Antifungal activity of plant extracts and oils against fungal pathogens of pepper (*Piper nigrum* L.), cinnamon (*Cinnamomum zeylanicum* Blume.), and turmeric (*Curcuma domestica* Val.)

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ABSTRACT

The antifungal and fungicidal effects of several water and ethanol extractions from plants and plant oils were studied in a series of *in vitro* and *in vivo* experiments against fungal pathogens of pepper (*Piper nigrum* L.), cinnamon (*Cinnamomum zeylanicum* Blume.), and turmeric (*Curcuma domestica* Val.). Spore germination of several fungi was completely inhibited by cinnamon bark and leaf oils, clove bud and leaf oils, lemon grass oil, and garlic oil (at concentrations of 0.1 – 3%), and by water and water/ethanol (50%) extracts of galangal rhizome, galangal stem, cardamom leaf, cinnamon bark, and lesser galangal rhizome (at concentrations of 500 mg fresh weight/mL).

The *in vitro* results revealed that ethanol extractions were more efficient than water extractions in inhibiting spore germination of several fungi. The highest inhibition of spore germination were provided by the oils of cinnamon (*C. zeylanicum*) and clove (*Syzygium aromaticum* [L.] Merr. et Perry). Cinnamon oil almost completely inhibited the germination of fungal spores. Extracts of galangal (*Alpinia galanga* [L.] Willd.) rhizomes and cardamom (*Elettaria cardamomum* Maton.) leaves (at concentrations of 500 mg fresh weight/mL) were the most effective in reducing spore germination of most of the fungi tested.

However, the data showed that extracts and oils were less effective in *in vivo* experiments. With papaya (*Carica papaya* L.) seedlings, germination of *Colletotrichum gloeosporioides* was variable. The effects of extracts and oils were qualitative rather than quantitative. Application of extracts and oils

reduced the symptoms of anthracnose caused by the fungus, but their effects against *C*. *gloeosporioides* were variable. It is suggested that this variability might be accounted for by the volatility of oil leading to a reduction of the concentration of active components on the leaves. Some extracts were phytotoxic at high concentrations and this had the unwanted effect of making infection easier for some fungi.

SEM observations revealed damaged spores and hyphae of *C. gloeosporioides* when cinnamon bark oil and galangal rhizome extract were present.

Finally, the data suggest that several plant extracts and oils may be a useful source of fungicidal preparations for agriculture use.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED AND THEIR MEANINGS

AF	Amistar fungicide
C	Cardamom plant sample
C21	*Pestalotiopsis* cf. *versicolor*
C24	*Curvularia inaequalis*
C26	*Helminthosporium* sp.
C30	*Curvularia* sp.
CaL	*Cassia* leaf
CDYE	Czapek-Dox Yeast Extract
CnB	Cinnamon bark
CnBO	Cinnamon bark oil
CnL	Cinnamon leaf
CnLO	Cinnamon leaf oil
ClBO	Clove bud oil
ClLO	Clove leaf oil
CmL	Cardamom leaf
CmLe	Cardamom leaf ethanol extract
CmLw	Cardamom leaf water extract
CmO	Cardamom oil
DF	Dithane fungicide
DW	Distilled water
EO	*Eucalyptus* oil
GcO	Garlic oil
GlL	Galangal leaf
GllesR	Lesser galangal rhizome
GllesO	Lesser galangal oil
GlR	Galangal rhizome
GlRe	Galangal rhizome ethanol extract
GlRw	Galangal rhizome water extract
GrR	Ginger rhizome
GrO	Ginger oil
GlS	Galangal stem
LgL	Lemon grass leaf
LgO	Lemon grass oil
LgS	Lemon grass stem
LmO	Lemon myrtle oil
NmO	Neem oil
OnO	Onion oil
P	Black pepper plant sample
P1	*Colletotrichum gloeosporioides*
P3	*Cladosporium* sp.
P6	*Curvularia* sp.
P8	*Fusarium* sp.
P10	*Pestalotiopsis* cf. *versicolor*
P13	*Colletotrichum* sp.
PL	Black pepper leaf
PO	Black pepper oil
RsO	Rosemary oil
T	Turmeric plant sample
T41	*Phoma* sp.
T48	*Exserohilum macginnisii*
T51	*Curvularia* sp.
T52	*Bipolaris spicifera*
TmL	Turmeric leaf
TO	Tea-tree oil
TmO	Turmeric oil
TmR	Turmeric rhizome

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 Plant diseases

Plant disease is an on-going limiting factor in crop production. Diseases of crops lead to yield losses and are of increasing importance as world population increases. A simple definition of plant disease is any disturbance that interferes with a plant's normal structure, function, or economic value (Persley 1993).

Plant diseases are of paramount significance to humans because they damage plants and plant products on which humans depend for food, clothing, furniture, and, in many cases, housing. Their influences on human welfare range from major catastrophes to minor annoyances. The potato late blight epidemic caused by *Phytophthora infestans* (Mont.) de Bary (responsible for the Irish potato famine of the 1840s), rice brown spot caused by *Helminthosporium oryzae* Breda de Haan (responsible for the Bengal rice famine of the 1940s), economic loss caused by southern corn leaf blight induced by *Helminthosporium maydis* in the U.S.A in 1970, and the aesthetic problems caused by Dutch elm disease from 1930s through the 1970s in the U.S.A, illustrate that diseases have been devastating on a large scale (Fry 1982). In the short term, plant diseases cause economic losses to growers, increase prices of produce to consumers, have direct and severe pathological effects on humans and animals, and destroy the beauty of the landscape. In order to control plant diseases around the world, billions of

dollars of toxic pesticides are utilized each year and the agents used may pollute water and terrestrial environments for long periods (Agrios 1997). Although it is extremely difficult to quantify losses due to such diseases, some authorities have estimated losses due to pathogens, weeds, and insects to be about 30% of worldwide food production (Fry 1982).

1.1.2 Plant diseases caused by fungi

Plant diseases can be divided conveniently into those caused by parasitic microorganisms or pathogens, and non-parasitic diseases or disorders. The major groups of plant pathogens are fungi, bacteria, viruses, and nematodes. Fungi are the most important common cause of plant disease (Persley 1993), since they are the most widespread and destructive parasites of plants (Ingold and Hudson 1993). More than 100,000 species of fungi are known to cause diseases in plants; some 50 species cause diseases in humans and about as many cause diseases in animals, most of them superficial diseases of the skin or appendages (Agrios 1997).

The effect of fungi on plants can be devastating. Cellular structure can be destroyed, physiological functions of the plant impaired, and rates of metabolism and metabolic pathways can be altered. These biological processes, have widespread consequences for infected plants because the impairment of any one function or organ has effects on others (Moore 1996). Prior to fungal penetration of a plant, many factors influence the sequence of events from germ tube emergence to attachment, adhesion, appresorium development, and growth on the plant surface. These may relate directly to endogenous factors such as influences from the environment, competition

from other microbes, or factors relating to the host plant such as leaf age, cultivar type, and physiological condition (Isaac 1992).

Fungi can be extremely destructive for several reasons (Strange 1993): 1) they sporulate prolifically; 2) the infection cycle (i.e., the time between infection and the production of further infectious propagules - usually spores) may be only a few days; 3) the fungi can rapidly mutate to develop resistance to fungicides; 4) the spores themselves may be carried long distances by the wind; 5) the fungus may produce compounds that are highly phytotoxic and/or a battery of enzymes that destroy the plant's structures; and 6) small lesions caused by some specialised parasites, such as rusts, act as powerful sinks, drawing nutrients away from the economically valuable part of the plant such as the grain, and thus depress yields.

The fungi and fungal-like organisms that cause plant diseases form a diverse group. The taxa with the largest number of plant pathogens are the Divisions of Deuteromycota and Ascomycota of the Kingdom Fungi (Moore 1996).

Fungal diseases still are an obstacle to the economic production of plants such as pepper (*Piper nigrum* L.), cinnamon (*Cinnamomum zeylanicum* Blume.), and turmeric (*Curcuma domestica* Val.). For example, an epidemic of foot rot disease caused by *Phytophthora palmivora* Leonian on pepper in 1956 destroyed about 50% of the pepper gardens in Kuching, Malaysia, and was estimated to have caused losses in Sarawak amounting to 7000 tonnes of pepper that contributed a significant part of the world's production at that time (Purseglove *et al.* 1981). Many researchers have described several fungal pathogens of pepper, cinnamon, and turmeric that

have become important disease-causing agents (Kumari and Gopalan 2000; Purseglove 1974; Purseglove *et al.* 1981; Sarma *et al.* 2000; Weiss 1997).

1.1.3 Synthetic versus natural product fungicides

At present, quick and effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic fungicides. Synthetic fungicides are being used on pepper, cinnamon, and turmeric for effective control of diseases in several countries (Anonymous 2000, 2003a). However, the indiscriminate application of chemicals has caused health hazards in animals and humans due to their residual toxicity (Satish *et al*. 1999; Strange 1993).

In recent years, a large number of synthetic pesticides has been banned in the Western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain, and an extension of their power to destroy both useful organisms and harmful pests (Strange 1993). In developing countries, they are still being used despite their harmful effects (Satish *et al*. 1999; Strange 1993). Many pathogenic microorganisms and insect pests have developed resistance against chemical pesticides. This seriously hinders the management of diseases of crops and agricultural products (Satish *et al*. 1999).

Considering these undesirable attributes of synthetic fungicides, there is an urgent need to develop alternative treatments that are less hazardous to humans and animals, and that impact less on the environment. Within this context, plant-produced compounds are of interest as a potential source of safer or more effective substitutes for synthetically produced antimicrobial

agents. Extracts isolated from several plants have been reported to have biological activity such as antimicrobial, antifungal, anti-inflammatory, and antioxidant activities (Yusuf *et al*. 2001).

The potential biological activity of extracts and essential oils from a wide range plants on various microorganisms has been assessed (Bhatm 2001; Hay and Waterman 1993; Kamalakannan *et al*. 2001; Letessier *et al*. 2001; Mahmoud 1999; Meena and Muthusamy 2002; Northover and Scheider 1996; Parveen and Kumar 2000; Suganda and Yulia 1998; Yusuf *et al*. 2001).

Therefore, the purpose of the present study was to evaluate the fungitoxic effect *in vitro* and *in vivo* of extracts and oils from pepper, cinnamon, and turmeric plants on their inhabitant phytopathogenic fungi. Similarly, extracts and oils from several medicinal plants also were screened for antimicrobial properties on the fungal pathogens of pepper, cinnamon, and turmeric.

1.2 RESEARCH AIMS

The aims of the research reported in this thesis were:

- To test *in vitro* and *in vivo* antimicrobial efficacy of plant extracts and oils on fungal pathogens from the same plant as that from the extracts and oils were prepared, and on fungal pathogens from plants of different origin.
- To compare the antifungal efficacy of natural plant-derived products with that of synthetic, commercial fungicides.

1.3 RESEARCH APPROACH

The research reported in this thesis was directed to:

- isolate fungal pathogens and leaf microflora from infected pepper, cinnamon, and turmeric plants, and to subject them to various concentrations of plant extracts and oils derived from those plants;
- identify plants that have potential antimicrobial properties for further research;
- investigate the antimicrobial activities of plant extracts and oils to satisfy the requirements of organic farming methods.

1.4 OUTLINE OF THE THESIS

A review of literature relevant to the nature and properties of medicinal plants and the use of plant extracts and oils as antimicrobial agents are presented in Chapter 2. Some of the characteristics and preparation of plant samples with potential antimicrobial activity are discussed in Chapter 3. The culturing of the pathogenic fungi is described in Chapter 4. The antifungal screening of selected plant extracts and oils is set out in Chapters 5 and 6. The more important results of the research are discussed in Chapter 7 where the overall conclusions from the research are presented and future research directions are explored. Where the reader is directed to figures or tables that are presented in Appendices, the figure or table number is prefixed with “A”.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Various plants have an important historical tradition in healing or are particularly valued for their medicinal, savoury, or aromatic qualities. They are considered as medicinal plants if they are collected only for their medicinal or aromatic properties (Anonymous 2001). Essential oils are aromatic, volatile extracts from components of such plants (leaves, flowers, fruit, bark, roots, rhizomes, and wood) that are usually obtained by the technologically simple processes of maceration and water solution or steam distillation (Brophy and Doran 1996). The extracts are used as flavours, fragrances, and for medicinal or health-care purposes (Brophy and Doran 1996). Nevertheless, the term 'volatile oil' is preferred because it refers to the fact that most components of the oils, which are stored in extracellular spaces in the epidermis or mesophyll, have low boiling points and can be recovered from the plant tissues by steam distillation (Hay and Waterman 1993).

Modern research has shown that medicinal plants act through a relatively small number of constituents called active principles. In a number of cases tannins have a more extensive action than the isolated active principles (Fluck and Schib 1976).

The general categories of plant natural products are as follows (Kaufman *et al*. 1999): 1) the lipids, including the simple and functionalised hydrocarbons, as well as terpenes; 2) aromatic compounds, including

phenols; 3) carbohydrates; 4) amines, amino acids, and proteins; 5) alkaloids; and 6) nucleosides, nucleotides, and nucleic acids.

There is no reason to doubt that evidence for the antifungal and antibacterial activity of some volatile oil components *in vitro* exhibit the same antibiotic activity in nature (Hay and Waterman 1993). The extent of recent interest in antimicrobial activity is shown by the wide range of organisms which have been tested against volatile oils, including filamentous fungi, yeasts, and plant viruses (Hay and Waterman 1993).

In general, terpenoid molecules predominate but many oils contain components originating from other biosynthetic pathways (Hay and Waterman 1993). The terpene component of oils is particularly significant (Weiss 1997). Volatile oils used for culinary, pharmaceutical, and perfumery purposes are composed almost entirely of two classes of compounds, terpenes and phenylpropenes. Of these, the terpenes are by far the more abundant but, where they occur, phenylpropenes are usually the major flavour/odour factors (e.g., anise, fennel, clove, cinnamon, and basil).

In this chapter, emphasis is given to the plant extracts and oils that are derived from commonly used medicinal plants, and to plant extracts and oils in relation to their use as antimicrobial agents, especially as controls over plant fungal pathogens.

2.2 PLANT EXTRACTS AND OILS

Plant extracts and oils have been used for a wide variety of purposes for many thousands of years. These purposes vary from the use of rosewood and cedar wood in perfumery, to flavouring drinks with lime, fennel

or juniper berry oil, and the application of lemon grass oil for the preservation of stored food crops. In particular, the antimicrobial activity of plant extracts and oils has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicines, and natural therapies (Hammer *et al*. 1999)

The prospect of using plant oils as fungicides is very appealing, because the refined oils are readily available and many are safe for human consumption. Their use as alternatives to synthetic fungicides is especially attractive to home gardeners and growers of organic produce (Northover and Scheider 1993).

Laboratory studies of extracts and oils of plants species have revealed powerful fungitoxicities in relation to many fungal pathogens (Hay and Waterman 1993). Such inorganic or organic chemicals either inhibit germination, growth and multiplication of pathogens, or are lethal to the pathogens. For example, oils obtained from seeds of several plants such as sunflower, olive, corn, and soybean have given excellent control against fungal pathogens (Agrios 1997).

Several researchers have shown that plant extracts may control anamorphic fungal plant pathogens (Agrios 1997; Bhatm 2001; Letessier *et al*. 2001; Mahmoud 1999; Parveen and Kumar 2000; Suganda and Yulia 1998). Plant extracts and oils have also been shown to be effective against other Ascomycota and Basidiomycota leaf pathogens (Kamalakannan *et al.* 2001; Meena and Muthusamy 2002; Northover and Scheider 1996). Some were also active against strains of bacteria (Dorman and Deans 2000; Muda *et al*. 2001; Yusuf *et al*. 2001).

Volatile oils can exhibit antifungal activity at very low concentrations in a growth medium. For example, inclusion of 1 – 10 μL mL^{-1} of marjoram oil in a culture broth reduced the growth of five species of filamentous fungi by up to 89% compared to the control (Hay and Waterman 1993).

Many plant oils have been tested for antibacterial and antifungal properties (Northover and Scheider 1993). Some of the natural products, such as cinnamon oil, clove oil, phenols, some spices, and many essential oils have been reported as effective inhibitors of fungal growth and aflatoxin production (Mahmoud 1999). Mycelial growth of the plant pathogenic fungi *Pyrenophora avenae* Ito & Kuribay and *Pyricularia oryzae* Cavara was completely inhibited by 0.4% hyssop (*Hyssopus officinalis* L.) oil (Letessier *et al*. 2001).

The volatile oil of lemon grass (*Cymbopogon citratus* [DC] Stapf.) has been confirmed against spoilage and mycotoxigenic fungi (Hay and Waterman 1993). The oil of basil (*Ocimum basilicum* L.) can inhibit the growth of a wide range of fungi (22 species) including mycotoxigenic strains of *Aspergillus flavus* Link ex. Fries and *A. parasiticus* Spear (Bennet and Christensen 1983) at a concentration of 0.15%. The effectiveness of the oil at this concentration, which is lower than the recommended application rates of several commercial fungicides, appears to be relatively unaffected by variations in temperature, conditions of storage, and inoculum density (Hay and Waterman 1993).

2.3 COMMONLY USED MEDICINAL PLANTS

Some commonly used medicinal plants come from the ginger family (Zingiberaceae), which contains plants from which essential oils are produced. These oils contain both terpenoids and phenylpropanoids (Katzer 1998). The grass family Poaceae includes about 40 species of aromatic tropical grasses of the genus *Cymbopogon* that are important for the production of essential oils (Anonymous 2003b).

Amongst the Angiosperms, the ability to accumulate terpenes is widely distributed in families of the temperate and tropical dicotyledons such as Piperaceae, Myrtaceae, Lauraceae, and Rutaceae, and in the monocotyledons mainly in the Araceae, Cyperaceae, Poaceae, and Zingiberaceae (Hay and Waterman 1993; Purseglove 1974).

2.4 BLACK PEPPER (*Piper nigrum*)

Pepper belongs to family Piperaceae and is a native of south India. The pepper plant is a large, woody, many-branched perennial shrub with a trailing or climbing stem. The leaves are smooth, dark green, nearly heart-shaped, and 10 to 15 cm long. The stem is divided into nodes and internodes. The nodes are slightly swollen joints from which arise the leaves, the flowers, and the roots. The terminal stem grows up tree supports or posts and clings to them by small finger-like roots, which are present at each node. The flowers arise at the nodes opposite the leaves on lateral stems or branches. The flowers are very tiny with 50 to 100 covering a 7 to 12 cm spike. When first in bloom, the flower spikes are yellow to light green. As the berries become mature, they turn dark green, and when fully ripe they

turn red. The unripe fruit is called a berry, and the dried berry, ready for marketing, is a peppercorn. The shallow root system of pepper is located within the uppermost 0.6 m of the soil. On average there are 3 to 6 main roots with many lateral or branching roots. Cultivated pepper is ideally planted from cuttings taken from terminal shoots of varieties of pepper that have a high ratio of hermaphrodite flowers having both male and female parts on the same flower. Pepper can be grown from seed, but growth from this source is very slow (Migvar 1967).

Pepper thrives in regions where the relative humidity is between 70 and 80%, and the annual rainfall is more than 2500 mm, and is evenly spread throughout the year. With its shallow root system, the pepper plant cannot survive prolonged drought of more than about 2 months. Pepper adapts to various soil types, but it prefers deep, and well-drained clay loam soils, rich in organic matter, and with a pH of 5.0 – 6.5. Pepper is usually planted on flat to undulating land not steeper than 25^0 and terracing is recommended where the slopes are steeper than 15^0. Good drainage is important to ensure good healthy growth (Meyer 1999).

