STUDIES ON THE EPIDEMIOLOGY AND OTHER ASPECTS OF *CHALARA ELEGANS*

BROWN P. J.

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Studies on the Epidemiology and Other Aspects of *Chalara elegans*.

Peter John Brown

Master of Applied Science (Biology)

Central Queensland University

School of Biological & Environmental Sciences

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ABSTRACT

Chalara elegans is a fungal pathogen of a wide range of plant hosts including several of economic importance such as citrus, tomato, legumes and lettuce. The pathogen elicits a range of symptoms, the overall syndrome commonly being referred to as black root rot. Although recognised as a common member of the soil mycobiota, it has been reported that more virulent strains have been introduced from overseas in sphagnum peat. This material is frequently used in seedling nurseries to raise seedlings prior to transplantation to field situations for crop maturation.

Studies were conducted in response to an outbreak of black root rot of lettuce in the Bundaberg region. They examined the method used by the pathogen to invade and infect lettuce. Invasion of the root and hypocotyl was facilitated by an appressorium and further growth and sporulation by this hemi-biotrophic pathogen only occurred while the host was alive. Mechanisms of resistance were not determined. Studies explored the influence of relative humidity, desiccation, and phialoconidial density on spore germination. Some factors influencing phialoconidial longevity were examined.

C. elegans was detected during screening of twenty samples from unopened New Zealand peat packages, the positive result confirming a route of transmission of the pathogen to lettuce production areas. Common weed and crop plants were inoculated with the Bundaberg isolate of *C. elegans* in order to gauge their susceptibility to the fungus. Chemical agents (chlorine, benzalkonium Cl + glutaraldehyde, benomyl) were assessed for their effectiveness in disinfecting polystyrene trays contaminated with *C. elegans*. Benomyl was most uniformly effective.

The change in inoculum density of *C. elegans* in the rhizosphere of successive plant groups, including lettuce, was simulated in a container trial during an eighteen month period. By the fourth crop cycle, soil propagule numbers were

highest in containers of susceptible lettuce. Propagule numbers were lower in plots with partially resistant cultivars, and still lower in more resistant weed plots, and fallow plots.

The trials demonstrated that *C. elegans* is transmitted to lettuce growing areas with peat in growing media. Many plant genera are susceptible to this pathogen. It is associated with many other plant genera which function as resistant hosts. Sustainable control of black root rot in commercial lettuce production may be difficult to achieve by soil cultivation, drying, fumigation, saprophytic competition, or crop rotation. More effective control will result from sterilisation of seedling growing media, baiting peat-based growing mixtures, quarantine of field growing areas from plants of unknown disease status, hygienic cultural practices, and growing resistant lettuce cultivars.

TABLE OF CONTENTS

DISEASE CYCLE

LIST OF ILLUSTRATIONS

LIST OF TABLES

ACKNOWLEDGEMENTS

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1.   Lettuce Production in Queensland

In Queensland, intensive production of 34 287 t of lettuce (*Lactuca sativa* L.) to the value of $24.8m (Australian Bureau of Statistics, *personal communication*) is concentrated in the southeast corner of the state. The principal areas are the Lockyer Valley, Toowoomba, the eastern Darling Downs, and the Granite Belt. Lettuce may be grown successfully on widely differing soils, and smaller levels of production in rotation with other smallcrops occur outside the areas listed above, if temperature ranges are appropriate.

The crop is essentially a cool season one, grown for its leaves. It is highly sensitive to changes in temperature. Lettuce grows best below 24°C and produce is nearly always available from some district in Queensland throughout the twelve month production period. The distribution of production is dependant not only on which groups of cultivars are grown, each with its specific climatic requirements, but also on the flexibility which variations in temperature due to differences in altitude and latitude provide. This flexibility, and overall production, have been augmented by the use of new cultivars which are resistant to leaf and root diseases. There is also a tendency for significant growers in one locality to move their operations to another area for part of the year in an attempt to increase their market share (Heisswolf *et al.*, 1997).

All of the major lettuce types are represented to varying degrees in Queensland production areas: crisphead types (e.g. the cool weather cultivar Yatesdale), butterhead types, cos types, and non-heading or fancy cultivars. Because of their thin leaves, lettuce wilts readily and must be supplied with regular adequate amounts of water by drip or sprinkler irrigation. Crop plants originate from seedlings grown in soil-less mixes in nurseries. In some cases, the nurseries belong to the farming enterprise, but usually the particular blend of cultivars is pre-ordered, grown to transplanting size by specialist seedling growers and then delivered to farms immediately prior to planting.

Plants are usually transplanted mechanically to the field at 25 to 30 cm spacings (Figures 1-3). This efficient, high-speed method obviates the transplant shock which occurs with bare-rooted seedlings. It also ensures rapid plant establishment, a uniform maturing of the crop, and a harvest exactly when scheduled. After five to eight weeks, depending on the season, harvest of field-grown plants is performed by cutting through the hypocotyl with a sharp knife, leaving the roots in the ground. After packing, produce is fast-cooled to remove field heat and despatched to market. After harvest has occurred in the field, the crop residues - essentially the roots and hypocotyl - are mechanically incorporated into the soil.

Figure 1 and 2. Mechanical transplanting of lettuce.

Figure 3. Transplanted lettuce ready for early summer production.

2. Review of Literature

2.1 The Fungus

2.1.1 Disease Initiation

The imperfect fungus *Chalara elegans* Nag Raj and Kendrick (syn. *Thielaviopsis basicola* (Berk. and Br.) Ferraris) is an unspecialised pathogen which causes black root rot on susceptible host plants in both tropical and temperate regions (Yarwood, 1974). Black root rot is a major disease of tobacco (*Nicotiana tabacum* L.), cotton (*Gossypium barbadense* L., *G. arboreum* L.), citrus (*Citrus* spp.), and legumes as well as many other genera (see Table 2). The ability of this fungus to survive and to tolerate unfavourable out-of-season periods makes it a formidable threat to industries based on susceptible crops.

Although a teleomorph stage is yet unknown for the fungus, different conidial spore types are produced. *C. elegans* produces thick-walled, pigmented propagules (chlamydospores) and smaller hyaline phialoconidia. They are often closely associated and often occur on the same hypha or phialophore (Nag Raj and Kendrick, 1975).

The infection process is initiated when plant exudates stimulate microbial activity in the rhizosphere and induce the growth of fungal hyphae toward nearby roots. Possibly too, fungal propagules which are randomly placed in the soil may be fortuitously reached by the host plant root (Foster, 1985). Penetration of dead root hairs has been observed in tobacco (Hood and Shew, 1997). Disease may also be initiated by air-borne inoculum, presumably phialoconidia, as was demonstrated in a greenhouse situation by Graham and Timmer (1991). Cool soil temperatures (16-18°C) enhance disease severity on cotton and tobacco (Holtz and Weinhold, 1994), and oxygen deficits predispose plant roots to infections (Schneider and Musgrave, 1992). Under favourable moisture and temperature regimes i.e. high soil moisture and low temperatures (Chittaranjan and Punja, 1993), and with cotton, especially in young seedlings

(Holtz and Weinhold, 1994), the spores germinate and germ tubes enter host root cells *via* root hairs (Christou, 1962; Prinsloo *et al.*, 1993).

Hyphae of *C. elegans* colonise the cortex and, commonly, the vascular tissue of the roots and hypocotyls. Infected cotton and other plants may recover to varying degrees after the initial infection because the pericycle remains unaffected, permitting cortical regeneration and secondary root growth (Mauk and Hine, 1988). Reports vary as to whether entry to host cells occurs through direct hyphal penetration (Christou, 1962), or whether an appressorium-like structure exists (Mauk and Hine, 1988; Prinsloo *et al.*, 1992). Mauk and Hine (1988) reported that both spore forms produce an appressorium in order to invade cotton. The presence or absence of appressoria and other adjuncts to infection may depend on the host genus and the infecting race of fungus. The latter has been described by Stover (1950).

Having penetrated the root epidermis, host cell walls are punctured by a long infection peg and the cytoplasm is filled by small constricted hyphae. These then spread to adjacent cells and intercellular spaces (Christou, 1962). A scanning electron microscope study of infected chicory (*Cichorium intybus* L.) roots found no evidence of invasion of the vascular cylinder (Prinsloo *et al.*, 1992; Lindsey, 1981) but a similar study of cotton detected hyphae and chlamydospores in the primary and secondary xylem and phloem of cotton under conditions of high inoculum density with cool temperatures (16-20°C) which are unfavourable for growth of the host (Mauk and Hine, 1988). The life cycle is quickly completed when reproductive hyphae develop inter- and intra-cellularly and produce numerous intracellular chlamydospores. Mauk and Hine (1988) observed that invasion of further cells only occurs after those already invaded are filled with hyphae.

Chlamydospores are produced in all tissues and also on the host surface together with phialophores (*sensu*, Nag Raj and Kendrick, 1975) which extrude abundant

phialoconidia. Phialoconidia are less commonly observed within host tissues. They have been reported as developing on root lesions when incubated under humid conditions (O'Brien and Davis, 1994). It is unclear whether chlamydospores germinate immediately within the host tissue to commence a new disease cycle, or whether they await tissue decomposition and then remain *in situ* for a subsequent host. Of the reports reviewed, only Mauk and Hine (1988) and Oyarzun *et al.* (1997) documented an active primary role for chlamydospores in disease initiation additional to the commonly accepted one of long term survival of the fungus.

Direct symptoms of black root rot are limited to the root. Generally, small, black to brown necrotic lesions result. Infected lettuce roots develop lesions which are small, discrete and brown in colour. These later turn black and coalesce to include all or substantial areas of the root. Small roots are often destroyed (O'Brien and Davis, 1994) and in severe cases, the whole root system is destroyed. In cotton, the syndrome consists of a swollen taproot, internal purplish black rot of the vascular tissue, and external black rot of the central cylinder of the root (Holtz and Weinhold, 1994). Usually, the infected taproot is smaller in diameter than those of the healthy seedlings, and the fungus seldom penetrates the endodermis, which remains white. Little evidence of infection remains on cotton plants after several weeks, when periderm tissue has been produced to replace the infected epidermal and cortical tissue (Lindsey, 1981). In citrus, rotting at the root tips causes sloughing of the cortex, exposing and desiccating the vascular tissue. Secondary symptoms such as chlorosis in leaves may result (Graham and Timmer, 1991). In root crops, such as highly susceptible chicory and carrot (*Daucus carota* L.), pre-harvest or postharvest root rot results in the unmarketability of affected roots.

2.1.2 Nomenclatural Aspects

Prominent among the genus *Chalara*, as interpreted by Nag Raj and Kendrick (1975), is a group of economically important fungi. The group includes

Thielaviopsis paradoxa, associated with the pineapple disease of sugarcane (*Saccharum officinarum* L.), soft rot of pineapple (*Ananas comosus (L.) Merr.*), blackhead of bananas (*Musa* spp.), and stem bleeding and premature fruit fall in palms. Prominent too are *Chalara quercina,* the conidial state of the oak-wilt fungus, *Ceratocystis fagacearum,* and *Chalaropsis thielavioides,* the aetiological agent of root rot of chinese elms (*Ulmus parviflora*) and roses (*Rosa* spp.).

The pleomorphic nature of *C. elegans* has been the source of nomenclatural problems. The species was originally described, and at that time named *Torula basicola* by Berkeley and Broome (1850, in Nag Raj and Kendrick, 1975). Only one spore type (chlamydospore) was included in that classification. Later, Ferraris (1910, in Shew and Meyer, 1992) described the phialoconidia of *Chalara* which are also produced by the related genera *Chalaropsis* and *Thielaviopsis,* and renamed the genus from *Torula* to *Thielaviopsis* on the basis of that spore type. Because chlamydospores were lacking in *Chalara,* subsequent authors continued to exclude *T. basicola* which produces both spore forms from that genus even though, as Nag Raj and Kendrick (1975) pointed out, the loss of the phialidic state would transfer either one of the other two genera into *Chalara* by default. Nag Raj and Kendrick (1975) elected to transfer *T. basicola* into *Chalara* on the basis that the name should be based on the state that is "most frequent and constant, most conspicuous and most readily identifiable". They concluded that the wider occurrence of a highly characteristic phialidic state should be the basis of classification in this case, without necessarily excluding the provision of a generic name for the chlamydosporic state. Consequently, Shew and Meyer (1992) are among those who use *T. basicola* as the name of the chlamydospore stage.

Variation also exists in the nomenclature of the spore types which are both readily produced. Many more phialoconidia are produced compared with chlamydospores. Both spore types form readily in many types of artificial cultures (Shew and Meyer, 1992). The optimum temperature range for growth

on media was 23-26°C and growth was negligible below 12°C and above 32°C (O'Brien and Davis, 1994). The hyaline, thin-walled phialoconidia, which generally develop first, are produced in large numbers from phialophores on the surface of diseased tissues (Christou, 1962). About four to five days after inoculation of a suitable medium, such as Malt Extract Agar or half-strength Potato Dextrose Agar, followed by incubation at 28°C in darkness, the colony has a moist, white or greyish appearance which later turns brownish black as chlamydospores are formed (Yarwood, 1946; Nag Raj and Kendrick, 1975). Phialoconidia are much more numerous than the chlamydospores and are rod-shaped, thin-walled, mucoid and hyaline, i.e. they are amerohyaloconidia produced profusely in a mucoid matrix.

The role of phialoconidia as infectious propagules in naturally infected soils is largely unknown (Tabachnik et al., 1979). Tsao and Bricker (1966) and Lechappe (1986, in Oyarzun, 1997) described their ability to change into a round "secondary chlamydospore" under adverse conditions. However, the conversion phenomenon has only been observed under laboratory conditions and may not occur in nature (Chittaranjan and Punja, 1994). Like mycelial fragments, they germinate rapidly, can initiate infections within 48 to 70 hours, and may permit short-term survival of the organism for several months (Lindsey, 1981; Chittaranjan and Punja, 1993; 1994) because the thin-walled phialoconidia and the mycelium are much more susceptible to lysis by microbial action than are chlamydospores (Tsao and Bricker, 1966). However, the latter authors were dubious about the role of phialoconidia as primary inoculum in natural soils or even in artificial cultures. Their research suggested that inoculating soils could be more reliably performed using chlamydospores alone.

In the case of *Thielaviopsis* and *Chalaropsis*, differing terms such as macroconidia, A-conidia, exospores, and aleuriospores have been used to describe the chlamydospores. Nag Raj and Kendrick (1975) suggested that the

term "chlamydospore" is the most appropriate. They are produced in rectilinear series of five to seven, with the terminal chlamydospore being conoid and the remainder short-cylindrical, dark brown or amber, with a thin smooth outer wall and a secondary inner wall one 1-2 μm thick.

Chlamydospores are slower to germinate than phialoconidia and are held to represent the inter-host survival mechanism of the organism (Chittaranjan and Punja, 1993). In the absence of a susceptible host, the fungus must survive adverse environmental conditions when they occur and such long-term survival is the role usually ascribed to chlamydospores. Tsao and Bricker (1966) demonstrated that, at least in selective agar media from soil dilution plates, chlamydospores were the origin of nearly all colonies, and in none of their investigations did colonies develop from phialoconidia or from mycelial fragments. Although earlier work (Tsao and Bricker, 1966) discounted the need for other than a carbon source to enable chlamydospores to germinate, Shew and Meyer (1992) recommended an exogenous source of thiamine for the production of chlamydospores on artificial cultures, especially those ones rich in carbon and nitrogen.

2.1.3 Methods of Detection

Bioassays   To rapidly and accurately isolate *C. elegans* from soils where no macroscopic evidence of its presence exists, Yarwood (1946) used the carrot bait technique. Baits consisting of 5 mm discs were cut from fresh carrots which are highly sensitive to the presence of *C. elegans*. Soil to be tested was spread thickly (and later, as the technique was refined, thinly) over the surface of the discs which were placed in petri dishes. After two to four days, the discs were washed to free them of soil and then incubated in moist chambers. If *C. elegans* was present, phialoconidia appeared in about six days, followed by chlamydospores. Later work involved drier slices of carrot (Yarwood, 1981; Graham and Timmer, 1991; O'Brien and Davis, 1994) and it was successful if

just enough water was added to keep the discs from drying out. Although pear (*Pyrus communis* L.) fruit was superior to carrot in supporting sporulating mycelia, carrot was a better general purpose medium (Yarwood, 1981). When compared to two direct isolation methods, the carrot disc technique was accurate and reliable and could be used for root samples as well as for soil (Yarwood, 1981). O'Brien and Davis (1994) compared the recovery rate of the carrot disc method with isolating infected root pieces directly on Potato Dextrose Agar and in 2 % Water Agar. The recovery rate of the direct isolation methods was lower than the carrot disc method. Oyarzun *et al.* (1997) found baiting with pea seedlings (*Pisum sativum* L. s.lat.), followed by microscopic examination as a sensitive test for the presence of *C. elegans* in soils. The use of carrot discs did not detect the pathogen at lower inoculum concentrations. They concluded that, in uninoculated soils, using bioassays, it may be impossible to quantify the inoculum density of certain fungal pathogens, including *C. elegans*, because individual propagules may be very isolated.

Tsao and Canetta (1964) modified the carrot disc method in two ways. The first was by hollowing out 10 mm carrot discs to enable them to hold 1 mL of soil suspension in distilled water and streptomycin. Coupled with a reference table and the appropriate dilution factor, this extremely time-consuming method gave a reasonably accurate estimate of the most probable number of phialoconidia and chlamydospores of *C. elegans* per volume of soil. Secondly, they estimated the area of sporulating fungus which developed on ten carrot discs of standard size which had been immersed in a soil sample for several days, washed, and then incubated at approximately 25°C. It was then found to be possible to extrapolate the soil inoculum concentration from the area of disc covered with mycelium, the latter being a linear function of the logarithm of the former. Although it could be performed more quickly, it did not produce data in terms of concentration figures, but merely degrees of colonisation.

