure 7-5 The effect of annealing temperature (36-40°C) on reproducibility of anchored primer AnCT and RAPD primer PA 10 amplified with *V. chlamydosporium* isolate 5B

7.4.4.4 Optimisation of buffer

Bands were more intense and more clearly visible on gel run in 1 x TAE (Fig. 7.6A) than those in 1 x TBE (Fig. 7.6B).

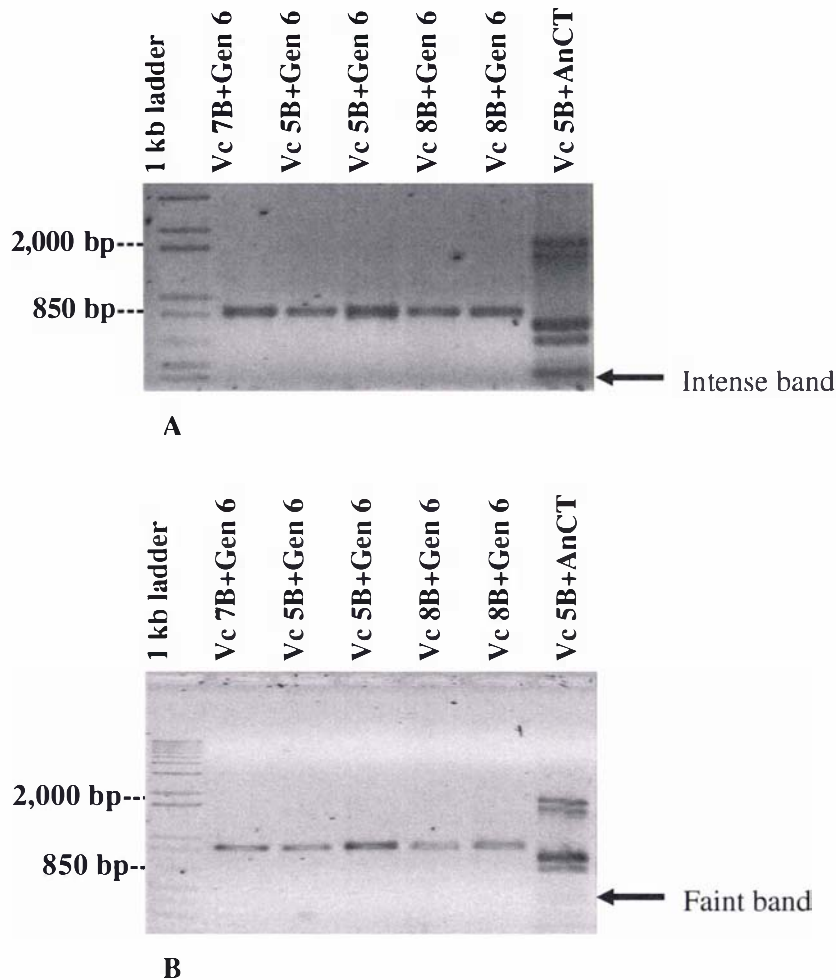
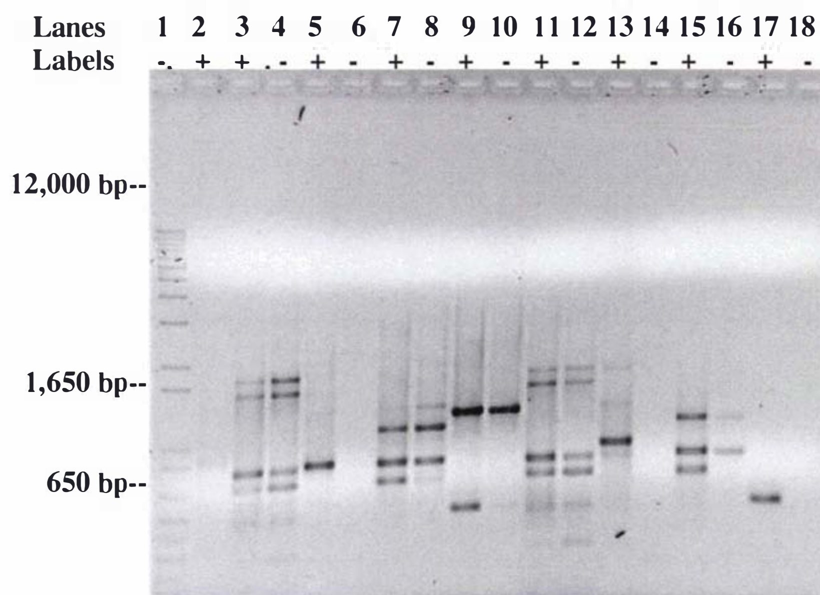


Figure 7-6 Agarose gel (1.2%) electrophoretic analysis of PCR amplification products of *V. chlamydosporium* isolates in TAE (A) or TBE buffer (B)

(A) 1 kb+ ladder (lane 1), PCR products of *V. chlamydosporium* isolate using RAPD primer Gen 3-60-6 with Vc 7B 3 µL (lane 2), Gen 3-60-6 with Vc 5B 3 µL, 5 µL, respectively (lane 3-4), primer Gen 3-60-6 with Vc 8B 3 µL, 5 µL, respectively (lane (5-6), PCR products of *V. chlamydosporium* isolate using Anchored CT (AnCT) primer with Vc 5B (lane 7) in TAE buffer, (B) Same as (A) but in TBE buffer.

The results varied from different PCR conditions and different concentrations of PCR reagents. Generally, reproducible and consistent amplification products were produced from these concentrations of PCR reagents in a total volume of 25 µL: 1 x PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primer, 0.8 U of *Taq* DNA polymerase, 20 ng of template DNA, Q solution (20% of total volume), and with these cycling conditions: an initial 4 min at 94°C, followed by 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min, a final 72°C elongation step for 6 min.

Adding Q solution to PCR reactions produced more clear bands than not adding Q-solution (Fig. 7.7). In some cases (for example, RAPD primer P. E03 with Vc 5B and Q solution produced one band but did not produce any bands without Q solution) (Fig. 7. 7, lane 5, 6), PCR products were not obtained unless Q solution was added.



+ indicates amplification with Q solution, - indicates without Q solution

Figure 7-7 Agarose gel (1.2%) electrophoretic analysis of RAPD PCR products of *V. chlamydosporium* isolates using Q-solution (+) and without Q-solution (-) 1 kb ladder (lane 1), Control (lane 2). Template DNA was Vc 5B with primer AnCT (lane 3-4), P. E03 (lane 5-6), P. PA 10 (lane 7-8), P. P05 (lane 9-10) or Vc 9B with primer AnCT (lane 11-12), P. E03 (lane 13-14), P. PA 10 (lane 15, 16), P. P05 (lane 17-18).

7.4.5 RAPD PCR (Anchored primers + RAPD primers)

In total, six out of 27 (Anchored + RAPD) primers used in this investigation successfully amplified multiple bands which showed some molecular variations among the *V. chlamydosporium* isolates tested (Table 7.2). Another eight out of 27 primers gave amplification products with all *V. chlamydosporium* tested but produced the same banding patterns (monomorphic). Another nine out of 27 did not give amplification products with all isolates and the rest (4 primers) gave amplified products with only one isolate out of two isolates used in a primer screen experiment. Possible reasons for the lack of amplification were that the primer itself did not amplify with this fungal species or that the optimisation of PCR and PCR cycling conditions were not appropriate for these primers.

Table 7-2 Amplification by 27 RAPD primers with 10 *V. chlamydosporium* isolates

	Primers which gave different banding patterns (polymorphic)	Primers which gave same banding patterns (monomorphic)	Primers which did not amplify with all isolates	Primers which amplified with one of two isolates tested
1	Anchored CT	Anchored AAG	P-B02	Anchored TG (Vc 8B)
2	P-E 03	OPC 15	OPF 11	P. PA 15 (Vc 8B)
3	P-E 15	P. PA 10	Gen 3-60-1	P. D 18 (Vc 9B)
4	P-P 05	Gen 3-60-2	Gen 3-60-4	P-P 06 (Vc 9B)
5	Gen-3-60-10	Gen 3-60-6	Gen 3-60-5	
6	Gen 3-60-3	P-E 08	Gen 3-60-7	
7		P-C 20	Gen 3-60-8	
8		P-P 14	Gen 3-60-9	
9			OPB 6	

Clear amplification banding patterns were produced by using the large gel (Fig. 7.9A) that was run overnight at 18 volts at 4°C compared with the bands resulting from a small gel in 1 x TAE, run at 80 volts at 4°C (Fig. 7.9B), even though the same PCR products were used. Probably, (1) DNA separated well because of less diffusion in a slow-run in the cold room, (2) better resolution was obtained by using sharp combs.

With the selected primers, amplification products ranging in size from 350-3400 bp and a total of 136 reproducible bands were generated (Fig. 7.8-7.13). Each of the six RAPD primers that gave polymorphic banding patterns separated the 10 *V. chlamydosporium* isolates into between 2 and 5 groups (Table 7.3).