2.4.1 Main constituents of pepper oil

Pepper has two main components: volatile oils and alkaloids. The pungency of pepper is conferred by the alkaloids of which piperine is the most important. Pepper contains about 0.6 – 2.6% essential oil that gives the aromatic flavour, which is dominated by monoterpene hydrocarbons (Anonymous 2003a; Katzer 1998; Vaughan and Geissler 1998).

2.4.2 Major diseases of pepper

Diseases caused by fungi, bacteria, mycoplasmas, algae, nematodes and phanerogamic parasites affect pepper in India (Kumari and Gopalan 2000; Sarma *et al.* 2000). The most common fungal diseases on pepper are foot rot (*Phytophthora capsici* Leonian), black berry (*Colletotrichum capsici* (Syd.) E.J. Butler & Bisby, *C. piperis* and *C. gloeosporioides* Penz.), pink disease (*Corticium salmonicolor* Berk. & Br.), velvet blight (*Septobasidium* sp.), and white root (*Rigidoporus lignosus* (Klotzsch) Imazeki). In black berry, which is caused by the alga *Cephaleuros parasiticus* Karsten, the affected fruits turn black and are shed prematurely (Purseglove 1974).

Other pathogens are root rot nematodes *(Meloidogyne* spp.), the fungus *Penicillium* sp., viruses, and combined infestation by the nematode *Radopholus similis* Cobb. with the fungus *Fusarium solani* (Mart.) Sacc. that causes slow decline or yellowing (Kumari and Gopalan 2000; Meyer 1999).

2.4.3 Pesticides used on pepper

Pesticides used to control foliar diseases on pepper are copper oxychloride (0.2%); potassium phosphorates (0.3%); metalaxyl (0.125%); Ridomil mancozeb (0.125%); Bordeaux mixture (copper sulphate and lime) (1%); carbendazim (0.1%); Phorate; and carbofuran. *Trichoderma* is a biologically active pesticide used for drench, foliar spray, or applied around the base of plants (Anonymous 2000, 2003a).

2.5 CINNAMON (*Cinnamomum zeylanicum*)

Cinnamon is a bushy, evergreen tree with numerous branches, long, leathery, bright green leaves, small yellow flowers, and ovoid blackish fruits. There is normally a single central stem to 20 m. The bark on young shoots is smooth and pale brown, on mature branches and stems is rough, dark brown or brownish grey. The bark contains 0.5 – 2.0% oil, of which the main constituent is cinnamaldehyde (60 – 70%). The leaves are opposite, 5 – 18 x 3 – 10 cm, with rounded bases and acuminate tips. They are green to bright green above, dull grey-green below, but young leaves may initially have a reddish tint. Green leaves yield about 1% essential oil, whose main constituent is eugenol (70 – 95%). The flowers are up to 3 mm diameter, pale yellow or cream, and have an unpleasant fetid smell. The fruit is a black or bluish, fleshy, ovoid drupe, 1.5 – 2.0 cm when ripe, and the enlarged calyx persists at its base. Fruits mature in 3 – 5 months, and the globular brown seeds contain approximately 33% fixed oil (Weiss 1997).

Cinnamon is a tree of the wet tropics with a somewhat restricted natural range in Sri Lanka, India, and South East Asia. It produces the finest bark in sunny regions with an average temperature of 27 – 30^0C. Annual rainfall of 2000 – 2500 mm with no pronounced dry season is optimal. It grows naturally from sea level to 1500 m, but for good quality commercial bark, 500 m is considered the maximum altitude in Sri Lanka, but it is somewhat higher in India (Weiss 1997).

2.5.1 Main constituents of cinnamon oils

The major oil constituents are cinnamaldehyde in bark oil, eugenol in leaf oil, and camphor in root-bark oil (Anonymous 2003a; Katzer 1998; Weiss 1997).

2.5.2 Major diseases of cinnamon

In India, Sri Lanka, and Indonesia, a most important disease is caused by the fungi *Corticium salmonicolor* Berk. & Broome (syn. *C. javanicum*) and *Phytophthora cinnamomi* Rands (Weiss 1997). Other stem diseases are caused by the fungi *Exobasidium cinnamomi* and *Diplodia* spp. Several root rots are damaging to some extent, such as brown rot caused by *Phellinus lamaensis* (Murrill), white rot (*Fomes lignosus* (Klotzsch) Bres.), and two black root rots (*Rosellinia* spp.). Leaf blight caused by *Pestalotia cinnamomi* Petch and *Glomerella cingulata* (Stoneman) Spaulding & V. Schrenk can cause severe damage and defoliation. Three other fungi causing leaf diseases have been recorded, namely, *Aecidium cinnamomi* Racib., *Leptosphaeria* spp., and *Gloeosporium* spp (Weiss 1997).

2.5.3 Pesticides used on cinnamon

The common pesticide used for cinnamon foliar diseases is Bordeaux mixture 1% (Anonymous 2000, 2003a).

2.6 TURMERIC (*Curcuma domestica*)

Turmeric is an erect perennial herb, but may be grown as an annual. The primary tuber at the base of the aerial stem is ellipsoidal, about 5 cm by

2.5 cm, bearing many rhizomes, 5 – 8 cm long, 1.5 cm thick, straight or a little curved, with secondary branches in two rows, which may have tertiary branches, and the whole forming a dense clump. The rhizomes are brownish and scaly on the outside and are a bright orange colour inside. The root is fleshy, often ending in a swollen tuber to 4 cm by 2 cm. The leafy shoots rarely exceed 1 m in height and are erect, bearing 6 – 10 leaves. Inflorescences are a cylindrical spikes, 10 – 15 cm long and 5 – 7 cm wide, that are terminal on the leaf shoot with the scape partly enclosed by the leaf sheaths (Purseglove *et al.* 1981).

Turmeric can be cultivated in most areas of the tropics and subtropics provided that rainfall is adequate or facilities for irrigation are available. It requires a hot, moist climate. It is usually grown in regions with an annual rainfall of 1000 – 2000 mm; below 1000 mm irrigation is required. It can be grown up to an altitude of 1220 m in the Himalayan foothills. Turmeric thrives best on loamy or alluvial, loose, friable, fertile soils and cannot stand waterlogging; gravelly, stony, and heavy clay soils are unsuitable for the development of the rhizomes (Purseglove *et al.* 1981).

2.6.1 Main constituents of turmeric oil

Turmeric contains an essential oil, which consists of a variety of sesquiterpenes. Most important for the aroma are turmerone, ar-turmerone, and zingiberene. Conjugated diarylheptanoids are responsible for the orange colour and probably also for the pungent taste (Katzer 1998).

2.6.2 Major diseases of turmeric

Most of the known diseases of turmeric are caused by fungi. A leaf spot, caused by *Taphrina maculans* Butl., is commonly present in India, wherever the crop is grown. Another leaf spot is caused by *Colletotrichum capsici* (Purseglove *et al.* 1981). A rhizome and root rot, caused by *Pythium graminicolum* L.S. Subram., has been reported in India, and a rhizome rot caused by *P. aphanidermatum* (Edson) Fitz. has been recorded in Sri Lanka (Purseglove *et al.* 1981).

2.6.3 Pesticides used on turmeric

Mancozeb (0.2%), zineb (0.3%) or Bordeaux mixture (1%) are used as leaf sprays. Dithane M-45 (0.3%) or Captofol (0.3%) are used for soil drenching or for dipping the rhizomes. The seed rhizomes are commonly treated with mancozeb (0.3%) and streptomycin. Bordeaux mixture (1%) or copper oxychloride (0.2%) are used to drench beds to control bacterial wilt (Anonymous 2000, 2003a).

CHAPTER 3

CHARACTERISTICS AND PREPARATION OF PLANT SAMPLES

3.1 INTRODUCTION

Some medicinal plants yield their bioactive compounds seemingly irrespective of growth condition (Weiss 1997). But the geographical area of production and weather conditions during the growing season, and particularly at harvest, can have a significant effect on the content of active ingredients found in a plant (Caldas 1998).

Each plant develops in a particular ecological zone where climatic conditions create very specific kinds of bioactive chemicals in the plants. For example, a medicinal plant grown in the high altitudes of the mountainous Himalayan regions may show substantial bioactive chemical profile differences when grown in the low land valleys (Caldas 1998).

The typical hot and humid tropical climate of north Queensland provides opportunities to produce a wide range of medicinal and aromatic plants. On account of the biodiversity found in such areas, it might be anticipated that many plants in the tropics would provide bioactive compounds. Meyer (2001) mentioned that if one were to choose randomly any pharmaceutical drug in the Western world, there would be a 25 per cent chance that the active ingredient came from some tropical plant. To illustrate the potential of tropical plants in the study regions, Setzer *et al.* (2001) found that crude extracts of 23 plants collected from the Paluma rainforest, near Townsville, north Queensland, showed antimicrobial activity.

The aims of the following section is to identify plants from north Queensland with promising antimicrobial activity, and to describe plant

samples collected from various regions of northern Queensland, methods used in plant samples preparations, and general characteristics of plant oils and synthetic fungicides.

3.2 PLANT SAMPLE PREPARATION

A range of plant materials was collected from their habitats in various regions of northern Queensland during September – December 2003 (Table 3.1). The specimens were stored at 4^0C in the cold room of the School of Tropical Biology, James Cook University.

Extracts of different plant parts were derived from nine species of plants and their antimicrobial activities were tested in the present study against a range of fungal species. Fresh mature specimens of greater galangal (*Alpinia galanga* [L.] Willd.), turmeric, pepper, and cinnamon were collected from the L & L Pepperfarm, owned by L. and L. Campagnolo, Walter Lever Estate, Silkwood, Queensland. Other plant extracts were derived from plants in the family Zingiberaceae, namely, ginger (*Zingiber officinale* Rosc.) that were collected from Gympie in southeasten Queensland, and lesser galangal (*Kaempferia galanga* L.) from the Innisfail area, and specimens of cardamom (*Elettaria cardamomum* Maton.) and lemon grass (*Cymbopogon citratus* [DC] Stapf.) from the Townsville area.

Table 3.1 Plants used as sources of extracts.

Species	Part of plant used	Code	Origins	Chemical properties (Sources*)
Cardamom (*Elettaria cardamomum* Maton.) Zingiberaceae	Leaves	CmL	Townsville	Oil in the seed contains α-terpineol, myrcene, limonene, menthone, β-phellandrene, 1,8-cineol, sabinene and heptane (1)
Cassia (*Cassia alata* L.) Fabaceae	Leaves	CaL	Townsville	Oil contains the phytochemicals antraquinone compound (e.g., dianthrone qlycosides) and flavonoids (2,3)
Cinnamon (*Cinnamomum zeylanicum* Blume.) Lauraceae	Barks, leaves	CnB, CnL	Silkwood (Fig.3.1A)	The essential oil in the bark is dominated by cinnamaldehyde and eugenol. Cinnamon leaves yield eugenol. Essential oil of cinnamon root bark is dominated by camphoric oil (1)
Galangal (*Alpinia galanga* [L.] Willd.) Zingiberaceae	Rhizomes, leaves, stems	GlR, GlL, GlS	Silkwood (Fig.3.1C)	The rhizome contains essential oil: 1,8 cineol, α-pinene, eugenol, camphor, methyl cinnamate and sesquiterpenes (1)
Lesser galangal (*Kaempferia galanga* L.) Zingiberaceae	Rhizomes	GllesR	Townsville	Main components of essential oil are ethyl cinnamate, ethyl-*p*-methoxycinnamate, and *p*-methoxycinnamic acid. 3-carene-5-one, 4-butylmenthol, β-phellandrene, α-terpineol, dihydro-β-sesquiphellandrene, pentadecane, 1,8-cineol, and cytotoxic principles (1)
Ginger (*Zingiber officinale* Rosc.) Zingiberaceae	Rhizomes	GrR	Gympie	The essential oil contains mostly sesquiterpenes, bisabolene, farnesene, and monoterpenoids (1)
Lemon grass (*Cymbopogon citratus* [DC] Stapf.) Poaceae	Stalks, leaves	LgS, LgL	Townsville	The essential oil consists mainly of citral (a mixture of two stereoisomeric monterpene aldehydes). Nerol, limonene, linalool and β-caryphyllene, and myrcene (1)
Pepper (*Piper nigrum* L.) Piperaceae	Leaves	PL	Silkwood	Essential oil is dominated by monoterpenes hydrocarbons: sabinene, β-pinene, limonene, furthermore terpinene, α-pinene, myrcene, Δ3-carene and monoterpene derivatives. Sesquiterpenes: β-caryophyllene, humulene, β-bisabolone and caryophyllene oxide, and ketones (1)
Turmeric (*Curcuma domestica* Val.) Zingiberaceae	Rhizomes, leaves	TmR, TmL	Townsville (Fig.3.1B)	Essential oil contains a variety of sesquiterpenes. turmerone, ar-turmerone, and zingiberene. Conjugated diarylheptanoids e.g., curcumin) are responsible for the orange colour and probably also for the pungent taste (1)

* 1 Katzer, 1998
2 Anonymous, 2005
3 Cowan, 1999

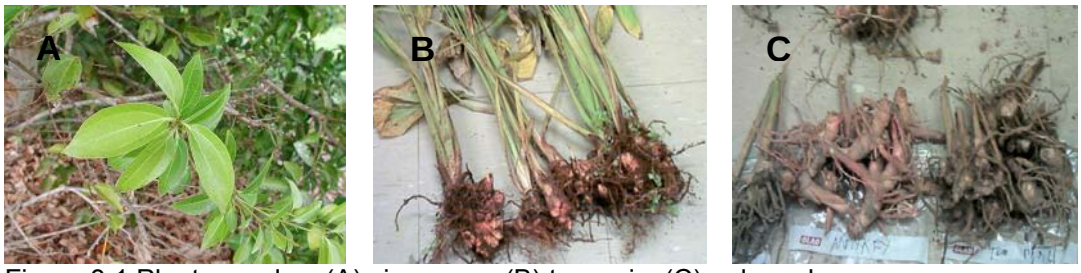

Figure 3.1 Plant samples: (A) cinnamon; (B) turmeric; (C) galangal.

3.2.1 Plant extractions

Samples of fresh plants used to prepare extracts were prepared variously from plant parts such as leaves, bark, stems, stalks, and rhizomes. Stevens *et al.* (2000) mentioned that the part of the plant which is harvested can have a significant effect on the subsequent efficacy of a medicinal plant product. Meanwhile, to eliminate any possible confusion of fundamental active ingredient profile differences with developmental maturity of the plant tissue, leaves of three different ages were mixed and used in the extractions from all of the plant samples. The ages chosen were young, mature (fully expanded leaves), and old leaves. Antifungal compound contents may be different in differently aged leaves. If protein content is used as a profile guide, the top leaves may contain around twice the amount of total soluble protein as the middle leaves, and the middle leaves, in turn, may contain about twice the amount as the basal leaves (Stevens *et al*. 2000).

3.2.2 Characteristics of water and ethanol extract suspensions

The extracts were made from 50 g fresh weight of plant material in 100 mL sterile distilled water or 50% ethanol, as follows. Various

concentrations of solutions were prepared by multiple dilutions. Appendix 5 shows the concentrations of the solutions used.

3.2.2.1 *Plant compounds extracted by water*

Fresh specimens of each plant were washed thoroughly 2 – 3 times with running tap water, followed with sterile distilled water, and then air-dried on a sterile blotter. An aqueous extract was prepared by blending 50 g of the selected plant part in 100 mL sterile distilled water in a Waring blender (Waring International, New Hartford, CT, USA) for 10 minutes. The macerate was filtered through four layers of cheesecloth, and then through Whatman No. 1 filter paper. The solution was sterilized by filtration through a bacteriological filter (0.2 μm pore size). The extracts were preserved aseptically in bottles at -18^0C until needed.

3.2.2.2 *Plant compound extracted by ethanol solution*

Plant materials were also extracted using organic solvents. Plant materials (50 g) were blended with 100 mL 50% ethanol for 10 minutes. The extracts were filtered through cheesecloth and filter paper, and stored in the same manner as were the water extracts.

The ethanol extractions resulted in thicker liquid, greater oil production, and stronger aromas and colour when compared with water extractions. Ethanol is the most efficient solvent for the extraction of herb bioactive principles (Hughes 2002).

3.3 PLANT OIL PREPARATION

Seventeen plant oils were tested in the present study; they were obtained commercially from two wholesale suppliers of 100% pure essential oils: New Direction (www.newdirections.com.au) and Perfect Potion (www.perfectpotion.com.au) (Table 3.2).

Appendix 5 shows calculations to determine the concentrations of the oils used.

Table 3.2 Oils used in the study.

Species	Part of plant used in preparing oil	Code
Black pepper (*P. nigrum* L.)	Dried unripe berries	PO
Cardamom (*E. cardamomum* Maton.)	Seeds	CmO
Cinnamon (*C. zeylanicum* Blume.)	Barks and leaves	CnBO, CnLO
Clove (*Syzygium aromaticum* [L.] Merr. et Perry)	Buds and leaves	ClBO, ClLO
Eucalyptus (*Eucalyptus fruticetorum* F.Muell. ex Miq.)	Herbs	EO
Garlic (*Allium sativum* L.)	Pods	GcO
Ginger (*Z. officinale* Rosc.)	Rhizomes	GrO
Lemon grass (*C. schoenanthus* (L.) Spreng.)	Leaves	LgO
Lemon myrtle (*Backhousia citriodora* F. Muell.)	Leaves	LmO
Lesser galangal (*K. galanga* L.)	Rhizomes	GllesO
Neem (*Azadirachta indica* A. Juss)	Seeds	NmO
Onion (*Allium cepa* L.)	Bulbs	OnO
Rosemary (*Rosmarinus officinalis* L.)	Herbs	RsO
Tea-tree (*Melaleuca alternifolia* Maiden & Benche)	Leaves and twigs	TO
Turmeric (*Curcuma longa* L.)	Rhizomes	TmO

3.4 SYNTHETIC FUNGICIDES

Fungicides chosen for the study were Amistar and Dithane M-45, which are used currently on the pepper farm. The former was obtained from the Syngenta Company and the latter from the L & L Pepperfarm.

Amistar is a broad spectrum and systemic fungicide produced by Syngenta. It has an active ingredient of 250 g/L azoxystrobin, which possesses a novel biochemical mode of action: it inhibits mitochondrial

respiration in fungi (Anonymous 2003c). It is active on all four classes of fungi that attack crops. It is registered for use on more than 80 crops in 85 countries worldwide. Today, farmers use Amistar for fungal control in cereals such as wheat and barley as well as in vines, fruits, vegetables, bananas, rice, soy beans, turf and ornamentals.

Dithane M-45 is a broad spectrum, contact fungicide produced by Dow AgroScience, Inc. Its active ingredient is 800 g/kg Mancozeb that has a multi-site mode of action that affects many enzymes in the fungi (Anonymous 2003d). Dithane M-45 is recommended for control of wide range of diseases. Optimal disease control is achieved when the fungicide is applied in a regularly scheduled, preventative spray program.

Fungicides usually are mixed on the farms and applied at the rate of 175 – 210 g/ha of Amistar (280 g mixed in 900 L water) and 3 – 4 kg/ha of Dithane M-45 (2 kg mixed in 900 L water) (L. Campagnolo, *personal communication*).

CHAPTER 4

CULTURE OF FUNGI

4.1 INTRODUCTION

Fungi must be cultured to increase the population of infective propagules for inoculation or to study their taxonomy. All microorganisms require a set of environmental conditions (aeration, light, moisture, temperature, etc.) under which they grow and sporulate best. The range of conditions permitting vegetative growth and sporulation is divided into minimum, maximum, and optimum ranges. The optimum range may be wide or narrow. There is no universal set of conditions for culturing pathogenic fungi (Dhingra and Sinclair 1985), and spores are often produced under conditions that are adverse to vegetative growth (Dhingra and Sinclair 1985).