Artificial Selective Media   While some workers have used water agar as the means of isolating fungi from contaminated material, it has generally proven unsatisfactory because fungi growing on water agar do not so readily produce identifiable fruiting structures, for which a threshold concentration of nutrients is required (Garrett, 1981). Artificial selective or semi-selective media contain optimum levels of nutrients and can be used to detect and accurately quantify micro-organisms in soil samples and in growth media, and to determine a relationship between a pathogen population and evidence of the disease. For *C. elegans,* the medium formulation is also based on work which demonstrated that propagules such as chlamydospores and phialoconidia were insensitive to antifungal agents such as nystatin and pentachloronitrobenzene (Papavivas, 1964; Tsao and Bricker, 1966). Inoculum suspensions, undiluted or diluted, were commonly incorporated with molten media augmented with carrot tissue, or spread on the surface of similar solidified media. Table 1 outlines the efficacy of various agar-based artificial media for recovery of *C. elegans*. Results with selective media have been reported to be unreliable at times (Papavivas, 1964).

Table 1. Selective media which have been tested for isolation of *C. elegans*. Constituents have been listed for one litre quantities.

AUTHOR/S	BASIC CONSTITUENTS	COMMENTS
Graham and Timmer (1991)	TB-CEN: carrot juice (from 100 g of tissue), etridiazole, nystatin, streptomycin sulphate, chorotetracycline, $CaCO_3$, agar 15 g	Capable of detecting low propagule numbers where the carrot disc assay could not
Chittaranjan and Punja (1993)	VDYA-PCNB: V8 juice 200 mL, yeast extract 2 g, glucose 2 g, pentachloronitrobenzene (PCNB) 0.5 g (a.i.) , oxgall 1 g, nystatin 30 mg, streptomycin sulphate 100 mg, chlorotetracycline Hcl 2 mg, $CaCO_3$ 1g, agar 20 g, pH 5.2	Most rapid growth of colonies; larger colonies; 83.6% recovery of spores. Poor recovery from soil.
	TBM-C: carrot juice (from 200 g of autoclaved roots in 1 L of water, 970 mL, yeast extract 2 g, PCNB 0.75 g (a.i.), oxgall 1 g, nystatin 50 mg, chloramphenicol 250 mg, penicillin G 60 mg, agar 20 g, pH 5.2	92.7% recovery of spores; smaller colonies; also rapid growth.
	TB-CEN: (from 100 g of blended and filtered tissue in 100 mL of water (unautoclaved) - 80 mL, etridiazole 400 mg (added as 1.2 g of Terrazole 35 WP ®), nystatin 250 000 units, streptomycin sulphate 500 mg, chlorotetracycline HCl 30 mg, $CaCO_3$ 1 g, agar 15 g, (pH 5.3)	Slowest growth; 76.1% recovery; smallest colonies; bacterial contamination a problem.
Holtz and Weinhold (1994)	TB-CENP: as above for TB-CEN, but with 200 mL of 33% fresh carrot extract (100 g of fresh carrots with 200 mL of distilled water), agar 17 g, and then combined with PCNB 1 g	Proved highly selective once PCNB added, incubation temperature lowered to 16°C, and incubation period lengthened.

2.1.4 Disease Control by Chemicals

Fumigants such as methyl bromide give complete control of the organism in artificial seedling mixes although increasing restrictions are being placed on the use of this chemical. Australia is expected to follow the lead of other developed countries such as The Netherlands, which no longer uses methyl bromide, and other northern European countries which have targets for phasing out its use soon after the year 2000 (Stirling et al., 1995). In culture plate tests, C. elegans was inhibited by benomyl at 0.01 μg.mL^{-1} and there was complete inhibition on media amended with benomyl 0.1 μg.mL^{-1} (O'Brien and Davis, 1994). Propiconazole completely reduced the growth of C. elegans when applied to culture plates at 1 μg.mL^{-1}. Drenches of both fungicides after planting gave a high level of control (O'Brien and Davis, 1994). Other references cited by those workers indicate that some chemicals more effective than benomyl may have potential for control of black root rot. Copes and Hendrix (1997b) demonstrated that while bromine and quaternary ammonium compounds were ineffective, captan (active ingredient captan 43.7%) significantly reduced viability of propagules of C. elegans after the chemical was sprayed on greenhouse surfaces of various textures. In the same study, sodium hypochlorite (5.25%, diluted 1:9) effectively disinfected galvanised metal surfaces of C. elegans, but a stronger solution as well as physical scrubbing was needed to significantly reduce propagule numbers on wood (a common component of bench materials) and polypropylene (greenhouse fabric cover).

2.2 The Host,

2.2.1 Host Range

C. elegans inhabits both cultivated and non-cultivated soils, surviving as a plant pathogen, a saprophyte, and a hemibiotroph in the rhizosphere of roots of a diverse range of host and non-host plant species, including weeds and cultivated plants (Stover, 1950; Yarwood, 1981; Shew and Meyer, 1992; Chittaranjan and Punja, 1993; Hood and Shew, 1997). Peat moss from Canada (Graham and Timmer, 1991) and New Zealand is a common source of *C. elegans* (R. O'Brien, *personal communication)*, while a related species, *C. thielavioides*, has also been found in two brands of Canadian peat (R. O'Brien, *personal communication)*.

Many susceptible host genera are found within the families Fabaceae and Malvaceae (Table 2). Particularly high populations of propagules are produced on leguminous crops (Shew, 1991). Otani (1967, cited in Shew and Meyer, 1992) found that of 300 species in 55 families tested for susceptibility to *C. elegans*, 137 were susceptible. The root rot severity varied quantitatively and qualitatively between these susceptible host genera, whereby degrees of severity are commonly recorded (e.g. Lucas, 1975, cited in Shew and Meyer, 1992).

In Queensland, the fungus was first recorded in 1930 as a pathogen of sweet pea *(Lathyrus odoratus* L.) (Simmonds, 1966 in O'Brien and Davis, 1994). Although unrecorded as a host of the black root pathogen in the United States, lettuce was recognised as such under field conditions during 1990-1992 in south-eastern Queensland (R. O'Brien, *personal communication)*. Black root rot of lettuce was found in crops in the eastern Darling Downs, Granite Belt, Brisbane metropolitan region and Bundaberg but not in the Lockyer Valley. In addition, the organism caused problems in hydroponically grown crops at Gatton and Mareeba, being introduced as a seedling in peat-based media (R. O'Brien, *personal communication)*. In all the above cases, severe disease and

production loss in the field resulted from the introduction of seedlings grown in peat-based media, as the organism is occasionally present in imported peat bales and is introduced through them (O'Brien and Davis, 1994). Marked differences in disease susceptibility were evident across a range of eleven lettuce cultivars inoculated with the organism (O'Brien and Davis, 1994). Three (Classic, Yatesdale and NKX030) were susceptible while others (Monaro, Kirralee, NKX029, and Centenary) were resistant.

Table 2. Economic hosts of C. *elegans*

Host	Disease Name	Source
Bean (*Phaseolus vulgaris* L.)	Black root rot	Christou, 1962
Chicory (*Cichorium intybus* L.)	Black root rot	Prinsloo *et al.*, 1993
Citrus spp.	Black root rot	Graham and Timmer, 1991
Daucus carota L.	Black root rot	Nag Raj and Kendrick, 1975; Yarwood, 1946; 1981
Cotton species including (*Gossypium arboreum* L., G. *barbadense* L. G. *hirsutum* L.)	Black root rot	Tabachnik *et al.*, 1979
Eucalyptus spp.		Nag Raj and Kendrick, 1975; Yarwood, 1946; 1981
Lettuce (*Lactuca sativa* L.)	Black root rot	O'Brien and Davis, 1994
New Zealand Blue Lupin (*Lupinus angustifolius* L.)	Black root rot	Nag Raj and Kendrick, 1975; Yarwood, 1946; 1981
Onion (*Allium* spp.)	Black root rot	Candole and Rothrock (1997)
Pea (*Pisum sativum* L. s.lat.)	Black root rot	Oyarzun *et al.*, 1997
Peanut (*Arachis hypogea* L.)	Black pod rot	Jones, 1991
Soybean (*Glycine max* (L.) Merr.)	*Thielaviopsis* root rot	Tabachnik *et al.*, 1979
Sweet potato (*Ipomoea batatas* (L.) Lam.)		
Tobacco (*Nicotiana tabacum* L.)	Black root rot	Shew and Shoemaker, 1993
Hairy vetch (*Vicia villosa* Roth)		Candole and Rothrock (1997)

C. *elegans* is not pathogenic to members of the family Poaceae and no records have been viewed where C. *elegans* has been found in association with

members of that family. In this thesis, such a lack of any relationship will be defined as non-host resistance. On the other hand, the fungus may also be associated with the roots of various plant species in the absence of any visible symptoms (Chittaranjan and Punja, 1994). Such genera, which attract and afford physical protection to propagules of *C. elegans*, will be described as resistant hosts here.

C. elegans is geographically widely-distributed. Yarwood (1981) tested 500 multiple collections of soil, roots, and leaves from numerous, mainly temperate climate locations, and found the organism moderately abundant in each location, regardless of geographic differences. Table 3 indicates the range of host genera, categorised according to their susceptibility or otherwise to the black root rot organism.

Table 3. Non-hosts, resistant or susceptible hosts of *C. elegans* (Nag Raj and Kendrick, 1975; Yarwood, 1946; 1981).

Non-host Genera	Resistant Hosts	Susceptible Hosts	
Acer macrophyllum	Begonia sp.	Aeschylus californica	Lupinus angustifolius
Avena fatua	Betula sp.	Arachis hypogaea	Lycopersicon spp.
Baccharis pilularis	Humulus sp.	Cichorium intybus	Malus sylvestris
Cirsium arvense	Ilex sp.	Citrus spp.	Medicago sativa
Eucalyptus sp.	Prunus	Crotalaria juncea	Nicotiana tabacum
Glycine max	laurocerrasus	Daucus carota	Phaseolus vulgaris
Pinus sp.	Prunus serrulata	Geranium dissectum	Pisum sp.
Quercus sp.	Rosa sp.	Gloxinia sp.	Primula sp.
Silybum mariana	Rubus idaeus	Gossypium hirsutum	Prunus avium
	Sequoia sp.	Hypochoeris radicata	Pyrus communis
	Vinca sp.	Ipomoea batatas	Ribes sp.

2.2.2 Qualitative Factors Affecting the Survival and Growth of *C. elegans*

Tests of roots, soil and leaves for presence of the black root rot organism, conducted over a 33 year period by Yarwood (1981), indicated that host associations and growth environments may have strategic influence on the presence or absence of *C. elegans* in soil. In various soil surveys, Yarwood (1981) investigated a number of environmental factors (apart from notable host plant associations) which may have a bearing on the survival of the organism. He found that *C. elegans* was generally never recovered at a soil depth more than sixteen centimetres, although the organism has been recovered at a depth of 54 cm (Klotz, 1965, in Yarwood, 1981). Tsao and Bricker (1966) also suggested a limited activity of the fungus in non-rhizosphere regions. The results of studies of tilled and untilled soils were significant in some situations, but inconclusive in others. Yarwood (1974) proposed that tillage possibly does reduce *C. elegans* incidence. In some cases, soil assays taken from an open field proved negative for the organism. In contrast, the organism was recovered from clay pots in a nursery situation. *C. elegans* was not recovered from natural or artificially wooded areas containing certain non-host genera, but was found in association with some of those genera in a nursery situation. Using selective media, more propagules of *C. elegans* were recovered from soils in the cooler coastal areas rather than the warmer inland parts of California (Yarwood, 1974).

Biological Factors   In the absence of a susceptible host, the fungus in the form of chlamydospores, phialoconidia, or hyphal fragments must survive less than ideal conditions such as the gradual drying of the soil matrix in the absence of plants and the loss of a readily accessible carbohydrate substrate after the decay of originally living hosts.

Oyarzun *et al.* (1997) noted that the concept of receptive soils - a quantifiable characteristic ranging from disease conduciveness to suppressiveness - has been

reported with relation to *C. elegans* and tobacco and bean, and demonstrated it with pea. Physical as well as biological factors were important and most soils were held to be relatively suppressive to *C. elegans*. This pathogen had greater sensitivity to biological factors in soil than *Fusarium solani* f. sp. *pisi*, the fungal wilt pathogen of pea, because more inoculum was required to produce an equivalent amount of disease in relation to the other pathogen in natural soil compared to sterile soil.

In a study of chicory, Prinsloo *et al.* (1993) reported no interaction between *C. elegans* and other common root rot organisms, including *F. oxysporum*, *Phoma* sp., *Pseudomonas marginalis*, *Sclerotium rolfsii*, and *S. sclerotiorum*. Yarwood (1981) reported *C. elegans* as "a host of amoeba" (giant soil amoebae), and Fravel and Engelkes (1994) ascribed a biocontrol function of hydrogen cyanide produced by fluorescent pseudomonads in controlling black root rot of tobacco. However, if antagonism by micro-organisms were conceivable, it might occur by mechanisms that non-pathogenic strains of *Fusarium oxysporum* use. These include saprophytic competition for nutrients, parasitic competition, and induced resistance to infection due to prior colonisation (Larkin *et al.* 1996). Soils which are conducive to growth of non-pathogenic *forma speciales* of *F. oxysporum* (for the purpose of natural suppression of Fusarium wilt) have common physical characteristics such as high pH, organic matter and clay content, and a large diverse population of antagonistic bacteria, including actinomycetes (Fravel *et al.*, 1996, Larkin *et al.*, 1996). Inhibition of chlamydospore germination and reduction of saprophytic growth of the pathogen was attributed to high clay and organic matter content in soils (Fravel *et al.*, 1996). Bacterial antagonism may increase or decrease according to the presence of buffering clays, because, although the latter have little effect on fungal growth, they greatly enhance the rate of respiration of soil bacteria. Foster (1985) noted that grinding a soil doubles the respiration rate of soil microbiota because of the presence of polysaccharides in soil pores less than 1 μm wide.

Soil microbiota may be manipulated by both changes in the crop grown and the form of soil amendment. For instance, a soil enriched with inorganic components will have different qualities to one which had been adjusted with organic components. It is conceivable that certain amendments may perform an antagonistic function in controlling pathogens. In various studies, Yarwood (1981) found that the presence of susceptible host genera increased *C. elegans* propagule recovery, even from the rhizosphere of non-hosts, and Holtz and Weinhold (1994) observed that propagule increase in the soil only occurs in the presence of an active host.

The presence of alternative plants or organic matter extracted from them can have a different effect. For instance, Chittaranjan and Punja (1994) trialed different soil amendments consisting of various plant extracts and host plants. Incorporation of unspecified plant tissues to organic soil gradually led to a reduction in recovery of propagules of *C. elegans*. Candole and Rothrock (1997) reported that soils amended with residues and also living plants (i.e. cover crops) of hairy vetch (*Vicia villosa* Roth) significantly suppressed the germination of chlamydospores of *C. elegans*. They compared the germination rate of chlamydospores placed in soils amended with susceptible plant residues and placed on the surface of TB-CEN medium agar, demonstrating that the key factor was the release of the volatile substance ammonia during decomposition of the residues. Soil planted with a living amendment, onions, susceptible to the black root rot fungus, also yielded far fewer propagules of *C. elegans*, although a higher frequency of species of fungi such as *Penicillium*, *Trichoderma*, and *Gliocladium* was recovered. A general increase in fungal presence, stimulated by the onions and the plant residues, may have caused competition with *C. elegans* which is generally regarded to be a poor competitor in soil. *Penicillium* spp., in particular, had been observed to reduce the survival of *C. elegans* within two weeks (Chittaranjan and Punja, 1994). In the same study, the propagule numbers did not increase in the presence of highly susceptible carrots than with

fallow ground, because black root rot of carrots usually occurs in nature as a postharvest disease, even when they are planted into heavily contaminated soil.

2.2.3 Host Resistance

Some pathogens are able to live and multiply in susceptible plants and cause disease under natural conditions. Other plants, even some belonging to the same genus, may partially or completely resist disease. As well as resistance, disease may be minimised by tolerance and avoidance. Avoidance may occur in plants as a separate mechanism to resistance and, although it occurs in plants prior to host-pathogen contact, the mechanisms by which it is manifested are heritable (Shew and Shew, 1994). Just as resistance-susceptibility is conceived as a continuum, so also may tolerance-intolerance represent a continuum (Shew and Shew, 1994).

Disease escape is a spatial phenomenon which may result from disease avoidance. In this case, a lack of disease results from reduced frequency of contact between infection court and inoculum. Escape may stem from a reduced root number, a sparse root distribution, slow rate of root growth, and root exudates which inhibit or fail to attract pathogens (Shew and Shew, 1994).

Non-host resistance to pathogens is exhibited by plant genera which are not considered to be hosts of the organism. Resistance by non-hosts and complete resistance by host plants results in a significant reduction in pathogen propagule numbers. This is most effective where there is no saprophytic phase, because the pathogen, being an obligate parasite, is unable to access plant substrates. A much more rapid increase in propagule number occurs in the rhizosphere of alternative hosts, although no or fewer signs of infection may be evident (Shew and Shew, 1994).