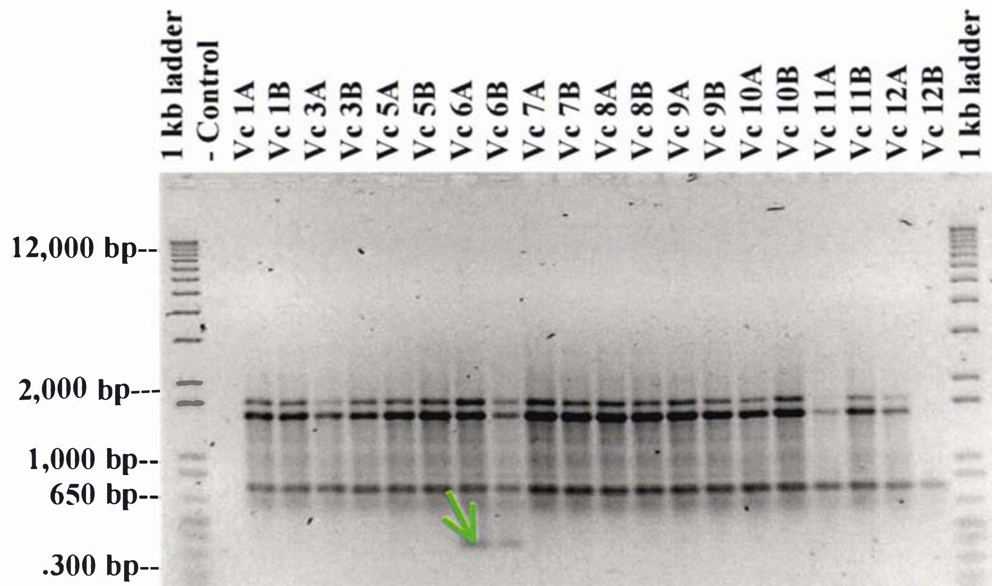


Figure 7-8 Anchored CT RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

Profiles from duplicates (A and B) of each isolate. The green arrow highlights an amplicon, approximately 350 bp in size, show polymorphism.

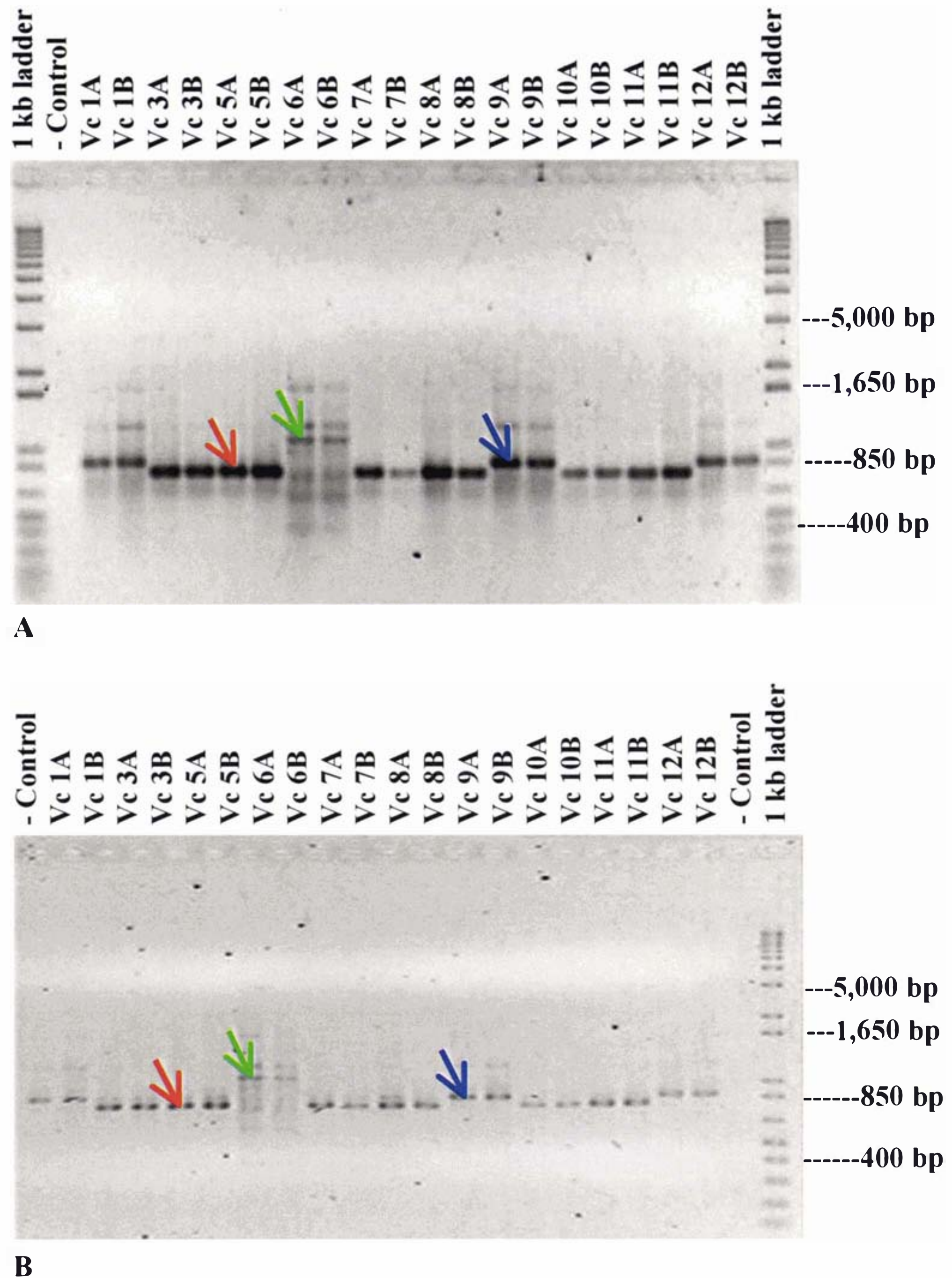


Figure 7-9 P. E03 RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

(A) Profiles from duplicates (A and B) of each isolate on a large gel (20 x 25 x 0.6 cm). The red arrow highlights an amplicon, approximately 800 bp in size, the blue arrow (850 bp), and green arrow (1200 bp), show polymorphisms, (B) Same as (A) but on a small gel (14.5 x 7 x 0.6 cm).

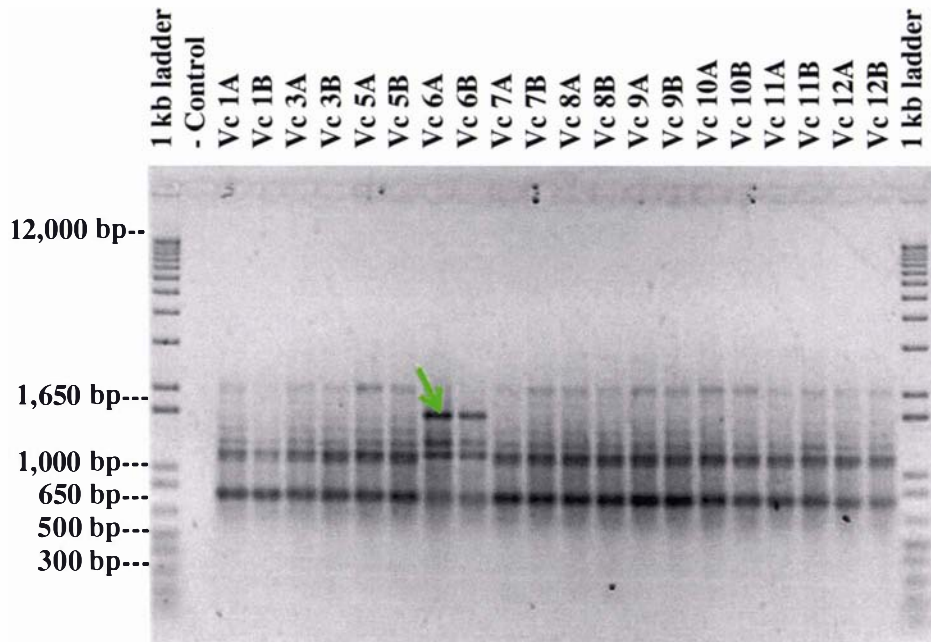


Figure 7-10 P. E15 RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

Profiles from duplicates (A and B) of each isolate. The green arrow highlights a 1600 bp amplicon, show polymorphism.