Spore germination is one of the most important life cycle stages of fungi as the spore is the principal agent of dispersal. Nutrients in the growth medium, especially carbon and nitrogen, have a major affect on the growth and sporulation of fungi (Dhingra and Sinclair 1985). Consequently, any consideration of the ecology or the spread of fungi must take fungus nutrition and spore germination into account. Hence, the control of plant diseases by protectant fungicides is, in essence, a matter of inhibiting the germination of spores (Cochrane 1963).

The aims of the following section of the present study were to isolate and cultivate fungi from pepper, cinnamon and turmeric crops at the L & L Pepperfarm, Silkwood, and to determine the times required by the fungi to germinate.

4.2 MATERIALS AND METHODS

4.2.1 Culture medium

The culture medium for fungal isolation was one-sixth strength Czapek-Dox Yeast Extract agar (CDYE agar) (Warcup 1955); Appendix 1 shows its composition. The pH of the medium was adjusted with hydrochloric acid (HCl) to 4.6 to restrict bacterial growth; appropriate growth media are slightly acid for most fungi, in contrast in the requirements for bacteria, which are generally intolerant of acid conditions (Fox 1993). Alternatively, chloramphenicol (0.19 g/L) was added to the medium and its pH adjusted to 6.5 (Pitt and Hocking 1991).

Other culture media such as water agar, potato dextrose agar (PD agar), and potato carrot agar (PC agar) (Dhingra and Sinclair 1985) were used at various times throughout the present project in order to stimulate fungal sporulation.

4.2.2 Isolating the microorganisms from plant leaves

4.2.2.1 *Fungal pathogens*

Pieces of fresh leaves of pepper, cinnamon, and turmeric plants from L & L Pepperfarm were cut from diseased and healthy parts of the leaves of each plant (Agrios 1997). These leaf pieces or whole pepper berries were dipped momentarily in 70% ethanol and then soaked in a solution of 1% sodium hypochlorite for five or ten minutes. The sterilized tissues then were washed once in sterile distilled water, placed in Petri dishes containing CDYE agar, and maintained in an incubator at 28^0C.

4.2.2.2 *Leaf microflora*

Leaflets (i.e. pieces of pepper, cinnamon, and turmeric plant leaves up to 10 x 10 mm in area) were prepared for assessment of their active fungal populations by serial washing with distilled water (Shipton *et al*. 1981). Washing schedules were 2, 4, 7, and 10 times (two replicates each). Twelve pieces of 100 mm^2 leaflets (four pieces from one leaf) were placed in Universal bottles for each washing schedule (two replications), and washed by shaking for 2 minutes first with 1 change of 10 mL sterile surfactant Tween 80 (2 mL/ litre water) using a Griffin Flask Shaker. This was followed by additional changes of sterile distilled water (10 mL).

Spread plate assessment of fungal fragments and spores recovered followed the method of Black (1999) by plating out 0.25 mL aliquots of selected washings on CDYE agar in three replications. Finally, 4 pieces of washed leaflets from each washing schedule were plated (leaf plate method; Black 1999) onto CDYE agar in three replications. After incubation for 5 days at 28^0C, the plates were examined and the species of each fungus present were counted under a bacterial colony counter (Black 1999) and identified.

4.2.3 Identification of fungi

The fungal colonies growing on the culture plates were identified morphologically on the basis of their colour, type of spores, the presence of sporodocia or appresoria, colony texture, and other growth characteristics of the fungi (Dhingra and Sinclair 1985).

4.2.4 Spore harvesting

The fungal pathogens were cultivated in order to produce viable spores in sufficient numbers (e.g. 50,000 spores/mL) to allow a range of tests to be conducted on spores of the same age and life history.

Spores were obtained by flooding 1 – 2 week old cultures with 5 mL of sterile distilled water, and the concentrations of spores were diluted to approximately 50,000/ mL with sterile distilled water (Dhingra and Sinclair 1985). Spore age was recorded since viability and germination potential are influenced by age. Spore counts were conducted using a haemocytometer (Mather and Roberts 1998).

4.2.5 Germination spore sampling times

A germination test was conducted for each fungal pathogen, over a period of 24 hours, to determine when germination reached a peak. Agar slides were prepared by pipetting 1 mL of 1.5% water agar onto slides to form a layer. The spores were harvested and 2 drops of spore suspension were placed and spread on the agar slides. The slides were incubated on humid, sterile, Petri plates. A count of germination was taken every 4 hours, starting at 6 hours after plating and finishing at 24 hours. Observations during the first six hours of incubation showed that most of the fungi started to germinate during this period.

4.2.6 Establishment of diseases

Pathogenicity tests were conducted to identify the abilities of the fungi to infect host plants. Isolated fungus was inoculated onto host tissue

(leaves) to determine if it was capable of producing visual symptoms or signs of plant diseases (symptom development). To inoculate the host tissue, a spore suspension was prepared in sterile water contain 0.5% concentration of surfactant Tween 80. The way chosen for breaking surface tension was to gently rub the leaf between moistened fingers and thumb without wounding the tissues (Dhingra and Sinclair 1985). Most fungal leaf pathogens do not require wounding for infection, since most penetrate through stomates, directly through epidermal cells, or at the junctions between epidermal cells (Dhingra and Sinclair 1985).

Spore suspensions were added to the leaves to the point of runoff. Alternatively, small plugs of mycelial inoculums were placed on the plant surface, and then a moist cotton swab was placed over the plugs. Inoculated host tissues were kept in a humid condition using plastic bags for intact tissues or moist boxes for detached tissues. Tissues were kept moist for at least 48 hours or until symptoms of the fungal infection appeared (Dhingra and Sinclair 1985).

4.2.7 Data analysis

Statistical analysis of the data was undertaken using an SPSS version 11.0 software package. The effect of serial washing schedules on fungal colonization was analyzed by one-way analysis of variance and the significance of the differences between means were calculated using the Duncan test at the 5% level (Zar 1999).

4.3 RESULTS AND DISCUSSION

4.3.1 Colony counts of leaf microflora

A variety of different organisms, especially bacteria and fungi, were isolated from pepper, cinnamon, and turmeric leaves. Several pigmented bacteria dominated the bacterial population. Fokkema (1986) and Lindow (2000) mentioned that large bacterial populations, as epiphytic bacteria, commonly colonize plant leaf surfaces. The mycelial fungal colonies were counted and the results are given in Table 4.1.

Table 4.1 Mean colony-forming units of fungi isolated from pepper, cinnamon, and turmeric under several washing cycles.

Plant samples	Washing cycles			
	Number of colony forming units per mL			
	2	4	7	10
Pepper	1827a	4000b	5227b	9000c
Cinnamon	2187a	4373b	5787c	6667c
Turmeric	1467a	4373b	7467c	8867c

Means followed by the same letter across the rows are not significantly different according to Duncan's test ($P<0.05$)

There was a strong trend in the number of isolated fungal colonies to increase from lower to higher washing cycles in all of the plant samples (Table 4.1). In general, the data show that microorganisms can be washed from a leaf surface, but with some apparent difficulty. The number of colony-forming units of fungi recovered increased with the number of washes. While washing presumably releases more microflora from the leaf surface, part of this increase may have been due to the fragmentation of leaves and fungal hyphae.

Washing 7 and 10 times did not significantly increase the colonies of microflora recovered from cinnamon and turmeric. All of the colonies recovered after 10 washes were significantly greater ($P<0.05$) than those found after seven. When extended beyond 10 washing cycles, there was no further increase in fungal recovery.

4.3.2 Fungi isolated from pepper, cinnamon, and turmeric leaves

A total of 37 fungal species were isolated from pepper, cinnamon, and turmeric plants from the L & L Pepperfarm (Tables 4.2 – 4.4; Fig. 4.1). They were identified using information from Arx (1981), Bailey and Jeger (1992), Barnett and Hunter (1972), Barron (1972), Carmichael *et al.* (1980), Ellis (1971), Ellis (1976), Kirk *et al*. (2001), and Rayner (1970). Those failing to produce identifiable structures were classified as unknowns. A laboratory code number was given to each fungal species with numbers 1 to 20 allocated to fungi isolated from pepper, numbers 21 to 40 to fungi isolated from cinnamon, and numbers 41 to 60 for fungi isolated from turmeric.

The macroscopic and microscopic characteristics of all the isolated fungi are shown in Appendix 2 and identifications are included where possible. Specimens of *Bipolaris*, *Exserohillum*, *Colletotrichum*, *Curvularia*, *Pestalotiopsis*, and *Phoma* were sent to Identification Services (CBS), Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands, to provide more detailed identification than was possible at JCU Townsville.

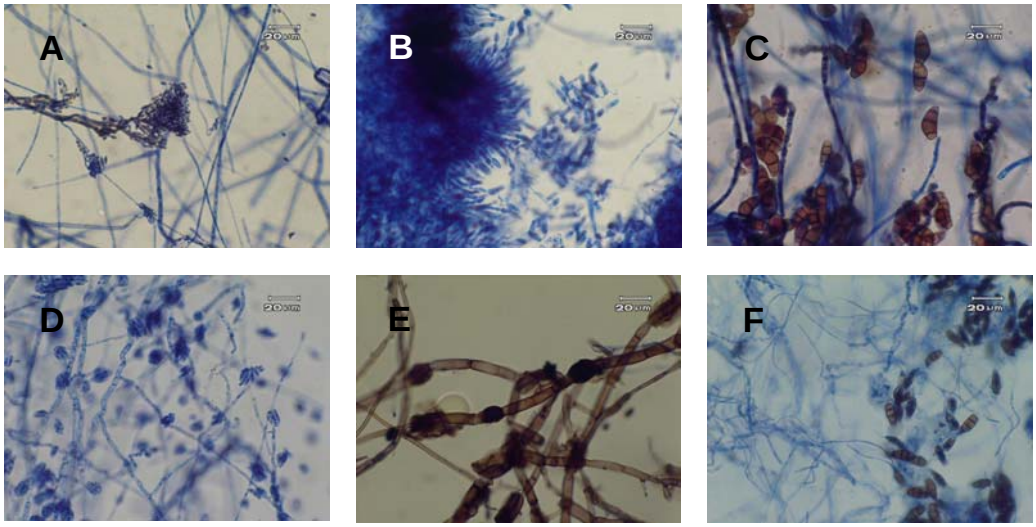

Figure 4.1 Isolated fungal species (A) *Cladosporium* sp.; (B) *Colletotrichum gloeosporoides*; (C) *Curvularia* sp.; (D) *Fusarium* sp.; (E) unknown species; (F) *Pestalotiopsis* sp.

Table 4.2 The fungi isolated from pepper leaves.

Fungi	Comments	Laboratory code no.
Fungi used in the study		
Cladosporium sp.††	Cause of leaf spot	P3
Colletotrichum orbiculare††	Cause of anthracnose	P13
Colletotrichum gloeosporioides††	Cause of anthracnose	P1
Curvularia sp.†	Cause of leaf spot	P6
Fusarium sp. B†	Cause of wilt disease	P8
Pestalotiopsis cf. *versicolor*†	Cause of leaf blight	P10
Fungi not used in the study		
Fusarium sp. A†	Not a leaf pathogen	P4
Nigrospora sp.††	Failed to germinate	P7
Trichoderma sp. A†	Not a leaf pathogen	P9
Trichoderma sp. B†	Not a leaf pathogen	P12
Unknown species A††	Failed to sporulate	P2
Unknown species B††	Failed to sporulate	P5
Unknown species C††	Failed to sporulate	P11

† Isolated from diseased part
† Isolated through serial washing
†† Isolated from diseased plant parts and through serial washing

Table 4.3 The fungi isolated from cinnamon leaves.

Fungi	Comments	Laboratory code no.
Fungi used in the study		
Curvularia inaequalis††	Cause of leaf spot	C24
Curvularia sp.††	Cause of leaf spot	C30
Helminthosporium sp.††	Cause of leaf spot	C26
Pestalotiopsis cf. *versicolor*†††	Cause of leaf blight	C21
Fungi not used in the study		
Aspergillus sp.††	Not a leaf pathogen	C23
Rhizopus sp.††	Not a leaf pathogen	C28
Cladosporium sp.††	Assessed on pepper	C22
Nigrospora sp.††	Failed to germinate	C25
Trichoderma sp.††	Not a leaf pathogen	C29
Unknown species A†	Failed to sporulate	C27
Unknown species B†	Failed to sporulate	C31

† Isolated from diseased part
†† Isolated through serial washing
††† Isolated from diseased plant parts and through serial washing

Table 4.4 The fungi isolated from turmeric leaves.

Fungi	Comments	Laboratory code no.
Fungi used in the study		
Exserohilum macginnisii††	Cause of leaf spot	T48
Curvularia sp.†††	Cause of leaf spot	T51
Phoma sp. (*Peyronellaea*)†††	Cause of leaf spot	T41
Bipolaris spicifera †††	Cause of leaf spot	T52
Fungi not used in the study		
Aspergillus sp.††	Not a leaf pathogen	T53
Cladosporium sp.††	Assessed on pepper	T42
Rhizopus sp.††	Not a leaf pathogen	T45
Nigrospora sp.†††	Failed to sporulate	T43
Paecilomyces sp.††	Not a leaf pathogen	T50
Pestalotiopsis sp.††	Assessed on pepper	T44
Trichoderma sp.††	Not a leaf pathogen	T46
Unknown species A†††	Failed to sporulate	T47
Unknown species B††	Failed to sporulate	T49

† Isolated from diseased part
†† Isolated through serial washing
††† Isolated from diseased plant parts and through serial washing

Thirteen fungal species were isolated from pepper, 11 species from cinnamon, and 13 species from turmeric, and seven fungal genera could not be identified because they failed to sporulate in pure cultures. The identified species of fungi belonged to the genera *Aspergillus*, *Bipolaris*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Exserohilum*, *Fusarium*, *Helminthosporium*, *Nigrospora*, *Paecilomyces*, *Pestalotiopsis*, *Phoma*, *Rhizopus*, and *Trichoderma*.

The fungal genera most frequently isolated from the plant samples were *Cladosporium*, *Curvularia*, *Fusarium*, *Helminthosporium*, and *Trichoderma*. These fungal genera, with the exception of *Trichoderma*, have been previously found to be the most common resident fungi isolated from medicinal plants under field conditions (Aziz *et al*. 1998). Members of the genera *Colletotrichum*, *Pestalotiopsis*, and *Phoma* are commonly pathogenic (Agrios 1997; Barnett and Hunter 1972). *Trichoderma* is recognized as a successful saprophytic fungus, besides being reported as a parasite on other fungi (Agrios 1997; Barnett and Hunter 1972). *Aspergillus* and *Rhizopus* are recognized as the most common contaminant fungi of stored plant materials (Aziz *et al*. 1998).

The colonization of leaf surfaces was dominated by some species, as shown in summary form in Table 4.5. The complete data are shown in Appendix 3.

The fungi *C. gloeosporioides*, *Pestalotiopsis* sp., and *Phoma* sp. are presumed as a major cause of the disease on pepper, cinnamon, and turmeric on account of their frequent occurrence and their known behaviour on other host plants.

Table 4.5 Dominance of fungal isolates isolated from pepper, cinnamon and turmeric.

Plant samples	Fungal species
Pepper	*Colletotrichum gloeosporioides*
	Unknown species A
	Cladosporium sp.
Cinnamon	*Pestalotiopsis* sp.
	Cladosporium sp.
Turmeric	*Phoma* sp.
	Cladosporium sp.

Several fungal species were not recovered form the higher washing cycles. These were fast-growing species in which the colonies grow quickly without producing spores or conidia. They were assumed to be either weak pathogens or saprophytic fungi on account of their fast growth. Fast growing fungi are commonly isolated as saprophytic contaminants (Barnett and Hunter 1972).

In order to avoid excessive work in maintaining fungal cultures and, more importantly, to maintain cultures with a minimum of genetic change (Cochrane 1963), the spores of all of the isolated fungi were preserved in microbeads (Fig. 4.2A) that were frozen at -73^{0}C (Madigan *et al*. 2000). Through this preservation technique, the fungi retain their abilities to grow, germinate, and sporulate well when they are regrown (Fig. 4.2B).

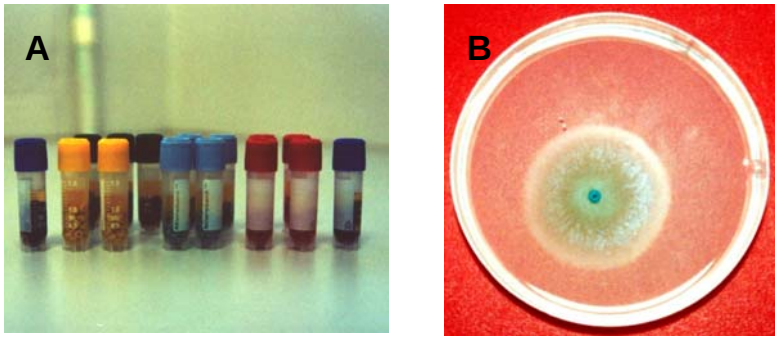

Figure 4.2 (A) A form of porous microbeads used for preservation; (B) fungal culture growing from a microbead in a Petri dish, diameter of 85 mm.

4.3.3 Germination spore sampling times

Germination tests were conducted for each fungal species, over a period of 24 hours in order to determine the best time to spot-sample for peak germination. An example of the kind of germination data collected is shown in Figure 4.3 for *C. gloeosporioides*; similar data for the other 13 species studied are shown in Figs. A.4.1 – A.4.13 in Appendix 4.

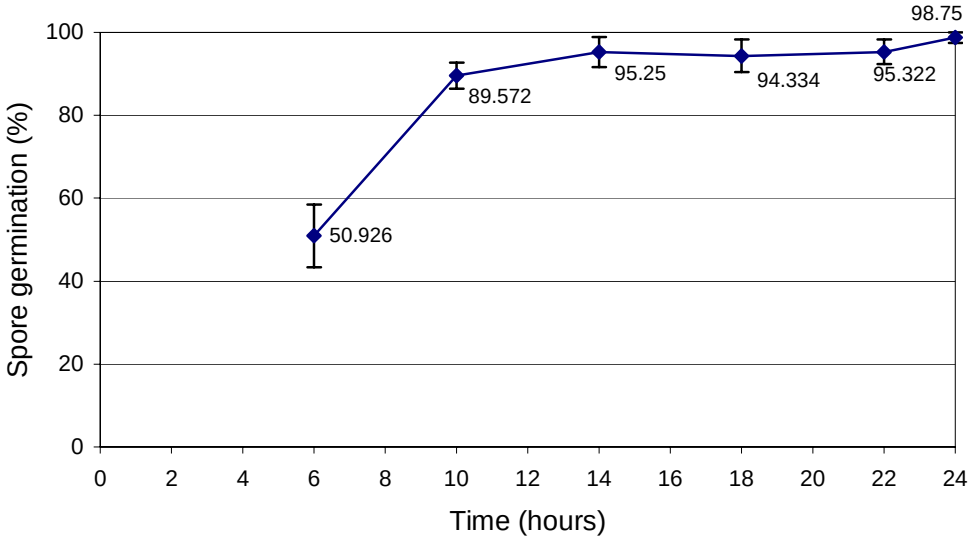

Figure 4.3 Germination of *Colletotrichum gloeosporioides* spores, isolated from pepper, after 6 to 24 hours after incubation.

Germination tests conducted in well-slides containing sterile distilled water resulted in a low percentage of germination compared to those performed on an agar medium; several fungi species (*Cladosporium* sp., *Colletotrichum orbiculare*, *Pestalotiopsis* sp.) failed to sporulate. For example, 24.9% of *C. gloeosporioides* spores and 63.3% of *Curvularia* sp.

spores had germinated after 24 hours compared to 100% germination of these fungi in the same time period on agar.

A number of factors are operating here, with aeration and nutrition being probably most significance. A preliminary test showed that application of 0.5% glucose solution resulted in 25 – 30% of *C. gloeosporioides* spores germinating instead of 0% in distilled water at 24 hours. Spores located at the extreme edge of the well slide germinated more strongly and uniformly than those in the centre. This indicated that oxygenation issues were significant.