A susceptible plant host will respond to fungal attack if recognition occurs between the two organisms at a genetic level. This is the basis of the gene-for-gene relationship and the development of so-called vertical and horizontal resistance (Zadoks and Schein, 1979). Accordingly, the nature of resistance may vary according to the race of the pathogen and possibly the virulence. Interaction between host and pathogen is attributed to the well-known gene-for-gene hypothesis of Flor, (1947, cited by Mills and Gonzalez, 1982): complementary genetic systems exist in the host and the pathogen. Only one of the combinations leads to an incompatible interaction, that being the interaction in which the host and pathogen carry complementary dominant genes for resistance and avirulence, respectively. Mutual recognition results in either the development of disease or complete resistance to the pathogen. The occurrence of races of the pathogen results from these genetic interactions. Such a relationship is possible for *C. elegans* and tobacco, as clearly-defined pathogen races and differences between host cultivars have been demonstrated to exist (Shew and Shew, 1994)

Distinct races of a pathogen may interact with differing components of specific plant resistance (Shew and Shoemaker, 1993; Shew and Shew, 1994). This has been noted above, with tobacco (Stover, 1950). In these cases, control of disease may be either completely effective or non-existent, depending on whether mutual recognition between host and pathogen occurs. Partial resistance (Shew and Shew, 1994), which is quantitatively expressed and incorporates mechanisms of resistance to both the pathogen and the disease, may provide superior disease control in a monocyclic (albeit systemic) system such as *F. oxysporum* (Ben-Yephet *et al.*, 1996). With *C. elegans*, partial resistance probably functions by increasing latent periods of infection and lowering secondary inoculum production with smaller lesions and rates of lesion expansion (Shew and Shew, 1994). Thus, partial resistance has the potential to augment or replace monogenic resistance in tobacco (*N. debneyi*) if the latter is overcome by a race of the pathogen.

C. elegans varies widely with regard to pathogenicity and virulence, as some strains show different host specificities. Genetic variation may be observed phenotypically, as when cultures exhibit sectoring on media high in carbohydrate, such as Potato Dextrose Agar, and isolates rapidly lose virulence on such media (Shew, 1991). Stover (1950) described the existence of different forms of *C. elegans* (grey wild and brown wild types), each having different pathogenicity on tobacco. The existence of physiological races was suggested by the reduced pathogenicity of some brown wild type cultures compared with other brown wild type cultures. The brown wild type subsisted as at least two physiological races, and on a nutrient-rich substrate was itself capable of spontaneous mutation to the less pathogenic grey wild type.

It was found that the pathogenic behaviour of the cultural type of *C. elegans* was markedly affected by the amount of inoculum added to the soil. If the initial infestation was too heavy or too light, an accurate assessment of the pathogenicity was very difficult or impossible to make. For instance, pouring inoculum over and about the roots of tobacco plants usually resulted in uniformly severe levels of root disease on most varieties, regardless of the wild type cultures used. At that stage it was conjectured that resistances inherent in plants from different growing areas stemmed from the cardinal temperatures of the races.

Shew (1992) noted that resistance in tobacco is found in two forms: partial resistance, varying from low to high, controlled by a group of recessive genes, in *Nicotiana tabacum*, and monogenic, complete resistance, in *N. debneyi*, controlled by a single dominant gene. Tabachnik et al. (1979) also found marked evidence of host specificity in nine isolates of *C. elegans* from different host plants (cotton, peanut, bean, pea, soybean) and soils from different geographic origins, and also in relation to inoculum concentration. Lloyd and Lockwood (1963, cited in O'Brien and Davis, 1994) reported that fungal strains from poinsettia (*Euphorbia pulcherrima*), orange and pea were moderately to

highly virulent on bean and pea but tobacco was unaffected. However, strains from tobacco were highly virulent on tobacco but non-pathogenic on bean plants.

Shew and Shoemaker (1993) noted that where tobacco plants with a single high resistance gene were inoculated, very little disease and inoculum production resulted, although lesions were present. Spores from these lesions caused no disease when reinoculated on the same plants, but severe disease developed in susceptible plants. It was concluded that the resistant plants were probably only colonised after damage by insects, fungi, or nematodes. The mechanism of resistance was unknown. Shew and Shew (1994) suggested that such strong selection pressure which is placed on a race of *C. elegans* may be ameliorated by the broad host range and high saprophytic ability of the pathogen.

Details of both the host range and the diversity of Australian races of *C. elegans* are incomplete. When O'Brien and Davis (1994) tested the pathogenicity of two southern Queensland lettuce isolates against a range of crop species, some lettuce cultivars were completely resistant, while other lettuce cultivars as well as a bean cultivar were severely affected. Watermelon (*Citrullus lanatus* var. *caffer* (Shrad.) Mansf.), cucumber (*Cucumis sativus* L.) and rockmelon (*Cucumis melo* L. s.lat.) were only moderately affected. Capsicum (*Capsicum frutescens* L.), cotton, radish (*Raphanus sativus* V.), and tomato cv. Floradade (*Lycopersicon esculentum* Miller) were unaffected. However, separate tests of isolates obtained from New Zealand peat moss samples proved non-pathogenic to even the previously susceptible lettuce cultivars (R. O'Brien, *personal communication*).

MYCOLOGICAL STUDIES

AIMS OF RESEARCH PROGRAMME

C. elegans is a serious and destructive organism because it is persistent in cultivated ground. In Queensland, the pathogen has a broad and economically significant host range. The vegetable, ornamental and citrus industries in Queensland may be vulnerable to black root rot because of the widespread use of peat-based seedling mixes and the fact that, in the USA, Canadian peat was shown to carry *C. elegans*, causing root rot in citrus seedlings (Graham and Timmer, 1991). The ease and extent of introduction of propagules of *C. elegans* in peat moss seedling mixtures to soils and the prevalence of container-based propagation compounds the threat to horticultural crops, for the fungus has been widely dispersed and is possibly a ubiquitous soil inhabitant in Queensland.

Although this much is known, little evidence exists on which to base a detailed explanation of how the fungus might be introduced to and transported within this country and, once this happens, how it survives during and between infection cycles. The literature review, then, raised questions which were addressed during a programme of research and actually formed the basis of that research. The questions were:

- How does the fungus *C. elegans* infect lettuce, a genus recently reported to be a susceptible host?
- Which physiological factors limit propagule viability and longevity in the soil?
- Which micro-environmental factors could affect propagule viability and longevity in the soil?
- In the light of similar scenarios in northern America, is it possible that *C. elegans* may be introduced to horticultural crops in Australia by the use of imported sphagnum peat in seedling mixes?
- Which common weed and crop genera are susceptible to *C. elegans?*

- Are some types of seedling container more likely to play a role in the transmission of C. *elegans* to field and nursery crops?

- How does the presence of common weeds and crop genera affect the population dynamics of C. *elegans*?

The venues for these studies were in Bundaberg (TAFE horticultural laboratory, Queensland Department of Primary Industries research station), and Rockhampton (Central Queensland University microbiology laboratory).

3. Method of Infection and Invasion of Lettuce

3.1 Introduction

As noted in the literature review, the method used by *C. elegans* to infect lettuce, including the deployment of appressoria, has not been described in the current literature. Although subsequent growth of hyphae may be halted or inhibited in resistant and tolerant lines, it has not been documented to date. Furthermore, although it is evident that chlamydospores enable long term survival of the fungus, their role in the infection process has not been confirmed. In work associated with this research phialoconidia were commonly observed as the initiators of infection in lettuce, but this by no means has been the experience of other workers. Tsao and Bricker (1966) demonstrated that, at least in selective agar media from soil dilution plates, chlamydospores were the origin of nearly all colonies, and in none of their investigations did colonies develop from phialoconidia or from mycelial fragments. This trial was initiated with the primary purpose of observing whether appressoria were produced by phialoconidia during the process of invading the roots of susceptible and resistant lettuce cultivars. However, it was also intended to document whether chlamydospores played an active role in the infection process.

3.2 Materials and Methods

Seven-day-old lettuce seedlings of susceptible, partially resistant, and resistant cultivars were examined to detect the method of invasion by *C. elegans*. These had been germinated on water agar in covered containers and inoculated with an aqueous suspension of phialoconidia (5×10^4 . mL^{-1}) after four days. The origin of the suspension was a Bundaberg isolate of *C. elegans* (4150) which had been obtained from infected lettuce and which had been continually maintained on Potato Dextrose agar. Phialoconidia used in this trial were obtained from three week old cultures grown at 22 ° C in 9 cm petri dishes, before the hyphae had reached the container margins. The culture surface was covered with 10 mL of sterile distilled water. Phialoconidia were released from the younger regions of the culture surface where they were most abundant, and where

chlamydospores had generally not yet been produced, by stroking with a sterile, right-angled glass rod. Phialoconidia were then washed from the culture surface with sterile distilled water into a container, and their concentration was adjusted. As expected, isolated chlamydospores were included with the phialoconidia which were much more numerous. Because prior testing showed phialoconidia to have a primary role, they were used as the basis of the inoculum, but it was accepted that chlamydospores might regularly initiate infection.

Seedlings of each cultivar were stained using the technique developed by Hood and Shew (1996). Seedling specimens were autoclaved for 15 min at 121°C in 50 mL of 1 M KOH, rinsed three times in deionized water, mounted on glass slides in 0.05% aniline blue solution and then viewed using a microscope equipped for epifluorescence microscopy.

3.3 Results

Hyphae of C. *elegans* were observed to invade susceptible and resistant lettuce root and hypocotyl tissue. Usual avenues were in between the laminae of root epidermal cells and through the lumen of root hairs, and so minimal disruption was apparent in host tissue. Leakage of callose from host cells could not be confirmed because of the particular fixing technique. Appressoria were commonly observed. Large numbers of phialoconidia and smaller numbers of chlamydospores were produced on the root surface of susceptible and resistant cultivars. In one case, a chlamydospore was observed germinating on the root epidermis of a resistant plant, but it could not be determined whether the spore had originated in the inoculum or had been recently formed. No differences were observed in the invasion method between susceptible and resistant lettuce.

3.4 Discussion

The study demonstrated that parasitic invasion of lettuce is accompanied by or is facilitated by a fungus-plant interface which can be described as an

appressorium. Two criteria exist on which such an identification may be based (Hood and Shew, 1997). The first is if the structure is delimited by a septum and consists of a significant swelling. It was not possible to determine the existence of a septum at the magnification used (x 400). However, the second criterion is confirmation that the purpose of the structure is to facilitate attachment. These structures were only ever observed to be in very close proximity to the root or hypocotyl surface, and were not seen to be adjacent to the lumen of infected root hairs. Therefore, circumstantial evidence is that their function must be to assist in the invasion of a surface barrier of significantly lower permeability than the lumen of root hairs. Nevertheless, such root hairs were not encountered frequently enough to make an unequivocal statement.

Because spore formation occurred on both resistant and susceptible cultivars, and no differences in spore quantity could be discerned between them, it is conceivable that whatever mechanism whereby resistance occurs must be activated at a further stage of colonisation. Copious numbers of phialoconidia were produced to continue the infection process until the demise of the host. However, the continual production of sparse numbers of chlamydospores from the time of establishment of infection should be understood as a means of ensuring the survival of the fungal organism in case the substrate is quickly exhausted.

This study was not intended to document saprophytic or biotrophic stages, and the conclusions that could be drawn will be stated later in the general discussion, based on records of fluctuations in propagule numbers during an extended period.

4. Spore Germination Studies

4.1 Introduction

In the presence of a suitable substrate, phialoconidia of *C. elegans* develop before chlamydospores and are produced in far greater numbers than them (Christou, 1962). Therefore, it is probable that their role is to initiate infections and to propagate the fungus during the limited period when a substrate is available. While this role may also be filled by mycelial fragments, phialoconidia, despite being thin-walled, are more likely to facilitate short-term survival of the organism for several months after the demise of the host (Lindsey, 1981; Chittaranjan and Punja, 1993).

Physical and chemical influences cause considerable variations in the micro-environment and the purpose of this series of tests was to gauge the effect of several physical influences upon phialoconidial viability. In the work reported here, the survivability and viability of phialoconidia were studied in relation to humidity and spore mass. Furthermore, the *in vitro* mycelial growth rate of the pathogen was assessed at different substrate water potentials.

4.2 Methods

Four tests were conducted. Replicated five times, they investigated the effect of:-

■   relative humidity on phialoconidial germination,

■   spore density on phialoconidial germination,

■   desiccation and spore density on phialoconidial germination,

■   different substrate water potentials on mycelial growth rates.

Relative Humidity and Germination of Phialoconidia   Aluminium rings (25 mm external diameter with a wall thickness of 2 mm) were placed on microscope slides to form a chamber, with a small amount of petroleum jelly on both upper and lower surfaces to provide a water-tight seal. Various super-saturated salt solutions were used to generate atmospheres of a specific relative humidity in the chamber, varying from 15% to 98%. (The salt solutions are

listed in Appendix 1.) The range of relative humidity (%) was as follows: 15, 20, 42, 52, 66, 75, 81, 86, 90, 92, 93, 95, 98, 100.

Sterile water (1mL) was placed in the base of some chambers to generate 100% relative humidity. Phialoconidia were harvested by gently rubbing the surface of a heavily sporulating culture of C. elegans which had been covered with 10 mL. sterile distilled water. The resulting spore suspension was adjusted to approximately 1 x 10^4 . mL^{-1} and small drops (approximately 0.05 mL) of this suspension were placed on glass cover slips which were then allowed to air dry in a room maintained at 24°C. The cover slips were then inverted and placed as a cover over the aluminium rings. All chambers were then incubated for either 20 or 30 hours at 24°C. After this incubation period the cover slips were removed, the spores stained with a drop of alcoholic lactophenol cotton blue, and stored prior to assessment of germination. A spore was considered to have germinated if it had produced a germ tube which was approximately as long as the spore was broad.

Spore Density and Germination of Phialoconidia   Phialoconidia were harvested as described previously and the suspension was diluted to give different spore densities (x 10^3 . mL^{-1}) as follows: 1700, 800, 100, 50, 40, 20, 10, 5, 1, 0.

Similar chambers to those constructed in the relative humidity experiment using sterile distilled water to generate 100% relative humidity were used. 50μL droplets of the relevant spore suspension were placed on the underside of the cover slips. A total of 50 chambers were prepared in this way and placed in a perspex box with a high internal relative humidity. After 20 and 30 hours incubation the boxes were opened, the spore droplets fixed and stained as described previously, and then stored until the germination rate of 100 spores per chamber was assessed.

Desiccation, Spore Density and Germination of Phialoconidia The spore suspensions used in the previous experiment were used to evaluate the effect of exposure to dry conditions on spore viability. Four spore densities were used: 800, 100, 50, and 10 x 10^3 mL. $^{-1}$. The cover slips were inoculated with three 50μL drops of a spore suspension, air dried as previously described and then stored in an environment with 65-70% relative humidity at 24°C for periods of time ranging from 1 to 20 days. Cover slips bearing spores were then removed, rehydrated by the addition of a drop of sterile distilled water and stored in a humid environment to arrest drying of the spores. After a further 24 hours the germination percentage of each group was assessed, using quantities of 100 spores, based on the same criterion as previously described.

Different Substrate Water Potentials and Mycelial Growth Rates A b a s a l medium was prepared using the method of Harrower and Nagy (1979), who adjusted the concentration of KCl in the agar medium to give different substrate water potentials (- Bar). The 1979 study demonstrated that an optimum level of potassium was necessary in the fungal cytoplasm. However, after that concentration had been attained, any differences in the growth rate could be attributed not to an embellished nutrient status but to the more or less restrictive effects of altered water potential. A similar effect might be induced by enhanced sugar levels in an agar-based medium. The water potential values (- Bar) were as follows: 8.7, 15.1, 23.6, 31.1, 38.5, 45.9, 53.4, 60.9, 68.3, 75.8.

Petri dishes (9 cm diameter) were filled with 20 mL. of medium and the centre of each plate was subsequently inoculated with a standard sized (approx 1mm^3) piece of medium excised from the periphery of a colony of C. *elegans* growing on half-strength Potato Dextrose Agar. The bases of each of eight replicate plates were marked with two diameters at right angles. After fifteen days, the mean radial growth was recorded from the edge of the inoculum cube along each of the four radii to the periphery of the colony.

