Fungal diseases in *Eucalyptus* and *Acacia* nurseries in South Africa

Submitted by

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University.

Lorenzo Lombard

February 2004

This thesis is dedicated to my parents, Lorenzo Z. C. Lombard and Lee E. Lombard

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The forestry nursery system in South Africa has undergone major changes in the past two decades, with the implementation of clonal forestry. Vegetative propagation of superior *Eucalyptus* hybrid clones have resulted in uniform, high – value plantations. However, losses to planting stock in nurseries can severely affect planting programmes. Most losses in South African forestry nurseries are caused by fungal diseases. *Acacia mearnsii* (black wattle) seedlings are severely affected by an unknown *Cylindrocladium* species in nurseries. Diseases of *Eucalyptus* cuttings and *A. mearnsii* seedlings are a serious threat in forestry in South Africa and require further study.

Chapter one of this dissertation presents a review on the pathogens affecting crops in hydroponics and possible implications of diseases in *Eucalyptus* hedge plants maintained in hydroponics. The aim of this review is to consider the prospect of *Eucalyptus* nursery pathogens becoming a limiting factor in hydroponic systems and it also treats the general topic of diseases in South African forestry nurseries. No information is available on pathogens related to *Eucalyptus* spp. in hydroponics. The possible pathogens, potential symptom expression and possible control measures are considered. This sets the stage for the rest of the dissertation and highlights the importance of nursery pathogens.

The experimental section of this dissertation focuses on nursery diseases of *Eucalyptus* and *Acacia mearnsii*. In chapter two, a survey of *Eucalyptus*

hedge plants, maintained in an ebb and flow hydroponic system, was conducted to determine which pathogens are present. Fungal isolates obtained during the survey were characterized based on morphology and where appropriate, identifications were supported by DNA sequence data comparisons.

Chapter three of this dissertation considers the importance of pathogens in *Eucalyptus* cutting production. A survey was conducted to determine which pathogens influence productivity in cutting production nurseries in KwaZulu – Natal, South Africa. A dominant pathogen was identified based on morphological characteristics and DNA sequence data comparisons. A pathogenicity test was also conducted to determine the susceptibility of *Eucalyptus* hybrids to the pathogen.

In chapter four, the identity of an unknown *Cylindrocladium* sp. affecting *Acacia meamsii* seedling production is identified. This fungus was characterized based on morphological characteristics and using DNA sequence data comparisons. The pathogenicity of this pathogen was also considered.

Chapter 1

An evaluation of the potential importance of pathogens to *Eucalyptus* plants propagated in hydroponics

Introduction

During the course of the past two decades, the South African forestry industry has made the major step of implementing clonal forestry (Van Wyk 1985, Denison & Quaile 1987). Thus, vegetatively propagated tropical and sub – tropical *Eucalyptus* spp. and their hybrids have been deployed widely in commercial plantations in South Africa (Denison & Kietzka 1993a,b). Clonal forestry allows for a small number of clones to be vegetatively propagated (White 1995) resulting in uniform plantations of selected, high value trees (Kulkarni & Lal 1995). The development of clonal hedge plants that are necessary for vegetative propagation is time consuming (Van Wyk 1985) and can take up to 18 months before they become productive (Wilson 1998, Aimers – Halliday *et al.* 1999). Mondi Forests in South Africa has thus embarked on the innovative step of speeding up clonal reproduction through the production of clonal – hedge plants using hydroponics.

Hydroponics represents the technology of growing plants in nutrient solution with or without the use of a substrate to provide mechanical support to the root system (Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Jensen 1997, Jensen 1999). In a liquid hydroponic system, no inert substrate is used, while in an aggregate hydroponic system inert substrates such as those composed of sand, gravel, peat, perlite, vermiculite and rock wool are utilized. Hydroponic systems are also further classified as being either open or closed systems. In closed systems, the nutrient solution is recovered, replenished and recycled

following the direct delivery to the root systems. In open systems, the nutrient solution is not reused (Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Jensen 1997, Jensen 1999). Various types of hydroponic systems are commonly used in commercial nurseries. These include the nutrient film technique (NFT), deep flow technique, trough culture, ebb and flow, rock wool culture, sand culture and bag culture (Graves 1983, Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Jensen 1997, Paulitz & Belanger 2001).

Hydroponic crop production is, in combination with greenhouses, a high - technology and capital-intensive approach (Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Jensen 1997, Jensen 1999). It allows for high-density maximum crop yield, production of crops in areas where unsuitable soils exist, a lack of dependence on ambient temperature and seasons, more efficient use of water and fertilizers and minimal land use. Hydroponics systems lend themselves to mechanization, which also enhances production. The plants can be isolated from the soil, which is often associated with disease problems, salinity, poor structure and drainage (Stanghellini & Rasmussen 1994, Jensen 1999). Major disadvantages of hydroponics include the high costs of capital and energy inputs as well as requiring a high degree of competence in plant science and engineering skills. High operation costs of hydroponic systems have tended to limit this technology to high economic value crops (Stanghellini & Rasmussen 1994, Jensen 1999).

Hydroponics is currently employed worldwide to produce flower, foliage, bedding plants and high value vegetable crops (Jensen & Collins 1985, Stanghellini & Rasmussen 1994). In South Africa, *Eucalyptus* clonal-hedge plants are grown in an ebb and flow system for cutting production (Dr. B. Janse, personal communication). These systems are based on a "flood and drain" principle that allows for the rapid production of *Eucalyptus* clonal cuttings.

One of the problems that can be associated with hydroponics is that of root diseases and especially diseases that are suited to spread in liquid media. Given that hydroponics is new to forestry in South Africa, virtually nothing is known regarding the possible impact of diseases in this system.

Several reviews and surveys have been published on diseases of *Eucalyptus* spp. in nurseries (Sharma *et al.* 1984, Sharma *et al.* 1985, Crous *et al.* 1991, Sharma & Mohanan 1992, Viljoen *et al.* 1992, Brown & Ferrreira 2000). These have included diseases in both seedling and clonal cutting systems. To the best of our knowledge, there have, however, been no reviews of diseases in nurseries where *Eucalyptus* are grown in hydroponics. The aim of this review is not to repeat the contents of previous reviews on diseases in *Eucalyptus* nurseries, but rather to consider the prospect of *Eucalyptus* nursery pathogens becoming a limiting factor in plants grown in hydroponics. The possible pathogens and potential symptom expressions that might occur in and on the hydroponically grown plants will be discussed and possible control measures that might be applied considered.

Eucalyptus Nursery Diseases

The nursery system for a *Eucalyptus* planting programme is a key component of any plantation industry. The planting programme can be severely affected by the loss of planting stock or by the production of inferior plants (Brown & Ferreira 2000). Both abiotic and biotic agents can cause damage to planting stock, with conditions in nurseries being very conducive to disease (Viljoen *et al.* 1992, Alfenas *et al.* 1997, Brown & Ferreira 2000).

Fungi are responsible for the majority of the diseases associated with *Eucalyptus* species in nurseries (Viljoen *et al.* 1992, Alfenas *et al.* 1997, Brown & Ferreira 2000). These diseases include death of germinating seedlings, foliar, stem and root diseases (Alfenas *et al.* 1997, Brown & Ferreira 2000). The most important diseases include damping – off, root rot, seedling, shoot or web blight, leaf spots, mildew and rust (Peterson & Smith 1975, Sharma *et al.* 1984, Sharma *et al.* 1985, Sharma & Mohanan 1992, Viljoen *et al.* 1992, Alfenas *et al.* 1997, Brown & Ferreira 2000).

Damping – off is a disease of young seedlings prior to lignification, and leads to the collapse of the seedling (Brown & Ferreira 2000). Infection takes place at the soil – air interface and will result in girdling diseases (Holliday 1990). Sharma *et al.* (1985) showed that a complex of pathogens could be responsible for the disease, which can occur prior to emergence (pre-emergence damping – off) or after seedlings have emerged (post-emergence damping – off). Damping – off of

Eucalyptus seedlings is caused by soilborne, seedborne or waterborne fungi belonging to genera *Botrytis* P. Micheli ex Pers., *Calonectria* de Not., *Cylindrocladium* Morgan, *Fusarium* Link, *Phytophthora* de Bary and *Rhizoctonia* DC. (Sharma *et al.* 1984, Sharma *et al.* 1985, Crous *et al.* 1991, Blum *et al.* 1992, Sharma & Mohanan 1992, Alfenas *et al.* 1997, Brown & Ferreira 2000, Sanfuentes *et al.* 2002).

Root rot is the second most commonly found disease in forestry nurseries (Sharma *et al.* 1985, Sharma & Mohanan 1992, Alfenas *et al.* 1997). This disease leads to the slow wilting of seedlings and cuttings (Sharma *et al.* 1985, Sharma & Mohanan 1992). Root rot is characterized by a change of pigmentation in the leaves from green to light purple. Within a week of infection, the pigmentation change moves downwards and is followed by wilting and eventual death of plants. The roots of plants are completely damaged with the roots turning light or dark brown. The infection usually begins in the lateral roots and proceeds to the main root system. The pathogens can then move into the root collar region causing decay and death (Sharma *et al.* 1984, Sharma *et al.* 1985, Crous *et al.* 1991, Crous *et al.* 1993, Sharma & Mohanan 1992, Viljoen *et al.* 1992). Some genera of pathogens responsible for root rot of *Eucalyptus* in nurseries include *Rhizoctonia* (Sharma *et al.* 1985, Sharma & Mohanan 1992, Viljoen *et al.* 1992, Alfenas *et al.* 1997, Brown & Ferreira 2000, Sanfuentes *et al.* 2002), *Phytophthora* spp., *Pythium* Nees spp. (Marks & Kassaby 1974, Von Broembsen 1984, Sharma & Mohanan 1992, Brown & Ferreira 2000) *Cylindrocladium* spp (Hodges & May 1972, Crous *et al.* 1991, Sharma &

Mohanan 1992, Crous *et al.* 1993, Alfenas *et al.* 1997, Brown & Ferreira 2000) and *Fusarium* spp. (Arya & Jain 1962, Sharma *et al.* 1985, Brown & Ferreira 2000).

Seedling and shoot blight are related diseases that share the same symptoms (Sharma *et al.* 1985, Crous *et al.* 1991). Seedling blight results from infection of either the stem of the seedlings or cuttings, or the leaves. Shoot blight is characterized by infection of the stem at soil level (Bolland *et al.* 1985, Sharma *et al.* 1985, Crous *et al.* 1991, Crous *et al.* 1993, Alfenas *et al.* 1997, Sanfuentes *et al.* 1999). Infection of the stem leads to the development of a grayish brown to dark brown lesion on the stem, which leads to desiccation of the seedlings or cuttings (Bolland *et al.* 1985, Sharma *et al.* 1985, Crous *et al.* 1991, Crous *et al.* 1993, Sanfuentes *et al.* 2002). Leaf infections usually begin at the tips of the leaves and extend down the length of the leaves towards the stems where lesions develop (Sharma *et al.* 1985, Sharma & Mohanan 1992). In conditions of high humidity, such as those encountered in greenhouses, these lesions can give rise to masses of spores and this increased inoculum significantly exacerbates disease conditions. Pathogens responsible for both seedling and shoot blight include *Rhizoctonia solani* Kuhn, *Botrytis cinerea* Pers., *Phytophthora* spp., *Cylindrocladium* spp. and *Cylindrocladiella* Boesew. spp. (Barnard 1984, Bolland *et al.* 1985, Sharma *et al.* 1985, Crous *et al.* 1991, Crous *et al.* 1993, Alfenas *et al.* 1997, Crous 2002, Sanfuentes *et al.* 2002).