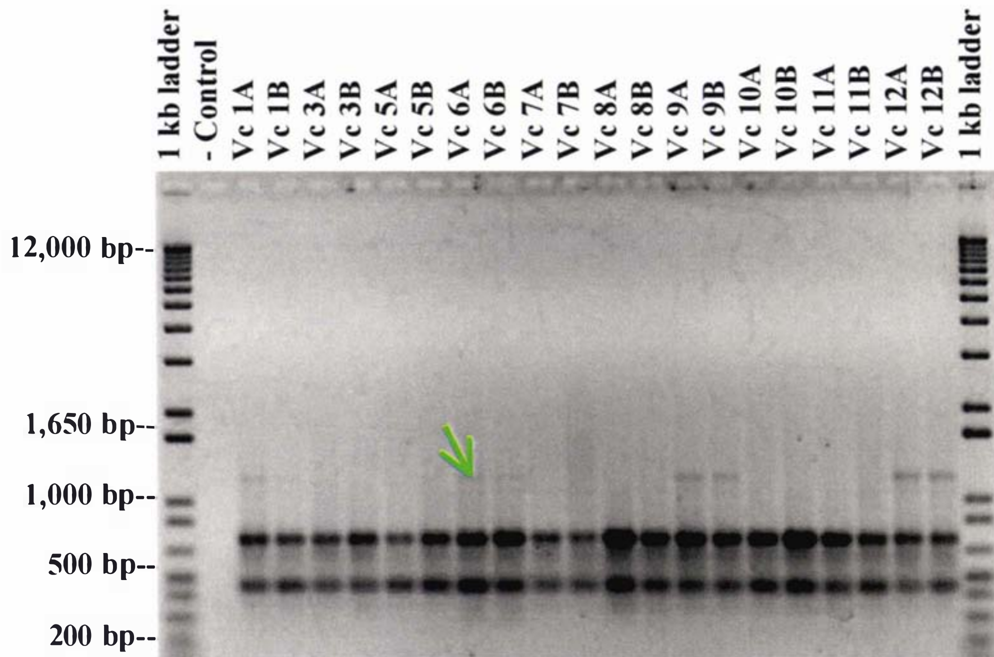


Figure 7-11 Gen 3-60-10 RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

Profiles from duplicates (A and B) of each isolate. The green arrow highlights an amplicon, approximately 1,300 bp in size, show polymorphism.

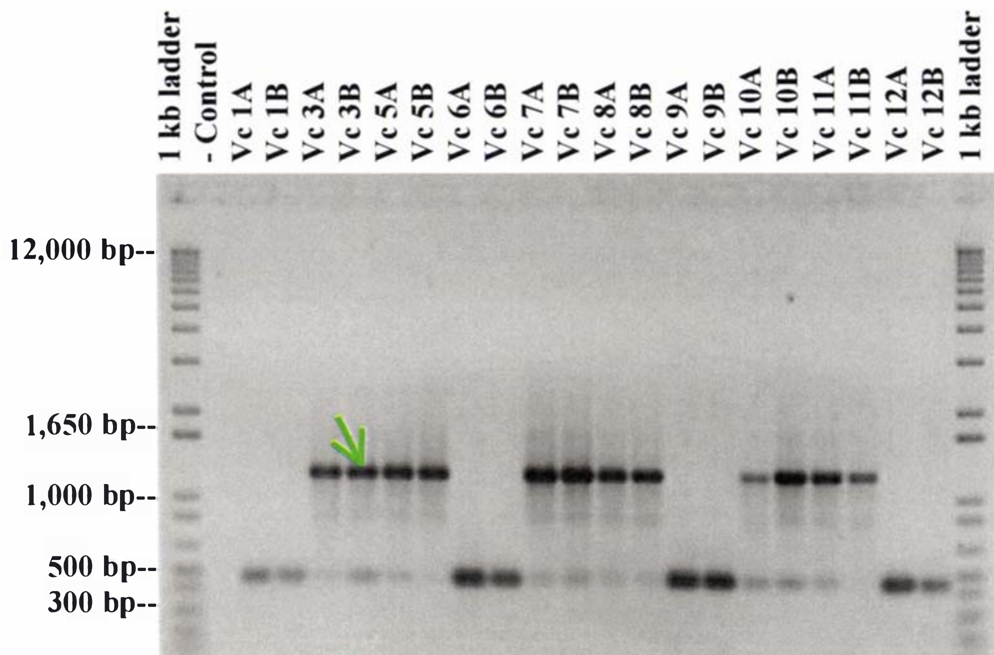


Figure 7-12 P. P05 RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

Profiles from duplicates (A and B) of each isolate. The green arrow highlights an amplicon, approximately 1300 bp in size, show polymorphism. The 500 bp band was not considered to be a reproducible polymorphism as it can be seen faintly in at least one replicate of all the isolates.

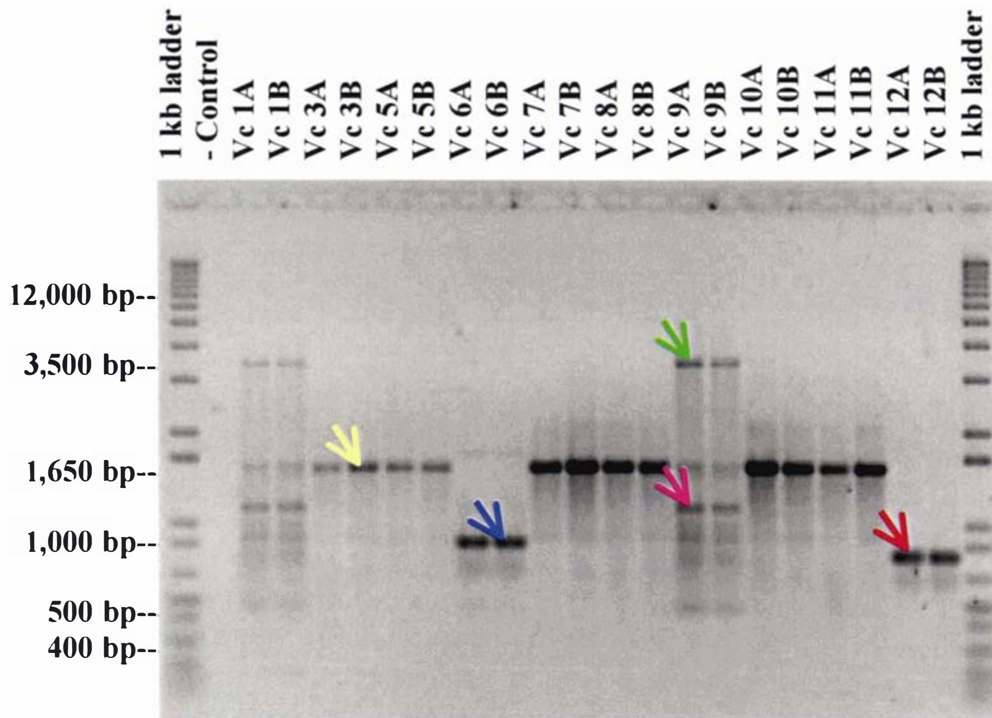


Figure 7-13 Gen 3-60-3 RAPD primer analysis of 10 *Verticillium chlamydosporium* isolates

Profiles from duplicates (A and B) of each isolate. The red arrow highlights an amplicon, approximately 800 bp in size, the blue arrow (850 bp), the pink arrow (1,250 bp), the yellow arrow (1,600 bp), and the green arrow (3,400 bp), show polymorphisms.

Table 7-3 Groups of *V. chlamydosporium* isolates by size of polymorphic amplification products from RAPD primers

Group	RAPD Primer					
	Anchored CT (Fig. 7. 8)	P. E 03 (Fig. 7.9)	P. E 15 (Fig. 7.10)	Gen 3-60-10 (Fig. 7.11)	P. P 05 (Fig. 7.12)	Gen 3-60-3 (Fig. 7. 13)
1	**350 bp** Vc 6	**1200 bp** Vc 6	**1600 bp** Vc 6	**1300 bp** Vc 1 Vc 6 Vc 9 Vc 12	**1300 bp** Vc 3 Vc 5 Vc 7 Vc 8 Vc 10 Vc 11	**3400, 1250 bp** Vc 1 Vc 9
2	**No amplicon** Vc 1 Vc 3 Vc 5 Vc 7 Vc 8 Vc 9 Vc 10 Vc 11 Vc 12	**850 bp** Vc 1 Vc 9 Vc 12	**No amplicon** Vc 1 Vc 3 Vc 5 Vc 7 Vc 8 Vc 9 Vc 10 Vc 11 Vc 12	**No amplicon** Vc 3 Vc 5 Vc 7 Vc 8 Vc 10 Vc 11	**No amplicon** Vc 1 Vc 6 Vc 9 Vc 12	**1600 bp** Vc 3 Vc 5 Vc 7 Vc 8 Vc 10 Vc 11
3		**800 bp** Vc 3 Vc 5 Vc 7 Vc 8 Vc 10 Vc 11				**850 bp** Vc 6
4						**800 bp** Vc 12

7.4.6 RAPD analysis

The sizes and scores of reproducible and consistent bands produced from each primer were used for RAPD analysis (Table 7.4).