Cochrane (1963) noted that many fungal spores germinate very poorly, or not at all, without added nutrients, and examples included conidia of several imperfect fungi. Other fungi germinated fairly well in water but did so more completely, or more rapidly in nutrient media, or in contact with complex biological products. The addition of low concentrations (0.1 – 0.2%) of nutrients in spore suspensions may provide the impetus needed for spore germination, and may also help the spores to adhere to leaf surfaces and prevent the spores drying out (Dhingra and Sinclair 1985).

The times taken for each fungal pathogen to achieved 80% $\pm$ 5 and 95% $\pm$ 5 germination are summarized in Table 4.6.

Previous experience indicates that where the germination percentage is lower than approximately 70%, the spores should not be used in germination inhibition trials (Edman *et al*. 2004).

Table 4.6 Fungal spores germination assessment over a 24 hour period.

Pathogen	Laboratory code no.	Time to germination (hours)	
		80% ± 5	95% ± 5
Cladosporium sp.	P3	14	18
Colletotrichum orbiculare	P13	18	22
Colletotrichum gloeosporioides	P1	10	14
Curvularia sp.	P6	6	10
Fusarium sp.	P8	8	14
Pestalotiopsis sp.	P10	14	18
Curvularia inaequalis	C24	6	10
Curvularia sp.	C30	6	10
Helminthosporium sp.	C26	<6	6
Pestalotiopsis sp.	C21	18	24
Exserohilum macginnisii	T48	<6	6
Curvularia sp.	T51	6	10
Phoma sp.	T41	14	22
Bipolaris sp.	T52	10	14

Fungi of *Curvularia*, *Bipolaris*, *Drechslera*, *Exserohilum* (those formerly included under 'Helminthosporium fungi' (Anonymous 2004b; Barnett and Hunter 1972; Elliott and Simone 2004) reached peak germination levels within the first six hours. Meanwhile, other groups of fungi that were most likely to be fungal pathogens reached peak germination levels at or after more than ten hours (Table 4.6). Germination commenced for some fungi within the first three hours after incubation whereas others took longer.

4.3.4 Establishment of diseases (Koch's Postulates)

An initial attempt to test the ability of *C. gloeosporioides* spores to infect intact pepper or detached leaves or berries was unsuccessful. Therefore, inoculation was conducted *in vitro* on pepper berries. The results

showed that the fungus infects the berries and allow Koch's postulates (Agrios 1997) to be fulfilled, thus establishing it as the causal agent of the disease. Figure 4.4 shows several stages of development of anthracnose symptoms on pepper berries caused by *C. gloeosporioides*. The initial symptom was black sunken spots. The whole berries later turned black, and then pinkish-orange areas formed when the fungus produced spore masses. The berries then became white as the germinated spores developed mycelia.

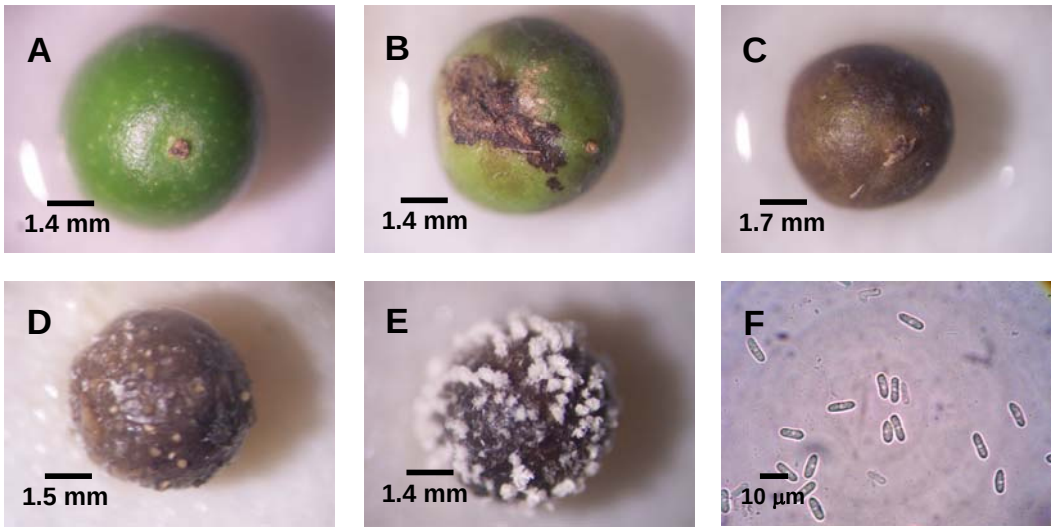

Figure 4.4 Anthracnose development on pepper berries: (A) an inoculated berry; (B) black sunken rot lesion at 6 days after inoculation; (C) black dry rot within 10 days after inoculation; (D) fungus sporulation within 12 days after inoculation; (E) mycelial growth development within 20 days after inoculation; (F) spores harvested from berries 12 days after inoculation. The pictures were captured using a Kodak DX4530 digital camera through a Carl Zeiss light microscope.

4.4 SUMMARY AND CONCLUSIONS

The number of fungal colony-forming units isolated from leaves increased with the frequency leaf of washing (Section 4.3.1). Increasing washing cycles from 2 to 4 led to significant increases in recovered colonies; there were few increases when washes were increased beyond seven.

Fungal recovery was not improved by an increase in the number of washes from 7 to 10 times for fungi isolated from cinnamon and turmeric, but increases were noted with pepper. The serial washing technique, led to the isolation of a variety of microorganisms from plant leaves. These were mainly colonies of bacteria, fungi, and yeast, although only the recovered colonies of fungi were counted in the present study. The isolated fungi were either pathogenic or saprophytic.

Dominance and co-dominance of fungal species was noticed in the microflora from all of the plant samples. Several fungi such as *Cladosporium* sp, *Curvularia* sp., *Fusarium* sp., *Helminthosporium* sp., *Paecilomyces* sp., *Rhizopus* sp. and *Trichoderma* sp., were easily isolated after serial washes; some of these grew very fast, and most were isolated through the spread plate method (Section 4.2.2.2). These fungi can be assumed to be saprophytic, contaminant fungi. Meanwhile, several species such as *Colletotrichum* sp., *Pestalotiopsis* sp., *Phoma* sp., and *Curvularia* sp., were mostly encountered after many washing cycles and also dominated the colonies recovered by the leaf plate method (Section 4.2.2.2), these are more likely to be pathogenic fungi.

Three species dominated the isolated fungi: *Colletotrichum gloeosporioides* on pepper, *Pestalotiopsis* sp. on cinnamon, and *Phoma* sp. on turmeric (Section 4.3.2). Meanwhile, *Curvularia* sp. was the most often commonly isolated fungus encountered in all of the samples observed.

Overall, the genera of fungi isolated in present study were *Aspergillus*, *Bipolaris*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Helminthosporium*, *Nigrospora*, *Paecilomyces*, *Pestalotiopsis*, *Phoma*,

Rhizopus, *Trichoderma*, and seven unknown species. However, the species of fungi used in subsequent germination inhibition tests were those fungi that were able to sporulate in pure cultures (Table 4.2); they are considered most likely to be leaf pathogens.

Assessment of spore germination was the focus of the present research. The time taken for spores to reach peak germination levels was determined for all tested fungal species (Section 4.3.3). Spore germination tests were conducted on an agar medium as this gave higher germination levels than tests carried out in distilled water in well-slides. Some fungi, such as *C. gloeosporioides*, responded to added nutrients, as has been shown previously for other species (Cochrane 1963; Dhingra and Sinclair 1985). However, on an agar surface with a high mineral content (Anonymous 2004a) and free access to oxygen, no carbon or nitrogen sources were needed to achieve maximum germination levels.

CHAPTER 5

ANTIFUNGAL SCREENING OF SELECTED PLANT EXTRACTS AND OILS

5.1 INTRODUCTION

Any chemical with fungicidal properties could be potentially useful to inhibit fungal growth or sporulation. Some chemicals derived from plants that have fungicidal properties may control plant fungal pathogens. Antifungal compounds come from volatile oil producers such as tea tree, *Eucalyptus*, cinnamon, and others (Carson and Riley 1998; Fluck and Schib 1976; Hay and Waterman 1993; Weiss 1997).

Laboratory screening of plant extracts for their biological activity using simple procedures has given encouraging results in earlier studies. For example, water extracts of several plants have been shown to have antibacterial activity against *Xanthomonas campestris* pv. *malvacearum*, *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. v*asicatoria* (Satish *et al*. 1999), and antifungal activity against *Didymella bryoniae* (Auersw.) and *Aspergillus flavus* Link ex. Fries (Fiori *et al.* 2000; Mahmoud 1999).

Similarly, salt extracts prepared from wheat kernels were found to have strong antifungal activity against pathogens such as *B. cinerea* Pers., *D. bryoniae* (Auersw.), *Colletotrichum acutatum* (Simmonds), *Fusarium culmorum* (W.G.Sm.) Sacc., *Septoria tritici* Berk. & M.A. Curtis, *Thielaviopsis basicola* (Berk. et Br.) Ferr., *Trichoderma viride* Pers. and *Verticillium dahliae* Kleb. (Chilosi *et al*. 2000).

Plant methanol extracts of several Zingiberaceae showed great potential in preparation of antibacterial agents (Muda *et al*. 2001; Yusuf *et al*. 2001). Extracts derived from *Inula viscosa* using several organic solvents (methanol, ethanol, ethyl acetate, acetone, chloroform, and n-hexane) were shown to have antifungal activity against *Phytopthora infestans*, *Colletotrichum cucumerinum*, *Botrytis cinerea*, and *Plasmophora viticola* (Cohen *et al*. 2003).

Plant oils also have great potential for controlling several fungal pathogens such as *Colletotrichum musae* (Berk. & M. A. Curtis) Arx, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Fusarium proliferatum* (Matsushima) Nirenberg (Fiori *et al*. 2000; Ranasinghe *et al*. 2002), and also for controlling many bacterial pathogens (Dorman and Deans 2000).

In the following section, the extent of antimicrobial activity of various plant extracts and oils is evaluated on the plant fungal pathogens that were isolated from pepper, cinnamon, and turmeric from the L & L Pepperfarm.

5.2 MATERIALS AND METHODS

5.2.1 Water and ethanol extracts

The methods used to prepare water and ethanol extracts from pepper, cinnamon, and turmeric plant components have been described above (Section 3.2).

5.2.2 Oil suspensions

Oil suspensions were prepared by adding commercial oils to sterile distilled water containing 0.01% Tween 80 (Letessier *et al*. 2001) to obtain

concentrations of 0.1, 0.2, 0.5, 1, 3 per cent. The emulsifier Tween 80 was added to enhance the solubility of the oils (Carson and Riley 1998; Fiori *et al*. 2000; Letessier *et al*. 2001).

The range of concentrations of the oils used in the present study (0.1 – 3%) was based on concentration ranges adopted from a previous study on tea tree (*Melaleuca alternifolia* Maiden & Benche) oil and at concentrations that are known to be safe for the host plants (0.04 – 3%; Clark and Gilrein 1992). Further guidance was sought from other studies. Hay and Waterman's (1993) study showed that oil from basil (*Ocimum basilicum* L.) could inhibit the growth of a wide range of fungi at a concentration of 0.15% (v/v), and that the inclusion of 1 – 10 μL/mL of marjoram oil in the culture broth reduced the fungal growth by up to 89% compared with an untreated control. Ranasinghe *et al.* (2002) found fungistatic and fungicidal effects of cinnamon and clove oils against *Colletotrichum musae*, *Lasiodiplodia theobromae*, and *Fusarium proliferatum* within a concentration range of 0.03 – 0.11% (v/v).

5.2.3 Synthetic fungicide solutions

Fungicide concentrations used were selected above and below the usual farm dose rates of 0.31 g/L of Amistar and 2.22 g/L of Dithane M-45 (Section 3.5). These concentrations were 12.5%, 25%, 100%, 2 times, and 5 times those of current farm use concentrations. The range of concentrations used was based on a preliminary germination test (Table 5.1).

Table 5.1 Germination responses of *Colletotrichum gloeosporioides* after different periods of time after incubation and specific synthetic fungicide treatments.

Fungicide (concentration)	Assessment period		
	24 hours	48 hours	72 hours
Dithane M-45† ($DF_{12.5}$)	Spore germinated	Spore germinated (1 – 5%)	Spore germinated (no change)
Dithane M-45† (DF_{25})	Complete inhibition	Complete inhibition	Complete inhibition
Dithane M-45† (DF_{100})	Complete inhibition	Complete inhibition	Complete inhibition
Amistar†† (AF_{100})	Spore germinated	Spore germinated (89 – 100%)	Spore germinated (100%)
Amistar†† (AF_{2x} recommended rate)	Spore germinated	Spore germinated (10 – 30%)	Spore germinated (up to 95%)
Amistar†† (AF_{5x} recommended rate)	Spore germinated	Spore germinated (0 – 3%)	Spore germinated (up to 50%)

† Recommended application rates = 2.22 g/L
†† Recommended application rates = 0.31 g/L

Therefore, the concentrations chosen for further tests were DF_{25} and AF_{100} because spores subjected to $DF_{12.5}$, AF_{2x} and AF_{5x} concentrations still germinated after 48 hours, and all of the assessments of the present study were made after 24 hours for ease of operations. Appendix 5 shows how the concentrations were determined for the solutions used in the present study.

5.2.4 Testing methods

5.2.4.1 *Laboratory testing protocols*

Experiments were conducted to find the best method to use in the germination inhibition trials of the present study. Initially, germination tests were conducted using the black pepper extract on *C. gloeosporioides* spores. The three methods tested were as follows:

1. *Mixed agar and spore suspension*

Agar (1 mL) was mixed with 1 mL of spore suspension, both at double their final concentrations. Then, 1 mL of the agar-spore suspension was spread over a slide to form a layer. A treatment solution of plant extract (40 μL) was applied to the surface of agar layer (the concentration was 40 μL of treatment solution/mL agar-spore suspension). Finally, the slide was incubated in a moist Petri dish.

The method attempted to replicate a natural condition where treatment solutions act as eradicative or protective fungicides. Concentrations of the bioactive agent did not change.

2. *Mixed agar and treatment solution*

Agar (1mL) was mixed with 40 μL of plant extract treatment solution (the concentration was 40 μL treatment solution/mL agar). Then 1 mL of agar-treatment solution suspension was spread over a slide to form a layer, and 40 μL of the spore suspension was placed onto the surface. The slide was then incubated as above.

Where agar was mixed with the treatment solutions, the solutions can be considered to mimic the protective action of fungicides. However, the concentration of treatment solutions changed from lower to higher values, since the spore suspension was added to the agar-treatment solution surface. The germinated spores were hard to count since most of agar-treatment solution layers formed an opaque film on the slide.

3. *Mixed spore suspension and treatment solution*

A spore suspension (1 mL) was mixed with 40 μL treatment solution. Then, 1 mL of the spore-treatment solution suspension was put on the surface of an agar layered slide, which was incubated as above.

In this method, the concentration of the treatment solution changed from higher to lower concentrations.

Results of those tests gave 100% spore germination in distilled water and 91.33%, 86.32%, 85.21% germination with methods 1 to 3, respectively. All methods gave satisfactory levels of germination, so other considerations were used to choose the method to be used in subsequent trials. Therefore, the first method, the mixed agar spore suspension approach, was chosen for future use as best represented the processes that function *in vivo*.

5.2.4.2 *Spore germination test - agar film method*

Germination tests were conducted using the agar film method described by Dhingra and Sinclair (1985) with slight modifications (Fig. 5.1). Spores were harvested using the method described in Section 4.2.4. The spores were diluted with sterile distilled water to approximately 50,000/mL (i.e. 100,000/mL at the doubled concentrations used; Section 5.2.4.1). The synthetic fungicide solutions, oils, and plant extracts were prepared at the concentrations shown in Section 5.2.1 – 5.2.3.

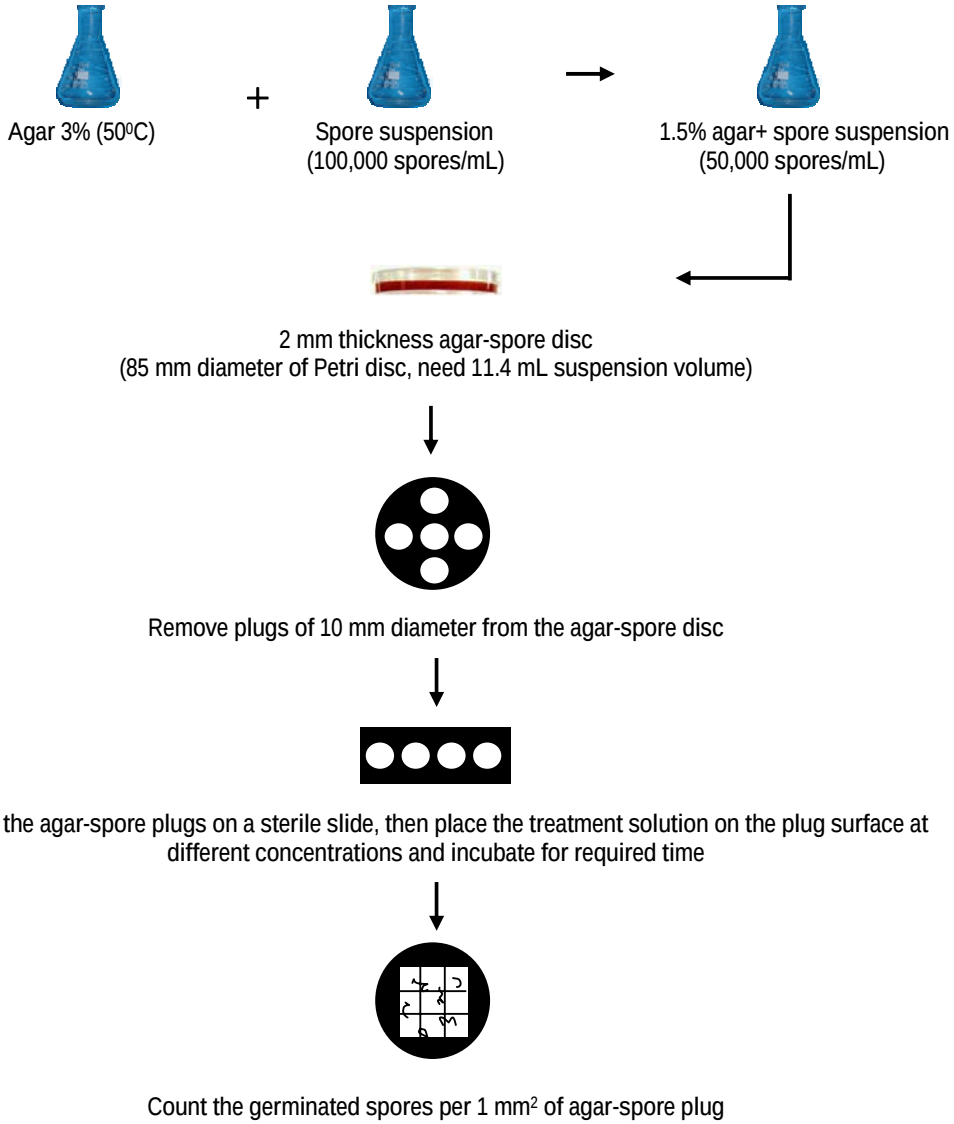

Figure 5.1 Illustration of the method of preparation of the spore germination test that was modified from Dhingra and Sinclair (1985).