Web blight is a common *Eucalyptus* seedling and cutting disease, occurring in conditions of over – crowding and high humidity (Sharma & Mohanan 1992, Alfenas *et al.* 1997). This disease is caused by *Rhizoctonia solani* and is characterized by mycelium emerging from the soil to grow epiphytically on the stems and leaves of plants. This mycelium gives rise to a cobweb – like profuse mycelial growth that entangles the infected seedlings or cuttings (Sharma *et al.* 1985, Sharma & Mohanan 1992, Alfenas *et al.* 1997, Sanfuentes *et al.* 1999, Sanfuentes *et al.* 2002).

Leaf spot diseases are commonly found on *Eucalyptus* seedlings and cuttings in nurseries. These leaf spots range in appearance from small, round water – soaked lesions, red to black in colour to larger irregular necrotic lesions. Several genera of pathogens are responsible for leaf spot and some of them are *Hainesia lythri* (Desm.) Hohn. (Palm 1991), *Mycosphaerella* Johanson spp., *Phaeophleospora* Rangel spp. (Crous *et al.* 1989a,b), *Cylindrocladium* spp. and *Cylindrolcadiella* spp. (Bolland *et al.* 1985, Sharma *et al.* 1985, Crous *et al.* 1991, Viljoen *et al.* 1992, Crous *et al.* 1993, Alfenas *et al.* 1997, Crous 2002).

Powdery mildew can cause significant nursery problems but rarely kills *Eucalyptus* seedlings and cuttings. Powdery mildews are responsible for symptoms that include leaf distortions, shoot discolouration and reduction in growth of nursery stock (Marks 1981, Marks *et al.* 1982, Brown & Ferreira 2000). Powdery mildews produce superficial mycelium on the surface of leaves and shoots and are regarded as obligate, biotrophic parasites on a wide host range

(Brown & Ferreira 2000). Infections are caused by air-dispersed conidia that germinate on dry surfaces in low humidity. The presence of free water inhibits germination (Sinclair *et al.* 1987). Seven species residing in the genera *Erysiphe* R. Hedw. Ex DC and *Sphaerotheca* Desv. cause powdery mildew of *Eucalyptus* (Brown & Ferreira 2000).

Eucalyptus rust, caused by *Puccinia psidii* Winter, is a potentially severe treat to *Eucalyptus* production worldwide (Alfenas *et al.* 1997, Coutinho *et al.* 1998, Alfenas *et al.* 1999). *Puccinia psidii* forms yellow uredinial pustules on juvenile *Eucalyptus* tissue that leads to necrosis and stunting of the infected tissues (Coutinho *et al.* 1998, Alfenas *et al.* 1999). Severe infections can occur under favourable conditions at mild temperatures and prolonged leaf wetness (Alfenas *et al.* 1999). Fortunately South Africa is still free of this pathogen but it has been reported from nurseries in South – and Central America and Asia (Coutinho *et al.* 1998).

Factors leading to disease development in hydroponics

The main driving force for the development of hydroponics in agriculture has been the avoidance of root diseases (Zinnen 1988, Stanghellini & Rasmussen 1994). Most common agricultural crop diseases are caused by soilborne pathogens (Stanghellini & Rasmussen 1994) infecting the roots and stems of these crops. Isolating the plants from these soilborne pathogens has become

necessary for many intensively grown crops where enhanced productivity has been required (Jensen & Collins 1985).

Hydroponic cultivation has led to a decrease in the diversity of root-infecting microorganisms (Stanghellini & Rasmussen 1994, Zinnen 1988) but a few pathogens have become more prominent and devastating in hydroponic systems (Paulitz 1997). Stanghellini & Kronland (1986) found that *Pythium dissotocum* Drechs caused a yield loss of more than 50% in lettuce grown in hydroponics, while this pathogen is not a problem in soil grown lettuce. This indicates that disease losses of hydroponically grown crops can occasionally be greater than soil grown crops.

Several characteristics of hydroponics can increase the occurrence of disease (Paulitz & Berlanger 2001). Generally, clones of one genotype are grown in hydroponics, thereby reducing genetic diversity. Furthermore, these plants are planted at high densities and this can favour the movement of pathogens. A similar situation exists in conventional forestry practices where genotypic uniform hedges are cultivated in soil. However, soil provides biological "buffering", thereby limiting root-infecting pathogens by antagonism from other microorganisms and is subjected to nutrient competition and fungistatis (Paulitz 1997). In recirculating hydroponic systems, pathogens are not limited by biological "buffering" and can easily spread between plants. This is especially true for the zoosporic fungi that produce spores that are motile in liquid (Zinnen 1988, Stanghellini & Rasmussen 1994).

A small amount of inoculum in hydroponics systems can lead to infection and disease losses. The substrates used in hydroponics sometimes lack the microbial diversity and buffering capabilities of natural soil. Hydroponic systems also lack antagonists that can be important in reducing the impact of diseases in nurseries using soil or other forms of medium (Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Paulitz 1997, Alsanius *et al.* 2001). Without nutrient competition and fungistatis in the substrate, pathogens can rapidly become established and cause severe disease outbreaks.

The physical environment in hydroponic systems can be favourable for the growth of pathogens (Jensen & Collins 1985, Zinnen 1988, Stanghellini & Rasmussen 1994). To allow for optimal plant growth, the temperature and moisture regimes are strictly controlled and these regimes are similar to those optimal for infection by the pathogens. A study by Bates & Stanghellini (1984), for example, showed that specific pathogens become dominant in the nutrient solution at specific temperatures.

Diseases of crops grown in hydroponics

Reports of diseases of plants grown in soil culture far exceed those of plants grown in hydroponics (Paulitz 1997). Currently no information is readily available on diseases of *Eucalyptus* clonal hedge – plants grown in hydroponics. This is despite the fact that hydroponics have been widely used to propagate these plants in Brazil for more than a decade. In this section of the review, common

pathogens causing diseases in hydroponically grown agricultural crops are considered in an attempt to illustrate their mechanism of spread and infection.

Zoosporic pathogens

Zinnen (1988) and Stanghellini & Rasmussen (1994) have reviewed many of the diseases occurring in agricultural hydroponic systems. They have shown that the most important pathogens of plants in hydroponics are zoosporic fungi, which is not surprising given the fact that these fungi spread by means of motile flagellate spores. These pathogens can cause root rot, seedling rot, stunting, yield loss and plant collapse. Reservoir and surface water, such as that from streams and rivers and inert substrates such as peat, provide a source of these pathogens in hydroponic systems. Some zoosporic fungi are also introduced by insects and these include *Pythium aphanidermatum* (Ebson) Fritzp. (Jenkins & Averre 1983, Bates & Stanghellini 1984, Stanghellini *et al.* 1988, Zinnen 1988, Moulin *et al.* 1994, Stanghellini & Rasmussen 1994, Stanghellini *et al.* 1996, Paulitz 1997, Wulff *et al.* 1998).

Most of the zoosporic pathogens reported in hydroponics are also well-recognized root pathogens of the same crops, grown in the field. There are more than 20 reported zoosporic pathogens in hydroponic systems (Zinnen 1988, Stanghellini & Rasmussen 1994). The most common species reside in the genera *Pythium*, *Phytophthora*, *Oplidium* Syd. and *Plasmopara* Schröt. (Zinnen 1988, Stanghellini & Rasmussen 1994).

The motile asexual zoospores encounter roots through chemotaxis; they attach, loose their flagella and encyst by forming a thick cell wall. A germ tube is produced from these encysted spores and this penetrates the roots. These events can take place within five minutes and depending on the fungus, the asexual life cycle can be completed within 12 hours. This can have serious consequences in a hydroponic system (Stanghellini & Rasmussen 1994, Paulitz 1997). Zoosporic pathogens rarely produce visible structures in the host tissue. The occurrence in plants is usually detected through the recognition of the macrosymptoms on the plants and is confirmed by laboratory analysis of the infected tissue (Bates & Stanghellini 1984, Zinnen 1988, O' Gara *et al.* 1997, Brown & Feirrera 2000).

Several *Pythium* and *Phytophthora* spp. are reported pathogens of *Eucalyptus* grown using traditional methods, e.g. soil culture. These include P. *aphanidermatum*, *P. debrayanum* R. Hesse, *P. intermedium* de Bary, *P. irregulare* Buisman, *P. myriotylum* Drechsler, *P. ultimum* Trow, *Ph. cinnamomii* Rands, *Ph. cryptogea* Pethybr. & Laff. and *Ph. nicotianae* de Haan (Marks & Kassaby 1974, Sharma *et al.* 1984, Von Broembsen 1984, Sharma *et al.* 1985, Sharma & Mohanan 1992, Belisario 1994, Brown & Ferreira 2000). All of these pathogens have also been reported from agricultural hydroponic crops (Jenkins & Averre 1983, Bates & Stanghellini 1984, Zinnen 1988, Stanghellini & Rasmussen 1994). Zoosporic pathogens usually attack only the young root tissue or undifferentiated root apices of *Eucalyptus* feeder roots. From these initial infections, the pathogens can spread to older roots and even the stems,

resulting in root and stem lesions or cankers (Brown & Feirrera 2000). The primary symptoms are rot of the young and fine roots and subsequently the larger roots. The secondary symptoms result in decline of the plant that can lead to plant death. The lesions formed are usually dicoloured and water – soaked, although asymptomatic infections also occur. Infection by *Pythium* spp. generally does not proceed beyond this stage, as these fungi are usually restricted to non – lignified tissues in the young roots. *Phytophthora* spp. can produce secondary symptoms since they are not restricted to the non – lignified tissues (O' Gara *et al*. 1997, Brown & Feirrera 2000).

Non – zoosporic pathogens

Non – zoosporic fungi important in agricultural hydroponic systems reside in the genera *Fusarium*, *Colletotrichum* Corda and *Verticillium* Nees. These are well – known soilborne microorganisms that act mostly as wilt pathogens. They are introduced into hydroponic systems through air, for example *Fusarium oxysporum* f. sp. *radicis – lycopersici* Schlechtend.:Fr., and accidental introduction of soil into the nutrient solution (Paulitz 1997, Stanghellini & Rasmussen 1994, Zinnen 1988, Jenkins & Averre 1983, Mihuta – Grimm *et al.* 1990, Duffy & Defago 1999).

Amongst the non – zoosporic fungi, *Fusarium* species are the most common causal agents of disease hydroponics (Jenkins & Averre 1984, Zinnen 1988, Stanghellini & Rasmussen 1994). The diseases include wilting and root rot of

specific hosts cultivated in a hydroponic system. The most predominant *Fusarium* species are *forma specialis* of *Fusarium oxysporum* Schlechtend. (Couteaudier & Alabouvette 1981, Jenkins & Averre 1984, Zinnen 1988, Stanghellini & Rasmussen 1994). *Fusarium oxysporum* f. sp. *radicis – lycopersici*, *F. o. lycopersici* Schlechtend.:Fr. and *F. o. cucumerinum* Schlechtend.:Fr. have been reported to cause wilting of several hydroponic crops that include tomatoes, cucumber and lettuce (Couteaudier & Alabouvette 1981, Jenkins & Averre 1984, Brammall & Lynch 1990).