4.3 Results

Relative Humidity and Germination of Phialoconidia The phialoconidia germination rate was greatest at the highest relative humidity with the majority of spores germinating after 20 hours incubation (Figure 4). A further incubation period of 10 hours slightly increased the percentage germination rate at all levels of humidity tested. No germination was evident even after 30 hours incubation at relative humidity values of 81% and below.
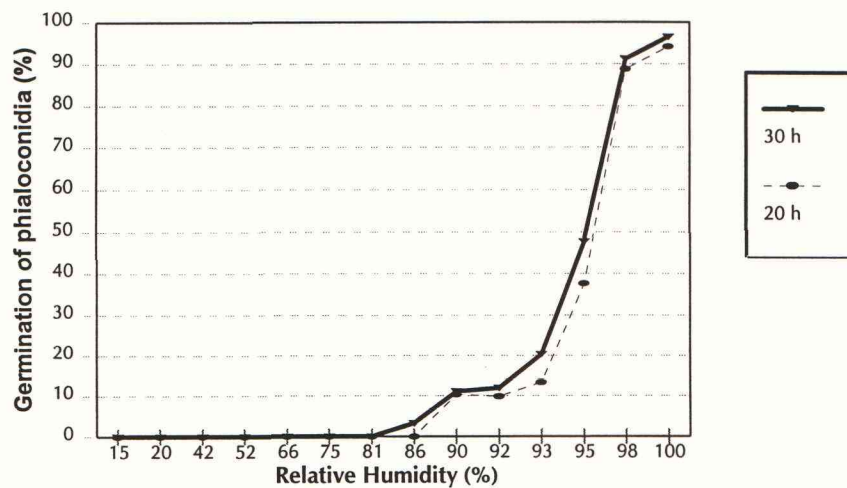
Figure 4. Effect of atmospheric relative humidity on germination of phialoconidia of *C. elegans*

Spore Density and Germination of Phialoconidia At spore densities of 10^5. mL^{-1} or greater spore germination was sparse, being 0.32 % and 0.36 % after 20 and 30 hours respectively (Figure 5). As the spore density decreased the germination rate increased with maximum observed rates being at the 5×10^2 . mL^{-1} density, being 93.84 % and 95.56 % after 20 and 30 hours respectively.
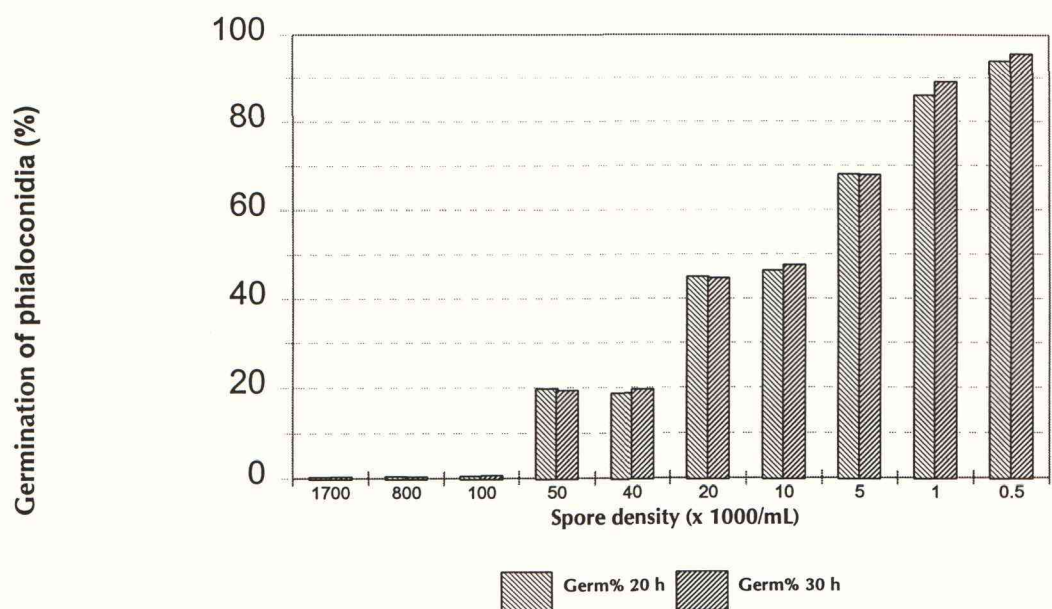
Figure 5.   Effect of spore density on germination of phialoconidia of *C. elegans*

Desiccation and Spore Density, and Germination of Phialoconidia Contrary to the previous experiment involving spore density tests, viability was prolonged in chambers with higher spore densities as time progressed. At the greatest spore density (800 x 10^3 . mL^{-1}), 84.4 % of phialoconidia germinated after a desiccation period of one day, and 20.8 % of the phialoconidia germinated after desiccation for 20 days. At the lowest spore density (10 x 10^3 . mL^{-1}), 78.2 % of the phialoconidia germinated after a desiccation period of one day, and only 3.6% of the phialoconidia germinated after the longest period of desiccation. Figure 6 demonstrates varying rates of decline in spore viability for periods of between 1 and 20 days, and differing spore densities.
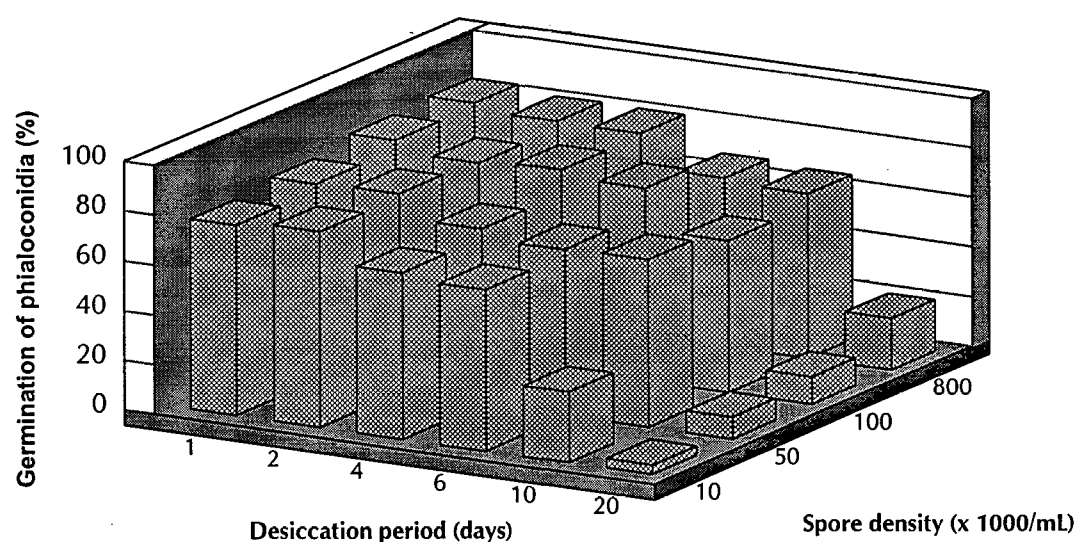
Figure 6. Effect of spore density and duration of desiccation on germination of phialoconidia of *C. elegans.*

Substrate Water Potentials and Mycelial Growth Rates   As water potential decreased from approximately -9 bar to approximately -75 bar the radial growth of all colonies of *C. elegans* decreased (Figure 7). Media with a water potential below -60 bar did not permit hyphal growth. Data differences between treatments were statistically highly significant (L.S.D. = 602.15, *P* < 0.001, Analysis of Variance).
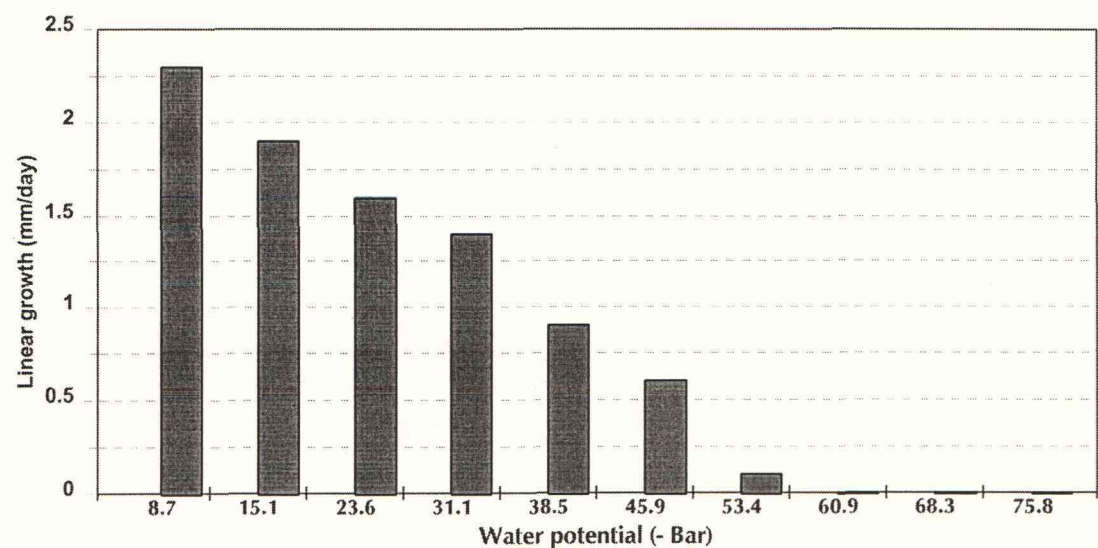
Figure 7.   Effect of decreasing water potential on mycelial growth rates of *C. elegans*.

4.4   Discussion.

In the fungal life cycle, water is necessary for germination of spores, vegetative growth, and reproduction. Phialoconidia of *C. elegans* must imbibe water, alter their rate and type of metabolism and germinate in order to produce (usually) one germ tube which elongates to form a hypha and subsequently to produce a foraging mycelium. The work reported here provides evidence of a relationship between air relative humidity, water potential of growing media (agar or soil), and spore density. It was demonstrated that an increasing level of relative humidity stimulates germination of phialoconidia provided that the density of phialoconidia is below a certain level, for the germination rate is probably controlled by autoinhibitory mechanisms. Such mechanisms have been reported in the phytopathogen *Leptosphaeria nodorum* Müll., the causal agent of glume blotch of wheat. *L. nodorum* overwinters by the production of pycnidiospores in a cirrus. Subsequent wetting and dilution of the cirrus material and spore mass has been reported to result in an increasing rate of germination below a threshold level of spore density and of an autoinhibitory compound associated with the pycnidiospores (Harrower, 1976). It is postulated that a similar autoinhibitory mechanism may exist in the massed clusters of phialoconidia produced by *C. elegans* on infected host tissue. Spore germination is enhanced as the spore mass is reduced in density (Figure 5). As this occurs the reduction in the level of autoinhibition will occur until a threshold level is reached below which most phialoconidia will germinate. As mentioned previously, it was the observation of Yarwood (1974) that tillage appears to reduce phialoconidia viability by introducing adverse conditions for the survival of the thin walled spores. These may include desiccation and lack of a suitable live host. However, perhaps germination in isolation from other spores and in the absence of a susceptible host would be sufficient to exhaust carbohydrate reserves and reduce field inoculum.

Furthermore, as in *L. nodorum*, the autoinhibitory agent also appears to protect the spore from the effects of desiccation. Therefore, the addition of water not

only breaks the effect of desiccation but also may reduce phialoconidia density sufficiently to enable germination to take place. That occurred consistently in this study, with germination rates ranging from 84.4 % to 76.2 % from the highest to the lowest density respectively after one day in the spore density/duration trial (Figure 6). The germination rate (76.2 %) at the lowest density (10 x 10^3 . mL^{-1}) was still consistent with the results of the density/germination tests (Figure 5), where, at the lower density (1 x 10^3 . mL^{-1}), germination percentage was 85.86 after 20 hours desiccation.

Other tests of viability have found, for instance, that lysis of phialoconidia at low densities was more rapid in a moist rather than a drier soil because germination was stimulated at an earlier stage, and so spore survival was reduced (Schippers, 1970, in Chittaranjan and Punja, 1994). Therefore, while the survival rate of phialoconidia is governed by the moisture level of the atmosphere and the substrate, the rate at which mycelial extension occurs in those which ultimately germinate is also determined, in part, by the external availability of water. This is a well established critical factor in the hyphal extension mechanism (Gooday, 1993).

In nursery and subsequent field situations, lettuce are routinely provided with adequate levels of water via drip irrigation to ensure that growth occurs and that roots can explore the surrounding soil matrix. Conditions of low soil water content arrest growth of the host and possibly decrease germination and phialoconidia longevity. The provision of adequate water benefits both the host and the pathogen (Shew and Meyer, 1992). Propagules may be dispersed throughout the soil matrix, and the same autoinhibitory substances diluted and germination enhanced. This would give the opportunity for colonisation of growing root tissue and plant debris to occur.

Soil contaminated with C. elegans could have its effective inoculum load reduced by being watered to saturation and then immediately dried. This is

clearly unlikely to be applicable to many field soils but, where pasteurisation or sterilisation of potting mixes is not practised, it may have a place as a nursery treatment. Media may be wet to field capacity, spread into shallow heaps and quickly dried in an attempt to reduce the effective inoculum load of this pathogen at minimal cost. Nevertheless, such a practice would be ineffectual through not knowing the inoculum density and its distribution in the growing medium. As well, it would be contraindicated for production nurseries which are accredited or seek to become accredited with the Nursery Industry Association of Australia (Australian Horticultural Corporation, 1994). Growers routinely use pathogen-free growing media. Although soil solarisation is recommended as a means of media pasteurisation to these producers, it is best carried out during periods of high temperature and intense solar radiation. Conditions in northern Australia favour such treatments for much of the year but are unreliable for other areas.

In field conditions, minimum tillage may delay dispersal of spores and inhibit their germination. However, in this industry the reverse usually occurs, with intensive secondary tillage such as rotary hoeing being practised for residue incorporation and seedbed formation. While such repeated tillage may hasten desiccation of phialoconidia, it would be pointless to do so for that purpose alone because of the damage it causes to the soil structure by crushing aggregates, stirring oxygen into the soil, and the formation of a hard pan. As well, the opportunity cost in leaving high value land unused discourages fallowing practices, and so when cultivation does occur it is generally followed by the regular irrigation of successive crops. Finally, it is difficult for producers to assess the soil inoculum concentration and distribution of C. elegans once disease has occurred. Even where only isolated plants have been affected and then removed by harvest or roguing, large numbers of phialoconidia are produced. Although the viability of phialoconidia may be reduced by dispersion, the uncertainty of success would provide little motivation for producers to initiate action.

DISEASE CYCLE

5. Survey of Unopened Peat Packages for Presence of *C. elegans*

5.1 Introduction

This series of tests was conducted in the Bundaberg area subsequent to an outbreak of black root rot in the lettuce crop of a local grower who used peat-based potting mixes. In order to confirm that *C. elegans* is commonly introduced to nurseries and fields via peat, it was necessary to test samples from commercial sources.

5.2 Materials and Methods

Twenty separate samples of New Zealand peat (Hauraki Gold ®, 14 samples; Warrior ®, 6 samples) were collected from five suppliers in the Bundaberg region. Peat was extracted from each unopened bale by grasping a handful using a new plastic bag as a glove. The peat was stored temporarily in the sealed plastic bag.

Each peat sample was tested for the presence of *C. elegans* with carrot baits using the following procedure. Fresh carrots were surface sterilised with 1% sodium hypochlorite, rinsed with sterile distilled water, and sliced into 4 mm thick transverse sections. Four sections were placed in each of three petri dishes on top of one dessertspoon of peat (approximately 5 g) which had been wetted with distilled water. After six days of incubating at ambient temperatures, the carrot discs were inspected for the presence of mycelia of *C. elegans*, confirmed by the presence of phialoconidia and/or chlamydospores.

Samples of peat from bales which were shown to be contaminated with *C. elegans* were mixed with equal portions of vermiculite and used to fill enough 60 cell size polystyrene trays to enable ten cells per contaminated sample. Therefore, only one tray was used for this pathological testing. Seeds of a

susceptible lettuce cultivar (Yatesdale) were sown and after a period of between four and six weeks the plants were inspected for black root rot lesions.

5.3   Results

One sample contained *C. elegans* (Hauraki Gold). A white mycelial growth on the carrot discs was examined and the presence of phialoconidia and chlamydospores confirmed the identity of *C. elegans*. Two other peat samples (one each of Hauraki Gold and Warrior) contained a related organism with greyish mycelia, *C. thielavioides* (R. O'Brien, 1997, *personal communication*).

Disease did not develop in the seedlings grown in the peat which was contaminated with *C. elegans*.

5.4   Discussion

The finding of *C. elegans* in one out of twenty bales is most significant and confirms suspicions that contaminated bales of peat are a possible source of many black root rot infections. Contamination of batches of peat-based growing media occurs because *C. elegans* is present at high levels in localized areas (Copes and Hendrix, 1997a). A pathogen found in this way may have been present in the original vegetation. It would survive only as chlamydospores as the studies by Yarwood (1981) and Tsao and Bricker (1966) point to a poor tolerance by the actively growing fungus of depths over 16 cm and non-rhizosphere environments.

Although the suppliers accessed in the Bundaberg area stocked only sphagnum peat of New Zealand origin, the conclusions which have been drawn below also stem from other Queensland-based surveys which tested Canadian peat (R. O'Brien, 1997, *personal communication*). It should be stressed that quantitative data concerning the frequency of transmission in peat cannot be generated from these qualitative tests which indicate merely the absence or presence of the pathogen. Indeed, the amount sampled is a minute fraction of the quantity

distributed around the State for use in seedling production and considerable potential exists for new infections of *C. elegans* in nurseries. Furthermore, the tests above were linked to other work ((R. O'Brien, 1997, *personal communication*) which demonstrated the presence of isolates of *C. elegans*, pathogenic to lettuce, in New Zealand peat, and isolates of *C. thielavioides*, non-pathogenic to lettuce, in Canadian peat.

Although lettuce was not susceptible to the strain of *C. elegans* isolated from the peat from the survey, it is known that the fungus exists as a range of pathogenic strains which affect different horticultural crops (see Section 2.2.3, e.g. Tabachnik *et al.*, 1979). Immense volumes of the two peat brands tested are used in nursery production, and peat is generally regarded in the nursery industry, with vermiculite and perlite, as a sterile or at least very clean component of propagation and growing media. Advisory publications (e.g. Australian Horticultural Corporation, 1994) state that peatmoss, perlite, vermiculite, and properly composted pine bark, prepared on clean surfaces, are most often free of the most common pathogens occurring in propagating facilities such as *Phytophthora* spp. and *Fusarium* spp. However, it may be necessary in future to warn producers about the risk of introducing the pathogen *C. elegans* to a production system in contaminated peat. And although its host range was not tested, it is feasible that this isolate may cause problems in other crops including tomato, citrus, pansy and viola which rely on container production.

C. thielavioides, a closely related organism, is not pathogenic to lettuce but causes black root rot in roses. Although further investigation and pathogenicity testing of that organism would be necessary, its presence in a small sample of peat from two bales in this trial suggests that it may be present in many others and that it poses a threat to the rose nursery industry.

The successful isolation of *Chalara* spp. confirms the findings of Graham and Timmer (1991) that peat may carry this pathogen. Its presence in New Zealand

peat, as reported here, and in Canadian peat indicates a widespread association and a common avenue of dissemination and infection.

6. Pathogenicity Tests on Various Alternative Plant Hosts

6.1 Introduction

A range of weeds and crop plants, common to southern Queensland, were tested to determine their susceptibilities to an isolate (4150) of *C. elegans* collected from infected lettuce in the Bundaberg region.

6.2 Materials and Methods

The trial was conducted on benches in a closed greenhouse. Plants to be tested were raised by seed, except for sugar cane (*Saccharum officinarum* L.), which was propagated by setts (stem cuttings) obtained from a local farm, and pigweed (*Portulaca oleracea* L.) which was propagated by nodal cuttings. Weed seeds were collected from plants in the Bundaberg region, while seeds of the crop plants such as oats (*Avena sativa* L.) were supplied by produce stores. Control plants were grown from seeds of the susceptible lettuce cultivar (cv. Yatesdale).