Table 7-4 The sizes and scores of reproducible RAPD PCR polymorphic amplification products for RAPD cluster analysis

Primer	AnCT	P-E03			P-E15	Gen 3-60-10	P-P05	Gen 3-60-3				
Isolate	350*	800	850	1200	1600	1300	1300	800	850	1250	1600	3400
1A	0	0	1	0	0	1	0	0	0	1	0	1
1B	0	0	1	0	0	1	0	0	0	1	0	1
3A	0	1	0	0	0	0	1	0	0	0	1	0
3B	0	1	0	0	0	0	1	0	0	0	1	0
5A	0	1	0	0	0	0	1	0	0	0	1	0
5B	0	1	0	0	0	0	1	0	0	0	1	0
6A	1	0	0	1	1	1	0	0	1	0	0	0
6B	1	0	0	1	1	1	0	0	1	0	0	0
7A	0	1	0	0	0	0	1	0	0	0	1	0
7B	0	1	0	0	0	0	1	0	0	0	1	0
8A	0	1	0	0	0	0	1	0	0	0	1	0
8B	0	1	0	0	0	0	1	0	0	0	1	0
9A	0	0	1	0	0	1	0	0	0	1	0	1
9B	0	0	1	0	0	1	0	0	0	1	0	1
10A	0	1	0	0	0	0	1	0	0	0	1	0
10B	0	1	0	0	0	0	1	0	0	0	1	0
11A	0	1	0	0	0	0	1	0	0	0	1	0
11B	0	1	0	0	0	0	1	0	0	0	1	0
12A	0	0	1	0	0	1	0	1	0	0	0	0
12B	0	0	1	0	0	1	0	1	0	0	0	0

* = Molecular weight (bp)

1 = PCR product of size stated

0 = no PCR product of size stated

The dendrogram (Fig. 7.14) produced from cluster analysis based on molecular weights amplified by six RAPD primers, separated two major clusters at Semi-Partial-R-Squared (minimum variance) 0.66: Vc 3, 5B, 7, 8, 10, and 11 formed cluster A. The remaining isolates were grouped as cluster B.

At minimum variance 0.24, groups C and D were divergent from group B: isolates Vc 1, 9, and 12 were under group C and Vc 6 was under group D.

At minimum variance 0.11, groups E and F were divergent from group C: Vc 12 was under E, and Vc 1 and 9 were under F.

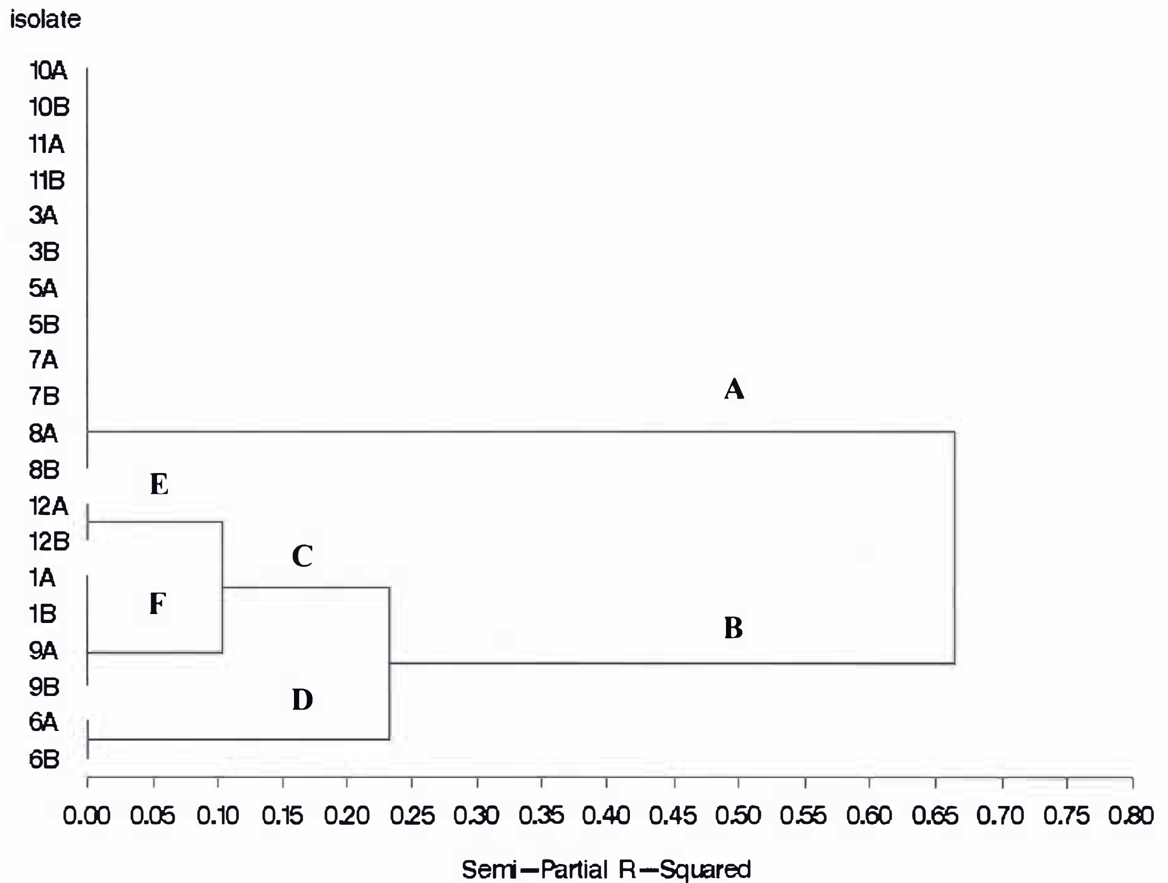


Figure 7-14 A dendrogram from cluster analysis of 10 *V. chlamydosporium* isolates [duplicates (A and B) in each isolate] based on RAPD data using six RAPD primers

Based on the cluster analysis, genetic variation was observed among the 10 isolates of *V. chlamydosporium*. Isolates Vc 1 and 9 were in the same group at the molecular level. Vc 1, 9, and 12 were closer to each other than Vc 6. Isolate Vc 1, 6, 9, 12 were closer to each other than Vc 3, 5, 7, 8, 10, and 11. Vc 3, 5, 7, 8, 10, 11 were in the same group.

Isolate Vc 6 was clearly different from the remaining isolates by RAPD PCR assay. It was also noted that fungal growth rate, viable spores count and spores size of Vc 6 (Table 7.5) were slightly bigger than others. However, the cluster analysis, in general, did not match with morphological characteristics such as fungal growth on PDA (Fig. 7.15), spores shapes (Fig. 7.16), fungal growth rates, viable spores counts

and sizes of chlamydospores of 10 *V. chlamydosporium* isolates (Table 7.5). The morphology of isolates may have changed after subculturing on agar or the RAPD primers may not have amplified areas of DNA associated with these morphological changes.