Fusarium oxysporum f. sp. *eucalypti* Arya & G. L. Jain is the only form of *F. oxysporum* found to cause root rot and wilting of *Eucalyptus* seedlings in nurseries (Arya & Jain, 1962). This pathogen causes a brown discolouration of the vascular tissue of the stem and roots of the plants. This subsequently results in the roots becoming sticky and the tips of the plants start to droop. Leaves turn yellow and abscise and whole plants then begin to wilt (Arya & Jain 1962).

Colletotrichum coccodes Penz. has been found to cause significant losses of tomato seedlings because of root rot in hydroponics (Schneider *et al.* 1978, Daughtrey & Schippero 1980, Jenkins & Averre 1983). However, this pathogen has not yet been found on the roots of *Eucalyptus* seedlings or cuttings. The only pathogen in the same genus reported on *Eucalyptus* is *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Viljoen *et al.* 1992, Smith *et al.* 1998, Brown & Ferreira 2000). *Colletotrichum gloeosporioides* causes anthracnose of *Eucalyptus* seedlings and cuttings. The disease is characterized by discrete,

round lesions surrounded by a red – purple border on *Eucalyptus* leaves. During wet weather, pink conidial masses exude from acervuli formed in the lesions (Viljoen *et al.* 1992).

Evans (1979) reported *Verticillium dahliae* Klebahn from the roots of wilted tomato plants grown using a nutrient – film technique. This pathogen has, however, not been reported on *Eucalyptus* plants. *Verticillium albo – astrum* Reinke & Berthold has been reported on *Eucalyptus* seedlings and is generally regarded as a seedborne pathogen that causes damping – off of seedlings (Harsh *et al.* 1992).

Disease control in hydroponics

Control of pathogens, once established in a hydroponic production system, is often difficult but can be successfully achieved. There are three primary categories of disease control available for use in hydroponics. They are biological methods, cultural and physical methods and chemical control.

Biological control

The most effective method of biological control is through use of resistant cultivars of plants. The choice of cultivar is dependant on the identification of the pathogens present (Stanghellini & Rasmussen 1994, Paulitz 1997, Paulitz & Bélanger 2001). Currently *Eucalyptus* breeding programmes select for resistance

to plantation diseases but not for resistance to nursery pathogens (Denison & Kietzka 1993a). The use of disease free plants is the one alternative and in the case of hydroponics, they can be obtained through tissue culture (Mitha – Grimm *et al.* 1990). Even if the plant starts off disease free, it is impossible to maintain it under aseptic conditions, thus disease resistance is extremely important.

The use of antagonistic microorganisms is another approach to biological control. Unfortunately only one antagonist, *Streptomyces griseoviridis* (Mycostop, Kemira Biotech, Finland) has been registered for use against *Fusarium* spp. in commercial hydroponic systems. (Stanghellini & Rasmussen 1994). Investigations into antagonists that might be useful in hydroponics are, however, ongoing and are revealing positive results (Stanghellini & Rasmussen 1994, Berger *et al.* 1996, McCullagh *et al.* 1996, Paulitz 1997, Stanghellini & Miller 1997, Ongena *et al.* 1999, Paulitz & Bélanger 2001, Grosch *et al.* 2001).

The use of biocides has provided excellent control of root – infecting zoosporic pathogens in hydroponics. Several bacteria and fungi in the genera *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Acinetobacter*, *Corynebacterium*, *Candida* and *Torulopsis* produce these biocides. These microorganisms produce species-specific rhamnolipids responsible for the surfactant activity (Stanghellini & Miller 1997).

Cultural and physical control

Sanitation is the most important component of cultural control of pathogens. The removal of infected plants, sterilization of equipment and disinfection of recycled aggregate substrates is essential for a pathogen – free hydroponic system (Stanghellini & Rasmussen 1994). These principles do not only apply to hydroponics but should be part of management of any nursery.

There are numerous methods used for the elimination of pathogens from hydroponic nutrient solutions. These methods include filtration (van Os *et al.* 1999, Schwartzkopf *et al.* 1987, Runia 1995, Goldberg *et al.* 1992), ozonation (Vestergárd 1988, Vanachter *et al.* 1988, Runia 1995), ultraviolet irradiation (Zhang & Tu 2000, Schwartzkopf *et al.* 1987, Runia 1995, Acher *et al.* 1997, Sutton *et al.* 2000) and thermal inactivation (Runia & Amsing 2001, Schuerger & Mitchell 1992, Runia 1995).

Chemical control

The use of chemicals and particularly fungicides has typically been the preferred means of disease control in agriculture. This is because they can be used relatively easily and cheaply. Unfortunately no fungicides have yet been registered for use in hydroponics. The reason for this is that a lag time is required between application and harvest in order for fungicide residue to be at an acceptable level. This is impossible in hydroponics because harvesting of

food crops is done daily. In forest crops such as *Eucalyptus*, a lag phase is not required since the products are not destined for human or animal consumption. However, the environmental impact of chemical application is a matter that is increasing in sensitivity.

Several investigators have found that the application of fungicides is very effective against some of the afore-mentioned pathogens. However, these chemicals could also have a negative effect on the crop grown in the hydroponic system. Price & Fox (1986) found that furalaxyl (Fongarid, Ciba – Geigy) inhibited the growth of *Phytophthora* and *Pythium* species in a NFT system. They also found a significant reduction in fruit development of cucumbers due to increased sodium levels but no disease development was noticed. The use of benomyl (Benlate, Du Pont) at very low concentrations (0.090 g a. i./ℓ) is very effective against *Fusarium* crown and root rot of tomatoes but can cause severe phytotoxic symptoms at higher concentrations (Mitha – Grimm *et al.* 1990).

Synthetic surfactants also occur in some fungicides, e. g. Manoxol, Triton X100, Sodium lauryl sulfate and Bavistin (Stanghellini & Miller 1997). Surfactants dissolve the unit membrane encasing zoospores and reduce their motility by disrupting the integrity and permeability of the plasma membrane (Stanghellini & Tomlinson 1987, Stanghellini & Rasmussen 1994, Stanghellini *et al.* 1996, Stanghellini & Miller 1997).

Addition of chitosan to nutrient solutions can increase the resistance of plants to infection by zoosporic pathogens. Chitosan (chitinase, chitosanase and β – 1,3 – glucanase) induce structural barriers in roots and stimulate the production of antifungal hydrolases in both roots and leaves. This chemical also causes wall loosening, vacuolation and protoplasm disintegration of zoosporic pathogens (Ghaouth *et al.* 1994).

Cherif & Belanger (1992) found that the addition of potassium silicate to nutrient solutions controls infections by zoosporic pathogens. This chemical also increases resistance in the plants by inducing structural barriers in the roots and also increases crop yields. However, Cherif & Belanger (1992) noted that the mechanism by which potassium silicate confers protection is still not fully understood.

Conclusions

Production using hydroponics is gradually increasing in both agriculture and horticulture. Hydroponics is an attractive yet over simplified technology, which is easier to promote than to sustain. This is indicated by the large number of failed applications of this technology (Jensen 1997). These failures can be attributed to several factors. One of these is disease caused by root infecting pathogens.
With the increase in demand for *Eucalyptus* products, forestry companies in South Africa must develop methods for the rapid production of plants. The new initiative of one of South Africa's major forestry companies to convert its

Eucalyptus production system to hydroponics is thus visionary. This system will clearly allow for the rapid production of large numbers of *Eucalyptus* cuttings in a small and environmentally controlled area.

There have been no previous investigations regarding the role of diseases that might cause damage to *Eucalyptus* plants grown in hydroponics. Several surveys of *Eucalyptus* nursery diseases have been conducted in the past (Sharma *et al.* 1984, Sharma *et al.* 1985, Sharma & Mohanan 1992, Viljoen *et al.* 1992, Brown & Feirrera 2000). Using knowledge gained from these surveys as well as information from hydroponically grown agricultural crops, it should be possible to identify potential pathogens relatively easily. This will not be sufficient to ensure that pathogens do not become important, but it will provide a foundation for the research that will be needed to reduce the impact of diseases. What is now required is a thorough survey of diseases in the emerging hydroponics nurseries in South Africa and in addition, potential pathogens will need to be evaluated for their relative importance.

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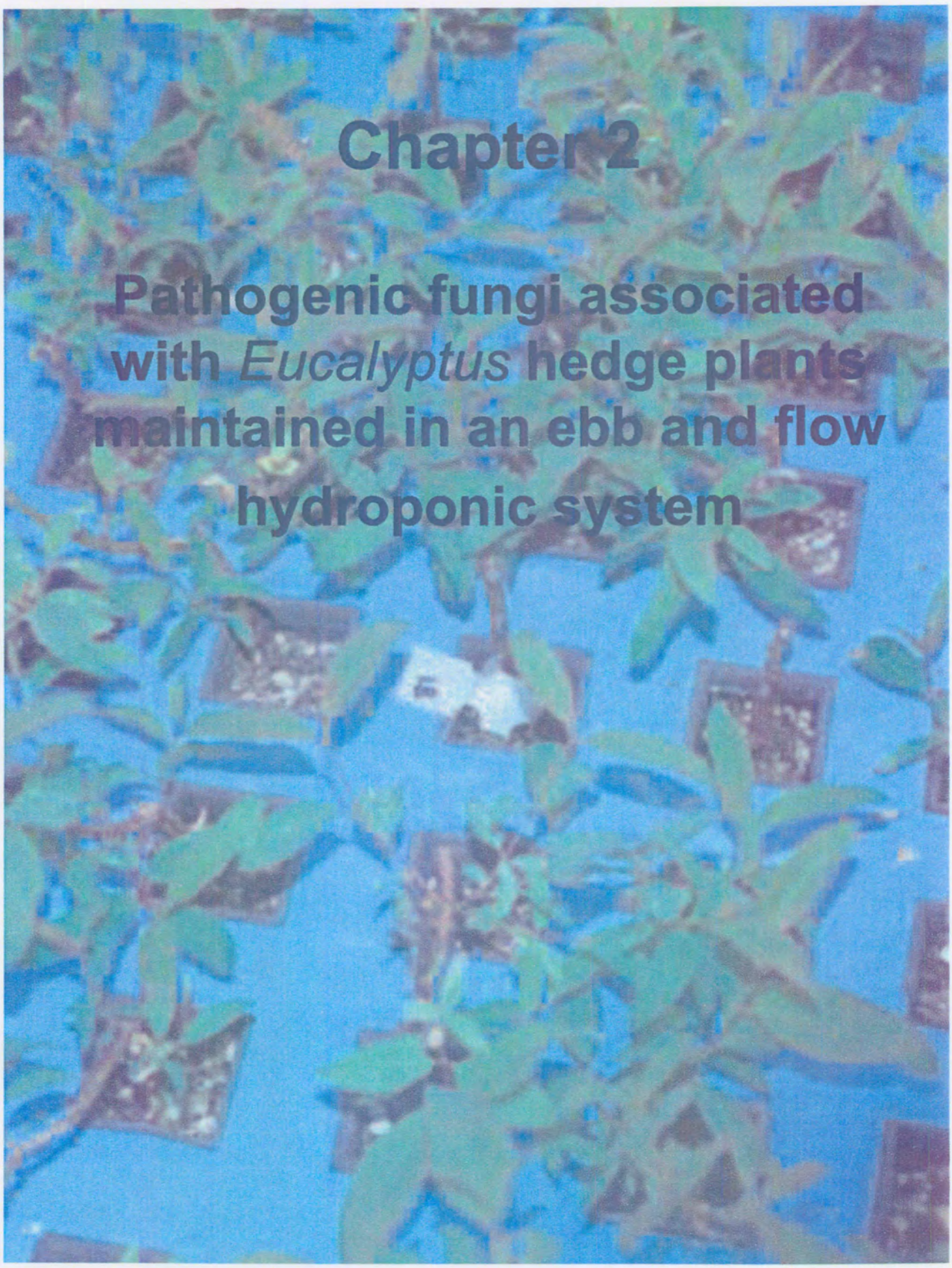

Chapter 2

Pathogenic fungi associated with *Eucalyptus* hedge plants maintained in an ebb and flow hydroponic system

Abstract

Vegetative propagation of *Eucalyptus* hybrid clones is a powerful tool contributing towards the establishment of uniform, high – value commercial plantations. However, the development and establishment of *Eucalyptus* hedge plants is a time consuming and space inefficient process. A new system to propagate and maintain these hedge plants in hydroponics has recently been developed for use in some South African forestry nurseries. No information is available on the presence of pathogens of *Eucalyptus* in hydroponics in this country. The aim of this study was, therefore, to determine which pathogens are responsible for the root rot symptoms observed on the hedge plants. A study was thus undertaken at a forestry nursery that maintains *Eucalyptus* hedge plants in an ebb and flow feeding system. Roots were randomly collected and direct isolations were done on standard and selective media. Nutrient solution was also collected, filtered and filters plated onto standard and selective media for the isolation of fungi. Several well – known fungal pathogens were collected that reside in the genera *Cylindrocladium*, *Fusarium*, *Pythium* and *Phytophthora*. All isolates were identified based on morphological characteristics and these identifications were confirmed using DNA sequence data comparisons.