The propagated plants were grown in medium contaminated with *C. elegans*. It was contaminated by soaking millet seed soaked in water for twelve hours. The seed mass was then autoclaved, and inoculated with agar blocks from the margin of growing cultures of *C. elegans*. After allowing approximately two weeks at approximately 20 ° C for complete colonisation of the millet seed, it was then incorporated at 1% volume per volume with a 1:1 peat-vermiculite potting medium (pH adjusted to approximately 6.5, nutrients added). Presence of *C. elegans* was confirmed by inoculating fresh, surface-sterilised, 3 mm thick slices of carrot with drops of soil solution and inspecting the resultant mycelia for phialoconidia and chlamydospores of *C. elegans*.

Apart from *S. officinarum*, which was grown in a separate container filled with identical contaminated medium, plants were grown in new 60 cell polystyrene trays (Speedling ®) with plant per cell. Ten cells were allocated to each genus tested. The test plants were watered by overhead sprinklers until the medium

was at field capacity. It was necessary to repeat some sowings because of seed germination problems. Table 4 lists the weeds and crop plants tested.

Only seedling lettuce were examined for root damage. The debilitating invasion of the black root rot pathogen results in symptoms on all or part of the root system. This causes poor plant growth and, in some cases, death of the plant. However, as noted in the Literature Review, plants may recover to varying degrees after the initial infection because of cortical regeneration and secondary root growth (Mauk and Hine, 1988). Therefore, basing pathogenicity ratings on the condition of mature plants may give grossly misleading results.

When root systems had fully explored the container (after 4 to 8 weeks), plants were removed from the trays. Their roots were gently washed and inspected for symptoms of black root rot. The degree of damage was rated visually, with a dissecting microscope, using the following scale:

0 = No disease
1 = Slight browning of small roots, tap root slightly affected
2 = Necrotic secondary roots, tap root slightly affected
3 = Tap root with large areas affected, most lateral roots affected
4 = Tap root severely affected, some lateral roots present
5 = Root system completely destroyed.

6.3   Results

The susceptible lettuce plants were most severely affected with a moderately severe rating of 3.6 (Table 4). Black root disease developed in three week old lettuce seedlings and was evident on the tap root and laterals, as the scale above indicates. Other crop and weed plants were also affected to a lesser degree, as per the rating scale listed above. Nine genera did not develop signs of root disease.

Table 4. Disease ratings on plants infected with isolate 4150 of *C. elegans*. Ratings are mean values for each genus tested, and range from 0 (no disease) to 5 (root system completely destroyed).

Botanical Name	Common Name	Disease Rating
Ageratum houstonianum Miller	Blue billygoat weed	0
Avena sativa L.	Common oats	0
Bidens pilosa L.	Cobbler's pegs	0
Cardamine hirsuta L.	Flickweed	0
Euphorbia prostrata Ait.	Caustic weed	0
Gomphrena celosioides Mart.	Gomphrena weed	0
Panicum laevifolium Hack.	Sweet panic	0
Portulaca oleracea L.	Pigweed	0
Saccharum officinarum L.	Sugar cane	0
Solanum nigrum L.	Blackberry nightshade	1
Sonchus oleraceus L.	Common sowthistle	1
Cucurbita maxima L. cv. Ken's Special	Pumpkin	1.6
Trifolium repens L. cv. Big Bee	White clover	1.7
Macroptilium atropurpureum (DC.) Urban	Siratro	1.9
Helianthus annuus L.	Sunflower	2
Sorghum halepense (L.) Pers.	Johnson grass	2
Cajanus cajan (L.) Millsp. cv. Dunn	Pigeon pea	2.5
Pisum sativum L. cv. Sugarsnap	Pea	2.8
Gossypium hirsutum L.	Cotton	3
Lablab purpureus (L.) Sweet	Lab lab bean	3.1
Lactuca sativa L. cv. Yatesdale	Lettuce	3.6

6.4 Discussion

As expected, the lettuce plants were most severely affected, and several legumes, pumpkin, and cotton were infected due to high concentrations of well-dispersed phialoconidia. Susceptibility of *S. oleraceus* was noted by O'Brien and Davis (1994) and it was confirmed in this trial where representatives of the families Asteraceae, Cucurbitaceae, Fabaceae, Malvaceae, Poaceae and Solanaceae were also susceptible. However, plants in the families Asteraceae, Brassicaceae, Euphorbiaceae, and Portulacaceae were unaffected. *Saccharum officinarum*, a valuable crop especially in Queensland, was not infected by this strain of the black root rot organism but *Sorghum halepense* was susceptible. Both genera are members of the family Poaceae in which very few other genera are susceptible. In the Literature Review section of this thesis, it was noted that no records of genera in the family Poaceae susceptible to *C. elegans* had been found. Sugar cane is grown in rotation with susceptible crops such as cucurbits, lettuce, tomatoes, capsicum, and legumes in Queensland regions. It is probable

that any effect of a non-host crop such as sugar cane, planted with the intention of decreasing soil infectivity in between susceptible crop cycles, would be nullified by the longevity of chlamydospores.

This set of results pertains to tests with a particular isolate of *C. elegans*. It should be noted, as discussed in the Introduction, that a different isolate may have a different pathogenic range and incite disease in a species which previously had been demonstrated to be resistant. It is also likely that the severity of symptoms in a species, shown to be susceptible, may alter if challenged by a different isolate of *C. elegans*. Hence the disease rating of lettuce was less severe than expected. All plants, including lettuce, were inoculated at a young stage when susceptibility is greatest and before mature plant resistance develops. Therefore, the lower disease rating may be attributed not to variations in the age of the plant but to variations in severity between different isolates of the one fungal organism.

7. Testing the Persistence of *C. elegans* on Sanitised Plant Containers

7.1 Introduction

These experiments tested the effectiveness of a limited range of disinfecting agents on polystyrene seedling containers contaminated by *C. elegans* during the seed raising process. It is feasible that contamination occurs through rootlets on soil particles attaching to the rough surface of polystyrene trays. The problem is often worse as trays become old and cracked. Commercial seedling nurseries commonly use chlorine-based disinfectants to treat containers, implements and foot-wear. Such products are difficult to use due to the chlorine vapours which are unpleasant and hazardous to human mucous membranes. Other products are available which could be suitable replacements.

7.2 Materials and Methods

The experiment was conducted in two parts. Firstly, inoculated seedlings were grown in seedling containers in order to contaminate them. Secondly, trays were washed free of seedling mix and then dipped in the disinfectants and the test plants were grown in them. These treatments of the second section are listed below.

The central 20 cells of 21 new, unused, polystyrene 110 cell size seedling trays (Speedling ® - multi-celled, tapered polystyrene containers) were filled with steam-pasteurised (65 ° C for one hour) UC medium (University of California potting medium; peat:sand, 1:1). Seedlings of lettuce cv. Yatesdale, a susceptible cultivar, were root dipped in a suspension of phialoconidia of *C. elegans* (isolate 2864) 1×10^5 . mL $^{-1}$ and were then transplanted one per cell in the trays. Uninoculated seedlings were also transplanted in three of these trays. Seedlings were then grown for a further 14 weeks in a glasshouse at ambient temperatures and watered by overhead sprinklers. The purpose of such an unusually long time period was to provide an opportunity for roots to grow into small cracks in the polystyrene. Many growers find it difficult to completely

remove such small root particles by washing alone and consider them an important source of contamination.

Seedlings were then removed and rated for disease severity, using a standardised scale (see Table 5). Mean severity figures were derived for each tray. These were then used to allocate trays to each of the three blocks in the second planting. The trays were gently hosed free of loose potting mix and then dipped for 10 minutes in the appropriate treatment. These were: Chlorine 100 ppm, Chlorine 1000 ppm, Aldespray 1%, Aldespray 0.5%, Benomyl 1g.L^{-1}, Control (water dip), Control (uncontaminated tray).

A commercial preparation of sodium hypochlorite, Hypo 10 $^{®}$ (100 g . L^{-1}) was diluted to each of the two concentrations. Aldespray 150 $^{®}$ containing glutaraldehyde was used in conjunction with the "accelerator" (phosphorous acid) at rates of 5 mL . L^{-1} and 10 mL . L^{-1}. Benlate $^{®}$ (500 g/kg benomyl) was used at the rate of 1 g . L^{-1} of dip.

After dipping, trays were lightly hosed and allowed to dry before refilling with steam sterilised UC mix. Lettuce seed cv. Yatesdale were sown and later thinned to one per cell. Plants were grown in a glasshouse for seven weeks, then roots were exposed as previously described and rated for disease severity.

7.3   Results

Disease occurred in all trays in the first planting with mean values for the twenty plants in each tray ranging from 2.8 - 4.9 (Table 5). In the three previously uncontaminated trays, 61.5%, 64.7%, and 90.9% of plants were infected, giving mean values of 1.4, 1.5, and 3.0 respectively (Table 5). The severity values of the deliberately contaminated trays were then used to allocate trays to treatments for the second part of the experiment. Block I (lowest severity) had trays containing plants ranging in severity from 2.8 - 3.7, Block II 3.8 - 4.3, and Block III 4.3 - 4.9. Treatments within each block were allocated at random as

indicated in Table 5. Compared to the other treatments, benomyl reduced the disease severity significantly (at $P=0.05$). None of the other treatments, including the standard chlorine dip were different from the control (water dip).

Table 5. Disease severity in trays at the end of the initial contamination period and allocation of trays to treatments for the decontamination experiment. The following scale was used: 0 = No symptoms, 1 = Slight discolouration of tap root, surface only, 2 = slight discolouration of tap root, some secondary roots missing, 3 = moderate discolouration of tap root, tap root affected, many roots still present, 4 = Tap root severely affected, some lateral roots left, 5 = No roots after washing.

Trays Allocated to Treatment	Disease Severity (0-5)			
	I	II	III	Mean
Chlorine 100 ppm	2.8	3.8	4.7	3.8
Chlorine 1000 ppm	2.8	3.9	4.8	3.8
Aldespray 1%	3.6	4.1	4.3	4.0
Aldespray 0.5%	3.2	4.3	4.9	4.1
Benomyl 1g.L^{-1}	3.7	4.2	4.5	4.1
Control (water dip)	3.6	4.1	4.5	4.1
Control (uncontaminated tray)	1.4	1.5	3.0	2.0

Table 6. The efficacy of chemical decontamination (10 min soak) treatments in controlling *C. elegans* in polystyrene seedling containers.

Trays Allocated to Treatment	Disease Severity (0-5)			
	I	II	III	Mean
Chlorine 100 ppm	2.7	2.7	3.5	2.97
Chlorine 1000 ppm	2.0	2.6	3.0	2.53
Aldespray 1%	2.8	1.9	2.8	2.5
Aldespray 0.5%	2.3	2.1	3.2	2.53
Benomyl 1g.L^{-1}	0.3	0	1.3	0.53
Control (water dip)	1.8	2.4	3.0	2.40
Control (uncontaminated tray)	1.8	1.8	3.5	2.37
L.S.D.[1] $P = 0.05$ $P = 0.01$				1.37 1.91

[1] Analysis of Variance

7.4   Discussion

The organism survived several chemical treatments which are widely used for decontamination purposes. This may be due to a combination of the resistant nature of the chlamydospores coupled with the coarse textured surface of the polystyrene seedling trays. The biocides chlorine and formaldehyde are hazardous and unpleasant to use. Residues contaminate their immediate environment on disposal. At the stated concentrations, they were surprisingly ineffective against propagules of *C. elegans,* probably due to the durable nature of chlamydospores and protection within root debris, which were not removed by a light hosing, as is normal treatment for polystyrene containers in production nurseries. Following the dipping treatments, there were no significant differences between treatments apart from benomyl, which greatly reduced disease severity. Copes and Hendrix (1997b) demonstrated the efficacy of sodium hypochlorite on greenhouse benches and fabric, especially non-porous ones, but did not extend their testing to seedling containers.

Benomyl, a member of the benzimidazole group, was outstandingly effective, probably because of a carryover of sufficient chemical in trays to provide root uptake and systemic protection against residual or later introduced disease propagules. The other chemicals, having no such systemic properties, did not provide the same degree of control. Although benomyl is not presently registered as a dip for seedling trays, it is widely used as a postharvest dip for a range of fruit and as a common spray for control of fungal diseases in many crops. It is possible that approval could be gained for its use as a dip for seedling containers.

Seedling nurseries which customarily rely on peat-based media, from which isolates of the black root rot organism commonly originate, and chlorine drenches of trays, may need to review disease prevention practices. These may be adjusted to incorporate more rigorous quarantine standards, systematic screening techniques such as baits, a different range of disinfection chemicals,

the use of steam sterilisation rather than steam pasteurisation for trays and containers, and possibly, the use of plastic trays which have been observed to be easier to clean of root and media debris.

Contamination occurred in all control trays. Disease had not been deliberately introduced to them. Contamination *via* the initial peat-based potting medium is possible but unlikely. More probable causes are water splash and aerosols from overhead watering. In the initial (contamination) trial, there was sufficient time for a number of disease cycles to be completed and for inoculum to be increased. Fungus gnats (Family Mycetophilidae) and cockroaches may become vectors for the pathogen. The former were observed in the greenhouse during the growth period and may have acted as an agent in pathogen transmission. Mucilaginous phialoconidia are produced in profusion, especially at and near the soil surface and it is possible that insects may come into contact with the propagules. Investigations are continuing at present to confirm whether insects and aerosols are implicated. Indeed, the possible role of aerosols in transmission of propagules of this pathogen serves to highlight another facet of the survival of *C. elegans* in different physical environments.

8. The Effect of Cropping Sequence on Inoculum Density

8.1 Introduction

Continuous cropping of susceptible hosts is generally recognised as being conducive to increased disease levels. A rotation with tolerant or resistant hosts has the potential of reducing inoculum density because of a lowered viability of propagules resulting from senescence or fungistasis. This study was designed to measure the change in inoculum density of C. *elegans* in containers of soil during four successive plantings of susceptible and resistant host plants and also a complete fallow treatment for that time period.

The trial was conducted over an 18 month period at the Queensland Department of Primary Industries Bundaberg Research Station, to determine the effect of various cropping cycles on disease potential. It was considered important to produce similar growing conditions to actual lettuce production. This open environment exposed the plants and soil to similar conditions of temperature, relative humidity, rainfall and sunlight as field plants. The use of microplots also gave the benefit of avoiding contamination of a field site with the organism. The study was carried out on a raised, concrete slab which was partially enclosed to protect plants from strong wind (Figure 8).

8.2 Materials and Methods

Establishment of Microplots   Large white plastic buckets (0.3 m diameter, 0.4 m height, 0.028 m³ capacity) known locally as 'tomato buckets' were filled with a friable red sandy clay soil classified as krasnozem. The soil was dug from the top 20 cm in scattered patches on a block of ground which had grown sugar cane until ten years previously. Since then, it had been continuously overgrown with a range of dicotyledon and grass weeds and had been periodically slashed or cultivated. No ameliorant treatments had been applied in the intervening period. Such deep soils with red tints prevailing and a gradual increase in clay content with depth are found widely in Australia on old plateaus and divides, often in association with basalt. In the Bundaberg district, krasnozem soils

occupy the higher portions of ridges and are often found in association with black prairie soils, also basaltic, on lower slopes. Krasnozems are volcanic in origin, although this is not necessarily so in other parts of Australia. Krasnozems are quite acidic throughout the profile, which is generally friable permeable clay, marked by a gradual change in texture with depth (Leeper and Uren, 1993). For intensive lettuce cultivation, growers commonly take advantage of the high residual fertility and workable texture of krasnozems and also the less acid, self-cracking black earths of the Lockyer Valley and Darling Downs.

The buckets, or microplots, were located in a position exposed to normal weather conditions. Five 12 mm drainage holes were bored in the base of each bucket. Each microplot thus had a contained microenvironment which could be altered by the imposed treatments. No precautions were taken against possible bucket-to-bucket contamination through the base because the base was considered isolated by 30 cm of soil as well as gravity. Although the soil was allowed to settle in the containers two months prior to planting, it was expected that all existing natural channels and pores in the soil would have been destroyed. This also occurs in the field with intensive cultivation.

The pH of a representative sample of soil was initially 4.8 (saturated paste method). Soil in the containers was adjusted with dolomite (0.25 kg/m^2) with the expectation that it would raise the pH to approximately 6.5 in the following six weeks. A mixed fertiliser (N:P:K 14.8:4.3:11.3) at the rate of 0.15 kg/m^2 was incorporated into the top 15 cm of the soil of each container prior to each planting of lettuce throughout the trial. Weed microplots, not being replanted for each cycle but instead being continuously cropped, were periodically top-dressed with the same mixed fertiliser. During the crop cycles, when nutrient deficiency signs became evident, plants were drenched with a liquid, high nitrogen fertiliser such as Aquasol ® (NPK 23:4:18). Weed control was mechanical (e.g. a trowel) or physical (hand weeding) only, and a granular

nematicide, Nemacur ® (50 g/kg fenamiphos) was applied by hand· when necessary.