For each test, the spore suspension was diluted (1:1) with 3% water agar held at 50^0C. The resulting mixture was poured immediately onto plates in equal quantities of 11.4 mL to produce a uniform thickness of 2 mm of agar medium in the culture plates. After drying, 10 mm diameter agar plugs were cut from the agar-spore plate and four plugs were placed on

sterile slides (Fig. 5.2A). The treatment solutions were applied in volumes of 5 μL to the agar-spore surface. Calculation of the volumes used and the resulting concentrations (μg extracts per mm^3 agar-spore) of extracts are shown in Appendix 5. As a control, sterile distilled water was used instead of the treatment solutions.

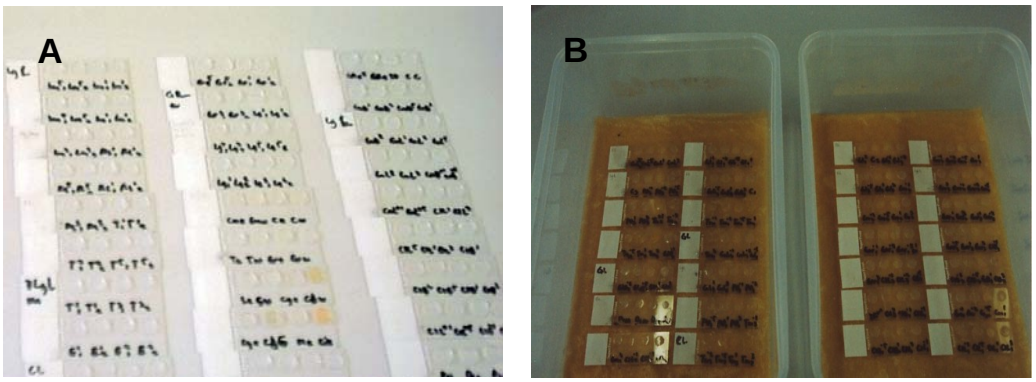

Figure 5.2 Spore germination test: (A) agar plugs on slides; (B) slides incubating.

The resultant slides were suspended in sterile plastic boxes containing moist, sterile blotting paper (Fig. 5.2B). The boxes were incubated at room temperature (28 ± 2^0C) for the periods set out in Section 4.2.5, after which, lactophenol-cotton blue was applied to stop spore germination (Fiori *et al*. 2000; Letessier *et al*. 2001).

Germinated spores were observed using a light microscope at 100x magnification. Germination percentages were calculated from 3 replicate samples observed in the area enclosed in 100 x 1 mm^2 grid cells in the ocular of the microscope (Anonymous 1943). A spore was deemed to have germinated if the length of the germ tube was one and a half times the length of the longest diameter of the spore (Dhingra and Sinclair 1985).

Images of several characteristics of spore germination in contact with plant extracts or oils were captured using an Olympus C5050 digital camera that was mounted on an Olympus BH-2 microscope operating at 100x magnification.

5.2.4.3 *Detached leaf bioassay*

The antifungal effects of plant extracts and oils at the concentrations determined in Section 5.2.1 and 5.2.2 were tested on pepper leaves using a detached leaf method (Agrios 1997). In this method, the inoculated detached plant parts were removed and maintained alive and turgid until disease symptoms developed (Dhingra and Sinclair 1985). The leaves were taken from pepper plants cultivated in a shadehouse at James Cook University, Townsville. Since leaf susceptibility changes with age, only young to middle age leaves were collected in the late afternoon when their carbohydrate and protein content was high (Dhingra and Sinclair 1985). The leaves were washed with sterile water, and dried between sterile filter papers; whole leaves were used instead of leaf discs. The leaves were placed in humid, transparent, plastic boxes with a layer of dampened paper towel in the base (Carlisle *et al.* 2002). Each box contained several leaves with three replicates of the solution treatments on each leaf. Alternatively, leaves were placed in Petri dishes (85 mm diameter) containing Whatman No. 1 filter paper, and wetted with sterile water, or a solution of 5% sucrose to which 1% agar was added (Dhingra and Sinclair 1985; Flors *et al*. 2004; Vallejo *et al*. 2001).

Drops of inoculum suspension or small blocks of mycelial inoculum were placed at predetermined sites on the leaves (Dhingra and Sinclair 1985). Before inoculation, however, treatment solutions (0.5% concentration of oils, 500 mg/mL concentration of extracts) were dropped onto specific sites on the leaves (5 μL), and control sites were treated with distilled water. Air-dried leaves were then inoculated with 10μL of fresh spore suspension (5×10^4 spores/mL) at each site.

Alternatively, mycelial plugs (0.5 cm diameter) of fungi were placed on leaves. Leaves were incubated at room temperature (28^0C). The diameters of the necrotic lesions on each leaf were measured 7 days after inoculation (Flors *et al*. 2004; Vallejo *et al*. 2001).

5.2.4.4 *In vivo fungicidal effects of plant extracts and oils*

The tests for the *in vivo* effect of plant extracts and oils against *C. gloeosporioides* were conducted on a sensitive host plant (papaya, *Carica papaya* L.) because the inoculation on pepper leaves produced no symptoms. *C. gloeosporioides* produces a major anthracnose disease on papaya (Dickman 1993; Latunde-Dada 2001; Nishijima 1999), with typical symptoms occurring on the fruit when it turns colour, and sometimes being exhibited as black spots on the plant. The first symptoms of papaya anthracnose on leaves are small, irregular-shaped, water-soaked spots. These spots eventually turn brown. The fungus also attacks the petioles of wilting leaves that hang along the trunk and become a major source of inoculum that may infect the green fruit still on the trees (Dickman 1993; Didier and Doumbia 2003).

Papaya seedlings were grown in a shadehouse at James Cook University, Townsville, and inoculated with *C. gloeosporioides*. The seedlings were grown in pots with one plant per pot; potted seedlings were exposed to several treatments per leaf per plant in three replications per treatment. The plants were of uniform size, age, and free of pathogens (Dhingra and Sinclair 1985). The seedlings were used for fungal studies when they had six leaves and were approximately 12 cm high. Concentrations of oils and extracts were 0.5% and 500 mg/mL, respectively. The extracts or oils were applied by dropper and allowed to run-off the leaf surfaces. A spore suspension (10^5 spores/mL) was then dropped onto the leaves and as the treatment solutions were dried for 10 – 30 minutes (Dhingra and Sinclair 1985). Then, the inoculated seedlings were kept in a humid atmosphere for 48 hours (Hong and Hwang 1998; Jhorar *et al*. 1998; Vloutoglou and Kalogerakis 2000) at room temperature (28^0C) in covered containers (Fig. 5.3). At the end of this period, the plants were uncovered, replaced in the shadehouse where the leaves were kept dry, and were maintained until disease symptoms appeared.

Figure 5.3 Wetness period on papaya seedling.

Disease development was assessed 7 days after inoculation by estimating the percentage of the leaf that was spotted by the disease symptoms. In addition, some observations were made using a whole-leaf clearing and staining technique to detect plant-pathogen interactions that helped to defined disease development (Dhingra and Sinclair 1985; Marte and Montalbini 1999; Shipton and Brown 1962; Soylu and Soylu 2003).

5.2.5 Data analysis

Statistical analyses were performed using SPSS version 11.0 computer package and the pooled mean values were separated on the basis of least significant difference (LSD) at the 0.05 probability level (Zar 1999). In the plant oil assessments, the experiments were conducted in a single factor, completely randomized design by carrying out three repetitions of 8 treatments for each fungus species. The other experiments were carried out in single factor, completely randomized designs with 17 treatments of plant water extracts, and 17 treatments of plant ethanol extracts for each fungus species.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of oils on spore germination

The effects of several concentrations (0.1 – 3%) of oils on the germination of *C. gloeosporioides* spores are shown in Figure 5.4. Similar data for the other 13 fungal species studied are shown in Figures A.6.1 – A.6.13 of Appendix 6.

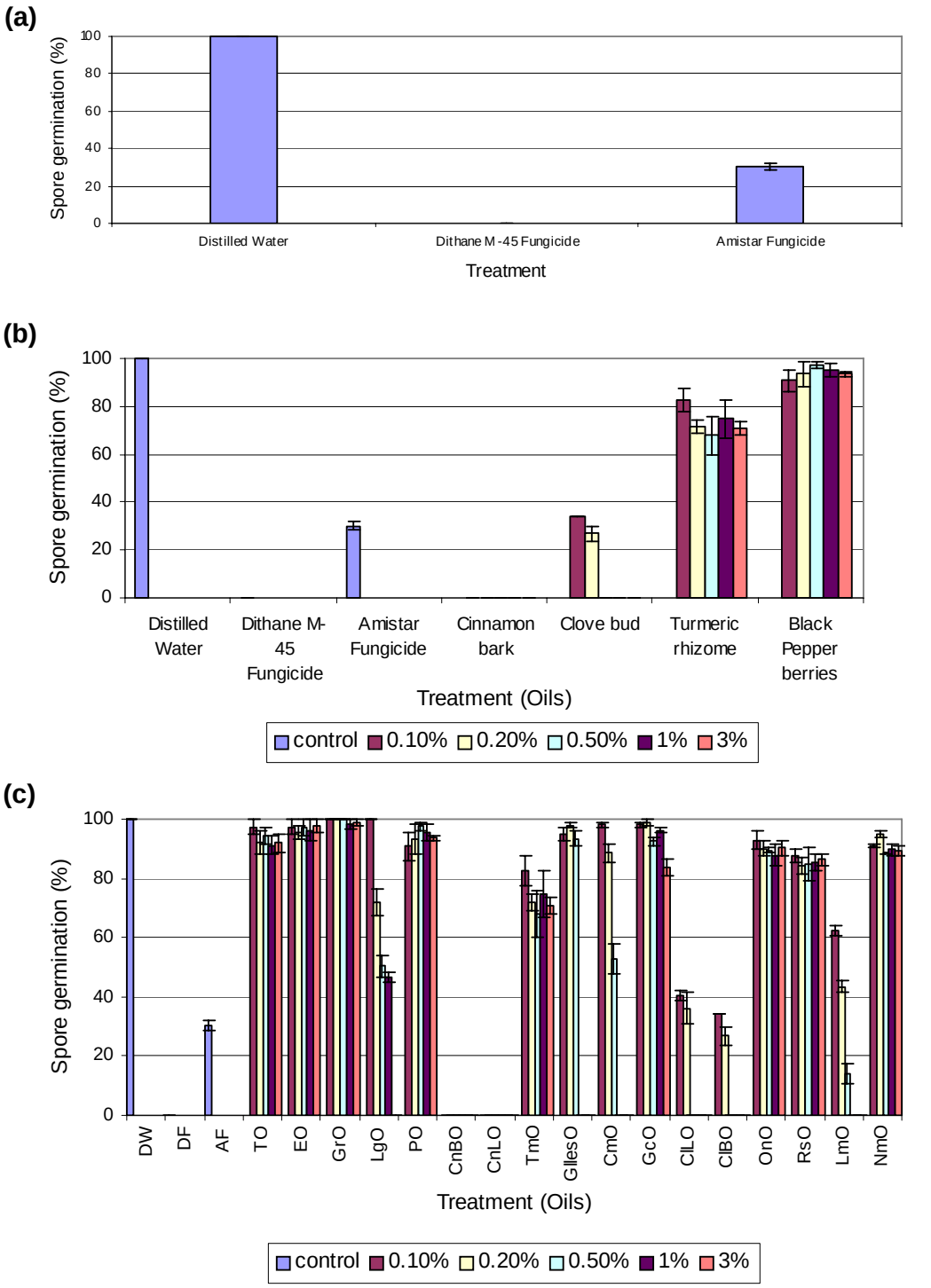

Figure 5.4 Germination after 24 hours of *C. gloeosporioides* spores in
(a) distilled water and synthetic fungicides;
(b) distilled water, synthetic fungicides and selected oils;
(c) distilled water DW, Dithane M-45 fungicide DF, Amistar fungicide AF, and all the oils tested: tea-tree TO, *Eucalyptus* EO, ginger GrO, lemon grass LgO, pepper PO, cinnamon bark CnBO, cinnamon leaf CnLO, turmeric TmO, lesser galangal GllesO, cardamom CmO, garlic GcO, clove leaf ClLO, clove bud ClBO, onion OnO, rosemary RsO, lemon myrtle LmO, neem NmO.

Germination of *C. gloeosporioides* was 100% in distilled water (DW), 0% in Dithane M-45 fungicide (DF), and 30.2% in Amistar fungicide (AF) at 24 hours (Fig. 5.4a). The efficiencies of some oils from cinnamon, clove, turmeric, and black pepper compared to control treatments of distilled water and synthetic fungicides are shown in Fig. 5.4b. Figure 5.4c shows the results of spore germination of *C. gloeosporioides* in the presence of all of the oils tested.

Oils of tea-tree (TO), eucalyptus (EO), ginger (GO), pepper black (PO), turmeric (TmO), garlic (GcO), onion (OnO), rosemary (RsO), and neem (NmO), at 0.1, 0.2, 0.5, 1, and 3% of concentrations, caused small reductions in germination of *C. gloeosporioides*. More noticeable reductions were observed through the application of 0.5 – 1% concentrations of oils of lemon grass (LgO), lesser galangal (GllesO), cardamom (CmO), and lemon myrtle (LmO). Low concentrations of clove bud and leaf oils (ClBO, ClLO), and all concentrations of the cinnamon bark and leaf oils tested (CnBO, CnLO) produced major reductions in fungal spore germination (Fig. 5.4c).

In general, the effects of the plant oils were similar for all tested fungi (Figs. A.6.1 – A.6.13 of Appendix 6), with the exception of an increased suppression of germination of *Cladosporium* sp. and *Phoma* sp. Garlic oil completely inhibited of *Phoma* sp. at all concentrations tested (Figs. A.6.1 and A.6.10).

The strongest inhibition of germination of *Curvularia* was shown by the cinnamon and clove oils; the latter completely inhibited germination of *Curvularia* over the entire range of concentrations. In addition, lesser galangal oil (GllesO) and turmeric oil (TmO) were superior germination

inhibitors for this fungus species in contrast to their effects on *C. gloeosporioides* (Compare Fig. 5.4 and Figs. A.6.2; A.6.7; A.6.9 and A.6.12). In two species of *Helminthosporium* (same fungal group as *Curvularia*), cinnamon and clove oils completely inhibited spore germination at all concentrations tested; lesser galangal oil (GllesO) also strongly suppressed germination (Figs. A.6.8 and A.6.11). Lesser galangal oil (GllesO) and turmeric oil (TmO) also showed strong suppression of germination of *Bipolaris* sp. (same group as *Curvularia*) (Fig. A.6.13).

On the other hand, however, both cinnamon bark and leaf oils (CnBO, CnLO) allowed germination of *Pestalotiopsis* at all concentrations (Figs. A.6.4 and A.6.6). However, lemon grass oil (LgO) showed strong (up to 100%) inhibition of this species isolated from cinnamon at all concentrations. Meanwhile, the germination of *Fusarium* spp. was restricted by cardamom oil (CmO) and lemon myrtle oil (LmO) oils in addition to strong inhibition of cinnamon and clove oils, followed by turmeric oil (TmO) and lesser galangal oil (GllesO) (Fig. A.6.3). *C. gloeosporioides* and *C. orbiculare* were similarly suppressed by several oils (Fig. A. 6.5), with the cinnamon and clove oils (CnBO, CnLO, ClBO, ClLO) being superior.

The effects of oils, at different concentrations, on spore germination were analysed statistically for each fungal species. The effect of tea-tree oil (TO), lemon grass oil (LgO), and cinnamon bark oil (CnBO) on germination of fungal species isolated from pepper, cinnamon, and turmeric are shown in Tables 5.2 – 5.10 (Section 5.3.1.1 – 5.3.1.3, below). Similar data for other fungal species and oils are shown in Tables A.7.1 – A.7.42 in Appendix 7.

5.3.1.1 *Germination of spores of fungi from pepper*

The results shown in Table 5.2 indicate that significant inhibition of germination of all of the fungi isolated from pepper plants by tea-tree oil (TO) occurred at selected concentrations. Complete inhibition of germination was not recorded. Germination was most restricted with *Cladosporium* sp., *Curvularia* sp. and *Fusarium* sp. where germination percentages of 43.7%, 64.8%, and 63.0%, respectively, were reached.

Table 5.2 Germination of fungi isolated from pepper subjected to tea-tree oil at a range of concentrations of 0.1 – 3%.

TO conc. (%)	P1		P3		P6		P8		P10		P13	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	92.5	4.5	100	0	100	0	100	0	89	4.5
DF	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	12.2*	5.2	29.3*	5.2	37.5*	2.2	22.4*	3.1	1.3*	1.3
0.1	97.3	2.7	55.3*	6.0	92.1	4.8	96.5	0.4	97.2	2.0	76.8	1.6
0.2	92.2*	4.0	50.2*	3.5	91.9	1.9	91.9*	1.0	92.5	4.7	70.9*	2.0
0.5	94.3	2.8	45.7*	5.1	87.2*	2.1	90.0*	1.1	96.7	4.1	74.1	9.6
1	91.1*	3.1	43.7*	2.0	85.9*	2.4	72.0*	1.5	87.4*	2.1	73.6	9.6
3	91.9*	2.9	44.0*	5.4	64.8*	2.5	63.0*	3.9	85.2*	2.6	73.2*	1.8

* Significant difference from the water ($p < 0.05$, df = 7)
TO = tea-tree oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, P1 = *C. gloeosporioides*, P3 = *Cladosporium* sp., P6 = *Curvularia* sp., P8 = *Fusarium* sp., P10 = *Pestalotiopsis* sp., P13 = *C. orbiculare*

Results shown in Table 5.3 indicate superior suppression of germination of fungi isolated from pepper by lemon grass oil (LgO). Suppression commenced at the lowest concentration tested (0.1%) with most fungi. Complete inhibition of germination was noted at the highest oil concentration (3%) with four fungal species whereas, for the other fungi, germination still occurred up to a concentration of 10.8% of lemon grass oil.

Table 5.3 Germination of fungi isolated from pepper subjected to lemon grass oil at a range of concentrations of 0.1 – 3%.

LgO conc. (%)	P1		P3		P6		P8		P10		P13	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	92.5	4.5	100	0	100	0	100	0	89	4.5
DF	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	12.2*	5.2	29.3*	5.2	37.5*	2.2	22.4*	3.1	1.3*	1.3
0.1	100	0	20.9*	3.6	81.3*	1.9	72.3*	1.0	67.1*	3.4	83.5	0.8
0.2	71.9*	4.3	20.7*	0.7	51.8*	4.3	67.4*	1.2	66.2*	2.3	51.6*	6.5
0.5	50.4*	3.5	17.3*	3.8	23.1*	3.9	40.0*	1.2	52.2*	2.2	32.1*	5.7
1	46.6*	1.8	8.2*	1.6	17.5*	4.3	0*	0	24.4*	4.4	10.1*	10.1
3	0*	0	8.1*	1.7	0*	0	0*	0	10.8*	5.8	0*	0

* Significant difference from the water ($p < 0.05$, df = 7)
LgO = lemon grass oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, P1 = *C. gloeosporioides*, P3 = *Cladosporium* sp., P6 = *Curvularia* sp., P8 = *Fusarium* sp., P10 = *Pestalotiopsis* sp., P13 = *C. orbiculare*

Tables 5.4 and A.7.12 indicate the effectiveness of cinnamon bark and cinnamon leaf oils to inhibit germination at most concentrations tested. The notable exception was with *Pestalotiopsis* sp. (P10) that still germinated in the two lowest concentrations of these oils. Nevertheless, the cinnamon-derived oils were found to be more effective fungicides than the commercial fungicide, Amistar, at its recommended application rate.

Table 5.4 Germination of fungi isolated from pepper subjected to cinnamon bark oil at a range of concentrations of 0.1 – 3%.