Introduction

In the past two decades, the *Eucalyptus* nursery system in South Africa has undergone major changes. The most significant of these was the rapid development of macropropagation and micropropagation of *Eucalyptus* hybrid clones from stem cuttings and tissue culture (Denison & Kietzka 1993a). Macropropagation, commonly known as vegetative propagation, is a powerful tool to capture specific family and clonal gains, not available from conventional tree breeding strategies using seed orchards (Denison & Kietzka 1993a).

In South Africa, vegetatively propagated tropical, sub – tropical and cold tolerant *Eucalyptus* spp. and their hybrids have been deployed in commercial plantations (Denison & Kietzka 1993b). This has resulted in uniform plantations of selected, high value trees (Kulkarni & Lal 1995). The development and establishment of conventional clonal hedge plants is time consuming, taking up to 18 months before they become productive (Wilson 1998, Aimers – Halliday *et al.* 1999). Mondi Forests, South Africa, has thus introduced a system to maintain clonal hedge plants in hydroponics. This has significantly reduced the time required for establishment of plants as well as increasing productivity of cuttings (Dr. B. Janse, pers. comm.).

Hydroponics encompasses the technology of growing plants in nutrient solution with or without the aid of a root – supporting substrate (Stanghellini & Rasmussen 1994, Jensen 1999). Several types of hydroponic systems are

available and used for a wide variety of plant products (Jensen & Collins 1985, Stanghellini & Rasmussen 1994, Jensen 1999). Currently in South African forestry, a recirculating liquid hydroponic system, known as an ebb and flow system, is used. This system is based on a "flood and drain" principle where the roots are submerged in nutrient solution for a few minutes then drained. This process is repeated approximately three to four times daily, depending on climatic conditions.

Although hydroponics has several advantages, one important disadvantage is that fungal pathogens capable of causing root diseases are easily spread in liquid medium. Several of these fungi are well – known plant pathogens while other fungi are only known to be pathogenic in a hydroponic system (Stanghellini & Rasmussen 1994, Paulitz 1997). Given that hydroponics is new to forestry in South Africa, little is known about the fungal pathogens present in this system. The aim of this study was, therefore, to determine which pathogens are present in newly established ebb and flow hydroponics systems used to grow *Eucalyptus* hedge plants for cutting production.

Materials and Methods

Ebb and flow system

The ebb and flow system used in this study is a liquid hydroponic system where the nutrient solution is re-circulated. The system consisted of four nutrient tanks (Batch 1 – 4; Fig. 1B) which each feed nutrient solution to eight

shallow tanks. The shallow tanks have removable lids containing holes in which the hedge plants are suspended in a plug.

Nutrient solution is pumped into the shallow tanks until the plugs are halfway submerged in the nutrient solution. This level is maintained for three to four minutes and the nutrient solution is then drained. This process is repeated three to four times daily. The nutrient solution consists of tap water and commercially available hydroponics fertilizers and is replaced every 20 days.

Survey of Eucalyptus *hedge plants*

Roots of hybrid *E. grandis* x *urophylla* hedge plants, in the ebb and flow system, were collected from one forestry nursery in KwaZulu – Natal. A sample of 20 root plugs was randomly collected from each of the four batches of the hydroponics system. Healthy and diseased roots (Fig. 1E) were air – pruned from the plugs and placed separately in sterile plastic tubes and transferred to the laboratory for further study. The aerial parts of the hedge plants showed no obvious signs of disease. These collections were made on five different occasions at two – month intervals. Thus, 80 samples were collected on each of five occasions, resulting in a total sample of 400 roots. Statistical analysis (ANOVA) of the survey data as means for each pathogen in each batch was carried out using SAS analytical programmes (1990).

Roots were surface – sterilized for one minute in 1% sodium hypochlorite and rinsed twice in sterile water. Isolations were made randomly directly from both

healthy and diseased roots onto 2% Malt Extract Agar (MEA; Biolab, Midrand, South Africa), *Fusarium* Selective Medium (FSM) (Nelson *et al.* 1983), Nystatin – Ampicillin – Rifampicin – Pentachloronitrobenzene Medium (NARP; 17g/l corn meal agar (Biolab, Midrand, South Africa) 100 000 units/ml, 0.1g/l ampicillin, 0.5g/l rifampicin, 0.1g/l, 0.1g/l pentachloronitrobenzene (Sigma, Steinheim, Germany) and NARP supplemented with Hymexazol (0.05g/l) (NARPH) as selective media for the isolation of *Fusarium* spp., *Pythium* spp. and *Phytophthora* spp. After isolation, the plates were incubated at 25°C in the dark for 3 – 5 days.

Nutrient solution was also collected from each batch of the hydroponics tanks, at regular intervals to determine the presence of pathogens. The nutrient solution (250ml) was filtered through four 0.45 µm cellulose – nitrate membrane filters (Whatman, Maidstone, England) for each batch. The filters were plated onto 2% MEA, FSM, NARP and NARPH and incubated at 25°C in the dark for 3 – 5 days.

The most commonly isolated fungi representing four different genera were selected for further study. Isolates were grouped based on their growth characteristics on the different media. Genera were then identified based solely on morphological characteristics. These cultures are maintained in the culture collection (CMW and FCC) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Morphological characteristics of Cylindrocladium *isolates*

A large number of isolates resembling *Cylindrocladium* spp. were obtained from the roots. Twenty randomly selected single conidial isolates were transferred onto Carnation Leaf Agar (CLA) (Crous 2002) to induce production of both anamorph and teleomorph structures. The plates were incubated at 25°C under continuous near – ultraviolet light and examined after 14 days. Morphological characteristics, described by Crous (2002), were used to identify cultures. Macroconidiophores were mounted in lactophenol and twenty measurements of vesicles, stipes and conidia were made for each of the selected isolates, using a light microscope. Measurements for these structures are presented as (min-)(average – standard deviation) – (average + standard deviation)(-max).

Morphological characteristics of Fusarium *isolates*

A large number of isolates resembling *Fusarium* spp. were obtained from the roots. Single conidial isolates were transferred onto CLA, half strength Potato Dextrose Agar (PDA; Biolab, Midrand, South Africa) and Synthetic Low Nutrient Agar (SNA) (Nirenberg &O'Donell 1998). The plates were incubated at 25°C under continuous near – ultraviolet light and examined after 14 days. Mycelium from PDA plates and conidia and conidiophores produced on CLA plates were mounted in lactophenol and SNA plates were studied directly with a light microscope. Morphological characteristics described by Nelson *et al.* (1983) and Nirenberg &O'Donell (1998) were used to identify cultures.

Morphological characteristics of Pythium *and* Phytophthora *isolates*

Pythium and *Phytophthora* isolates were grown for five days on half strength PDA and Corn Meal Agar (CMA) at 25°C in the dark. Four 3 mm discs taken from the actively growing margins of the cultures, growing on PDA, were placed in 12 ml 20% clarified V8 broth and incubated at 25°C for three days with continuous light. Mycelial mats were washed twice with sterile Petri's salt solution (Ribeiro 1978) amended with 0.002g E.D.T.A. disodium salt (Roche, Indianapolis, USA) and 0.002g $MnSO_4$ (Sigma, Steinheim, Germany). Mycelial mats were then incubated in 12 ml Petri's salt solution at 25°C under continuous light. After 48h, the mycelial mats were harvested and examined using a light microscope for the presence of sporangia. Morphological characteristics described by Newhook *et al.* (1978) and Van der Plaats – Niterink (1981) were used to identify cultures.

DNA sequence comparisons

Seven *Cylindrocladium* isolates (Table 1) were randomly selected and utilized for the DNA sequence comparisons. Single conidial isolates were grown on 2% MEA plates from which mycelium was collected and freeze dried. The freeze – dried mycelium was ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted using the technique described by Möller *et al.* (1992).

Nine morphologically distinct *Fusarium* isolates (Table 1) morphologically distinct were randomly selected and utilized in the DNA sequence comparisons. The single conidial isolates were grown on half strength PDA plates from which mycelium was collected and freeze dried. The freeze – dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was extracted using the technique described by Möller *et al.* (1992).

Three *Phytophthora* and ten *Pythium* isolates (Table 1) representing morphologically distinct groups were randomly selected and utilized in the DNA sequence comparisons. Cultures were grown in 20ml sterile 20% chlarified V8 broth for five days at 25°C. Mycelium was harvested and rinsed twice with sterile water before freeze drying. The freeze – dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was extracted using the technique described by Möller *et al.* (1992).

A 507 base pairs (bp) fragment of the β - tubulin gene was amplified using primers T1 (O'Donell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) for the *Cylindrocladium* isolates. For the *Fusarium* isolates a 280 bp fragment of the Elongation 1 - α gene (EF1 - α) was amplified using primers EFFF and EFFR (Dr. K. Jacobs, pers. comm.). For the *Phytophthora* and *Pythium* isolates a 877 bp. and 851 bp. fragment of the internal transcribed spacer regions were obtained, respectively using primers ITS4 (White *et al.* 1990) and ITS6 (Cooke & Duncan 1997). The PCR reaction of 25μl comprised of 2.5 units Taq enzyme (Roche Molecular Biochemicals, Almeda, California, USA),

10x buffer, 1mM $MgCl_2$ (as supplied by manufacturer), 0.25 mM deoxynucleoside triphosphate, 0.5 μm primers and approximately 30 ng of fungal DNA as target. The amplified fragments were purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, California, USA).

Each DNA strand of the PCR products was sequenced in both directions with the primers used for the PCR amplification. Sequence reactions were done using an ABI PRISM™3100 DNA Autosequencer (Applied BioSystems). Sequence data were processed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California, USA). The nucleotide sequences were aligned manually by inserting gaps where necessary and phylogenetic relationships were determined using PAUP version 4.0b10 (Swofford 2002). Gaps were treated as missing data and confidence intervals were determined using 1000 bootstrap replications.