Each container was irrigated with a 360 degree microspray, the flow rate of which could be adjusted to produce a wider or narrower trajectory. Initially, the frequency of irrigation for all microplots was governed by two tensiometers (Irrometer ®), one installed at root zone level and one at approximately 30 cm depth. Tensiometers indicate the increase and decrease in soil water potential by a manometer reading of the gradient between water in a tube and the soil water. When a higher reading (40 kPa) in the gauge of the tensiometer in the root zone was reached the containers were irrigated for several minutes. This method proved difficult to calibrate because of the confined space in the containers and the thin leaves of the lettuce, causing the plants to be over-watered or to wilt rapidly. The irrigation scheduling was altered to twice daily waterings of a fixed duration. As the season changed and plants grew, it was necessary to adjust the settings correspondingly. It was difficult to do so efficiently because of the risk of changing the schedule of numerous·adjacent trials, and a process evolved whereby the trajectory of the sprinklers was periodically adjusted to accommodate evaporation, rainfall, and developmental stage of plants. This process was mainly effective. Furthermore, when heavy rain occurred, the containers were covered with a large sheet of plastic in an attempt to avoid leaching of inoculum.

Figure 8. Trial site at Queensland Department of Primary Industries, Bundaberg Research Station.

Figure 8. Trial site at Queensland Department of Primary Industries, Bundaberg Research Station. In the upper photograph, lettuce seedlings are growing in contaminated soil in the container. In the lower photograph, five lettuce plants in the container are approaching maturity and harvest.

Testing for C. *elegans* propagules   Prior existence of *C. elegans* in the 24 microplots was tested with a method based on the carrot test described by Yarwood (1981). Clean fresh carrots were surfaced sterilised with 2% sodium hypochlorite and sliced into 3 mm discs which were placed on sterile moistened filter paper in petri dishes. A small amount of soil (50 mL) was withdrawn from each container, mixed with 150 mL of sterile distilled water, and shaken. After the suspension had settled, two drops of fluid were applied to each disc. Untreated discs were included as controls. The baits were incubated at 16° C in darkness for ten days and inspected after that time for mycelia of *C. elegans*.

Contamination of Microplots with C. *elegans*   One 9 cm diameter petri dish of a Potato Dextrose agar culture of *C. elegans* (Bundaberg isolate 4150), about ten days old, was macerated in a blender with 200 mL of distilled water for one minute at high speed. The macerate was made up to 500 mL and the phialoconidia concentration was estimated to be 1.7×10^6 . mL^{-1} with a haemocytometer. The chlamydospore concentration was estimated to be 8×10^4. mL^{-1} with a haemocytometer. These spores appear to be the main propagule responsible for the long-term survival of this fungus in soil (Holtz and Weinhold, 1994). In a long term study such as this, phialoconidia were treated as the main constituent of the primary inoculum. However, chlamydospores were added with this inoculum to ensure that the fungus was given a chance to persist in all plots, especially those ones where survival was expected to be more difficult.

It was noted that Holtz and Weinhold (1994) used *C. elegans* chlamydospore soil inoculum concentrations of 500 cfu. g $^{-1}$ of soil in selective medium recovery work. However, Papavivas (1964) used an phialoconidial suspension of 5×10^4 . g $^{-1}$ of oven-dried soil in small plots. Although some lettuce growers commonly fumigate lettuce crop land with methyl bromide every second year or so, it was intended that the microplot soil remained unsterilised so that a normal range of micro-organisms could be represented. This fact and also that a lengthy study was intended resulted in a significantly higher number of propagules

being added to the soil to enable a detectable level in the long term. Thus, 1 x 10^4 cfu. mL^{-1} was considered adequate in this case, and the soil in each bucket was uniformly contaminated with *C. elegans* propagules in the following way.

The top 10 cm of soil was collected from each bucket and samples were combined in a cement mixer. Inoculum was added, during mixing, to give a final propagule density of 1 x 10^4 cfu. mL^{-1} of soil. The contaminated soil was replaced in equal quantities in the buckets as a layer over uncontaminated soil.

Planting of Microplots   Four crop cycles were planted. Each crop cycle was followed by sampling of the soil in the rhizosphere and evaluation of roots to determine damage. From July 4th, 1995 (the initial planting of lettuce) to October 11th, 1996 four cycles were completed and they will be referred to as follows:

1st	July - October 1995	(lettuce only, stabilisation cycle)
2nd	November 1995 - January 1996	(mixed plants and fallow)
3rd	February - April 1996	(mixed plants and fallow)
4th	May - October 1996	(mixed plants and fallow)

All containers were initially planted with seeds and also seedling transplants of the susceptible lettuce cultivar Yatesdale. The determination of lettuce only for the first planting was in order to moderate differences in soil propagule number between microplots that the mixing procedure could not overcome. As well, most lettuce producers use seedling transplants but some sow seed in the field directly. Therefore, in order to test for differences in disease susceptibility, the ten plants per container in the first cycle consisted of five from direct seeding and five introduced as 21 day old transplants. This practice was repeated, but with only five plants for lettuce plots, transplanted or directly seeded, during the later three cycles, as described below.

In cycles 2 to 4, the 24 containers were arranged in a randomised block design of six treatments with four replications. The treatments comprised:

1st   A partially resistant lettuce cultivar (Warrior, direct-seeded)

2nd   A susceptible lettuce cultivar (Yatesdale, as 21 day old transplants)

3rd   A susceptible lettuce cultivar (Yatesdale, direct-seeded)

4th   A susceptible weed genus, *Solanum nigrum*

5th   A susceptible weed genus, *Sonchus oleraceus*

6th   A fallow treatment, where irrigation, fertilisation, and weed control occurred as normal during the first normalisation cycle; complete weed control continued throughout the three subsequent cycles.

In cycles 2 to 4, lettuce cv. Yatesdale was now either directly seeded and thinned to five plants per container, or grown separately in a peat/vermiculite medium and transplanted to the containers at the same density. Lettuce cv. Warrior was direct seeded only. Since weed seeds are dispersed randomly in natural conditions, a thicker growth was allowed to develop in the weed plots by selecting volunteer seedlings and removing others at the start of each cycle. In this way, the leaf canopy covered about half the soil area of each microplot at maturity.

Evaluation of Root Damage   When the plants were mature (Figure 9), root damage ratings were made from all plot types. All lettuce were removed and five from each microplot were evaluated after each of the four cycles. Because the first cycle was planted with ten lettuce plants per plot, a random five from each plot were selected and evaluated. For weed plots, having higher plant populations, five plants were chosen at random for testing and the remainder were retained. Both weed genera had strong tap roots. All plants were then transferred to a covered location where soil was gently washed from their roots and they were placed in order for evaluation. Using a dissecting microscope, the root condition was rated according to the following scheme:

0 = No disease
1 = Slight disease on secondary roots only: < 10% damage
2 = Moderate disease on secondary roots: 10-25%, not on tap root
3 = Severe disease on secondary roots: >25-50%, not on tap root
4 = Very severe on secondary roots and some on tap root: >50-75%
5 = Very severe on secondary roots and severe on tap root: >75-90%
6 = No tap root functioning: >90%

Lettuce roots were assessed for disease severity after the first cycle. They were then bulked together, chopped to small pieces, returned to the plots and incorporated into the soil to assist with an even rise in the black root rot inoculum level in all plots. After successive cycles, this process was not repeated and roots were discarded after assessment.

Testing of Soil Propagule Density Using Selective Medium   The selective medium (TB-CENP) modified by Holtz and Weinhold (1994) was tested prior to the main trial and shown to foster growth of colonies of C. *elegans* with a small amount of contamination when incubated at 16°C. The same method was followed after each of the four cycles. It is outlined below, and a complete list of ingredients for the TB-CENP medium is provided in Appendix 2.

The procedure is straightforward but time-consuming. Nevertheless, it is less prolonged than other techniques reviewed in Section 2.1.3. All anti-microbial substances were added after autoclaving the medium and after it had cooled below 48 ° C. A small trowel was used to remove a representative soil sample from each container, i.e. from five sites, close to a rhizosphere if possible and at a depth of approximately 6 cm. Soil from each microplot was bulked and air-dried at 25 ° C. Clay lumps were crushed with a spatula. A 0.5 g subsample was removed and mixed with 100 mL TB-CENP medium.

Each suspension was agitated for several minutes on a rotary shaker, and poured into five 9 cm petri dishes. The soil suspension was poured while swirling to keep the fungicide and soil uniformly suspended. The cultures were incubated

in darkness at 16°C. Colonies were counted when they developed on the surface of the medium between 14 and 21 days. Populations of C. elegans were calculated as the total value per 0.5 g of soil, representing each microplot, and mean values were later derived for each plot.

Selective media prepared for the first and third cycles (and also for the test sample) suffered minor fungal contamination, but only in a few plates and not sufficient to occlude C. elegans colonies. Contamination was worse for the second cycles and for subsequent attempts, with species of Fusarium appearing most frequently. Few other chemicals were available which would be effective against Fusarium and not affect C. elegans. It was decided to substitute hymexazol (Tachigaren ®) at the rate of 1.2 g per litre for etridiazole but this made no difference to the level of contamination during several more soil assays. Therefore, a semi-quantitative approach was used to test soil populations after the fourth and final cycle.

Testing of Soil Propagule Density: Semi-quantitative Method   The semi-quantitative method of Baxter (1990) was adopted at the completion of the fourth cropping cycle. It utilises discs cut from fresh carrots (Figure 10). The following procedure was followed:

The soil sub-sample was air-dried at 25°C. After being passed through a 2 mm sieve, it was mixed with a 0.05% streptomycin sulphate solution to make a paste. The soil paste was applied to completely cover the cut surface of replicated 4 mm thick carrot slices taken from whole carrots previously surface-washed with a 70% ethanol solution. Circles of blotting paper were dipped in a 2.0% solution of diclosan (Dicloran ®, 750 g/kg a.i.) and then used to line 24 petri dishes. The carrot slices were placed in these dishes to form three replicates corresponding to each plot. They were incubated at 24°C ± 1°C for 24 hours. The soil was then removed with a gentle jet of water. The slices were then re-incubated for a further 48 hours after which they were examined

microscopically for the presence of colonies of C. *elegans*. The average number of colonies per disc was recorded.

Statistical Analysis The inoculum density results obtained by the semi-quantitative method and the root damage assessments were tested by one-way analyses of variance. Data was not transformed.

Figure 9. Microplots with mature lettuce plants prior to harvest.
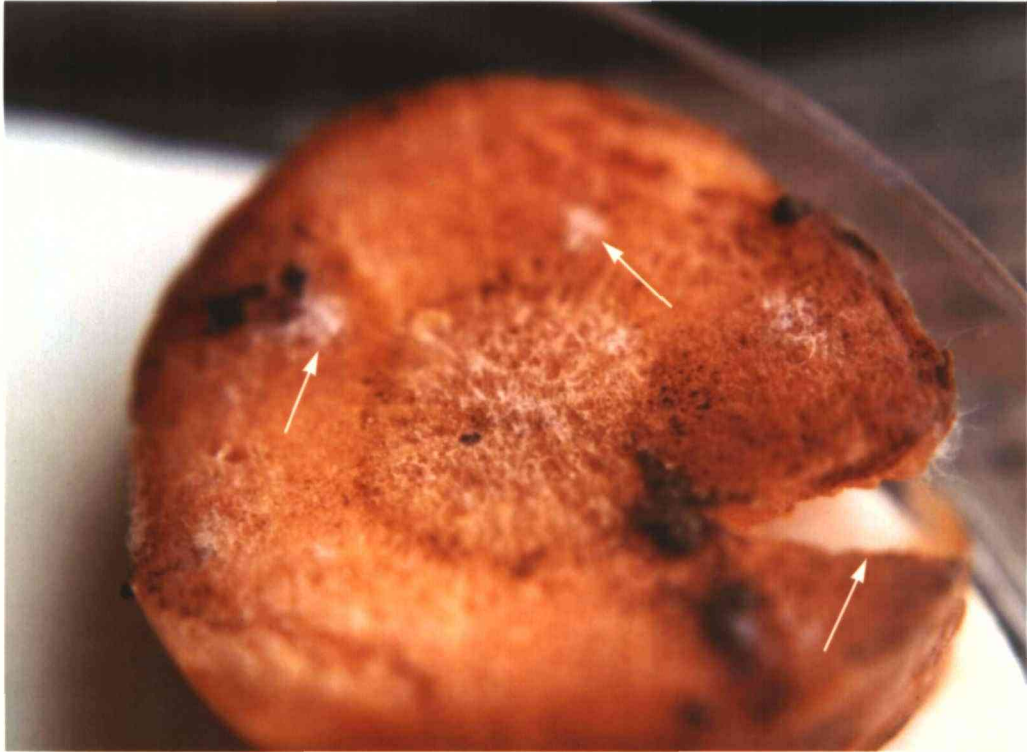
Figure 10. Semi-selective method of assaying soil for colony numbers of *C. elegans*. The carrot disc is pictured close to the completion of the test. Several greyish-white colonies of *C. elegans* are visible (arrowed). The disc has split on the edge due to desiccation.

8.3   Results

General Growth Comments   The soil samples that were tested prior to inoculation were shown to be uncontaminated with *C. elegans*. Lettuce grew readily in this form of large container and tolerated the plant spacings imposed. Root knot nematodes (*Meloidogyne* spp.) were already present in the unsterilised soil and their damage was evident at the end of the first crop cycle. Downy mildew (*Bremia lactucae* Regel) was an occasional problem because of frequent overhead watering. Nutrient disorders developed regularly. Nitrogen deficiency was common, but lack of calcium (tip burn) was evident at hot periods and when watering was inadequate. Regular hand weeding and scuffling with implements maintained lettuce, weed, and fallow plots. It was necessary to regularly remove flowers and seeds from weed plants and to trim them to size.

During the months of higher rainfall (April and May 1996; Figure 11), it was necessary to cover the containers with plastic sheeting several times. However, heavy rain occurred during May 1, 1996. Total rainfall on that day was 155 mm. Because the containers were not covered in time, all overflowed copiously. Monthly totals of rainfall over the period were obtained from the Bureau of Meteorology, based on a weather station adjacent to Bundaberg Research Station, and are represented in Figure 11.

Plants of the second cycle (November 1995 - January 1996) grew through conditions where the average maximum temperature was about 30° C (Figure 12). Average minimum temperatures for the period were 20 - 22° C. Temperatures were milder during the third and fourth cycles but the April - May period was one of heavy rain. Monthly air temperature recordings are represented in Figure 12. It was necessary to use data from Bundaberg Airport, 10 kilometres further inland, after it became impossible to access data from a weather station adjacent to Bundaberg Research Station.
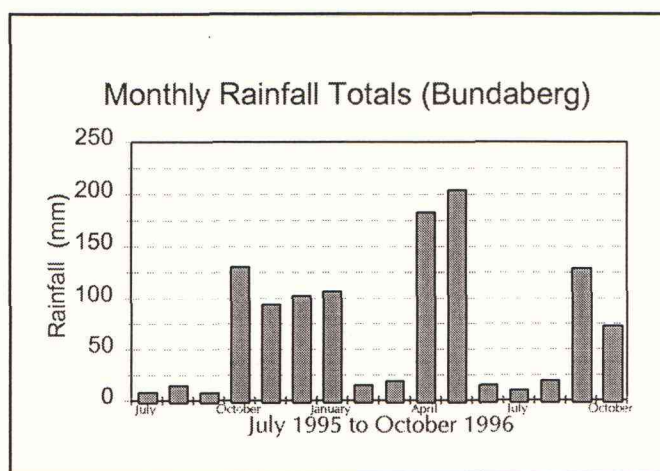
Figure 11. Monthly average rainfall recordings (July 1995 to November 1996) for Bureau of Sugar Experiment Stations, Kalkie Research Station, adjacent to QDPI, Bundaberg Research Station. Data derived from Commonwealth of Australia, Bureau of Meteorology, 1998.
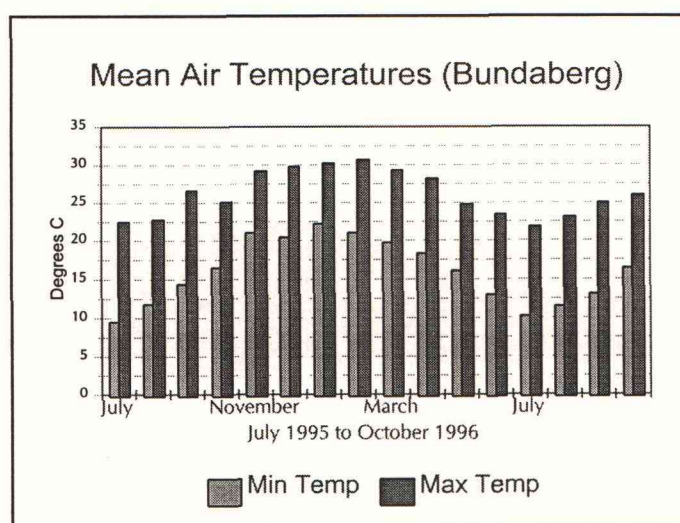
Figure 12. Monthly air temperature recordings for Bundaberg airport, 10 kilometres from Bundaberg Research Station (July 1995 to November 1996). Data derived from Commonwealth of Australia, Bureau of Meteorology, 1998.

Soil Propagule Density The difficulties encountered with fungal contamination and the need to change to a semi-quantitative medium have been detailed above. The results of the TB-CENP selective medium tests are reported

in Table 7. Results for the first cycle of susceptible lettuce only, column A, depict much variability in inoculum density between plots.

Column B depicts the change in inoculum density by April 1996 at the conclusion of the third cycle. At this stage, plots with the susceptible lettuce cultivar Yatesdale contained the highest concentrations of propagules, followed by those with the weeds Solanum and Sonchus, lettuce cultivar Warrior, and fallow plots. Ranked scores for each plot type, derived from the second set of results (Column B) in Table 7, are listed in Table 8. Variability of this data as per analysis of variance was too great to attribute significance to treatments.

Table 7.	Fungal colony counts per gram of soil for each of 24 soil containers after a planting of susceptible lettuce only (July - October 1995) and after two plantings of lettuce cultivars and weeds genera (November 1995 to April 1996). Data for the second cycle were not obtained due to contamination difficulties with the selective medium. Therefore, the column B is an estimate of the six month period from November to April 1995. The data comprises totalled scores for five petri dishes for each container using the TB-CENP selective medium.