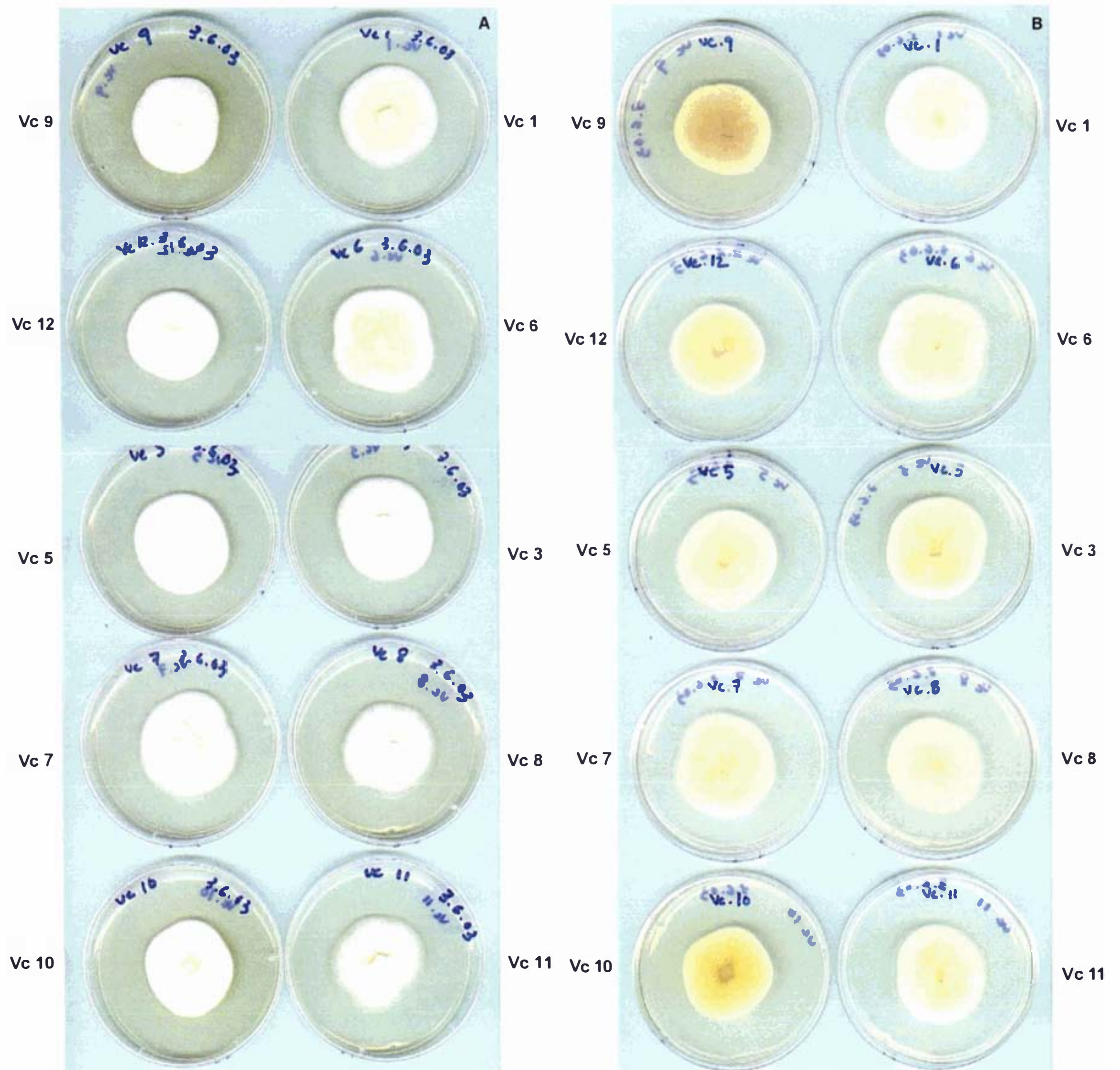


Figure 7-15 The growth of *V. chlamydosporium* on PDA (2 week-old culture)
(A) Colony colour of 10 *V. chlamydosporium* isolates as viewed from above,
(B) Colony colour of 10 *V. chlamydosporium* isolates as viewed from below.

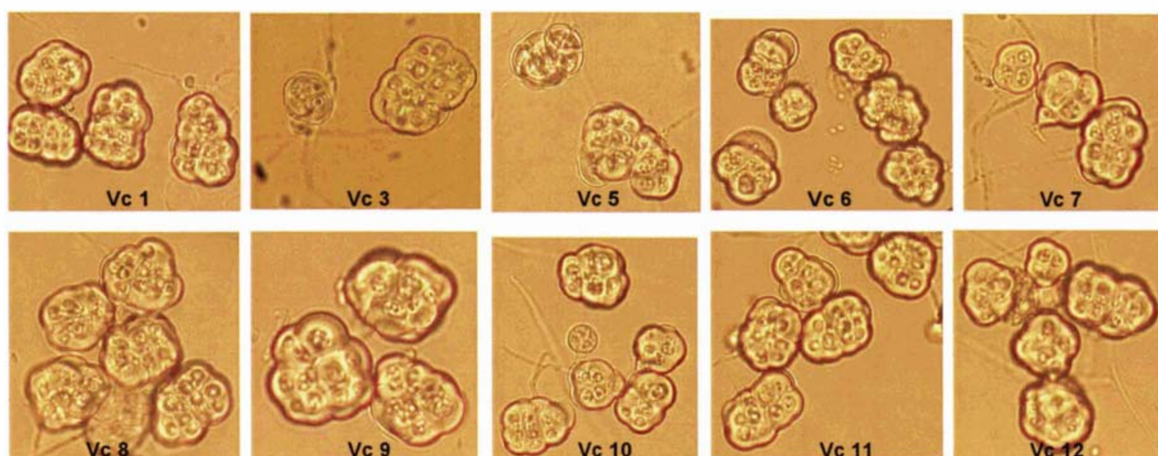


Figure 7-16 Chlamydospores (x 400) of 10 *V. chlamydosporium* isolates on WA (1 month old culture)

Table 7-5 Mean fungal growths, numbers of viable spores and sizes of chlamydospores of 10 *V. chlamydosporium* isolates

Vc isolates	Growth rate on PDA (mm in diameter, 3 wk old)	No. of viable spores/ml (3 wk old on PDA)	Spore size (μm) (1 month old on WA)
Vc 1	70	2.64×10^{8}	5x7 - 7x9
Vc 3	68	2.22×10^{8}	6x7 - 7x9
Vc 5	73	3.49×10^{8}	7x8 - 8x9
Vc 6	78	8.40×10^{8}	8x11 - 10x13
Vc 7	69	3.35×10^{8}	7x9 - 8x10
Vc 8	69	2.98×10^{8}	5x7 - 6x8
Vc 9	74	6.72×10^{8}	8x10 - 9x11
Vc 10	70	3.98×10^{8}	6x7 - 6x8
Vc 11	76	4.35×10^{8}	6x7 - 8x10
Vc 12	68	3.08×10^{8}	6x9 - 8x10

7.5 General Discussion

This is the first molecular variation study carried out with RAPD analysis using RAPD primers on *V. chlamydosporium* populations isolated from females and young cysts of clover cyst nematode *H. trifolii* from Manawatu pasture.

The ITS sequences of fungal isolates from *H. trifolii* in the current study are identical to that of *V. chlamydosporium* from the UK according to the GenBank database. This confirms that the fungus used in this study is *V. chlamydosporium*.

Several attempts were made to get optimal RAPD PCR amplification products. The reproducibility of the RAPD technique is influenced by several factors including the source and procedure used for DNA isolation, the occurrence of contaminants, extraction of DNA, concentration of PCR reagents and primers, PCR conditions, and the amplification of different DNA sequences of the same size. Therefore, these factors were strictly controlled to produce reproducible results in RAPD PCR. Once optimisations for RAPD PCR were set up, the procedures were not difficult for reproducible results but it was necessary to work with care in each step. In the current study, prominent, faint bands, and some primers were not always amplified to the same extent so using different RAPD primers instead of just one, and repeated experiments, gave a broader picture of the diversity and similarity among *V. chlamydosporium* isolates.

Adding Q solution to the PCR reaction enhanced reproducibly distinct bands and using a large gel, slow-run overnight, instead of a small gel gave distinct and clear profiles that were more easy to score for RAPD analysis.

RAPD PCR proved to be an efficient method for detecting the genetic variability of *V. chlamydosporium* isolates tested. Isolate Vc 6 was separated from other *V. chlamydosporium* isolates by the six RAPD primers tested. RAPD primers Gen 3-60-3 separated four groups: (1) Vc 1 & Vc 9, (2) Vc 6, (3) Vc 12, and (4) the remaining isolates.

In summary, the DNA polymorphisms detected by the RAPD PCR assay were consistent in duplicate cultures of each isolate and the cluster analysis (SAS, version 8.2) of polymorphic bands based on molecular weight did not match with fungal morphological characteristics on PDA.

7.6 Future work

(1) The present study was conducted on a small number of isolates that represent seasonal samples from one pasture paddock in Palmerston North and tested with a small number of RAPD primers, and thus may not represent the entire range of intraspecific genetic variation of this fungal species. Therefore, there remains a need to detect genetic diversity among the *V. chlamydosporium* isolates nationwide.

(2) Detection of molecular variation among enzymes produced from *V. chlamydosporium* isolates for distinguishing the virulent isolate to clover cyst nematode on the basic of their ability to produce chitinases would be worthwhile, perhaps using the method of Bidochka et al. (1999).

(3) Detection of this fungal species on nematode-infected clover roots grown in soil to enable the monitoring of this fungus in the rhizosphere by using direct DNA extraction, thereby, avoiding the need for fungal culture, would be a useful technique to develop.

(4) Designation of primers that distinguished the most effective biological control isolates of *V. chlamydosporium* from indigenous isolates in field trials would be an extremely useful tool for monitoring populations of biocontrol isolates in trials and following field releases. Polymorphic RAPD bands can be cloned and sequenced. Specific PCR primers based on the sequence can then be used at high stringency to detect polymorphisms in a reliable and reproducible way. These are called SCARS (sequence characterised amplified regions).

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8 GENERAL DISCUSSION AND CONCLUSION

Clover cyst nematode, *H. trifolii*, is one of the economically most important pests in New Zealand pastures. Chemical control of *H. trifolii* has a quick effect, but it causes environmental pollution and is also uneconomic. Alternative strategies, such as cultural control methods, use of resistant cultivars, and use of biological control agents have been investigated. The aim of this study was to control *H. trifolii* by using fungi that naturally infect it in the soil. To achieve this aim, the population dynamics of this nematode species and of the natural causes of mortality, were studied. An investigation of relationships of environmental factors (soil moisture and soil temperature), plants, *H. trifolii*, and fungi that appeared to parasitise *H. trifolii*, was a first step. The fungi most associated with *H. trifolii* were tested for their pathogenicity to white clover plants to ensure they were not phytopathogenic. Then they were screened for pathogenicity to *H. trifolii*. The development of monoxenic culture of *H. trifolii* on *T. repens* was attempted for the purpose of screening a range of *V. chlamydosporium* isolates obtained from *H. trifolii* over a 2 year period for their potential as biocontrol agents. RAPD-PCR was used to assess the variation among those *V. chlamydosporium* isolates.

In chapter two, the frequency of soil sampling was important for the population dynamics study. In this study, fortnightly sampling showed distinct peaks and seasonal trends of all stages of *H. trifolii*. Although there are geographically differences between this study and that of Yeates (1973) in the Wairarapa and Yeates & Risk (1976) in Southland, all three studies used fortnightly sampling and found there were two reproductive cycles of *H. trifolii* per year.