Phylogenetic relationships and identities were established for the *Cylindrocladium* isolates by including 12 sequences of known *Cylindrocladium* spp. (Table 1) from GenBank, in the alignment. *Cylindrocladiella infestans* Boesew. (AF320190) was used as the out – group taxon in the analysis. The same was done for the *Fusarium* isolates by including nine sequences of known *Fusarium* spp. (Table 1) with *Botryosphaeria dothidea* Ces. & De Not (AY 236899) as the out – group taxon. To establish the phylogenetic relationships and identities of the *Phytophthora* isolates, five sequences of known *Phytophthora* spp. (Table 1) were obtained from GenBank and

included in the alignment. *Pythium aphanidermatum* (Edson.) Fitzp. (AJ 233438) was used as out – group taxon in the analysis. For the *Pythium* isolates, eight sequences of known *Pythium* spp. (Table 1) were used from GenBank and included in the alignment. *Phytophthora cinnamomi* Rands (AF 266764) was used as out – group taxon in the analysis.

Results

Survey of Eucalyptus *hedge plants*

Several well – known *Eucalyptus* pathogens, represented by 717 fungal isolates, were collected from both healthy and diseased *Eucalyptus* hedge plant roots and nutrient solution at the forestry nursery surveyed (Table 2). *Cylindrocladium* isolates were the most commonly isolated pathogens (Fig. 1F) in the ebb and flow system and the next most common pathogens resided in the genus *Pythium* (Table 2). Several *Fusarium* isolates and *Phytophthora* isolates were also obtained in this study (Table 2). These possible pathogens were all isolated from diseased roots of the hedge plants and the nutrient solution. Significant differences existed ($P < 0.0001$) between the pathogens isolated in the survey (Table 3). No significant difference existed ($P > 0.6$) between the batches and pathogens isolated from each batch and indicate no changes ($P > 0.9$) over time (Table 3). However, the interactions between pathogens were significant (Table 3 - 4). Change in pathogen density over time and between batches was not significant ($P > 0.8$). Most of the fungi isolated in this study have been reported previously as pathogens of

Eucalyptus in South Africa, with exception of a few isolates in the genera *Fusarium* and *Pythium*.

Morphological characteristics of Cylindrocladium *isolates*

All *Cylindrocladium* isolates were morphologically similar. The stipes and extensions to the stipes (Fig. 2A) were septate, straight, hyaline, (79)104 – 149(200) μm in length and terminated in obpyriform to ellipsoidal vesicles, (11)16 – 23(31) x (2)5 – 7(9) μm (Fig.2 D & E). Each terminal branch of the conidiophores produced approximately 4 – 6 phialides (Fig. 2A). Phialides were doliiform to reniform, hyaline and aseptate. Conidia were cylindrical, straight, (37)45 – 57(65) x (3)4 – 6(7) μm, 1 – septate and held in parallel cylindrical clusters by colourless slime (Fig. 2B). These characteristics are typical of *C. pauciramosum* C. L. Schoch & Crous as described by Schoch *et al.* (1999).

Morphological characteristics of Fusarium *isolates*

Several *Fusarium* spp. were isolated from the *Eucalyptus* hedge plant roots (Table 2). Based on morphology, five *Fusarium* spp. were identified (Table 5, Fig. 3A - G) according to Nelson *et al.* (1983) and Nirenberg & O'Donnell (1998). They were *F. lateritium* Nees, *F. nygamai* Nirenberg & O'Donnell, *F. oxysporum* Schlect. Emend. Snyd. & Hans., *F. solani* (Mart.) Appel & Wollenw. Emend. Snyd. & Hans. and *F. verticillioides* Nirenberg & O'Donnell.

Morphological characteristics of Phytophthora *and* Pythium *isolates*

Two *Phytophthora* spp. were isolated from the hedge plant roots (Table 6) and identified following the suggestions of Newhook *et al.* (1978) (Fig. 4A – D). These were *P. cinnamomi*, characterized by large, non – papillate, ellipsoid sporangia (Fig. 4C), and *P. nicotianae* van Breda de Haas with papillate, obpyriform sporangia (Fig. 4A & B). Six *Pythium* spp. were isolated from the hedge plant roots (Table 6) and identified using the keys of Van der Plaats – Niterink (1981) (Fig.4E – I). These were *P. aphanidermatum*, *P. deliense* Meurs, *P. dissotocum* Drechsler, *P. intermedium* de Bary, *P. helicoides* Drechsler and *P. vexans* de Bary.

DNA sequence comparisons

***Cylindrocladium* spp.** A data set of 12 in-group taxa and one out-group taxon, *Cylindrocladiella infestans*, was analyzed for the *Cylindrocladium* isolates. The alignment of the β-tubulin gene fragments gave rise to a data set of 520 characters of which 372 were constant, 80 were parsimony – uninformative and 68 parsimony – informative (Appendix II). One parsimonious tree was generated and chosen for presentation (Fig.5). The tree had a consistency index (CI) = 0.852, retention index (RI) = 0.830, and rescaled consistency index (RC) = 0.707 while gaps were treated as missing data. The phylogenetic tree (Fig.5) clearly showed that all seven randomly selected *Cylindrocladium* isolates grouped in the clade representing *C. pauciramosum* (64% bootstrap support).

***Fusarium* spp**. A data set of nine in-group taxa and one out-group taxon, *Botryosphaeria dothidea*, was analyzed for the *Fusarium* isolates. The alignment of the EF1 – α gene fragments gave rise to a data set of 280 characters of which 82 were constant, 75 were parsimony – uninformative and 123 parsimony – informative (Appendix II). One tree from two most parsimonious trees was chosen for presentation (Fig.6). The trees had a CI = 0.867, RI = 0.926, and RC = 0.803 while gaps were treated as missing data. The phylogenetic tree (Fig.6) clearly showed that all nine selected *Fusarium* isolates grouped in clades representing the different *Fusarium* spp. identified (Table 5).

***Phytophthora* spp.** A data set of five in-group taxa and one out-group taxon, *Pythium aphanidermatum*, was analyzed for the *Phytophthora* isolates. The alignment of the ITS gene fragments gave rise to a data set of 814 characters of which 444 were constant, 235 were parsimony – uninformative and 135 parsimony – informative (Appendix II). One most parsimonious tree was generated and chosen for presentation (Fig.7). The trees had a CI = 0.885, RI = 0.754, and RC = 0.667 while gaps were treated as missing data. The phylogenetic tree (Fig.7) clearly showed that all three selected isolates grouped in the clades representing the different *Phytophthora* spp. identified (Table 6).

***Pythium* spp.** A data set of eight in-group taxa and one out-group taxon, *Phytophthora cinnamomi*, was analyzed for the *Pythium* isolates. The alignment of the ITS gene fragments gave rise to a data set of 851 characters

of which 370 were constant, 106 were parsimony – uninformative and 375 parsimony – informative (Appendix II). One most parsimonious tree was generated and chosen for presentation (Fig.8). The tree had a CI = 0.748, RI = 0.819, and RC = 0.620 while gaps were treated as missing data. The phylogenetic tree (Fig.8) clearly showed that all ten selected *Pythium* isolates grouped in the clades representing the different but known *Pythium* spp. identified (Table 6).

Discussion

This study represents the first survey of pathogens to be conducted on *Eucalyptus* hedge plants maintained in a recirculating ebb and flow hydroponic system in South Africa. A relatively large number of potential pathogens or fungi previously shown to be pathogenic were isolated from the hedge plant roots. Some of these have not been reported previously on *Eucalyptus*. No disease symptoms were observed in the aerial parts of the plants but root rot was evident. The lack of symptoms could be attributed to the fact that the hedge plants were in a nutrient rich environment created by the hydroponic fertilizers that are used. Thus the plants were under less stress than those grown using conventional hedge bank methods and this could have resulted in lower levels of symptom development.

An interesting result of this study was finding the dominant presence of *Cylindrocladium pauciramosum* on *Eucalyptus* hedge plant roots. *Cylindrocladium pauciramosum* is an important *Eucalyptus* pathogen in

forestry nurseries in South Africa (Crous 2002). It is a soilborne pathogen responsible for diseases such as root rot, stem cankers and leaf spots (Schoch *et al.* 1999, Crous 2002). This study showed that *C. pauciramosum* is present in a liquid hydroponic system and can spread to other plants through this solution. No stem cankers or leaf spots were observed on the hedge plants during the survey, even though they are commonly found elsewhere. However, *C. pauciramosum* was readily isolated from diseased roots. Other *Cylindrocladium* spp. might also become a limiting factor in hydroponic systems due to their large host range in the future (Crous 2002).

Fusarium spp. are the most common and destructive non – zoosporic pathogens in hydroponic systems (Stanghellini & Rasmussen 1994). *Fusarium oxysporum, F. solani* and *F verticilioides* (syn.= *F. moniliforme* J. Sheld.) are well – known pathogens of *Eucalyptus* seedlings responsible for damping – off, wilting and root rot (Sharma *et al.* 1985, Brown & Ferreira 2000). In this study, these *Fusarium* spp. were isolated together with *F. nygamai* and *F. lateritium,* neither of which have previously been reported as pathogens of *Eucalyptus*. Both *Fusarium* spp. are reported to occur on woody hosts (Nelson *et al.* 1983, Nirenberg & O'Donnell 1998).

Zoosporic fungi are known as the most important plant pathogens in hydroponics. This is because they are water – borne pathogens and they spread by means of motile flagellate spores (Zinnen 1988, Stanghellini & Rasmussen 1994). Both *Pythium* and *Phytophthora* spp. were isolated from the hedge plant roots during this study. *Phytophthora cinnamomi* and *P.*

nicotianae are both important pathogens of *Eucalyptus* causing root rot in nurseries and plantations (Sharma *et al.* 1985, Shearer & Smith 2000). All *Pythium* spp., with the exception of *P. dissotocum* and *P. helicoides*, are known pathogens of *Eucalyptus* seedlings responsible for damping – off and root rot (Sharma *et al.* 1985, Brown & Fereirra 2000, Shearer & Smith 2000). *Pythium helicoides* causes root diseases of ornamental plants (Van der Plaats – Niterink 1981) and *P. dissotocum* is responsible for stunting of lettuce in hydroponics (Stanghellini & Rasmussen 1994). Although pathogenicity tests have not been done at this stage, we expect that these fungi could contribute to reductions in tree health.

DNA sequence comparisons were used in this study to confirm the identity of the different species of pathogens associated with *Eucalytpus* hedge plants. This was necessary as species in these genera are morphologically similar and difficult to identify. The sequence data used in all cases confirmed identifications based on morphology when the β – tubulin gene was used for *Cylindrocladium* isolates (Schoch *et al.* 1999), EF1 – α for *Fusarium* isolates (Nirenberg & O'Donnell 1998) and ITS for *Pythium* and *Phytophthora* isolates (Wang & White 1997, Cooke *et al.* 2000).

Several well – known and possible new pathogens have been isolated from the *Eucalyptus* hedge plants in the ebb and flow hydroponics system considered in this study. Pathogenicity is suggested based on a knowledge of the fungi involved, but pathogenicity tests in an ebb and flow system need to be conducted in order to determine the impact of these pathogen on the

hedge plants. Sound and cost effective management strategies can then be developed to reduce losses.

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Table 1. List of fungal isolates used in DNA sequence comparisons.