No	A. July - Oct 1995	Plot Type	B. November 1995 to April 1996
1	158.0	Yatesdale (t)	133.0
2	148.0	Solanum nigrum	73.3
3	56.0	Fallow	16.6
4	190.0	Solanum nigrum	Missing [c]
5	180.0	Warrior	6.6
6	58.0	Warrior	26.6
7	500.0	Yatesdale (d)	180.0
8	16.0	Solanum nigrum	240.0
9	258.0	Sonchus oleraceus	136.6
10	204.0	Yatesdale (t)	176.6
11	20.0	Fallow	0.0
12	134.0	Warrior	6.6
13	266.0	Sonchus oleraceus	90.0
14	310.0	Sonchus oleraceus	53.3
15	404.0	Sonchus oleraceus	23.3
16	274.0	Yatesdale (t)	110.0
17	370.0	Fallow	50.0
18	260.0	Yatesdale (d)	253.0
19	252.0	Yatesdale (d)	203.3
20	118.0	Yatesdale (t)	260.0
21	8.0	Warrior	156.6
22	172.0	Solanum nigrum	3.3
23	500.0	Fallow	53.3
24	134.0	Yatesdale (d)	56.6

(d) = direct seeded   (t) = transplanted   [c] = heat damaged sample

Table 8.	Averaged and ranked colony numbers of *C. elegans* per gram of soil for each treatment at April, 1996 (end of third cycle).	
	Nature of Plot	**Colony Numbers**
	Yatesdale (d)	173.2
	Yatesdale (t)	169.9
	Solanum nigrum	105.5
	Sonchus oleraceus	75.8
	Warrior	49.1
	Fallow	29.9
	L.S.D. (*P* < 0.05)	2.5459 (Analysis of Variance)

At the conclusion of the four planting cycles, colony numbers using the semi-quantitative method showed greatest density in the soil in the lettuce treatments. Propagule numbers were lowest for the fallow and weed treatments and progressively higher for the Warrior, Yatesdale transplant and Yatesdale direct seeded treatments with the semi-quantitative test (Table 9). No significant differences (*P* < 0.05, Analysis of Variance) existed between both Yatesdale replicates (direct-seeded or transplanted) but they were found between the Yatesdale lettuce and the remaining treatments.

Table 9.	Relative density of propagules of *C. elegans* in soil tested in October 1996 with the semi-quantitative test.	
	Nature of Plot	**Mean**
	Yatesdale (t)	86.15
	Yatesdale (d)	85.25
	Warrior	40.40
	Solanum nigrum	25.65
	Sonchus oleraceus	20.90
	Fallow	8.63
	L.S.D. (*P* = 0.05) [1]	35.40
	(d) = direct seeded (t) = transplanted	[1] = Analysis of Variance

Root Disease Ratings   In the series of four root disease ratings, each made when a planting reached maturity, the highest disease severity for five of the six treatments occurred after the second cycle (Table 10). There was variability from cycle to cycle within each treatment. In general, the susceptible cultivar Yatesdale showed moderate to high disease levels in both the direct-seeded and

transplanted treatments. The less susceptible lettuce cultivar Warrior showed lower levels of disease than the Yatesdale lettuce throughout, but its ratings were much lower after the third cycle in April 1996 than any other time. Root disease ratings of both weed genera were initially low (January 1996) and decreased to zero (October 1996). Significant differences were not present between either Yatesdale treatment but they were found between them and remaining treatments. After the final cycle, significant differences existed between the lettuce treatments on one hand, and the remaining plot types on the other.

Table 10. Root disease severity ratings (0-6) on susceptible and tolerant lettuce cultivars and two weed genera over four crop cycles from October 1995 to October 1996.

Date Evaluated and Plot Type	Treatment Means				Mean Rating	L.S.D. [1]
	1	2	3	4		
October 1995						$P = 0.05$
Yatesdale (d)		3.05			3.05	
January 1996						
Yatesdale (d)	1.50	5.00	5.00	4.20	3.93	
Yatesdale (t)	5.00	5.00	3.80	5.00	4.70	
Warrior	0.60	3.00	3.00	1.40	2.00	
Solanum nigrum	1.00	1.00	0.20	0.00	0.55	
Sonchus oleraceus	0.20	0.40	0.00	0.20	0.20	1.46
April 1996						
Yatesdale (d)	4.00	3.80	3.75	1.80	3.38	
Yatesdale (t)	4.00	3.80	3.80	4.00	4.00	
Warrior	0.40	0.60	0.80	1.00	0.70	
Solanum nigrum	0.00	0.00	0.00	0.00	0.00	
Sonchus oleraceus	0.00	0.00	0.00	0.00	0.00	0.83
October 1996						
Yatesdale (d)	4.00	2.00	4.25	3.00	3.31	
Yatesdale (t)	2.00	3.80	4.00	2.00	2.95	
Warrior	3.00	2.00	2.00	3.00	2.50	
Solanum nigrum	0.00	0.00	0.00	0.00	0.00	
Sonchus oleraceus	0.00	0.00	0.00	0.00	0.00	1.40

(d) = direct seeded   (t) = transplanted   [1] Analysis of Variance

8.4 Discussion

Black root rot is a major disease of important crops such as cotton and tobacco and of several less important crops such as chicory. Studies on aspects of soil populations of *C. elegans* generally have found that the continued cultivation of susceptible hosts increases the soil population (Bateman, 1963; Yarwood, 1981; Holtz and Weinhold, 1994; Chittaranjan and Punja, 1994).

Crop rotation with non-hosts such as safflower, wheat, and barley, and other practices such as summer soil flooding caused soil populations to decline in cotton soils of the San Joaquin Valley (Holtz and Weinhold, 1994). A strong link between soil populations and black root rot severity has been found in studies with cotton and tobacco. Holtz and Weinhold (1994) reported a positive linear correlation between the inoculum density of *C. elegans* and black root rot disease.

The disease is only newly reported on lettuce and there have been no previous studies with a similar focus to these. The studies reported here were designed to test the effect of three cycles of susceptible and partially susceptible crops and fallow treatments on soil populations of *C. elegans* and disease levels in the plants.

The results from this study conducted in microplots generally support the finding of others (Yarwood, 1981; Holtz and Weinhold, 1994; Chittaranjan and Punja, 1994) which were based on field data.

The basis of this study was to introduce a similar number of propagules to each microplot, allow a stabilisation cycle when each was planted to a susceptible lettuce cultivar, and then follow populations through three crop cycles when different treatments were imposed.

Using the TB-CENP method of Holtz and Weinhold (1994), the initial cfu's in the microplots were found to vary between 8 and 500. This was more variable than expected. It was anticipated that initially there would have been a decline due to natural attrition of propagules but the variability would seem to stem from a different cause. Perhaps there was uneven mixing. It is possible that, due to their sticky nature, spores did not mix through the soil as expected. As well, it could not be expected that sampling of soil would be uniform in each container.

With the TB-CENP selective medium, problems were encountered with contaminants which competed with and in some cases overgrew the C. elegans colonies. Similarly, Chittaranjan and Punja (1993), in the process of modifying selective media, encountered much contamination with recovery of C. elegans propagules from soil. Papavivas (1964) used high artificial inoculum levels in soil recovery work with C. elegans. Holtz and Weinhold (1994) modified the VDYA-PCNB medium described by Papavivas with the addition of the fungicide etridiazole for naturally contaminated cotton soils of the San Joaquin Valley, which contain low inoculum levels. Those soils are quite finely textured and therefore different from the krasnozem of Bundaberg. In the initial testing of TB-CENP (see Section 8.2), despite mild levels of contamination, it seemed to be suitable for use in these studies. Problems with non-selectivity of the medium in later tests could be due to changes in the soil microflora to species of Fusarium which were not sensitive to suppression by the anti-microbial ingredients, despite a substitution of dichloran for etridiazole. It is also likely that the density of Fusarium propagules may have increased greatly in the containers. Similar problems of selective medium contamination were reported by G. Baxter (personal communication) in Victoria, who found the use of selective media to be less effective in following propagule density in tobacco soils than a modification of Yarwood's (1946) carrot baiting technique. Due to the continued problems experienced with TB-CENP and the satisfactory performance of Baxter's semi-quantitative carrot disc technique in preliminary tests, this was used in the final assessment of propagule density.

Root Disease Ratings   At the end of the first, stabilisation, cycle the root disease levels in lettuce were moderately severe (3.05 mean for all plants rated) with a range of 1 to 5 for individual plants. Although the plate test showed variability in soil cfu density, these were sufficient to cause moderate levels of disease in all plots. The fact that root disease severity was less variable than plate tests indicates some doubt on the accuracy of plate tests, or possibly the soil sampling technique, since a direct relationship has been found by others. For instance, Bateman (1963) suggested that propagule numbers of C. *elegans* depend upon the amount of lesion surface on the roots.

There were no significant differences in disease severity between transplanted and direct seeded cv. Yatesdale in any cycle. Although it could be expected that direct seeding should increase disease levels through a longer period of contact between root system and pathogen, this did not happen. Black root rot thus differs from the bacterial disease corky root (caused by *Rhizomonas suberifaciens*) which elicits more severe disease symptoms on direct seeded lettuce (van Bruggen and Rubatzky, 1992). In the case of corky root, the lower disease level on transplants is thought to be due to a form of mature plant resistance. The "root nibbling" symptom of black root rot apparently affects the majority of small roots of both direct seeded and transplanted Yatesdale lettuce. This debilitating invasion causes poor plant growth following both methods of plant establishment.

In the first crop cycle (January 1996), root disease levels were as anticipated with the Yatesdale treatments being significantly more affected than any other. It was expected that the two Yatesdale lettuce treatments would incur significantly more damage than the cv. Warrior lettuce and weed plots. Similarly, after the second crop cycle in April, root damage ratings showed a strong trend of moderate damage in the Yatesdale plants and mild damage in Warrior plants. This was generally in agreement with the selective medium results at that time.

At the conclusion of the third crop cycle (October 1996), root damage ratings grouped Warrior lettuce with the more susceptible lettuce plots, but narrowly so. However, while the soil assay test for that period confirmed the general ranking of susceptibility from previous cycles, it grouped the lower propagule numbers for Warrior lettuce with weeds and fallow plots as previously. It is possible that the more severe damage sustained by Warrior plants at the conclusion of the third cycle, sustained during the winter period, may be due to an interaction between the cultivar, the season, and the pathogen because, as noted in the Introduction, lettuce are highly sensitive to changes in temperature.

Overall, cfu numbers showed a strong trend of increase in the presence, and much lower populations in the absence, of a susceptible host. Similarly, Bateman (1963) reported an increase in populations in rhizosphere soils of host plants (bean) for a period of up to thirteen weeks. However, in non-rhizosphere soils of bean, and rhizosphere and non-rhizosphere soils of a non-host plant (corn), no increase was recorded and no difference between the three environments was noted. In this trial, rhizosphere soil was sampled, and it is likely in this case, as Bateman (1963) and Reddy and Patrick (1989) also suggested, that the increase in pathogen numbers resulted from sporulation in lesions on the surface of diseased roots, rather than saprophytic growth of the fungus in soil.

Detectable propagule numbers in weed plots, higher than those in fallow plots, but with the absence of visible root damage suggest a level of saprophytic activity by the pathogen. Perhaps, because weed plants in containers were self-sown, thinned, and not re-introduced from a seed bank external to the trial, more robust and therefore more resistant plants were unwittingly selected to be retained. A tendency for C. elegans to associate with the roots of various plant species in the absence of any visible symptoms has been noted above in Section 2.2.1., citing the work of Yarwood (1946) and Chittaranjan and Punja, (1994).

Fallow plots contained lower but still detectable populations of propagules after a twelve month period. Their ability to survive in the absence of a susceptible host as chlamydospores has been frequently recorded (e.g. Mauk and Hine, 1988; Chittaranjan and Punja, 1993; Oyarzun et al., 1997). Evidently, it would not be possible to eradicate or even effectively reduce populations of C. elegans propagules by bare fallowing alone for a year. However, if soil amendments were incorporated such as rye (Secale cereale L.), a significant population decrease due to microbial antagonism and the release of volatiles and other substances might be expected (Reddy and Patrick, 1989; Chittaranjan and Punja, 1994; Fravel et al. 1996). Nevertheless, the work here has shown that planting of a susceptible lettuce crop after even a long fallow period would likely result in a rapid increase of propagules of the black root rot pathogen.

9. General Discussion

The research of this thesis considered questions concerning the nature and dissemination of *C. elegans*. In particular, investigations were made into the introduction of the pathogen to this country, and to specifically to propagation and growing areas *via* sphagnum peat, and the further spread of infection by contaminated nursery containers and other susceptible genera. The major agronomic component of the thesis was to investigate the change in propagule numbers with respect to susceptible and resistant host genera, and bare fallows. Finally, mycological studies included studies of the infection process and of the effect of micro-environmental factors upon phialoconidial viability. The results of these studies are conceptualised in Figure 13. Central elements of the epidemiology of *C. elegans* will be discussed below. They centre upon the introduction and transmission of the pathogen, and the black root rot disease cycle.
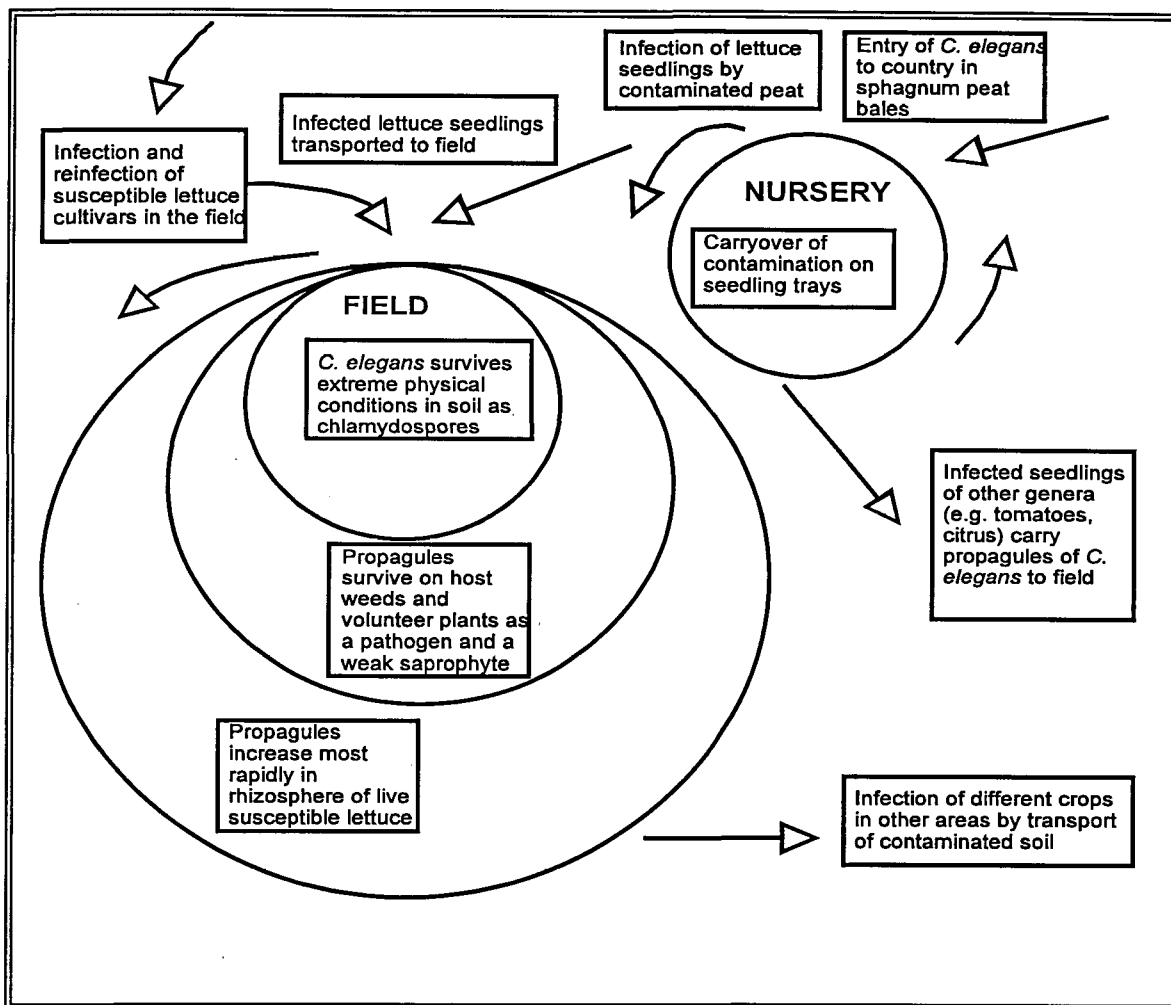
Figure 13. Probable epidemiological cycle of *C. elegans* in seedling nursery and field.

9.1 Introduction and Transmission of Propagules of *C. elegans*

Tests which were described earlier in this thesis demonstrated that sphagnum peat bales may contain *C. elegans* propagules which are transported directly to areas of propagation media preparation. The low pH (4.0 in all bales tested in the Bundaberg region) and low moisture content of the peat provide unusually harsh conditions for active plant and fungal growth, and existence of the propagule would be passive until the appropriate stimulus was provided in the nursery or field for the primary infectious propagule, probably the chlamydospore, to germinate.

Large numbers of phialoconidia, densely massed, germinate only sparsely until the propagules become isolated by some means. In the nursery situation, cross-infection between containers by water splash or aerosols produced during overhead watering is possible. It was noted that C. elegans propagules may have an ability to spread in a glasshouse situation because in some way, uninoculated containers became contaminated during the course of the seedling container experiment (Section 7.). It is suggested that conidial transfer by splash or aerosol may have occurred, and although such an agency was not actually demonstrated, the presence of fungus gnats foraging in the surface layers of the seedling mix was observed. Prior contamination of the uninoculated trays by the peat-based potting mix used to grow the uninfected plants is doubtful because of the high proportion of infected plants. Furthermore, although the gnats may have assisted the movement of inoculum to uninoculated containers, the lengthy time in the glasshouse was sufficient time for several disease cycles to have been completed.