Peak numbers of J2 in soil (J2 emergence) coincided with start of root growth and this in turn appeared to reflect soil moisture levels. Rising or falling numbers of J2 in soil were followed by those of J2 in roots then of females in soil. The latter coincided with high spring temperatures in both 1999 and 2000. Although there was a clear relationship between the numbers of females and the subsequent numbers of cysts in the studies of Yeates (1973) and Yeates & Risk (1976), it did not show in the year 2000 sampling in this study as the trend of numbers of new cysts followed that of females but numbers were low and old cysts were stable at low numbers in 2000. One of the possibilities is parasitism of fungi on females, especially *V. chlamydosporium* as it

parasitised females *in vitro* or in pot trials in this study. Kerry and Crump (1977) found the population decline was due to the failure of many *H. avenae* to form cysts and fecundity was reduced because eggs and females were parasitised frequently by *V. chlamydosporium.*

The range of fungi obtained from young cysts and females were similar to those obtained by Hay & Skipp (1993). However, the isolation frequency of the frequently found fungal species; *Fusarium* spp. and *G. roseum* did not appear to match the trend of abundance of any cyst stages in this study. Only the peak percentages of *V. chlamydosporium* in spring/summer coincided with the peak numbers of females in both 1999 and 2000. This result did not follow those of Waipara et al. (2001) who found greater numbers of *Verticillium* spp. in autumn. One of the possibilities was that they did not specify whether 'cysts' referred to young cysts, old cysts or both from Waikato and they plated onto water agar amended with chlortetracycline but in this work, young cysts and females from Manawatu and plated on water agar amended with oxytetracycline.

The low numbers of new cysts in the current study might reflect the high parasitism of *V. chlamydosporium* or females on the *T. repens* roots and may also reflect the longer dry period in 2000 than in 1999 which affected, in sequence, root growth, J2 in soil, subsequent numbers of J2 in roots and finally numbers of females. There was a spring mortality of J2 in roots in 2000 in this study and that of Yeates (1973) in the Wairarapa and Yeates and Risk (1976) in Southland. The reason is not known but one possibility is high rainfall levels that could have affected the relationship between the host plant and *H. trifolii.*

Chapter three gave details of several fungal genera isolated from the young cysts and females, with the most common species being; *Fusarium oxysporum*, *Verticillium chlamydosporium*, and *Gliocladium roseum*. These fungal species were tested for potential pathogenicity on white clover seedlings *in vitro* before testing for biocontrol activity in pots in the glasshouse. The results *in vitro* showed that *F. oxysporum* and *G. roseum* were virulent to the *T. repens* seedlings grown on 1.0% water agar but *V. chlamydosporium* was not. These three fungal species plus *P. lilacinus*, and *Trichoderma* sp. were tested for their pathogenicity to wounded or unwounded *T. repens* plants in pots in the glasshouse. *F. oxysporum* and *G. roseum* were not as virulent to the plants as in the *in vitro* tests, possibly because agar plate tests may have

created conditions in which roots were unnaturally susceptible to the isolates because of the non-competitive environment (Skipp & Christensen 1989). *P. lilacinus*, *Trichoderma* sp. were not pathogenic to the clover plants but they did not frequently associate with *H. trifolii* cysts and females compared with other fungal species. *F. oxysporum*, *G. roseum* and three isolates of *V. chlamydosporium* (Vc1, Vc2, and Vc6) therefore, were tested for their pathogenicity to *H. trifolii* on *T. repens* in pots.

The soil used in the first two trials described in Chapter four was first frozen to eliminate residual clover cyst nematode populations. However, live *H. trifolii* were found even in the control checks where *H. trifolii* was not added. It would appear that the frozen soil did not kill the existing *H. trifolii*. However, numbers were too low compared with those added to influence the results.

The numbers of females were significantly reduced in the *V. chlamydosporium* isolates Vc6+*H. trifolii* and Vc 1+ *H. trifolii* treatments compared with any of *H. trifolii* added treatments. Control+ *H. trifolii* had significantly more females than remaining treatments, including oatmeal+ *H. trifolii*. The most likely reason was that poor plant growth in the oatmeal+ *H. trifolii* treatment could have affected the egg hatching and J2 development which then reduced parasitism of white clover by *H. trifolii*.

Although *F. oxysporum* could have some positive effect on plant growth as *V. chlamydosporium* had, *F. oxysporum* appeared to enhance the production and development of female nematodes compared with the rest of the fungal treatments. This result was similar to those of Zahid et al. (2002) who reported that *F. avenaceum* not only increased nematode penetration of white clover plants but also increased their development in the plants. *G. roseum*+*H. trifolii* and Vc 2+ *H. trifolii* did not reduce the numbers of females lower than those of the control+ *H. trifolii*. Only Vc6 significantly reduced the numbers of females, possibly through egg parasitism. It appeared to minimise the adverse effect of oatmeal on plant growth, probably by utilising or breaking down the toxic materials. It therefore has the greatest potential as a biological control agent of *H. trifolii*.

Although, the fungal isolates grew well on oatmeal and the plants grew well in soil mixed with fungal culture/oatmeal in this test, the plants had poor growth in soil mixed with oatmeal without fungal inoculum. The possible cause was microbes that were already present in the soil and had exploited the nutrient from the oatmeal medium leaving less nutrient available for the plants which then showed poor growth. The poor

plants in oatmeal without fungal inoculum could have affected the egg hatching and J2 development, alternatively, toxic products produced by microbe in oatmeal alone could have affected the *H. trifolii* reproduction.

Oatmeal was not suitable for *T. repens* plant growth, hence other media were investigated as a substrate for the Vc6 isolate and to test its pathogenicity to *H. trifolii* on *T. repens* in microwaved sand instead of frozen soil in pots in the glasshouse. Microwaving killed existing cysts in soil and microwaved soil was used to test isolate Vc6 cultured on alginate beads (AB), wheat bran beads (BB), potato dextrose beads (PB), potato dextrose/dry sand (DS) or wheat/sand (WS). The DS and WS inocula were not bead-based. BB and PB were bead-based and they would have had residual nutrients in the bran to support fungal growth and colonisation in soil, but AB had no such nutrient base. In general, AB, BB, or PB media themselves did not reduce the numbers of females but Vc grown on these media did reduce them. Siddiqui et al. (2000) and Bharadwj & Trivedi (1996) found that *V. chlamydosporium* multiplied on wheat bran reduced cyst numbers of *H. avenae* on wheat and *Meloidogyne incognita* on tomato, respectively and it also improved plant growth in these two studies.

Vc6 cultured on bran bead-based medium reduced the numbers of 2nd generation females more than of the 1st generation females. Presumably 1st generation females increased the release of nutrients into the rhizosphere, which the fungus was then able to exploit (Dourhourt et al. 1993). The ability to produce chlamydospores might be more important in determining levels of parasitism in the soil (Kerry et al. 1986). Plant shoot weights were significantly higher in Vc6 on bran beads+*H. trifolii* as compared to the rest of the treatments. Overall, Vc6 cultured on this medium had the greatest effect both on plant growth and on parasitism to *H. trifolii* in this pot trial.

V. chlamydosporium was re-isolated from about 85% of the females in the Vc6 spores+*H. trifolii* treatment. The numbers of females were reduced but the fungus did not have a significant effect on plant growth compared with the addition of Vc6 grown on alginate beads or bran beads colonised with *H. trifolii*. One possible reason is that the bead-based media had residual nutrients which could support fungal growth. However, this experiment clearly showed that *V. chlamydosporium* reduced the numbers of *H. trifolii* females as Vc spores without medium reduced the numbers of females. *V. chlamydosporium* isolate (Vc6) showed greater parasitism on *H. trifolii* than *F. oxysporum*, *G. roseum*, Vc1 or Vc2. There were variations in pathogenicity to *H.*

trifolii among these three *V. chlamydosporium* isolates in this trial and variations in pathogenicity between the isolates required further investigation.

More than 100 *V. chlamydosporium* isolates (22.2% of fungal isolates from infected *H. trifolii* cysts) were obtained from *H. trifolii* young cysts and females in the two years of field study. Irving & Kerry (1986) observed that there is considerable variation between isolates of *V. chlamydosporium* in their cultural characteristics, chlamydospore production and pathogenicity, therefore screening of isolates with biological control potential is particularly important. Screening of these isolates as potential biocontrol agents of *H. trifolii in vitro* was done before carrying out further tests under glasshouse and field conditions.

These results clearly showed that microwaving was strong enough to disinfect the existing *H. trifolii* cysts in soil for potential pathogenicity test of fungi that naturally infected *H. trifolii* in the soil but freezing was not. Wheat bran bead-based was the most suitable medium for growth of *V. chlamydosporium* isolate (Vc6) and clover plant growth.

In chapter five, monoxenic culture was developed for use in screening *V. chlamydosporium* isolates. Since most of the life cycle of *H. trifolii* is in the soil, there are many microorganisms on the surface or inside the cysts and eggs, or on the surface of J2. Sterilisation of the J2 surface is therefore essential before use in monoxenic culture. Use of fresh cysts and freshly hatched J2 reduced the chances of growth of contaminants.