CnBO conc. (%)	P1		P3		P6		P8		P10		P13	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	92.5	4.5	100	0	100	0	100	0	89	4.5
DF	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	12.2*	5.2	29.3*	5.2	37.5*	2.2	22.4*	3.1	1.3*	1.3
0.1	0*	0	0*	0	0*	0	0*	0	20.1*	1.9	0*	0
0.2	0*	0	0*	0	0*	0	0*	0	4.1*	4.1	0*	0
0.5	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
1	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
3	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0

* Significant difference from the water ($p < 0.05$, df = 7)
CnBO = cinnamon bark oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, P1 = *C. gloeosporioides*, P3 = *Cladosporium* sp., P6 = *Curvularia* sp., P8 = *Fusarium* sp., P10 = *Pestalotiopsis* sp., P13 = *C. orbiculare*

Results for *Eucalyptus* oil (EO) are shown in Table A.7.1 and indicate that significant inhibition of germination of all test fungi, except *C. gloeosporioides*, occurred at oil concentrations of 0.1%, 0.2%, 1%, and 3%. At all concentrations tested, however, the effect of *Eucalyptus* oil on the germination of *C. gloeosporioides* was not significantly different from distilled that of water.

The effect of ginger oil (GrO) almost paralleled that of *Eucalyptus* oil (Table A.7.2). The main difference was that the oil showed strong inhibition of germination of *Pestalotiopsis* sp. (31.9% of germination) and *Cladosporium* sp. (10.4% of germination) at the highest concentration of 3%.

In general, treatment with oils of tea-tree, *Eucalyptus*, and ginger were not particularly inhibitory. Higher oil concentrations did not always lead to lower percentages of spore germination. This differed from all of the other oil treatments such as lemon grass, cardamom, lesser galangal, and lemon myrtle, which showed greater fungal germination inhibition at higher concentrations.

Other oils, such as black pepper (PO), garlic (GcO), onion (OnO), rosemary (RsO), and neem (NmO) (Tables A.7.3; A.7.7; A.78; A.7.9 and A.7.11), exhibited a similar inhibitory behaviour to tea-tree oil (TO), with the exception of turmeric oil (TmO) against *Cladosporium* sp. which failed to germinate at an oil concentration of 0.5% (Table A.7.4). Garlic oil, onion oil, and neem oil had no inhibitory effect on *C. orbiculare* (Tables A.7.7; A.7.8 and A.7.11). In some cases, the mean germination of oil-treated spores was higher than the mean germination on the distilled water (discussed in Section 5.3), especially at concentration of 0.1 – 0.5%.

The results shown in Table A.7.5 and Table A.7.10 indicate that complete inhibition of germination of the most fungi occurred when lesser galangal oil (GllesO) and lemon myrtle oil (LmO) were applied at various concentrations. Inhibition by cardamom oil (CmO) was complete for *Colletotrichum*, *Curvularia,* and *Fusarium* at concentrations of 1% to 3%, whereas *Cladosporium* and *Pestalotiopsis* spores still germinated (7.8%) in the highest oil concentrations (Table A.7.6). With the two clove oils (ClBO, ClLO) at concentrations above 0.5%, spore germination was completely inhibited for most fungi (Tables A.7.13 and A.7.14).

Inhibition of fungi isolated from pepper was noted with oils of lesser galangal (GllesO), cardamom (CmO), and lemon myrtle (LmO) at all of the oil concentrations tested (Tables A.7.19; A.7.20 and A.7.24). Complete inhibition of the test fungi by lesser galangal oil (GllesO) occurred with an oil concentration of 0.5% (Table A.7.19). Cardamom oil (CmO) showed lower potential of fungal suppression than lesser galangal (GllesO) since complete inhibition of germination either did not occur, or occurred at the higher concentration levels (Table A.7.20).

5.3.1.2 *Germination of spore of fungi from cinnamon*

The results shown in Table 5.5 indicate that tea-tree oil (TO) significantly inhibited germination of all of the fungi isolated from cinnamon at concentrations of 0.1% or 0.2%, with the exception of *Curvularia inaequalis* which responded only at the highest concentration (3%).

The results for lemon grass oil (LgO) shown in Table 5.6 indicate that complete inhibition of *Pestalotiopsis* (C21) spore germination occurred at all

concentrations tested. Meanwhile, complete inhibition of germination on *Curvularia* sp. (C30) occurred at 1% concentration. Significant inhibition of spore germination of the other fungi was noted at oil concentrations of 0.1% or 0.2%. Generally, lemon grass oil showed the greatest suppression of germination of fungi isolated from cinnamon.

Table 5.5 Germination of fungi isolated from cinnamon subjected to tea-tree oil at a range of concentrations of 0.1 – 3%.

TO conc. (%)	C21		C24		C26		C30	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	84.7	3.9	95.8	1.8	87.8	0.5	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	21.9*	5.2	3.3*	2.7	16.4*	1.8	6.3*	3.2
0.1	73.6	1.6	95	2.4	71.6*	2.9	88.0*	3.3
0.2	60.6*	4.3	93.0	2.0	68.0*	2.3	80.8*	1.7
0.5	47.1*	8.5	89.7	2.5	73.4*	2.1	68.7*	3.1
1	13.1*	5.5	89.9	1.5	73.4*	1.1	67.6*	3.3
3	0*	0	60.1*	5.0	71.2*	1.9	32.6*	6.4

* Significant difference from the water ($p < 0.05$, df = 7)
TO = tea-tree oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, C21 = *Pestalotiopsis* sp., C24 = *Curvularia inaequalis*, C26 = *Helminthosporium* sp., C30 = *Curvularia* sp.

Table 5.6 Germination of fungi isolated from cinnamon subjected to lemon grass oil at a range of concentrations of 0.1 – 3%.

LgO conc. (%)	C21		C24		C26		C30	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	84.7	3.9	95.8	1.8	87.8	0.5	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	21.9*	5.2	3.3*	0	16.4*	1.8	6.3*	3.2
0.1	0*	0	71.3	1.6	59.6*	2.9	56.6*	5.1
0.2	0*	0	58.3*	10.3	56.7*	1.3	26.9*	4.0
0.5	0*	0	36.6*	16.8	48.4*	0.8	6.5*	3.4
1	0*	0	27.3*	13.6	34.2*	1.4	0*	0
3	0*	0	4.3*	3.5	13.0*	1.7	0*	0

* Significant difference from the water ($p < 0.05$, df = 7)
LgO = lemon grass oil, DW = distilled water, DF = Dithane M-45 M45 fungicide, AF = Amistar fungicide, C21 = *Pestalotiopsis* sp., C24 = *Curvularia inaequalis*, C26 = *Helminthosporium* sp., C30 = *Curvularia* sp.

Table 5.7 and Table A.7.26 show complete inhibition of fungal spore germination, with the exception of those of *Pestalotiopsis*, by cinnamon bark oil and cinnamon leaf oil (all concentrations), *Pestalotiopsis* fungus isolated from cinnamon required cinnamon bark oil concentrations above 0.5% to substantially reduce its germination potential (Table 5.7).

Table 5.7 Germination of fungi isolated from cinnamon subjected to cinnamon bark oil at a range of concentrations of 0.1 – 3%.

CnBO conc. (%)	C21		C24		C26		C30	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	84.7	3.9	95.8	1.8	87.8	0.5	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	21.9*	5.2	3.3*	0	16.4*	1.8	6.3*	3.2
0.1	52*	5.6	0*	0	0*	0	0*	0
0.2	44.0*	7.6	0*	0	0*	0	0*	0
0.5	13.2*	7.0	0*	0	0*	0	0*	0
1	0*	0	0*	0	0*	0	0*	0
3	0*	0	0*	0	0*	0	0*	0

* Significant difference from the water ($p < 0.05$, df = 7)
CnBO = cinnamon bark oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, C21 = *Pestalotiopsis* sp., C24 = *Curvularia inaequalis*, C26 = *Helminthosporium* sp., C30 = *Curvularia* sp.

The results shown in Tables A.7.15 and A.7.16 indicate significant inhibition of germination of fungi isolated from cinnamon mostly occurred at concentrations of *Eucalyptus* and ginger oils of 0.1% or 0.2%. The highest concentration of ginger oil (GrO) (3%) completely inhibited germination of *Pestalotiopsis* sp. (Table A.7.16). Data shown in Tables A.7.17, A.7.18, A.7.22 and A.7.23 indicate a medium level of inhibition of spore germination for most of the fungi isolated from cinnamon by black pepper oil (PO), turmeric oil (TmO), onion oil (OnO), and rosemary oil (RsO).

The results shown in Table A.7.21 for garlic oil (GcO) indicate that significant inhibition of germination of all fungi isolated from cinnamon

occurred at all the concentrations tested. *Pestalotiopsis* was completely inhibited at 1% concentration; similar spore germination suppression was shown by neem oil (NmO) at a concentration of 0.5% (Table A.7.25).

Germination of spore of two species of *Curvularia* were completely inhibited at all concentrations of both of the clove bud and clove leaf oils (Tables A.7.27 and A.7.28). However, *Helminthosporium* and *Pestalotiopsis* were not completely inhibited by the lowest concentration of either of the clove oils (Table A.7.27 and A.7.28).

5.3.1.3 *Germination of spore of fungi from turmeric*

The results shown in Tables 5.8 – 5.10 and Tables A.7.29 – A.7.42 indicate that significant inhibition of germination of all of the fungi isolated from turmeric occurred at all of the oil concentrations tested. The major exceptions were that onion oil (OnO), rosemary (RsO), and neem (NmO) were ineffective against *Helminthosporium* sp. These three oils permitted spore germinations greater than 60% in all fungi at the lowest concentration tested (Tables A.7.36; A.737 and A.7.39). With few exceptions, however, significant inhibition of spore germination occurred at the lowest concentrations of oils tested (0.1%) (Tables A.7.29; A.31; A.35; A.7.36 and A.7.37).

Inhibition of germination by oils of lemon grass (LgO), lesser galangal (GllesO), cardamom (CmO), and lemon myrtle (LmO) mostly occurred at the high concentrations of 1% and 3% (Tables 5.9; A.7.33; A.7.34 and A.7.38).

Table 5.8 Germination of fungi isolated from turmeric subjected to tea-tree oil at a range of concentrations of 0.1 – 3%.

TO conc. (%)	T41		T48		T51		T52	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	93.3	6.7	100	0	100	0	98.0	1.3
DF	0*	0	0*	0	0*	0	0*	0
AF	6.7*	6.7	36.1*	7.8	10.6*	0.8	17.3*	3.4
0.1	73.7*	5.7	86.8*	0.7	96.0*	0.8	78.1*	2.9
0.2	64.6*	2.8	75.8*	1.9	95.1*	1.9	75.7*	2.3
0.5	52.5*	6.4	65.2*	4.8	94.9*	1.1	76.0*	2.6
1	49.2*	4.5	62.3*	4.7	69.5*	2.3	74.8*	1.8
3	48.2*	4.5	64.5*	4.6	68.2*	1.0	71.0*	0.4

* Significant difference from the water ($p < 0.05$, df = 7)
TO = tea-tree oil, DW = distilled water, DF = Dithane M-45fungicide, AF = Amistar fungicide, T41 = *Phoma* sp., T48 = *Exserohilum* sp., T51 = *Curvularia* sp., T52 = *Bipolaris* sp.

Table 5.9 Germination of fungi isolated from turmeric subjected to lemon grass oil at a range of concentrations of 0.1 – 3%.

LgO conc. (%)	T41		T48		T51		T52	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	93.3	6.7	100	0	100	0	98.0	1.3
DF	0*	0	0*	0	0*	0	0*	0
AF	6.7*	6.7	36.1*	7.8	10.6*	0.8	17.3*	3.4
0.1	12.3*	6.2	66.4*	3.4	67.5*	0.8	68.5*	1.2
0.2	10*	5.8	50.7*	3.2	27.1*	4.4	69.6*	2.5
0.5	0*	0	46.1*	6.6	23.3*	3.0	58.3*	1.0
1	0*	0	25.3*	5.3	2.9*	2.9	43.5*	3.5
3	0*	0	0*	0	0*	0	13.0*	1.9

* Significant difference from the water ($p < 0.05$, df = 7)
LgO = lemon grass oil, DW = distilled water, DF = Dithane M-45fungicide, AF = Amistar fungicide, T41 = *Phoma* sp., T48 = *Exserohilum* sp., T51 = *Curvularia* sp., T52 = *Bipolaris* sp.

Stronger inhibition of germination of the fungi isolated from turmeric was again shown by the cinnamon oils and clove oils. Treatment with either of the cinnamon oils resulted in no spores germinating with the exception of 9.8% germination for *Bipolaris* at the lowest concentration of cinnamon leaf oil (Tables 5.10 and A.7.40). Meanwhile, the two clove oils gave complete inhibition of spore germination at all concentrations tested only for

Exserohilum sp. With the other fungi, complete inhibition was achieved generally at the higher oil concentrations, and spore germination at the lowest concentrations of these oils were lower than 40% (Tables A.7.41 and A.7.42). Interestingly, the garlic oil (GcO) caused 100% inhibition of germination of *Phoma* isolated from turmeric at all concentrations tested (Table A.7.35) and turmeric oil (TmO) showed great inhibition of germination especially of *Exserohilum* sp. (Table A.7.32).

Table 5.10 Germination of fungi isolated from turmeric subjected to cinnamon bark oil at a range of concentrations of 0.1 – 3%.

CnBO conc. (%)	T41		T48		T51		T52	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	93.3	6.7	100	0	100	0	98.0	1.3
DF	0*	0	0*	0	0*	0	0*	0
AF	6.7*	6.7	36.1*	7.8	10.6*	0.8	17.3*	3.4
0.1	0*	0	0*	0	0*	0	0*	0
0.2	0*	0	0*	0	0*	0	0*	0
0.5	0*	0	0*	0	0*	0	0*	0
1	0*	0	0*	0	0*	0	0*	0
3	0*	0	0*	0	0*	0	0*	0

* Significant difference from the water ($p < 0.05$, df = 7)
CnBO = cinnamon bark oil, DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, T41 = *Phoma* sp., T48 = *Exserohilum* sp., T51 = *Curvularia* sp., T52 = *Bipolaris* sp.

5.3.1.4 *Summary of effects of oils*

The effects of several oils on the fungi were qualitative rather than quantitative. Shorter length of germ tubes and slower hyphal development were found especially after the application of clove, cardamom, lemon myrtle, lesser galangal, and turmeric oils. Lemon grass oil and clove oil caused physical damage to the spores of the fungus *Fusarium* sp., lesser galangal oil caused abortion of germination of *Curvularia* sp., and cardamom oil caused the malformation of hyphae of this species.

The oils capable of causing complete inhibition of spore germination of selected fungal species, at all concentrations tested, are listed in Table 5.11.

Table 5.11 Fungal species whose germination was found to be inhibited significantly by exposure to plant oils at concentrations of 0.1 – 3%. The fungal species codes are listed in Tables 4.2 – 4.4.

Oil	Fungal species (species code)
Cinnamon bark	*C. gloeosporioides* (P1) *Cladosporium* sp. (P3) *Curvularia* sp. (P6) *Fusarium* sp. (P8) *C. orbiculare* (P13) *Curvularia inaequalis* (C24) *Helminthosporium* sp. (C26) *Curvularia* sp. (C30) *Phoma* sp. (T41) *Exserohilum* sp. (T48) *Curvularia* sp. (T51) *Bipolaris* sp. (T52)
Cinnamon leaf	*C. gloeosporioides* (P1) *Cladosporium* sp. (P3) *Curvularia* sp. (P6) *Fusarium* sp. (P8) *C. orbiculare* (P13) *Curvularia inaequalis* (C24) *Helminthosporium* sp. (C26) *Curvularia* sp. (C30) *Phoma* sp. (T41) *Exserohilum* sp. (T48) *Curvularia* sp. (T51)
Clove bud	*Curvularia* sp. (C30) *Exserohilum* sp. (T48)
Clove leaf	*Curvularia* sp. (C30) *Exserohilum* sp. (T48)
Garlic bud	*Phoma* sp. (T41)
Lemon grass	*Pestalotiopsis* sp. (C21)

5.3.2 Effect of plant extracts on spore germination

The effects of several plant extracts (concentration of 500 mg/mL) on germination of spore of fungi isolated from pepper, cinnamon, and turmeric are shown in Tables 5.12 – 5.17.

5.3.2.1 *Germination of spores of fungi isolated from pepper*

The extracts responsible for the greatest inhibition of germination of spores of fungi isolated from pepper were the galangal rhizome (GlR) extracts in both water and ethanol solvents. These extracts completely inhibited germination of all of the fungi tested, with the exception of 25.4% germination by *Cladosporium* in the water extract (Tables 5.12 and 5.13).

The second most effective spore germination inhibitor was cardamom leaf (CmL) extract in water and ethanol solvents. However, these extracts were more effective on putative pathogenic fungi (P1, P8, P10, and P13) rather than on putative saprophytic fungi (P3 and P6 – Tables 5.12 and 5.13). Complete inhibition of germination of *Colletotrichum* was noted only in the ethanol extract of cardamom leaf that maintained low (less than 20%) spore germination in the other fungi.

Ethanol extracts of cinnamon bark (CnB), galangal stem (GlS) and lesser galangal rhizome (GllesR) were efficient germination inhibitors (Tables 5.13 – 5.17). Cinnamon bark (CnB) extract worked well against *Cladosporium*, *Curvularia*, and *C. orbiculare* with reduced spore germination (0%, 21.1%, and 12.8% germination, respectively; Table 5.13), but this extract was less efficient against the other fungi. The galangal stem (GlS) extract showed strong inhibition of most fungi especially with *C. orbiculare*, whereas the lesser galangal rhizome (GllesR) extract inhibited spore germination strongly only on *Cladosporium* with 16.9% germination being reached (Table 5.13).

The spore germination inhibition potential of the plant extracts was disappointing. No significant differences from water controls were found with the exception of significant inhibition by:

- ethanol and water extractions of lesser galangal rhizome (GllesR) and ginger rhizome (GrR) against *Curvularia* and *Cladosporium*, respectively,
- ethanol extraction of *Cassia* leaf (CaL) against *C. gloeosporioides*, *Cladosporium* and *Curvularia*, and
- water extractions of galangal stem (GlS) against *Fusarium*.

Several of the plant extracts, such as those of pepper black leaf, lemon grass, and turmeric, showed no inhibitory effects. The significant difference noted between these extracts and distilled water against *Pestalotiopsis* and *C. orbiculare* (Tables 5.12 and 5.13) were considered to be anomalous since the mean spore germination rates recorded for these extract treatments were higher than the mean germination under distilled water (See Section 5.3.2.4, below). Application of these extracts stimulated fast germ tube and mycelial growth, and all of the spores germinated, responses that did not occur even in the distilled water treatment.

Table 5.12 Germination of spores of fungi isolated from pepper subjected to several plant **water** extracts (500 mg/mL). The highlighted values indicate effective inhibition of spore germination.