Transfer of propagules also occurred between successive batches of lettuce plants as a result of incomplete disinfection of seedling containers. Especially for polystyrene containers, which are rough textured, more difficult to physically clean than smooth plastic containers and more likely to afford protection to pathogens, only sterilisation with fumigants or steam or an application of benomyl is likely to be completely effective against C. elegans. Although sodium hypochlorite has been demonstrated to be efficacious in other studies (Copes and Hendrix, 1997b), it failed to prevent carry-over of C. elegans in the polystyrene containers commonly used in Queensland in this experiment. Because sanitisation also controls other fungal pathogens, bacterial pathogens and nematodes, it is the preferred treatment where containers are suspected or known to be contaminated and must be reused. However, the practice of disinfecting equipment against this serious, successful and long-lived pathogen is really the last line of defence. Instead, seedling growers should be advised to institute preventive measures such as avoiding use of peat-based seedling mixes

or steam sterilising the potting mix. The simple carrot disc test on which the survey hinged was most effective and its routine use as a bait is recommended in production nurseries for tests of seedling mixes and soil to detect the black root rot pathogen.

Investigation of the infection process confirmed the presence of appressoria in facilitating entry to the epidermis of lettuce roots (Figure 14). Both spore forms initiated invasions of resistant and susceptible cultivars without discrimination (Figure 15). The mechanism of resistance by lettuce against C. elegans does not depend on host-pathogen mutual recognition. Instead, it becomes effective in that period after the initial growth of hyphae and production of spores. Methods of resistance and the degrees of resistance might be envisaged in a number of ways. The microscopic basis for partial resistance to C. elegans usually involves some mechanical isolation of the pathogen soon after penetration of the root. This has been described as lignified cells in peanuts (Jones, 1991) and cork layer formation in tobacco (Shew and Shew, 1994). During the preparatory trials for this project, hypersensitive reactions in lettuce were not observed and no microscopic evidence was obtained of cell wall strengthening in response to fungal invasion. More probable mechanisms include the secretion by the root cortex of compounds which actually preclude fungal colonisation and perhaps, phytoalexin production. This suggestion is made with the assumption that the compounds are not too readily detoxified by the pathogen (Kema et al., 1996).

O'Brien (1997, personal communication) described the larger root bulk of highly resistant lettuce cultivars which had been challenged by C. elegans and had, far from suffering less root mass destruction, been stimulated to produce bulkier roots with no disease. This is in accord with the observation of Yarwood and Karayiannis (1974) that, in different situations, C. elegans may be pathogenic to one species but symbiotic to another species of a higher plant genus. The scenarios which underlie what is possibly a symbiotic relationship
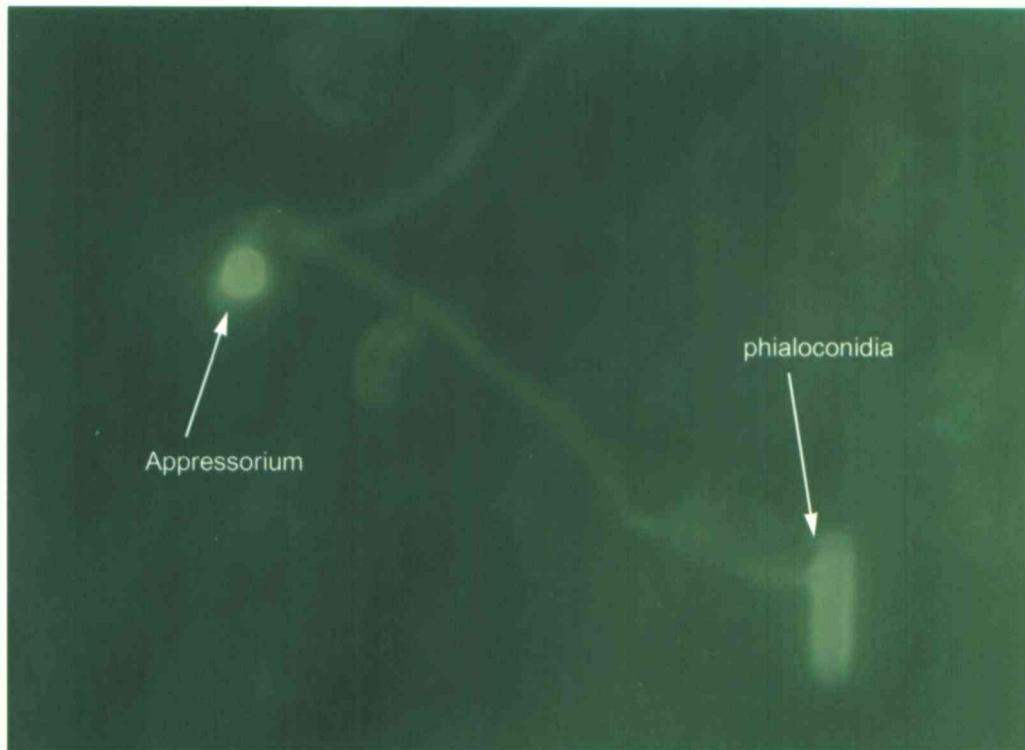
Figure 14. Appressorium produced by infection peg of *C. elegans* immediately prior to invasion of the root of a susceptible cultivar of lettuce.
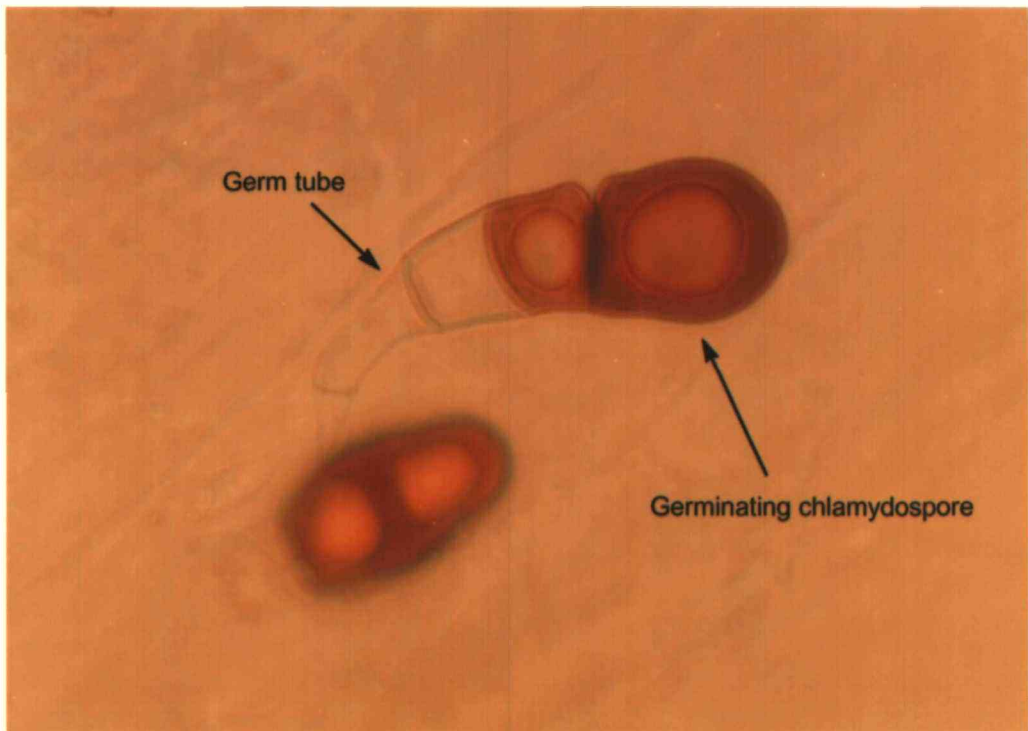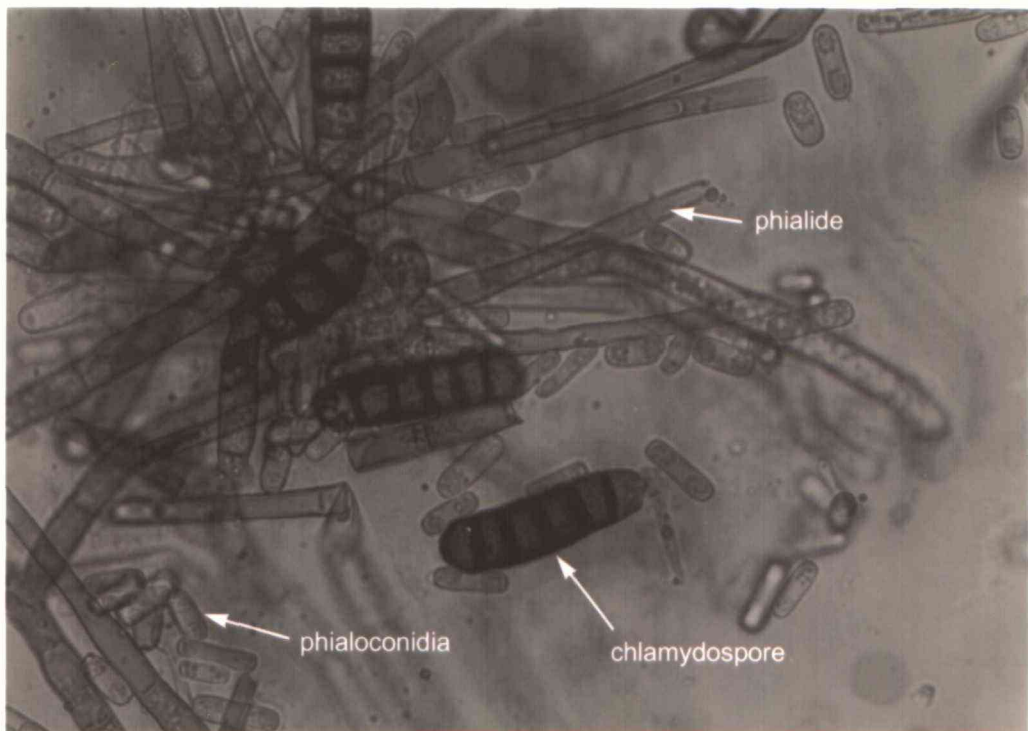
Figure 15. Phialoconidia, chlamydospores and hyphae of *C. elegans*.

9.2   The Black Root Rot Disease Cycle

Some fungal pathogens e.g. *Puccinia* spp., *Phytophthora* spp., produce inoculum which is multiplied step-wise during the growing season. Vanderplank (1963, in Zadoks and Schein, 1979) described this cycle as polycyclic disease. On the other hand, monocyclic root pathogens such as *Cylindrocladium crotalariae* disperse no secondary inoculum (Zadoks and Schein, 1979). Pathogens such as *C. elegans* are in an intermediate or heterogeneous category because initial infections may result in limited production of secondary inoculum capable of initiating additional disease cycles (Shew and Shew, 1994). Heterogeneous disease cycles are more likely to be controlled by sanitation than compound interest cycles, as suggested earlier in this thesis, because the final disease level is more directly related to the initial inoculum level.

Clear trends were found in the work described here for highest propagule numbers in microplots containing susceptible genera and decreasing with an increase in host resistance. Fallow microplots yielded fewer but still detectable numbers of propagules.

The trends found here generally concur with other work. For instance, Holtz and Weinhold (1994) found that the inoculum of the black root rot organism builds up gradually each year only in the presence of a host, and decreases in soil planted with non-hosts or in fallow soil. They recorded a positive correlation between inoculum densities of *C. elegans* and the number of years spent growing cotton, a susceptible host. Likewise Bateman (1963) reported an increase of inoculum in the rhizosphere of susceptible host plants, most probably produced by sporulation on lesions upon the root and hypocotyl. During the study reported in this thesis, a wide range of susceptible hosts of *C. elegans* was tested. If present as weeds or used as rotational crops in field situations, they are likely to foster an increase in the level of soil inoculum, and facilitate seasonal carryover of disease to new plantings.

Regarding the survival of C. *elegans* in fallow soil, Bateman (1963) suggested that non-host plants such as wheat and corn did not reduce populations any more effectively than fallow treatments. Experience in the work reported in this thesis is that fallow treatments foster the lowest number of propagules. However, as has been suggested in the preceding section, even an extended period of fallow would be ineffectual in eliminating propagules of C. *elegans.*

This research has also demonstrated the hemi-biotrophic nature and activity of the black root rot fungus. Although propagule numbers did not increase in a fallow environment, the conditions were not sufficient to destroy them during a twelve month period. During the course of investigations related to this project, hyphae of C. *elegans* have not been observed to invade dead root hairs. To observe the infection process, lettuce roots were placed in a small amount of tap water in a covered petri dish and the process of infection was observed over several days. Hyphal growth and profuse sporulation occurred on the tissues until necrosis, at which stage different microflora always appeared and predominated. It may be that if C. *elegans* were the only organism present, colonisation of dead roots may occur, but it is considered unlikely that under natural circumstances, C. *elegans* would be able to compete with faster growing saprophytes.

Hood and Shew (1997) observed invasion of dead root hairs of tobacco seedlings by C. *elegans*, although the hypha did not continue to invade further and did not form spores inside the host cells. More importantly, in those studies the behaviour of C. *elegans* appeared to be characteristic of invasion of a living host because mutual host/parasite recognition was evidenced by movement of host cytoplasm. Sporulation occurred in necrotic tissue once cytoplasmic viability had been compromised. Thus, biotrophic infection was followed by necrotic sporulation.

It is unlikely that the survival of *C. elegans* propagules for twelve months can be attributed to acquisition of residues of lettuce and successful competition in the soil as a saprophyte. Furthermore, in view of the argument of the preceding paragraphs, it is doubtful that control of *C. elegans* can be achieved by saprophytic competition or antagonism by other fungi, bacteria, and actinomycetes. This statement is made notwithstanding the fact that enhanced microbial activity of fungi and bacteria is known to reduce the extent of survival of chlamydospores of *C. elegans*. In fact, saprophytic competition between various micro-organisms is probably already encouraged in lettuce soils, for the same physical conditions which are induced to foster the rapid growth of lettuce are those characteristic of the saprophyte-rich "suppressive" soil described by Reddy and Patrick (1989), Fravel *et al.* (1996), and Larkin *et al.* (1996). They include high pH, water potential, organic matter, clay content and possibly the presence of residues originating from decomposing pasture plants such as rye (*Secale cereale* L.). However, if *C. elegans* was a facultative saprophyte, it would be dependent upon the acquisition of residues in the absence of a living host. Then, in natural or unsterilised soil in the field situation, it would directly compete for those residues with other saprophytes which are quite likely to prove more successful in doing so.

Although black root rot disease re-emerges in susceptible hosts during successive growing seasons, the success of *C. elegans* propagules in surviving the absence of host plants to initiate disease can be attributed to two particular qualities - the enduring nature of chlamydospores and the hygroscopic properties of the mucilage surrounding phialoconidia. It is timely to agree with Lucas (1975, in Reddy and Patrick, 1989) that the fungus is capable of indefinite survival in soil, even in the absence of host plants.

Table 11 summarises the survival methods of *C. elegans* which may be concluded from the results of these trials. Although the two weed genera (*Solanum nigrum* and *Sonchus oleraceus*) used in some studies of the work

reported here were initially found to be susceptible to *C. elegans* (rating 1 out of a very susceptible 5), disease levels later in the container trial decreased to zero, and the two weed genera were now regarded as resistant to Isolate 4150 of *C. elegans*. As has been noted in Section 8, this may have resulted from unintentional selection of more resistant seedlings from the existing volunteers.

Table 11.   Effect of host status on survival of propagules of *C. elegans*.

Host Plant	Status	Remarks
Lettuce cv. Yatesdale	Susceptible host	No resistance and little tolerance; ideal environment for disease replication by production of secondary inoculum in lesions on root and hypocotyl surface
Lettuce cv. Warrior	Less susceptible host	A level of resistance and also disease tolerance afford a more modest increase in propagule numbers
Sonchus oleraceus	Resistant host	Organism survives as a hemibiotroph in rhizosphere with a smaller increase in propagule numbers
Solanum nigrum	Resistant host	Organism survives as a hemibiotroph in rhizosphere with a smaller increase in propagule numbers
Bare fallow		Cfu's slowly decline; organism survives indefinitely as chlamydospores and for a shorter period of time as phialoconidia

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11. Appendices

Appendix 1

Super-saturated Solutions and Corresponding Atmospheric Relative Humidity (Griffin, D., 1972)

Salt	Humidity
$LiCl.H_2O$	15
$KCl.3H_2O$	20
$CaCl_2.6H_2O$	42
$NaHSO_4.H_2O$	52
$NaNO_2$	66
$NaClO_3$	75
$(NH_4)_2SO_4$	81
$KHSO_4$	86
$ZnSO_4.7H_2O$	90
K_2HPO_4	92
$NH_4H_2PO_4$	93
$Na_2HPO_4.12H_2O$	95
$CuSO_4.5H_2O$	98

Appendix 2

Modified TB-CEN-pentachloronitrobenzene (TB-CENP) Selective Medium

(Holtz and Weinhold, 1994)

INGREDIENTS PER LITRE

- Fresh carrot extract (200 mL) prepared by blending 100 g fresh carrots at high speed for 2 min with 200 mL of distilled water
- 1.2 g of Terrazole (35% wettable powder)
- 1 g of nystatin
- 500 mg of streptomycin sulphate
- 30 mg of chlortetracycline HCl
- 1 g of $CaCO_3$
- 17 g of agar

pH $\approx$ 5.2

16 $^{\circ}$ C, 21 day incubation

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