Surface-sterilisation of *H. trifolii* (Hay 1994) was used as a basic technique. There were many difficulties in sterilising the surface of J2 as they were small and difficult to handle. Use of disinfectant is the main approach for surface-sterilisation of J2. In the first step, 5% Hibitane and antibiotics, antibiotics alone (Penicillin-G and Streptomycin sulphate BP, both at 0.1% w/v), or 0.3% NaOCl were used as disinfectants to sterilise the J2 surface using a syringe filter holder. Some J2 were lost during the sterilisation process and one hour after surface-sterilisation most J2 died. Adherence of J2 to the plastic filter holders could have accounted for the loss in numbers of nematodes. Those J2 that survived sterilization with 5% Hibitane in the filter holder developed into females on 0.5% Hoagland and Knop's agar but their eggs did not hatch because J2 did not fully develop inside the eggs. This could have been

caused by a residual chemical toxicity from the Hibitane or it could have been the result of physical damage from too high a pressure in the filter holder.

To overcome these problems, Eppendorf tubes were used instead of plastic filter holders and the percentage of Hibitane was reduced to 0.25 %. Antibiotics alone or NaOCl alone did not give control of the contaminants on the J2. Hibitane alone, from 0.25% to 4.0% removed the contaminants and did not affect J2 survival so they developed into females on Hoagland & Knop's agar. However, some J2 were still lost in the Eppendorf tubes, probably because of an electrostatic reaction within the plastic Eppendorf tubes. J2 were more active at low concentration of Hibitane and were removed accidentally during the process in Eppendorf tubes.

Centrifugation of the Eppendorf tubes or using Eppendorf tubes stored in water were tested for reduction of possible electrostatic charges but neither technique solved the entire J2 loss. Glassware, such as test tubes, therefore, were used instead of plasticware. Although use of glass test tubes reduced J2 loss compared to the plasticware, the tubes were too small to use for the J2 surface-sterilisation process and sorting under microscope. Also there were no gradation marks on the glass test tubes and it was difficult to obtain the correct concentration of Hibitane. Lower concentrations of Hibitane than 0.25% appeared insufficient to kill the contaminants of J2 as bacteria were found on the agar 2-3 days after surface sterilisation.

For a margin of safety, 0.5% Hibitane in solid watch glasses was used. Although there were no gradation marks on the watch glass, as it was easier to handle J2 during the process of surface-sterilisation under the microscope than in test tubes. There were also fewer J2 lost in the solid watch glass and at least 3 generations of females could be produced on Hoagland & Knop's agar under a mixture of lights on a 12 h / 12 h day / night regime, at 20°C.

Hay (1994) found that 20% of Hibitane for duration of up to 1 h using a filter holder was not successful in freeing J2 of bacteria on White's medium but a combination of 20% Hibitane and Penicillin + Streptomycin sulphate gave nematodes free of bacteria. In this study, concentration of Hibitane from 0.25% for 15 min in watch glass was enough to kill the contaminants of J2 on Hoagland & Knop's agar. The differences were use of a syringe filter holder (Hay 1994) and watch glass (current study) and different agar media; White's medium (Hay 1994) but Hoagland & Knop's agar (current study).

J2 were successfully multiplied monoxenically and 18 *V. chlamydosporium* isolates were screened for their pathogenicities to *H. trifolii* using surface-sterilised *H. trifolii* J2 on *T. repens* seedlings on Hoagland & Knop's agar or in sand in 1 ml pipette tips in Chapter six. Their parasitism on eggs of *H. trifolii* was also tested on 1.0% water agar supplemented with oxytetracycline (10 µg/ml). There were no significant differences in their pathogenicity to *H. trifolii* among these isolates in monoxenic culture. This raised the possibility that these isolates might be from a limited, possibly clonal, population or the methods used in the screening tests were not sufficiently sensitive to screen fungal activities against *H. trifolii.* Although there were no significant differences among the test isolates, considerable time can be saved by using appropriate *in vitro* screening procedures that eliminate many isolates which show insufficient activity to justify further testing (Kerry 1991).

Barron and Onions (1966) distinguished *Diheterospora* from *Verticillium* because of the presence of dictyochlamydospores. The recent molecular studies by Zare et al. (2000) and Sung et al. (2001) showed the phylogenetic distinctness of the parasites of nematode cysts and eggs and the justification of a separate genus *Pochonia* from *Diheterospora* (Zare et al. 2001). The circumscription of this genus *Pochonia* deviates considerably from the previous concept of *Diheterospora* and, therefore, it was changed to the simpler and nomenclaturally correct name, *Pochonia*, rather than conserving the name *Diheterospora* (Gams & Zare 2001). *Pochonia chlamydosporia* (*V. chlamydosporium*) is found to be rather heterogeneous in molecular analyses and in their ecological qualities (Arora et al. 1996, Kerry et al. 1986, 1993).

In chapter seven, PCR-based techniques were used to detect the variations among these isolates at a molecular level. ITS sequences of four isolates tested were identical with the sequence of *V. chlamydosporium* from the UK (Genbank accession number AJ292397) and showed that they were indeed *V. chlamydosporium*. RAPD-PCR was used in this study because of its simplicity and wide applicability. Several attempts were made to obtain optimum conditions by adjusting concentrations of the PCR mix, especially concentrations of $MgCl_2$ and DNA, annealing temperatures, PCR cycles, addition of Q solution, and 27 different RAPD primers to obtain reproducible bands.

Six of 27 RAPD primers gave reproducible bands on agarose gel. Among the isolates, Vc6 amplified with those six primers and produced different, consistent

reproducible bands. RAPD primer Gen 3-60-3 separated the 10 isolates of *V. chlamydosporium* into four groups. Therefore, RAPD-PCR would appear to be an efficient tool for detecting variation between the isolates and the results showed that isolates of *V. chlamydosporium* tested in this study varied at a molecular level. The present study was conducted on a small number of isolates that represent seasonal samples from one white clover/ryegrass pasture paddock in Palmerston North and were tested with a small number of RAPD primers. The entire range of intraspecific genetic variation of this fungal species is almost certainly much greater than found in the small study.

In conclusion, this work confirmed that there are two reproductive cycles of *H. trifolii* per year and that soil moisture levels affect clover plant growth and hence populations of *H. trifolii*. Possible causes of *H. trifolii* population declines were due to fungal parasitism on females and a longer dry period in 2000. Monitoring of microorganisms in the natural environment is useful for biological control studies. *V. chlamydosporium* Goddard (*P. chlamydosporia var. chlamydosporia* Zare et al.) isolate Vc6 is a promising, potential biocontrol agent of *H. trifolii*. Bran bead-based medium is suitable for growth of both clover plant and *V. chlamydosporium*. Second stage juveniles of *H. trifolii* can be produced in monoxenic for the purpose of nematological study. RAPD-PCR showed that there was variation among *V. chlamydosporium* isolates at a molecular level.

8.1 Future work

The importance of fungi in the decline of cereal cyst nematode populations in monocropped cereals and their involvement in the sustained suppression of nematode populations below the economic threshold has been clearly demonstrated by Kerry 1984, Kerry & Crump 1998). The ubiquitous distribution of many antagonists of nematodes and the increasing number of examples where naturally occurring antagonists are known to provide a degree of nematode control suggest that this approach deserves further consideration. Successful biological control agents are only obtained if their interaction with the pathogen or nematode target, the crop, the natural, resident microbiota and environment are understood (Kerry 1995). In the future, therefore, efforts need to be directed towards choosing organisms with characteristics

likely to favour survival in a hostile environment and towards developing techniques of inoculum preparation that can be applied in the field. The inoculum also needs to be cost-effective. Furthermore, testing for 1) a suitable shelf life of fungal inoculum, 2) effect on non.target organisms, 3) consistent activity of the fungi under a range of different conditions and locations on different host plants are necessary to develop a successful biological control agent. Molecular-based techniques (Section 7.6) should be considered in future studies of variability.