Species	Isolate number	GenBank Assencion number
Cylindrocladium candelabrum	STE-U 1677	AF210858
	STE-U 1674	AF210857
C. colhounii	STE-U 307	AF232855
	STE-U 705	AF232854
C. gracile	STE-U 623	AF333405
	IMI 167580	AF333404
C. insulare	STE-U 3211	AF44951
	STE-U 3219	AF44950
C. pauciramosum	[a]**CMW 12689**	
	[a]**CMW 12697**	
	[a]**CMW 12702**	
	[a]**CMW 12707**	
	[a]**CMW 12709**	
	[a]**CMW 13039**	
	STE-U 3207	AF449448
	CSL 2021133	AY162320
C. scoparium	STE-U 1722	AF210875
	STE-U 1720	AF210874
Fusarium avenaceum	MT – F 150	AY337423
F. equiseti	MT – F 151	AY337424
F. lateritium	MT – F140	AY337435
	[a]**FCC 3117**	
	[a]**FCC 3119**	
F. nygamai	FRC M 1374	AY337445
	[a]**FCC 3113**	
F. oxysporum	UG L6886#44	AY337428
	[a]**FCC 3109**	
	[a]**FCC 3115**	
F. proliferatum	MT – F14	AY337436
F. sambucinum	MT – F149	AY337422
F. solani	MT – F116	AY337438
	[a]**FCC 3110**	
	[a]**FCC 3114**	
F. verticillioides	FRC M 1325	AY337450
	[a]**FCC 2957**	
	[a]**FCC 2964**	
Phytophthora cinnamomi	UQ 881	AF266764
	CMW 13804	
P. cryptogea	IMI 045168	AF266796
P. infestans	IMI 66006	AF266779

Table 1. (Continued) List of fungal isolates used in DNA sequence comparisons.

Species	Isolate number	GenBank Assencion number
P. nicotianae	UQ 848	AF266776
	[a]CMW 13800	
	[a]CMW 13816	
P. palmivora	UQ 1294	AF266780
Pythium aphanidermatum	TOc 159	AJ233438
	[a]CMW 13815	
	[a]CMW 13810	
P. deliense	MAFF 305568	AJ233442
	[a]CMW 13805	
P. dissotocum	MAFF 305576	AJ233443
	[a]CMW 13820	
	[a]CMW 13807	
P. helicoides	RoPh3 C14	AB108061
	[a]CMW 13808	
	[a]CMW 13817	
P. intermedium	MAFF 305570	AJ233447
	[a]CMW 13796	
	[a]CMW 13822	
P. irregulare	MAFF 305572	AJ233448
P. ultimum	OF 231	AB108064
P. vexans	MAF 305905	AJ233462
	[a]CMW 13819	

[a] Isolates listed in bold were sequenced in this study. Other sequences originated from Genbank.

Table 2. Percentage of fungi isolated from *Eucalyptus* hedge plant roots and nutrient solutions from an ebb and flow hydroponic system.

Fungus	**[a]Roots**	**[b]Nutrient solution**
Cylindrocladium pauciramosum	17.2	12.1
Fusarium lateritium	1.5	0.3
F. nygamai	1.1	0.3
F. oxysporum	8.2	6.1
F. solani	5.2	4.0
F. verticillioides	4.0	2.8
Phytophthora cinnamomi	0.1	0
P. nicotianae	0.3	0
Pythium aphanidermatum	13.1	5.2
P. deliense	3.1	0.8
P. dissotocum	4.7	1.7
P. intermedium	1.8	1.3
P. helicoides	0.8	0.8
P. vexans	2.0	1.4
Total number of isolates		**717**

[a] Percentage isolated from the *Eucalyptus* hedge plant roots.

[b] Percentage isolated from the nutrient solution.

Table 3.: Combined ANOVA for interaction of the pathogens isolated from the *Eucalyptus* hedge plants and the interactions between the pathogens and the batches.

Source	**SS**	**d.f.**	**MS**	***F***	***P***
Pathogen	11.32	3	3.77	19.16	<0.0001
Batch	2.56	3	0.85	4.33	0.0183
Time	0.0019	4	1.37	0.01	0.995
Batch x Pathogen	1.28	9	0.14	0.72	0.682
Time x Batch x Pathogen	0.49	12	1.77	0.42	0.855

Table 4: The means of each pathogen in each batch to indicate differences between pathogens and batches.

Batch	Pathogen	Mean	Std. Dev.
1	*Cylindrocladium,*	2.60	0.36
	Fusarium	1.60	0.20
	Phytophthora	1.40	0.69
	Pythium	1.27	0.29
2	*Cylindrocladium,*	2.66	0.83
	Fusarium	2.07	0.13
	Phytophthora	1.89	0.44
	Pythium	0.92	0.80
3	*Cylindrocladium,*	2.69	0.22
	Fusarium	1.94	0.14
	Phytophthora	1.89	0.17
	Pythium	1.77	0.27
4	*Cylindrocladium,*	2.26	0.12
	Fusarium	1.25	0.64
	Phytophthora	1.36	0.59
	Pythium	0.90	0.82

Table 5. Morphological characteristics of *Fusarium* isolates obtained from the *Eucalyptus* hedge plants and nutrient solution.

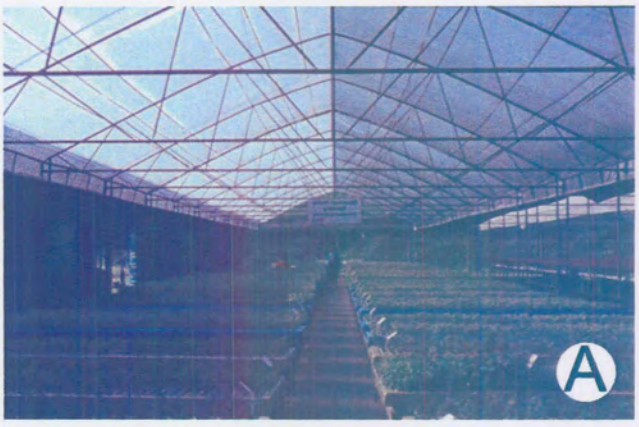
***Fusarium* spp.**	**[a]Colony colour**	**Macroconidia**	**Microconidia**	**Conidiophores**	**Chlamydospores**
F. lateritium	Orange; tan	Abundant; 4 – 6 septate; cylindrical	Abundant in false heads; ellipsoidal	Branched and unbranched monophialides	Present; single or in chains
F. nygamai	White; light pink	Abundant; 4 – 6 septate	Abundant in false heads; oval to kidney shaped	Monophialides and polyphialides	Present; single
F. oxysporum	White/purple; purple	Abundant; 4 – 6 septate; sickle - shaped	Abundant in false heads; oval to kidney shaped	Branched and unbranched monophialides	Abundant; single and pairs
F. solani	White/blue; white	Abundant; 3 – 5 septate; cylindrical	Sparse; oval to kidney shaped	Branched and unbranched monophialides	Present; single
F. verticillioides	White/purple; purple	Absent	Abundant in false heads; oval to club - shaped	Branched and unbranched monophialides	Absent

[a]Colour of colony determined on half – strength PDA (Nelson *et al.* 1983). Indicated as aerial mycelium; below culture.

Table 6. Morphological characteristic of *Phytophthora* and *Pythium* isolates obtained from the *Eucalyptus* hedge plants and nutrient solution. ([a]Colony morphology described from CMA)

Species	[a]Colony morphology	Sporangia
Phytophthora cinnamomi	No aerial mycelium; rosette pattern; large hyphal swellings	Terminal; non – papillate; internally proliferating; ellipsoidal to ovoid
P. nicotianae	Scant aerial mycelium; hyphae uniform	Terminal and intercalary; papillate; obpyriform to obturbinate
Pythium aphanidermatum	Cottony aerial mycelium	Inflated; filamentous; complex of terminal swollen hyphae
P. deliense	Loose aerial mycelium	Terminal and intercalary; Inflated; filamentous
P. dissotocum	Submerged mycelium	Non – inflated; filamentous; dendroidly – shaped
P. intermedium	Submerged mycelium; terminal and intercalary hyphal swellings	None produced
P. helicoids	Cottony aerial mycelium	Terminal; subglobose to obovoid; papillate
P. vexans	Cottony aerial mycelium	Terminal and intercalary; ovoid; spherical; proliferating

Figure 1. The ebb and flow system used to maintain *Eucalyptus* hedge plants in a forestry nursery. A. The ebb and flow system. B. Nutrient solution tanks. C. Shallow tanks containing the hedge plants. D. Feeder roots of hedge plants extending from plugs in the shallow tank. E. Healthy and diseased roots extending from plug. F. Infected root tip with sporulating macroconidiophores of *Cylindrocladium pauciramosum*.


A


B


C


D


E

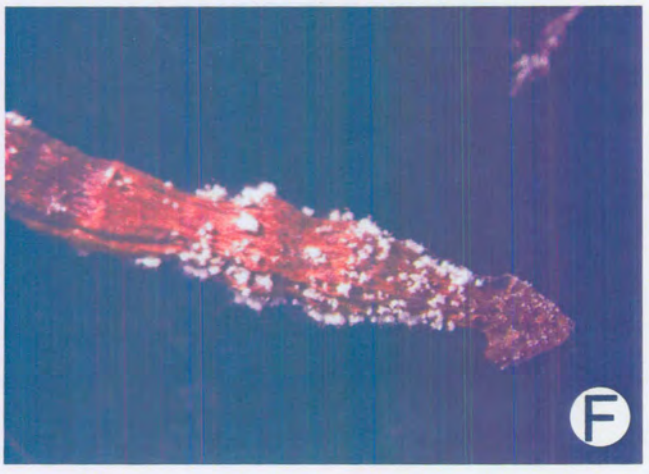

F

Figure 2. Macroconidiophores, vesicles and conidia of *Cylindrocladium pauciramosum* isolated from *Eucalyptus* hedge plant roots. A. Macroconidiophores. B. Conidia. C. Chlamydospores forming a sclerotium. D & E. Vesicles (Scale bars = 10 µm).