Extract	P1		P3		P6		P8		P10		P13	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	100	0	100	0	100	0	93.3	3.3	83.8	1.9
DF	0	0	0*	0	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	15.6*	3.6	8.7*	1.4	27.4*	6.2	25.7*	2.9	1.3*	1.3
PL	100*	0	100	0	100	0	100	0	93.3	6.7	100* [a]	0
CnB	100	0	**87.4***	**3.5**	100	0	93.9	1.9	93.0	3.5	95.1* [a]	3.1
CnL	100	0	100	0	100	0	97.5	2.5	96.3 [a]	3.7	100* [a]	0
GlR	**0***	**0**	**25.4***	**4.5**	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**
GlL	100	0	100	0	100	0	96.9	3.1	96.7 [a]	3.3	100* [a]	0
GlS	100	0	97.6	1.2	100	0	**83.2***	3.5	95.8 [a]	4.2	100* [a]	0
LgL	100	0	97.6	2.4	100	0	100	0	97.0 [a]	3.0	92.1* [a]	2.1
LgS	100	0	100	0	100	0	98.2	1.0	96.3 [a]	3.7	97.7* [a]	1.2
TmR	100	0	100	0	100	0	100	0	97.2 [a]	2.8	100* [a]	0
TmL	100	0	96.1	2.1	100	0	100	0	98.2 [a]	1.2	97.1* [a]	1.5
GllesR	100	0	100	0	91.7*	4.8	94.3	2.9	97.0 [a]	3.0	100* [a]	0
GrR	100	0	93.1*	1.4	99.5	0.5	100	0	100* [a]	0	94.3* [a]	3.8
CaL	98.4	1.6	97.4	2.6	99.5	0.5	95.0	2.7	95 [a]	0.9	91.4* [a]	1.5
CmL	**43.0***	**5.5**	95.0	3.0	98.3	1.0	**28.1***	**3.0**	**36.2***	**3.1**	**19.2***	**3.1**

* Significant difference from the water ($p < 0.05$, df = 16), [a] Anomalous value = unusual data since the mean spore germination rates for these extract treatments were higher than the mean germination under distilled water

DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, PL = black pepper leaf, CnB = cinnamon bark, CnL = cinnamon leaf, GlR = galangal rhizome, GlL = galangal leaf, GlS = galangal stem, LgL = lemon grass leaf, LgS = lemon grass stem, TmR = turmeric rhizome, TmL = turmeric leaf, GllesR = lesser galangal rhizome, GrR = ginger rhizome, CaL = *Cassia* leaf, CmL = cardamom leaf, P1 = *C. gloeosporioides*, P3 = *Cladosporium* sp., P6 = *Curvularia* sp., P8 = *Fusarium* sp., P10 = *Pestalotiopsis* sp., P13 = *C. orbiculare*

Table 5.13 Germination of spores of fungi isolated from pepper subjected to several plant **ethanol** extracts (500 mg/mL). The highlighted values indicate effective inhibition of spore germination.

Extract	P1		P3		P6		P8		P10		P13	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	100	0	100	0	100	0	93.3	3.3	83.8	1.9
DF	0*	0	0*	0	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	15.6*	3.6	8.7*	1.4	27.4*	6.2	25.7*	2.9	1.3*	1.3
PL	100	0	100	0	100	0	99.4	0.6	92.6	3.7	41.1*	4.3
CnB	100	0	**21.1***	**4.9**	**0***	**0**	93.3	2.4	93.9	3.1	**12.8***	**3.6**
CnL	100	0	94.4	2.8	100	0	96.7	3.3	93.3	3.3	59.6*	3.6
GlR	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**
GlL	100	0	97.1	2.9	100	0	96.5	3.5	96.3 [a]	3.7	98.3* [a]	1.7
GlS	**77.1***	**7.6**	**76.3***	**2.0**	**52.1***	**2.1**	**82.0***	**2.5**	**80.8***	**3.6**	**12.7***	**0.9**
LgL	100	0	94.1	2.2	100	0	96.7	3.3	96.7 [a]	3.3	100* [a]	0
LgS	100	0	98.9	1.1	100	0	96.1	3.1	97.3 [a]	2.7	100* [a]	0
TmR	100	0	100	0	100	0	100	0	97.2 [a]	2.8	94.5* [a]	1.0
TmL	100	0	95.9	2.3	100	0	100	0	98.2 [a]	1.2	94.5* [a]	1.1
GllesR	100	0	**16.9***	**3.5**	91.7*	4.8	93.3	3.3	96.3 [a]	3.7	94.3* [a]	5.7
GrR	100	0	90.9*	1.0	98.9	0.6	100	0	100 [a]	0	96.3* [a]	2.2
CaL	90.6*	2.6	85.5*	3.1	94.0*	2.4	92.6	2.7	89.0	0.8	90.1 [a]	1.6
CmL	**0***	**0**	89.3*	4.7	97.5	1.2	**3.1***	**1.6**	**17.7***	**2.8**	**0***	**0**

* Significant difference from the water ($p < 0.05$, df = 16), [a] Anomalous value = unusual data since the mean spore germination rates for these extract treatments were higher than the mean germination under distilled water

DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, PL = black pepper leaf, CnB = cinnamon bark, CnL = cinnamon leaf, GlR = galangal rhizome, GlL = galangal leaf, GlS = galangal stem, LgL = lemon grass leaf, LgS = lemon grass stem, TmR = turmeric rhizome, TmL = turmeric leaf, GllesR = lesser galangal rhizome, GrR = ginger rhizome, CaL = *Cassia* leaf, CmL = cardamom leaf, P1 = *C. gloeosporioides*, P3 = *Cladosporium* sp., P6 = *Curvularia* sp., P8 = *Fusarium* sp., P10 = *Pestalotiopsis* sp., P13 = *C. orbiculare*

5.3.2.2 *Germination of spores of fungi isolated from cinnamon*

Galangal rhizome extracts (water and ethanol) inhibited spore germination in all of the fungal species isolated from cinnamon (Tables 5.14 and 5.15). Ethanol extract of galangal stem (GlS) were less inhibitory and ethanol extractions of lesser galangal rhizome (GllesR) were moderately effective only against *Helminthosporium* sp. (Table 5.15).

Cinnamon bark (CnB) ethanol extract was more suppressive of the putative saprophytic fungi especially two species of *Curvularia* (C24 and C30) and *Helminthosporium* sp. (C26) rather than the putative pathogenic fungi; cardamom leaf (CmL) extract was more effective in the suppression of *Pestalotiopsis* sp. (C21) (Tables 5.14 and 5.15). *Pestalotiopsis* development was stimulated by some extracts such lemon grass, turmeric rhizome, and galangal leaf. However, this observation needs clarification, as fungal germination in the distilled water treatment was low in these trials.

Table 5.14 Germination of spore of fungi isolated from cinnamon subjected to several plant water extracts (500 mg/mL). The highlighted cells indicate effective inhibition of spore germination.

Extract	C21		C24		C26		C30	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error
DW	88.4	1.2	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	21.9*	5.2	3.3*	3.3	16.4*	2.2	6.3*	3.2
PL	98.4*[a]	1.6	100	0	100	0	100	0
CnB	93.2 [a]	1.6	100	0	100	0	100	0
CnL	93.5 [a]	1.7	100	0	100	0	100	0
GlR	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**
GlL	98.2* [a]	1.8	100	0	100	0	100	0
GlS	95.0* [a]	2.5	97.8	2.20	100	0	100	0
LgL	96.9* [a]	1.6	100	0	100	0	100	0
LgS	98.3* [a]	1.7	100	0	100	0	100	0
TmR	96.7* [a]	3.3	100	0	100	0	100	0
TmL	96.3* [a]	2.1	100	0	95.9*	1.5	100	0
GllesR	96.7* [a]	3.3	93.5*	0.6	100	0	100	0
GrR	92.9 [a]	2.0	100	0	97.7	2.3	100	0
CaL	92.5 [a]	1.4	100	0	97.6	1.7	100	0
CmL	**32.6***	**2.1**	100	0	97.9	1.4	100	0

Table 5.15 Germination of spore of fungi isolated from cinnamon subjected to several plant ethanol extracts (500 mg/mL). The highlighted cells indicate effective inhibition of spore germination.

Extract	C21		C24		C26		C30	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error
DW	88.4	1.2	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	21.9*	5.2	3.3*	3.3	16.4*	2.2	6.3*	3.2
PL	89.7 [a]	1.2	100	0	100	0	85.6*	4.7
CnB	95.4* [a]	0.4	**5.8***	**3.2**	**66.1***	**3.1**	**8.8***	**2.2**
CnL	97.0* [a]	1.5	100	0	100	0	81.0*	2.5
GlR	**0***	**0**	**0***	**0**	**0***	**0**	**0***	**0**
GlL	92.5 [a]	1.7	100	0	74.1*	3.8	94.1*	1.2
GlS	**81.4***	**1.0**	**10.7***	**1.4**	**3.3***	**3.3**	**3.3***	**3.3**
LgL	97.0* [a]	3.0	100	0	100	0	98.3*	1.7
LgS	97.1* [a]	1.5	100	0	100	0	100	0
TmR	98.2* [a]	1.8	100	0	100	0	96.5	3.5
TmL	94.2* [a]	1.5	100	0	89.8*	1.9	100	0
GllesR	96.7* [a]	3.3	**79.2***	**3.4**	**50***	**0**	**23.6***	**2.6**
GrR	81.8	2.5	100	0	99.1	0.8	100	0
CaL	86.5	0.8	97.7	1.2	96.5	1.9	95.7	2.1
CmL	**13.5***	**3.3**	98.7	1.3	91.8	2.4	96.5	1.8

* Significant difference from the water (α = 0.05, df = 16), [a] Anomalous value = unusual data since the mean spore germination rates for these extract treatments were higher than the mean germination under distilled water

DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, PL = black pepper leaf, CnB = cinnamon bark, CnL = cinnamon leaf, GlR = galangal rhizome, GlL = galangal leaf, GlS = galangal stem, LgL = lemon grass leaf, LgS = lemon grass stem, TmR = turmeric rhizome, TmL = turmeric leaf, GllesR = lesser galangal rhizome, GrR = ginger rhizome, CaL = *Cassia* leaf, CmL = cardamom leaf, C21 = *Pestalotiopsis* sp., C24 = *Curvularia inaequalis*, C26 = *Helminthosporium* sp., C30 = *Curvularia* sp.

5.3.2.3 *Germination of spores of fungi isolated from turmeric*

The effect of the plant extracts on germination of the spores of the fungi isolated from turmeric was similar to their effects on the fungi isolated from cinnamon. Cinnamon bark (CnB) extracts (water and ethanol) were the most effective (Tables 5.16 and 5.17).

Table 5.16 Germination of species of fungi isolated from turmeric subjected to several plant water extracts (500 mg/mL). The highlighted cells indicate effective inhibition of spore germination.

Extract	T41		T48		T51		T52	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error
DW	100	0	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	14.0*	4.4	46.1*	2.6	10.6*	0.8	17.3*	3.4
PL	100	0	100	0	100	0	100	0
CnB	**58.4***	**1.8**	**0***	**0**	93.5*	1.8	100	0
CnL	100	0	88.1*	2.4	100	0	100	0
GlR	**0***	**0**	**0***	**0**	**7.1***	**1.8**	**0***	**0**
GlL	100	0	100	0	100	0	100	0
GlS	81.3*	2.1	85.2*	3.7	98.4	1.6	100	0
LgL	100	0	100	0	100	0	100	0
LgS	100	0	100	0	100	0	100	0
TmR	100	0	100	0	100	0	100	0
TmL	99.4	0.6	100	0	100	0	100	0
GllesR	100	0	100	0	100	0	100	0
GrR	80.5*	2.2	80.6*	4.3	95.9*	0.8	100	0
CaL	96.7	3.3	100	0	100	0	100	0
CmL	**61.6***	**2.3**	**37.2***	**3.9**	98.4	1.0	99.4	0.6

* Significant difference from the water ($p < 0.05$, df = 16)

DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, PL = black pepper leaf, CnB = cinnamon bark, CnL = cinnamon leaf, GlR = galangal rhizome, GlL = galangal leaf, GlS = galangal stem, LgL = lemon grass leaf, LgS = lemon grass stem, TmR = turmeric rhizome, TmL = turmeric leaf, GllesR = lesser galangal rhizome, GrR = ginger rhizome, CaL = *Cassia* leaf, CmL = cardamom leaf, T41 = *Phoma* sp., T48 = *Exserohilum* sp., T51 = *Curvularia* sp., T52 = *Bipolaris* sp.

Table 5.17 Germination of spore of fungi isolated from turmeric subjected to several plant ethanol extracts (500 mg/mL). The highlighted cells indicate effective inhibition of spore germination.

Extract	T41		T48		T51		T52	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ (%)	Std Error
DW	100	0	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	14.0*	4.4	46.1*	2.6	10.6*	0.8	17.3*	3.4
PL	80.7*	2.6	100	0	93.4	0.5	100	0
CnB	**0***	**0**	**0***	**0**	**27.4***	**3.9**	**0***	**0**
CnL	100	0	72.5*	2.4	89.8*	0.7	93.3*	3.8
GlR	**0***	**0**	**0***	**0**	**9.4***	**1.8**	**0***	**0**
GlL	**19.6***	**3.6**	89.6*	4.4	78.8*	4.0	91.3*	4.3
GlS	**0***	**0**	**45.2***	**4.0**	**28.0***	**3.6**	**0***	**0**
LgL	100	0	100	0	98.9	1.1	100	0
LgS	100	0	100	0	96.7	3.3	100	0
TmR	100	0	100	0	98.7	1.3	100	0
TmL	98.5	1.5	92.8*	3.6	100	0	100	0
GllesR	**7.4***	**0.7**	100	0	83.5*	4.5	**0***	**0**
GrR	67.7*	5.1	80.2*	4.6	89.6*	3.8	96.4*	1.2
CaL	98.0	1.2	86.9*	0.6	96.8	1.9	98.8	1.2
CmL	**54.8***	**4.7**	**15.6***	**2.9**	94.9	1.0	90.0	0.6

* Significant difference from the water ($p < 0.05$, df = 16)
DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, PL = black pepper leaf, CnB = cinnamon bark, CnL = cinnamon leaf, GlR = galangal rhizome, GlL = galangal leaf, GlS = galangal stem, LgL = lemon grass leaf, LgS = lemon grass stem, TmR = turmeric rhizome, TmL = turmeric leaf, GllesR = lesser galangal rhizome, GrR = ginger rhizome, CaL = *Cassia* leaf, CmL = cardamom leaf, T41 = *Phoma* sp., T48 = *Exserohilum* sp., T51 = *Curvularia* sp., T52 = *Bipolaris* sp.

5.3.2.4 *Summary of the effects of plant extracts*

A summary of extracts capable of causing complete inhibition of spore germination of the tested fungal species is provided in Table 5.18.

The galangal rhizome extracts (both solvents) provided excellent suppression of spore germination of all of the fungal species tested (Table 5.18). The ethanol extract of cinnamon bark (CnB) and galangal stem (GlS) gave better reductions of germination than the water extract with most fungi, whereas the ethanol extract of lesser galangal rhizome (GllesR) worked well only on particular fungal species. The ethanol extract of cardamom leaf (CmL) showed greater inhibitory effects of spore germination than the water

extract, and it worked well on putative pathogenic fungi, but showed low inhibition against the *Helminthosporium* fungus group.

Table 5.18 List of fungal species whose germination was found to be inhibited significantly by exposure to water and ethanol extracts from plants (500 mg/mL).

Extracts	Fungal species
Galangal rhizome water extract	*C. gloeosporioides* (P1) *Curvularia* sp. (P6) *Fusarium* sp. (P8) *Pestalotiopsis* sp. (P10) *C. orbiculare* (P13) *Pestalotiopsis* sp. (C21) *Curvularia inaequalis* (C24) *Helminthosporium* sp. (C26) *Curvularia* sp. (C30) *Phoma* sp. (T41) *Exserohilum* sp. (T48) *Bipolaris* sp. (T52)
Galangal rhizome ethanol extract	*C. gloeosporioides* (P1) *Cladosporium* sp. (P3) *Curvularia* sp. (P6) *Fusarium* sp. (P8) *Pestalotiopsis* sp. (P10) *C. orbiculare* (P13) *Pestalotiopsis* sp. (C21) *Curvularia inaequalis* (C24) *Helminthosporium* sp. (C26) *Curvularia* sp. (C30) *Phoma* sp. (T41) *Exserohilum* sp. (T48) *Bipolaris* sp. (T52)
Galangal stem ethanol extract	*Phoma* sp. (T41) *Bipolaris* sp. (T52)
Lesser galangal rhizome ethanol extract	*Bipolaris* sp. (T52)
Cardamom leaf ethanol extract	*C. gloeosporioides* (P1) *C. orbiculare* (P13)
Cinnamon bark water extract	*Exserohilum* sp. (T48)
Cinnamon bark ethanol extract	*Curvularia* sp. (P6) *Phoma* sp. (T41) *Exserohilum* sp. (T48) *Bipolaris* sp. (T52)

Ethanol extractions tended to be inhibitory and caused some abortion of germinating spores, the germ tubes were swollen and abnormal hyphal growth developed, especially with *Curvularia* sp. and *Fusarium* sp. exposed to the cinnamon bark extract. With many fungi, shorter germ tubes were noted under the ethanol extract than under the water extract. This was noted particularly with lesser galangal rhizome and galangal stem extracts.

Germination tests conducted on an agar medium instead of in distilled water in the present study ensured ideal conditions for spore germination up to 100%. However, several fungi did not achieve 100% germination within 24 hours. This condition seemed to be influenced by genetic or environmental factors (Section 4.3, above).

Another improved experimental outcome initiated in the present study was the method of applying the extracts or oils. The results indicated that treatment suspensions could be absorbed into the agar medium and then significantly influence spore germination. Figure 5.5 shows several characteristics of spore germination of *C. gloeosporioides* in contact with plant extracts or oils after 24 hours. Hyphal growth and branching characteristics were markedly different in distilled water (sparse growth no branching, Fig. 5.5 A) compared to growth in a chilli-ethanol extract (restricted growth but frequent branching, Fig 5.5 B), and to growth in a chilli-water extract (frequent branching, vigorous growth, Fig. 5.5 C).

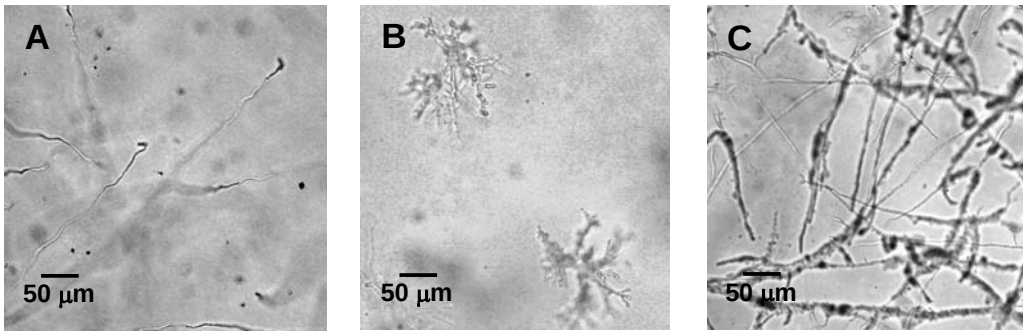

Figure 5.5 Germinated spores of *C. gloeosporioides* at 24 hours after incubation: (A) germination in distilled water; (B) germination in a chilli-ethanol extract; (C) germination in a chilli-water extract.

It was noted that increases of spore germination occurred in contact with particular plant extracts (e.g., lemon grass, turmeric, ginger, and galangal leaf extracts) against *C. orbiculare* and *Pestalotiopsis* sp. (Tables 5.12 – 5.15). This condition may be attributed to the increase of some growth factor, which, in high concentrations in the crude extract, stimulate fungal development (Fiori *et al*. 2000). Where oils were involved (e.g., garlic and neem oils against *C. orbiculare*; Tables A.7.7 and A.7.11), another explanation may apply. When the oil is present at a low concentration, it stimulates germination, but inhibits germination increasingly, as the oil concentration is raised. This type of response has been noted with the detergent Tween 80 by Plotkin *et al*. (1996) who showed that enzymatic activity and cell growth of the yeast *Malassezia furfur* (Robin) Baillon were first induced, then later inhibited, by increasing concentrations of Tween 80.

The role of nutrition availability in spore germination was explained by Agrios (1997) in living plants. He found that spore germination is often favored by nutrients diffusing from the plant surface, and that the more nutrients (sugars and amino acids) exuding from the plant, the more spores

germinate and the faster they germinate. Agrios (1997) also showed that, in some cases, spore germination of a certain pathogen is stimulated only by exudates of plants susceptible to that particular pathogen. In other cases, spore germination may be inhibited to a lesser or greater extent by materials released into the surrounding water by the plant, by substances contained within the spores themselves (especially when the spores are highly concentrated), and by saprophytic microflora present on or near the plant surface (Agrios 1997).