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APPENDIX I. NUMBERS OF *H. TRIFOLII* (CHAPTER 2)

Numbers of *H. trifolii* J2 / 100 ml soil, numbers of J2 / g of wet root weight, numbers of white females / 100 ml soil, numbers of new cysts / 100 ml soil, numbers of old cysts / 100 ml soil, white clover wet root weights / 100 ml soil and soil moistures recorded from fortnightly sampling in the study area, soil temperatures and rainfall from the meteorological station, AgResearch, Fitzherbert West, Palmerston North, New Zealand, over a 2-year period (March 1999- March 2001)

	Sampling date	J2 in soil	J2 in roots	Female	New cyst	Old cyst	Root weight	Soil moisture %	Soil temperature °C	Rainfall (mm)
1	12.3.99	85.1	49.4	2.2	0	228.2	0.26	NR	19.2	6.5
2	1.4.99	62.9	91.4	2.8	0	307.5	0.17	NR	18.4	0
3	19.4.99	74	54.8	20.2	43.3	612.2	0.36	20.84	10.1	0
4	3.5.99	18.5	29.7	4.4	18.9	463.1	0.16	14.24	12.6	0
5	17.5.99	207.2	24.7	30.9	72.3	730.6	0.51	26.2	13	0
6	31.5.99	125.8	27.3	3.1	23.5	512.3	0.17	27.57	14.2	1.1
7	14.6.99	25.9	6.7	3	18	376.5	0.19	26.43	11.2	17.2
8	28.6.99	29.6	451	2.6	19	430.9	0.13	28.1	4.8	0
9	12.7.99	118.4	136.7	9	53.7	338.4	0.16	27.46	6.1	0.9
10	26.7.99	70.7	140.8	7.8	29.2	245	0.22	30.6	9.2	9.2
11	16.8.99	1398.6	332.2	7.4	9.8	420.9	0.33	31.06	9.8	11.1
12	30.8.99	1036	125	4.3	34	242	0.21	27.27	7	0
13	13.9.99	1816.7	1049.3	3.3	13.7	418.1	0.12	25.83	10.8	1
14	27.9.99	455.1	1377.7	3.9	8.7	224.1	0.1	26.4	12.1	0.3
15	11.10.99	192.4	259.4	66.4	3.9	200	0.17	17.78	16	0
16	25.10.99	329.3	314.2	71.8	18	158.9	0.18	25.51	9.8	0
17	8.11.99	162.8	226.6	72.7	6.9	139.3	0.15	28.01	17.7	0
18	22.11.99	148	50.4	26.7	27.4	184.7	0.14	27.96	16.5	0
19	6.12.99	140.6	1.9	19.1	11.3	149.9	0.17	23.3	18.6	0
20	20.12.99	63	47.5	8	3	105.5	0.17	32.73	18.9	0
21	3.1.00	255.3	8.4	20	21.6	180	0.15	30.4	18	3
22	17.1.00	107.3	45.6	5.2	5.4	96.9	0.04	20.2	19.9	0.2
23	31.1.00	55.5	72.9	5.9	21.1	171.1	0.2	32	17.7	2.3
24	14.2.00	162.8	24.7	14.8	11.65	121.53	0.11	13.84	19.5	1.1
25	28.2.00	96.2	2.4	19.1	7	128.75	0.2	9.63	18.3	0
26	13.3.00	632.7	350	30.5	20.2	109.2	0.27	17.3	17.4	10.9
27	27.3.00	81.4	211	1.1	7.4	98.6	0.19	17.3	14.5	0
28	17.4.00	118.4	86.8	13.7	8	254.7	0.13	26.27	14	0
29	1.5.00	88.8	725.5	8.5	8.1	227.9	0.19	20.5	12.8	0
30	15.5.00	74	32.3	18.5	28.1	158.8	0.24	31.1	11.7	4.5
31	29.5.00	162.8	109.4	9.8	17.5	147.8	0.23	32	6.9	3.2
32	12.6.00	285	45.4	5.6	23	210.4	0.22	34.2	8.3	2.5
33	26.6.00	203.8	53	3.4	13	136.3	0.08	27.8	8.3	0
34	17.7.00	370.1	25.6	0.9	24.8	139.1	0.15	27.2	8	0
35	31.7.00	1095.2	177.7	0.6	8.4	159.7	0.17	34.5	8	0
36	14.8.00	774.8	214.6	3.7	24.2	195.8	0.2	32.8	6.2	0
37	28.8.00	1024.9	312.1	2.4	10.4	163	0.15	32.3	10.4	0
38	11.9.00	477.3	197	0	6.3	177.4	0.11	30.2	10.9	0
39	25.9.00	469.9	43	0	1.9	125.8	0.1	25.3	10.6	11.3
40	9.10.00	325.6	24.1	4.1	3.3	163.7	0.18	34.2	13.4	15.6
41	23.10.00	225.7	241.1	12.6	5.4	184.8	0.13	23.5	14.8	0
42	6.11.00	125.8	273.4	38.3	1.65	151.6	0.15	23.5	14.1	0.8
43	20.11.00	74	148.1	16	4.6	115.6	0.14	22.3	11.7	0.5
44	4.12.00	370	139.8	44.2	10.7	150.6	0.21	26.9	13.4	0
45	18.12.00	122.1	163.3	13	2	176.1	0.11	12.6	21.3	0
46	1.1.01	59.2	96.7	12.6	11.1	156.4	0.06	27.4	13.8	1
47	15.1.01	122.1	79.1	16.5	12.6	145.4	0.17	10.5	15.2	0
48	29.1.01	62.9	1.1	22.2	10.4	80.7	0.12	10.8	17.5	0
49	12.2.01	114.7	38.9	3	12.6	98.3	0.14	28	19	3.1
50	26.2.01	151.7	83.7	11.5	20.9	126	0.13	10.5	18.2	0
51	12.3.01	51.8	22	38.9	12.2	107	0.12	5.7	13.9	0

APPENDIX II. SYSTEMATIC POSITIONS OF NEMATODES (CHAPTER 2)

Systematic positions of nematodes recorded from fortnightly sampling in the study area over a 2-year period (March 1999 – March 2001)

	Systematic position of nematodes	Genus
1	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Aphelenchina Superfamily: Aphelenchoidea Family: Aphelenchidae Subfamily: Aphelenchinae	*Aphelenchus* spp.
2	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Hoplolaimidae Subfamily: Rotylenchoidinae	*Helicotylenchus* spp.
3	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Heteroderidae Subfamily: Heteroderinae	*Heterodera trifolii*
4	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Meloidogynidae Subfamily: Meloidogyninae	*Meloidogyne* spp.
5	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Criconematoidea Family: Tylenchulidae Subfamily: Paratylenchinae	*Paratylenchus* spp.
6	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Pratylenchidae Subfamily: Pratylenchinae	*Pratylenchus* spp.
7	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Tylenchidae Subfamily: Tylenchinae	*Tylenchus* spp.
8	Class: Secernentea Subclass: Diplogasteria Order: Tylenchida Suborder: Tylenchina Superfamily: Tylenchoidea Family: Tylenchidae Subfamily: Tylenchinae	*Cephalenchus* spp.
9	Class: Adenophorea Subclass: Enoplia Order: Dorylaimida	
10	Class: Secernentea Subclass: Rhabditia Order: Rhabditida	

APPENDIX III. BUFFERS, SOLUTIONS AND MEDIA (CHAPTER 7)

Gel loading buffer (GLB)

20% Sucrose	4.0 g
5 mM EDTA	0.2 ml of 0.5 M
1% SDS	2.0 ml of 10%
0.2% Bromophenol blue	40.0 mg
Xylene cyanol	40.0 mg
Distilled water	20.0 ml

Preparation of λ DNA standards (GIBCOBRL, LIFE TECHNOLOGIES)

Stock λ DNA is 500 μL/ml.

To get 50 ng/5 μL, dilute 20 μL stock with 180 μL TE buffer (solution A).

To get 25 ng/5 μL, dilute 5 μL solution A with 5 μL TE buffer (solution B).

To get 10 ng/5 μL, dilute 5 μL solution A with 20 μL TE buffer (solution C).

For use (to make 50 μL)

10 μL solution A + 10 μL GLB + 30 μL TE buffer.

10 μL solution B + 10 μL GLB + 30 μL TE buffer.

10 μL solution C + 10 μL GLB + 30 μL TE buffer.

Preparing of buffer for fluorometer

10x TNE pH 7.4

Tris base	12.1 g/L
EDTA.Na_2	3.7 g/L
NaCl	58.4 g/L

Electrophoresis buffer 10 x TAE buffer

Tris	48.4 g
EDTA (NA salt)	7.4 g
Glacial acetic acid	11.4 ml
Distilled water	1 L

Adjust pH to 8.5 with NaOH

10 x TBE buffer

Tris (0.89 M)	108.0 g
EDTA (Na salt) (0.01 M)	9.3 g
Boric Acid (0.89 M)	55.0 g
Distilled water	1 L

Adjust pH to 8.2 with 1N HCl

Lysis Buffer (Al-Samarrai & Schmid 2000), (for total 50.0 ml)

3.0 M Sodium acetate	330.0 μL
0.5 M EDTA	100.0 μL
1 M Tris acetate	50.0 μL
10% SDS pH 7.8	5.0 ml
Distilled water	44.52 ml

Ethidium Bromide solution (1 μg/ml)

10 mg/ml ethidium bromide.. 1.0 μL

Distilled water .10.0 ml

RNase A (DNase free) 10 mg/ml

10.0 mM Tris-HCl (pH 7.5)

15.0 mM NaCl

Hoescht's fluorescent dye solution

This solution contained 1 x TNE and a final concentration of 0.1 μg/ml Hoescht 33258 dye. This solution was not sterilised, so it was necessary for this solution to be prepared fresh when needed.

Corn Meal Agar (CMA)

Corn meal agar (Difco) 17.0 g/L distilled water

Potato Dextrose Agar (PDA)

PDA (Difco) 39.0 g/L distilled water