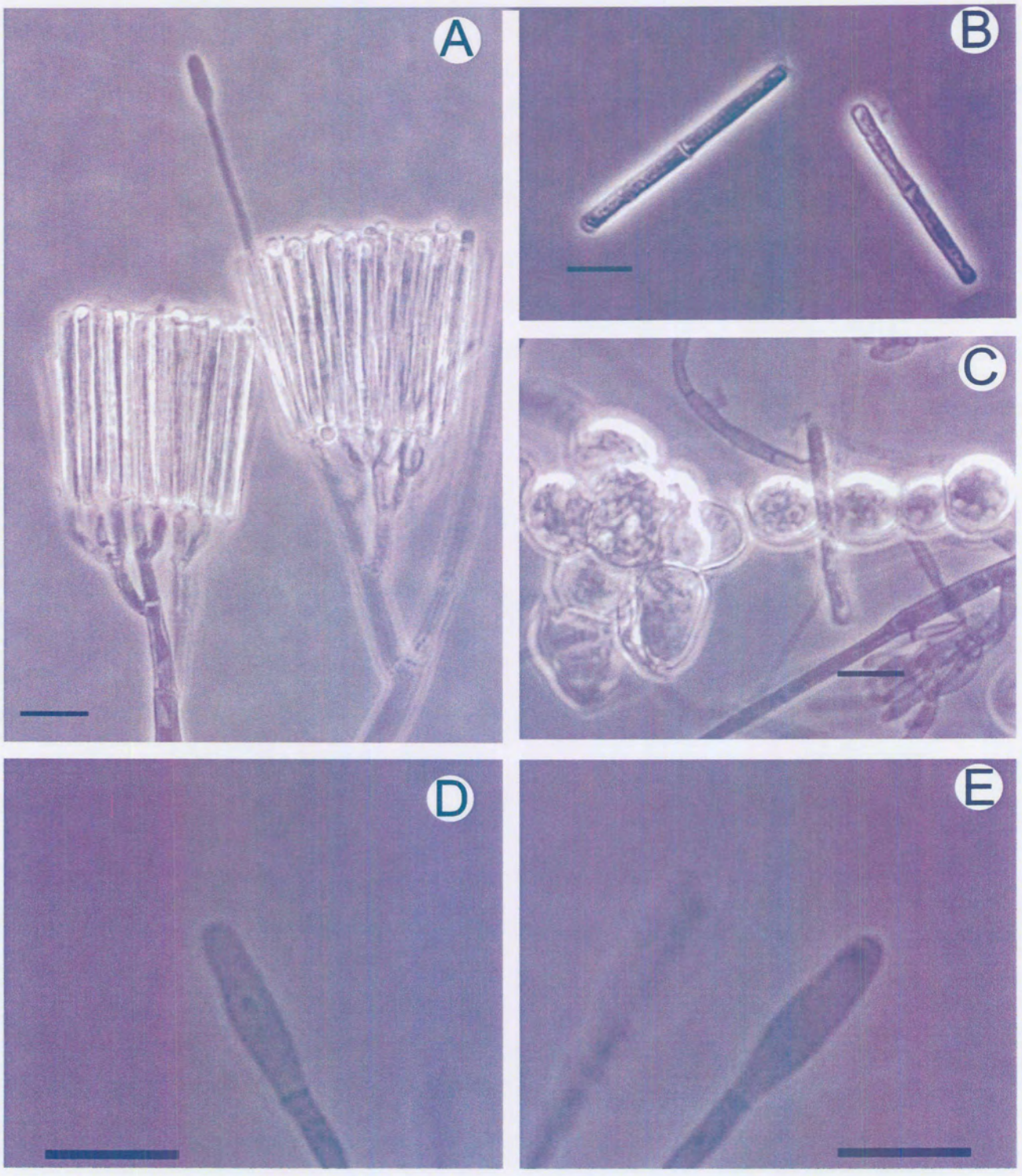


Figure 3. Some morphological structures of *Fusarium* spp. isolated from the roots of *Eucalyptus* hedge plants maintained in an ebb and flow hydroponic system. A. Microconidia of *F. lateritium* carried in false heads. B. Microconidia of *F. nygamai* carried in false heads. C. Monophialide of *F. solani*. D. Monophialide and polyphialide of *F. nygamai*. E. Intercalary chlamydospores of *F. oxysporum*. F. Sickle – shaped macroconidia of *F. oxysporum*. G. Microconidia of *F. verticillioides* (Scale bars = 10 µm).

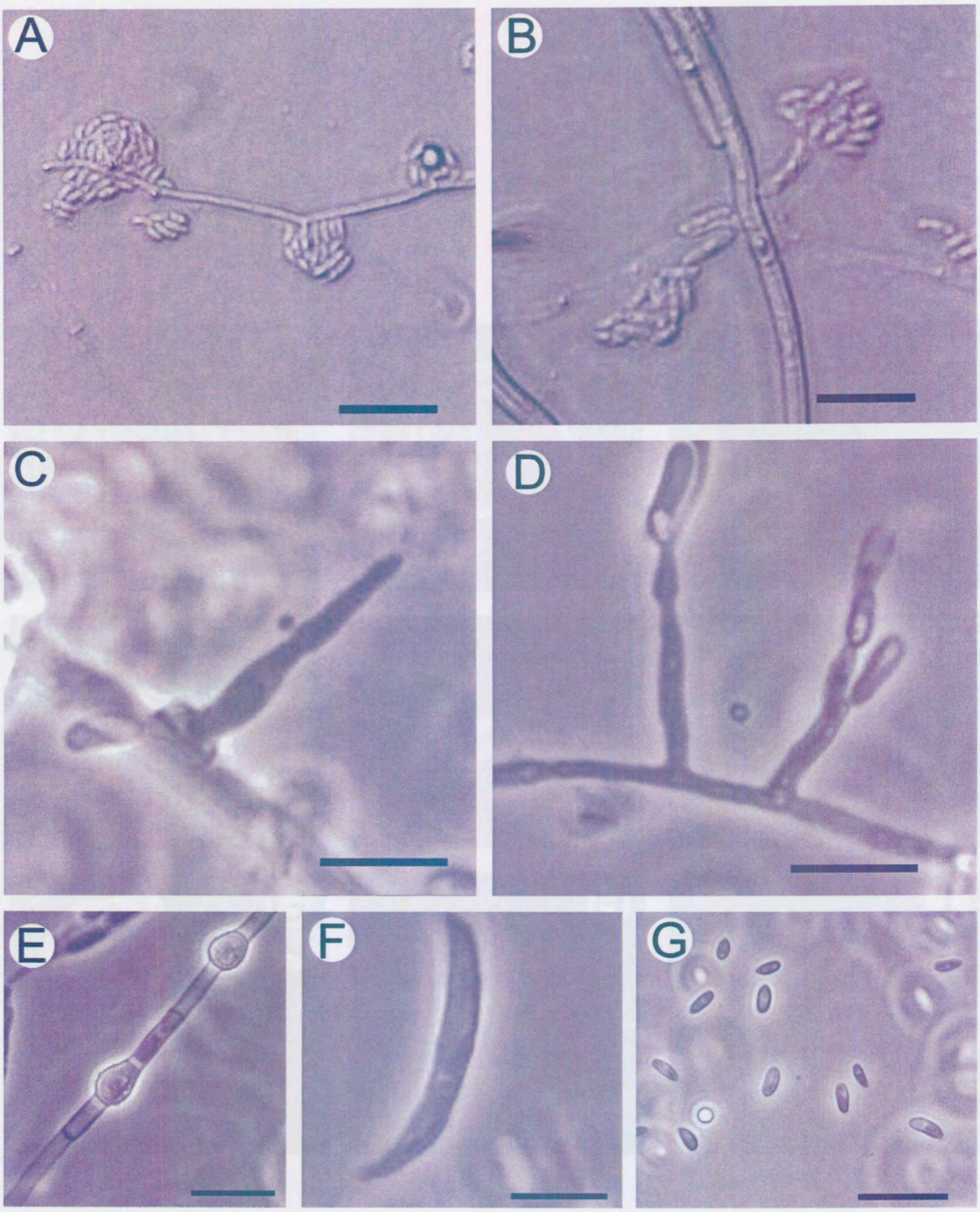


Figure 4. Sporangiophores of the Oomycetes isolated from the roots of Eucalyptus hedge plants maintained in an ebb and flow hydroponic system. A & B. Papillate sporangiophores of *Phytophthora nicotianae*. C. Sporangiophore of *P. cinnamomi*. D. Chlamydospores and oogonia of *P. cinnamomi*. E. Sporangiophore of *Pythium dissotocum*. F. Sporangiophore of *P. helicoids*. G. Sporangiophore of *P. aphanidermatum*. H. Sporangiophore of *P. deliense*. I. Sporangiophore of *P. vexans* (Scale bars = 10 µm).

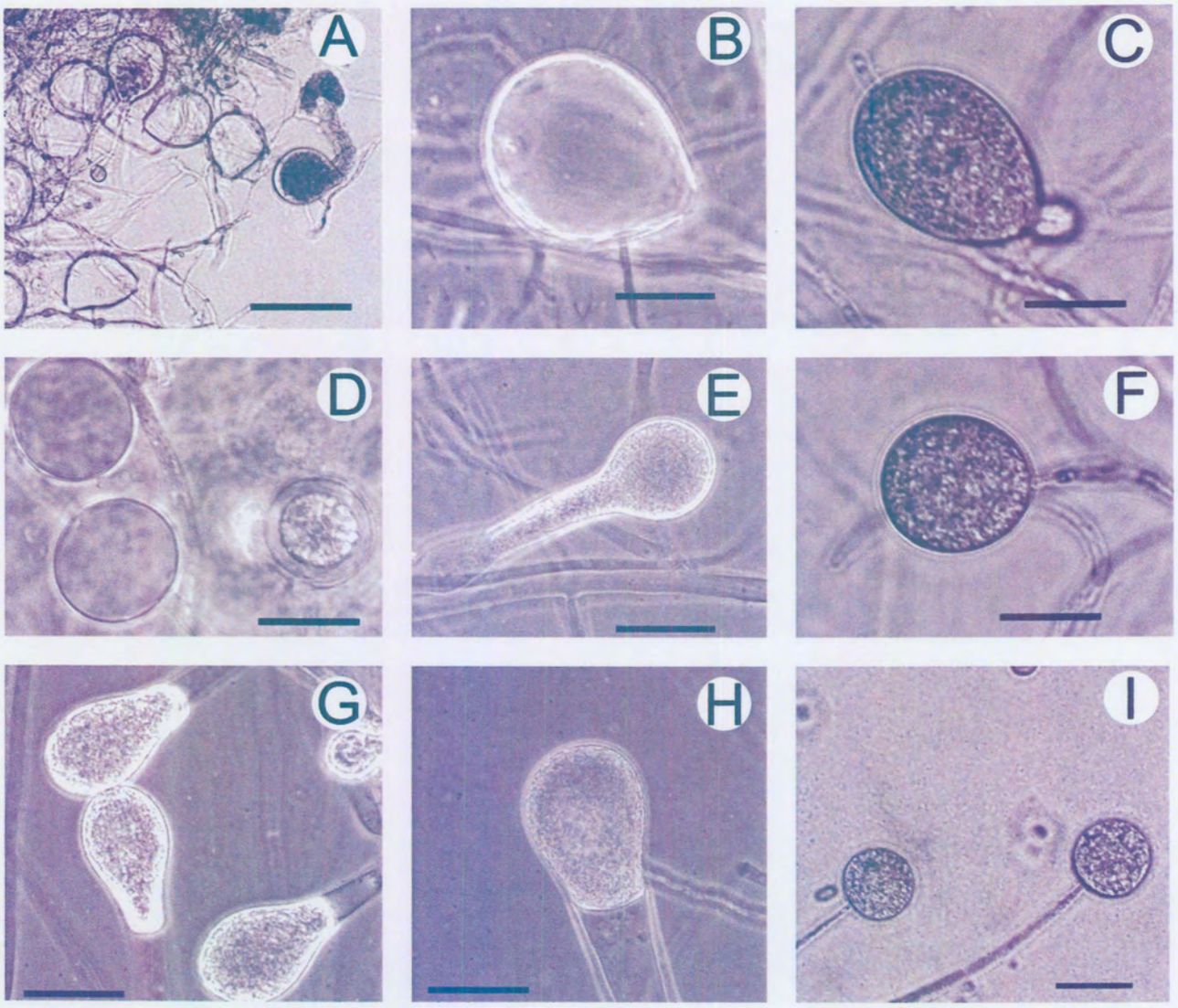


Figure 5. The most parsimonious tree obtained from a subset of *Cylindrocladium* isolates (520 steps, CI = 0.852, RC = 0.707, RI = 0.830) generated with a heuristic search in PAUP version 4.0b1 from aligned sequences of the 5' end of the β - tubulin gene. Gaps were treated as missing. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown below branches and decay indices above. A *Cylindrocladiella infestans* (GenBank accession number AF320190) was used as out – group.

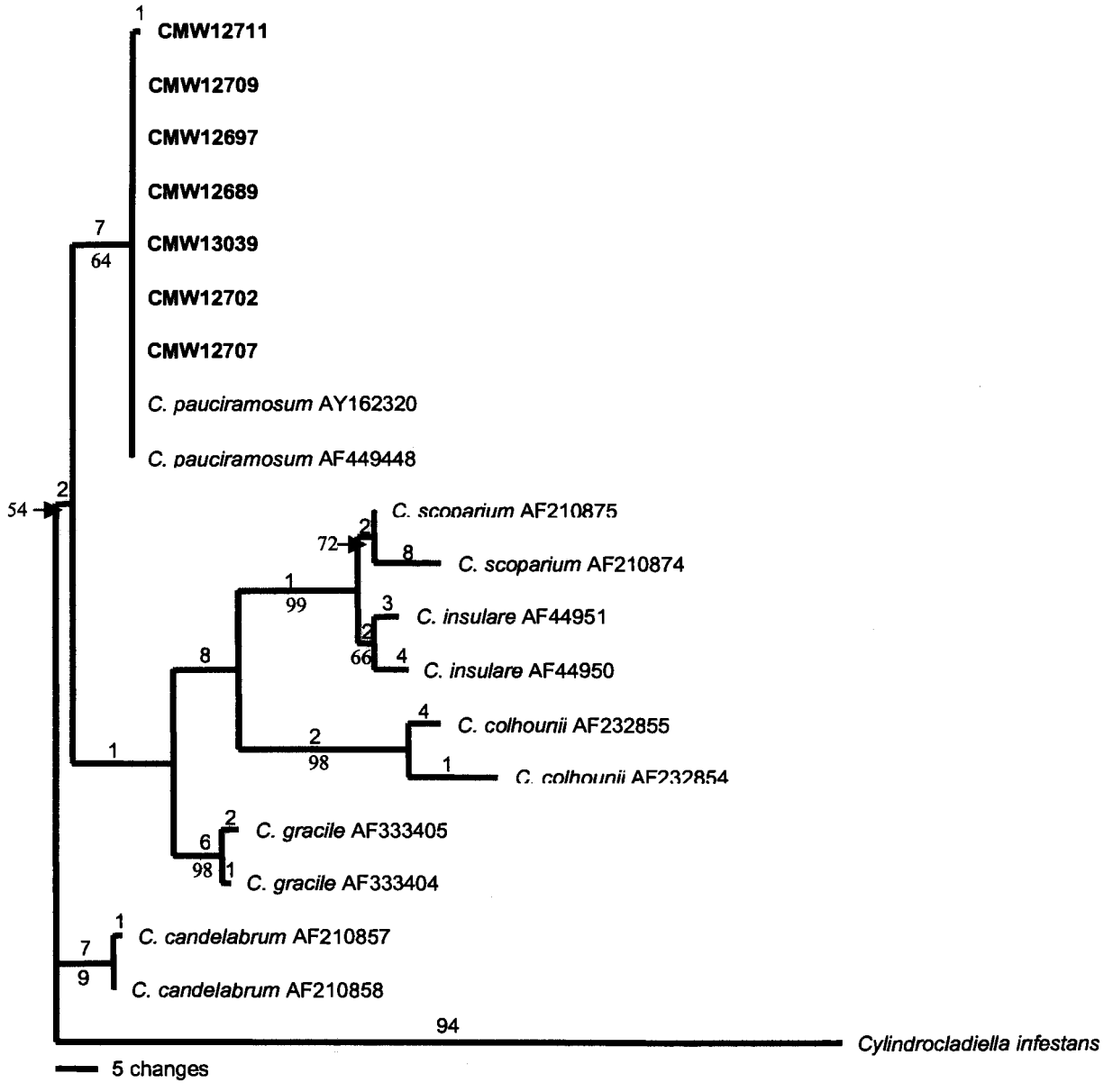


Figure 6. The most parsimonious tree obtained from a subset of *Fusarium* isolates (280 steps, CI = 0.867, RC = 0.803, RI = 0.926) generated with a heuristic search in PAUP version 4.0b1 from aligned sequences of the 5' end of the EF - 1α gene. Gaps were treated as missing. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown below branches and decay indices above. A *Botryosphaeria dothidea* (GenBank accession number AY236899) was used as out – group.

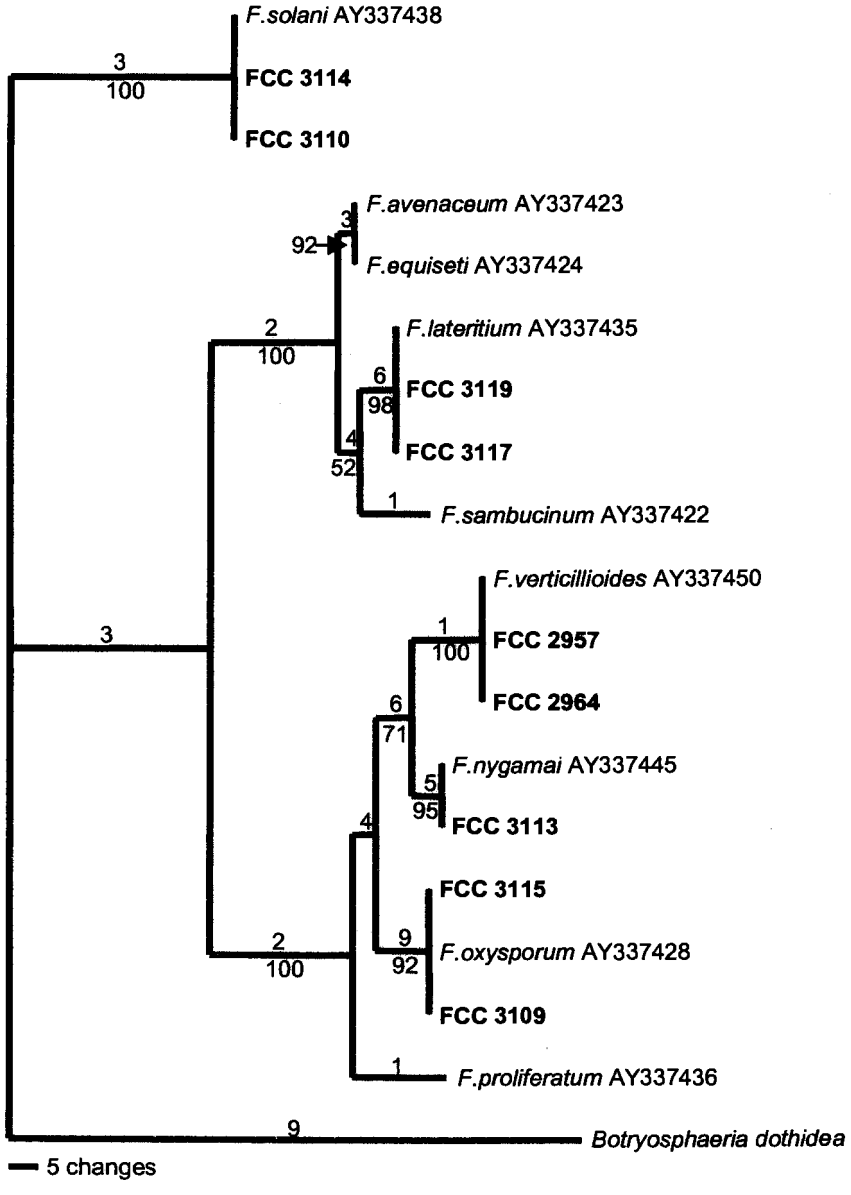


Figure 7. The most parsimonious tree obtained from a subset of *Phytophthora* isolates (814 steps, CI = 0.885, RC = 0.667, RI = 0.754) generated with a heuristic search in PAUP version 4.0b1 from aligned sequences of combined ITS1, 5.8s subunit and ITS2 regions of the genomic RNA gene. Gaps were treated as missing. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown below branches and decay indices above. A *Pythium aphanidermatum* (GenBank accession number AJ233438) was used as out – group.

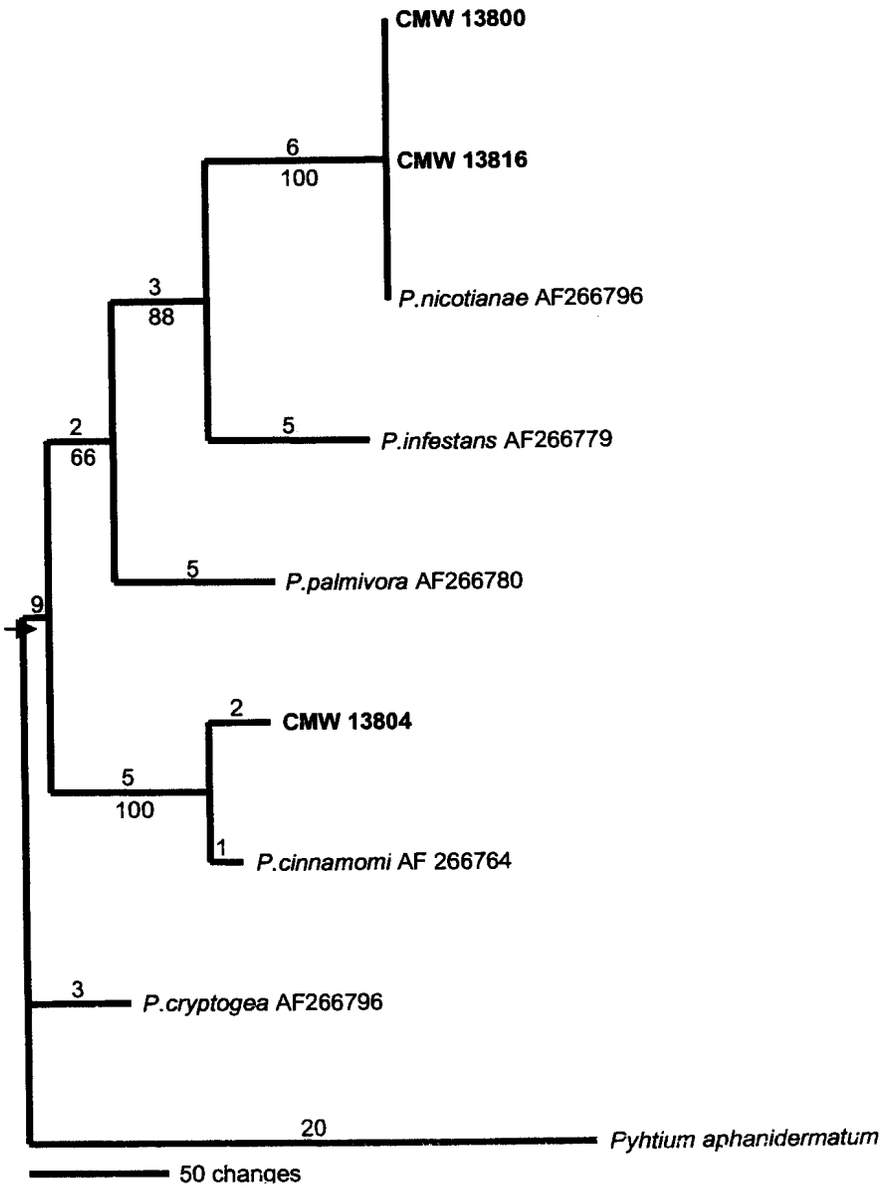


Figure 8. The most parsimonious tree obtained from a subset of *Pythium* isolates (851 steps, CI = 0.748, RC = 0.620, RI = 0.819) generated with a heuristic search in PAUP version 4.0b1 from aligned sequences of combined ITS1, 5.8s subunit and ITS2 regions of the genomic RNA gene. Gaps were treated as missing. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown below branches and decay indices above. A *Phytophthora cinnamomi* (GenBank accession number AF266764) was used as out – group.