5.4 SUMMARY AND CONCLUSIONS

The data suggest that a range of plant oils over the concentration range of 0.1 – 3% is capable of inhibiting fungal spore germination on pepper, cinnamon and turmeric. Several oils were shown to be more efficient than the synthetic fungicide Amistar at field concentrations. The oils of cinnamon bark, cinnamon leaves, clove buds, and clove leaves provided the greatest inhibition of spore germination of most of the fungal species tested. Cinnamon oils completely inhibited spore germination of most fungi over the whole range of concentrations tested, and especially at low concentrations. Although spore germination still occurred following the application of the two clove oils, these oils completely inhibited germination of one species of *Curvularia, Exserohilum* and *Helminthosporium*.

The oils responsible for the greatest inhibition of spore germination, but at relatively high concentrations (0.5 – 1%) were lemon grass leaf oil, lesser galangal rhizome oil, cardamom seed oil, and lemon myrtle leaf oil. Lemon grass leaf oil completely inhibited spore germination of *Pestalotiopsis*

sp. at all concentrations tested. Cardamom seed oil and lemon myrtle leaf was particularly effective against *Fusarium* spp. Less inhibition was shown at all of the concentrations tested of tea-tree oil, *Eucalyptus* oil, ginger rhizome oil, pepper berry oil, turmeric rhizome oil, garlic pod oil, onion bulb oil, rosemary oil, and neem seed oil.

Cladosporium sp. and *Phoma* sp. were more susceptible to inhibition by plant oils compared with the other fungal species. For example, garlic oil completely inhibited the spore germination of *Phoma* sp., but was less efficient on all other fungal species.

Other oils of potential interest were lesser galangal rhizome oil and turmeric rhizome oil. These worked well on *Bipolaris* sp., *Curvularia* sp., *Fusarium* sp., and *Helminthosporium* sp.; lesser galangal rhizome oil showed better suppression of *Bipolaris* sp. than did the clove oils.

The extracts responsible for the greatest inhibition of spore germination were from galangal rhizomes and cardamom leaves. Preparations derived from plants by ethanol extraction methods outshone the corresponding water extracts in terms of their ability to inhibit spore germination.

The galangal rhizome extract worked well whether prepared by ethanol or water extraction methods. The ethanol extract completely inhibited spore germination on all fungi, while the water extract was equally effective, except for *Cladosporium* sp. (24% germination).

Ethanol extractions of galangal stem and cinnamon bark also inhibited spore germination. Galangal stem extract worked well against most of the fungi, as did the galangal rhizome extract; they completely inhibited spore

germination of *Bipolaris* sp. and *Phoma* sp. Meanwhile, cinnamon bark extract tended to work well on putative saprophytic fungi. This means that the application of this extract was more efficient on the fungi isolated from cinnamon or turmeric leaves, most of which were from the genera *Curvularia* and *Helminthosporium*. The cinnamon bark extract was less efficient on *Pestalotiopsis* sp., however, which is the species, most frequently isolated from cinnamon leaves.

The ethanol extract from cardamom leaves was more efficient than the corresponding water extract. This extract suppressed spore germination of *C. gloeosporioides*, *C. orbiculare*, *Fusarium* spp., and *Pestalotiopsis* sp. (putative pathogenic fungi) better than *Cladosporium* sp. and *Curvularia* sp. (putative saprophytic fungi). Since most of the fungi isolated from pepper came from genera of putative pathogenic fungi, application of the ethanol extract of cardamom leaves was more efficient on fungi isolated from pepper leaves rather than those from cinnamon or turmeric.

Several extracts did not inhibit fungal spore germination. For example, up to 100% spores still germinated on the application of galangal leaf, ginger rhizome, pepper leaf, lemon grass, and turmeric extracts. Several extracts from lemon grass and turmeric occasionally stimulated spore germination or hyphal growth.

CHAPTER 6

STUDIES ON *COLLETOTRICHUM GLOEOSPORIOIDES*

6.1 INTRODUCTION

Colletotrichum species have been implicated in plant diseases throughout the world and especially as the cause of pre-harvest and post-harvest problems in the tropics (Bailey and Jeger 1992). Their ability to cause latent or quiescent infections places them amongst the most important of the post-harvest fungal pathogens (Bailey and Jeger 1992).

Anthracnose disease is caused by the genus *Colletotrichum*, which is a very common group of plant pathogens (Roberts *et al*. 2001). The fungus has an extremely wide host range including vegetables, field and forage crops, fruit trees, and ornamentals and it infects foliage and fruits, particularly in warm, wet weather (Baird 2005). Many species of *Colletotrichum* infect more than one host, and more than one *Colletotrichum* species may be present on the host (Roberts *et al*. 2001).

Colletotrichum can affect stem tips causing die back and stem lesions. It commonly infects young succulent stems, petioles, and fruits, but all aboveground plant parts are susceptible (Baird 2005). In general, leaf lesions are dark brown to black and may be associated initially with a leaf vein. Lesions initially appear as small, sunken water-soaked spots. Orange-pink to brownish spore masses may be seen on older lesions. A diagnostic feature of some *Colletotrichum* infections that can be seen with a hand lens is the production of dark tufts of setae (hair-like structures) within the lesion. *Colletotrichum* produces spores within an acervulus (fungal fruiting

structure). The disk or cushion shaped acervuli break through the surface of host tissue; short, simple, colourless conidiophores produce abundant conidia; and long, black setae may or may not be produced among conidiophores. Ovoid to cylindrical and single celled conidia are colourless when viewed alone, but may appear pink or salmon coloured in mass (Baird 2005).

This fungus became a particular interest because of its widespread occurrence in the host plants of the present study, and the potential for its control by plant oils or extracts.

6.2 LONG TERM FUNGAL GROWTH

6.2.1 Time effects on fungal growth

6.2.1.1 *Prolonged observation periods: 48 hours after incubation*

In germination tests of *C. gloeosporioides*, all of the treatments that gave zero germination at 24 hours were incubated for longer time periods. Delayed germination was found especially when Amistar fungicide treatments were applied and observations were made up to a week later. Amistar fungicide treatments gave spore germinations up to 100% after as little as 48 hours. However, extract and oil treatments essentially gave unchanged results after prolonged incubation. For example, after 48 hours incubation, there was no further germination over that of 24 hours for *C. gloeosporoides* in the ethanol or water extract of galangal rhizome (GlR) and ethanol extract of cardamom leaf (CmL) treatments.

Oil treatments showed some changes in germination behaviour when longer testing times were used (Table 6.1).

Table 6.1 Changes in spore germination under oil treatments at 48 hours compared with that after 24 hours.

Oils	Concentration (%)	Change in performance
CnBO	0.1 – 3 (all)	No germination
CnLO	1 – 3	No germination (no change)
	≤ 0.5	22 – 25% germination, change from 0%
ClBO	0.5 – 3%	No germination (no change)
ClLO	0.5 – 3%	10% germination, change from 0%
LgO	1 – 3	No germination (no change)
LmO	1 – 3	No germination (no change)
CmO	1 – 3	No germination (no change)
GllesO	3	No germination (no change)
	1	12.5% germination, change from 0%

CnBO = cinnamon bark oil, CnLO = cinnamon leaf oil, ClBO = clove bud oil, ClLO = clove leaf oil, LgO = lemon grass oil, LmO = lemon grass oil, CmO = cardamom oil, GllesO = lesser galangal oil

6.2.1.2 *Prolonged observation periods: 7 days after incubation*

To test fungal development after prolonged observation periods, *C. gloeosporioides* and *Curvularia* exposed to distilled water, galangal rhizome water extract, and Amistar fungicide treatments were regrown for one week after the initial inoculation by placing the agar plugs on a new agar medium. Both fungi were able to recover and to grow from the galangal rhizome extract and Amistar plugs as the concentration of the active fungicidal ingredient declined, as a result of diffusion into the surrounding agar. However, different characteristics were noted in the growth of hyphae. Hyphae emerging after exposure to the Amistar treatments developed extravasation of the cytoplasmic contents in comparison with normal hyphae developing in the distilled water and galangal rhizome treatments.

6.2.2 Potency groups for extracts and oils

The potential of extracts and oils to inhibit spore germination of *C. gloeosporoides* is particularly interesting owing to the significance of this fungus as a widespread plant pathogen.

The potency of oils to inhibit *C. gloeosporioides* germination can be grouped into three categories that gave high, moderate and low inhibition of spore germination. These general groups of oils are shown in Table 6.2, and the potency of galangal rhizome and cardamom leaf extracts to inhibit this fungus are shown in Table 6.3.

Significant differences in inhibition between the cinnamon extract and cinnamon oil treatments were noted but these are considered to reflect failures in extract preparations as some considerable difficulty was experienced in preparing the extractions from finely blended barks.

Table 6.2 Potency of plant oils based on inhibition of germination of spores of *C. gloeosporioides*.

Inhibition of spore germination	High inhibition	Moderate inhibition	Low inhibition
> 50%	Cinnamon Clove Lesser galangal Lemon grass Lemon myrtle		
20 – 50%		Neem Onion Rosemary Turmeric	
< 20%			*Eucalyptus* Garlic Ginger Black pepper Tea-tree

Table 6.3 Potency of plant extracts based on inhibition of germination of spores of *C. gloeosporioides*.

Inhibition of spore germination	High inhibition	Low inhibition	No effect
60 – 100%	Water and ethanol extracts of: Galangal rhizome Cardamom leaf		
10 – 30%		Ethanol extract of: Galangal stem *Cassia* leaf	
Nil			The 22 other water and ethanol extracts tested

6.2.3 Lethal concentration (LC_{50})

Galangal rhizome extracts, cardamom leaf extracts, cinnamon oils, and clove oils were tested at low concentrations to determine their lethal concentrations for *C. gloeosporioides*. The extracts and oils were mostly completely inhibitory to the germination of spores of *C. gloeosporioides* over the range of concentrations previously tested. Water extracts of galangal rhizome (GlRw) and cardamom leaf (CmLw), and ethanol extracts of galangal rhizome (GlRe) and cardamom leaf (CmLe) were tested at concentrations of 5, 10, 50, 100, 200, 300, and 400 mg/mL; concentrations of 0.005, 0.01, and 0.05% were used for the oil treatment. The results are shown in Tables 6.4 and 6.5.

Table 6.4 Germination of *C. gloeosporioides* in galangal rhizome extracts and cardamom leaf extracts over the range of concentrations 5 – 500 mg/mL.

Cont. (mg/mL)	GlRw		GlRe		CmLw		CmLe	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	30.2*	1.6	30.2*	1.6	30.2*	1.6	30.2*	1.6
500	0*	0	0*	0	43.0*	5.5	0*	0
400	0*	0	0*	0	100	0	60.6*	0.9
300	16.3*	4.0	0*	0	100	0	62.5*	1.0
200	11.9*	2.4	0*	0	100	0	66.9*	1.8
100	28.1*	2.5	0*	0	100	0	64.8*	0.5
50	52.8*	4.3	58.0*	4.0	100	0	81.5*	3.6
10	100	0	97.9	1.3	100	0	79.3*	0.1
5	100	0	100	0	100	0	81.3*	3.7

* Significant difference from the water ($p < 0.05$, df = 10)
DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, GlRw = water extractions of galangal rhizome extract, GlRe = ethanol extractions of galangal rhizome extract, CmLw = water extraction of cardamom leaf extract, CmLe = ethanol extractions of cardamom leaf extract

Table 6.5 Germination of *C. gloeosporioides* in cinnamon oils and clove oils over the range of concentrations 0.005 – 3%.

Cont. (%)	CnBO		CnLO		ClBO		ClLO	
	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error	Germ. (%)	Std Error
DW	100	0	100	0	100	0	100	0
DF	0*	0	0*	0	0*	0	0*	0
AF	2.7*	1.3	2.7*	1.3	2.7*	1.3	2.7*	1.3
0.005	100	0	100	0	100	0	100	0
0.01	100	0	100	0	100	0	100	0
0.05	40.9*	3.7	93.1*	1.9	100	0	100	0
0.1	0*	0	0*	0	34.3*	0	40.4*	1.8
0.2	0*	0	0*	0	26.8*	3.0	36.2*	5.2
0.5	0*	0	0*	0	0*	0	0*	0
1	0*	0	0*	0	0*	0	0*	0
3	0*	0	0*	0	0*	0	0*	0

* Significant difference from the water ($p < 0.05$, df = 10)
DW = distilled water, DF = Dithane M-45 fungicide, AF = Amistar fungicide, CnLO = cinnamon bark oil, CnLO = cinnamon leaf oil, ClBO = clove bud oil, ClLO = clove leaf oil

The lethal concentration at which 50 per cent of spores fail to germinate (LC_{50} value) was determined for the extracts and oils by regression analysis. The galangal rhizome extracts behaved in a logarithmic

fashion (Fig. 6.1), that is, an increase in the concentration of extract was followed by a rapid decrease in germination percentage. The cardamom leaf extracts behaved in a quadratic fashion (Fig. 6.1) with steady spore germinations observed at the lower extract concentrations; only at the higher concentrations were significant reductions in germination seen. In all these cases the mathematical relationships accounted for 79 – 97% of the variation in the data (Fig. 6.1).

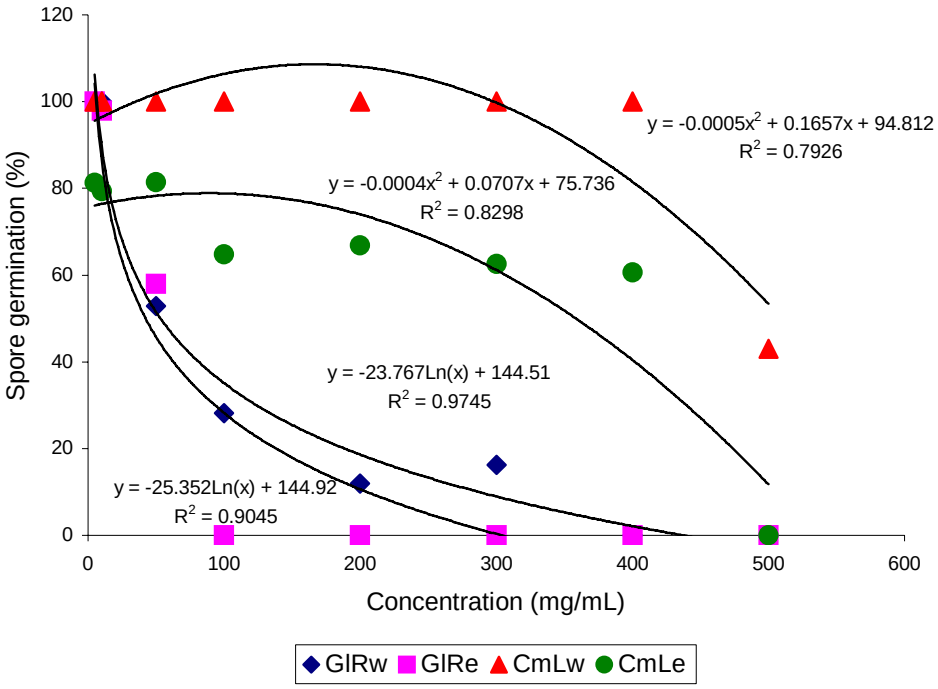

Figure 6.1 Spore germination of *C. gloeosporioides* in the presence of water extracts of galangal rhizome (GlRw), ethanol extracts of galangal rhizome (GlRe), water extracts of cardamom leaf (CmLw), and ethanol extracts of cardamom leaf (CmLe).

All the oils behaved in a logarithmic fashion with determination coefficients between 0.72 and 0.95 (Figs. 6.2 and 6.3). That is, an increase in the concentration of extract was followed by a marked decrease in germinations.

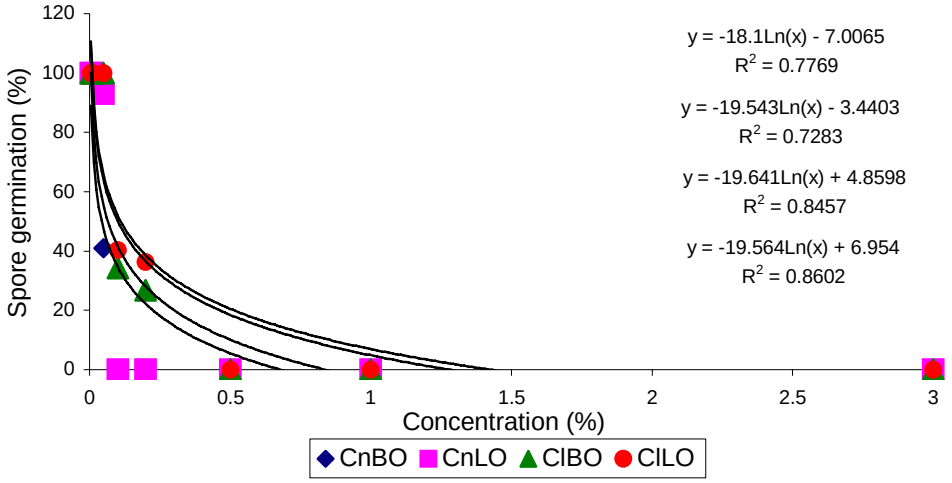

Figure 6.2 Spore germination of *C. gloeosporioides* in the presence of cinnamon bark oil (CnBO), cinnamon leaf oil (CnLO), clove bud oil (ClBO), and clove leaf oil (ClLO). The order of the equations corresponds with the legend order.

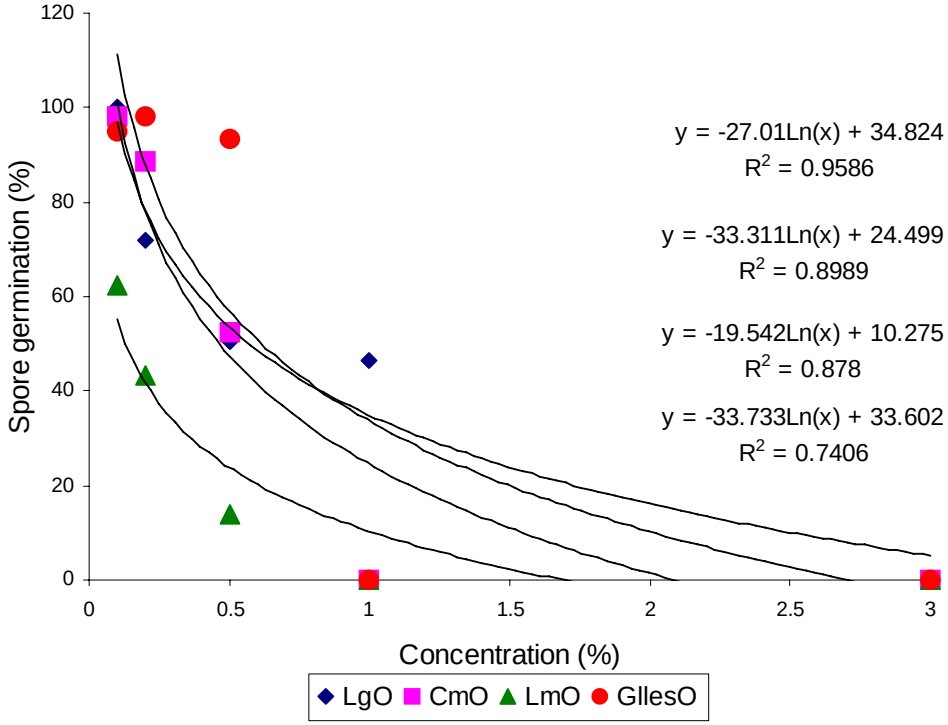

Figure 6.3 Spore germination of *C. gloeosporioides* in the presence of lemon grass oil (LgO), cardamom oil (CmO), lemon myrtle oil (LmO), and lesser galangal oil (GllesO). The order of the equations corresponds with the legend order.

The mathematical relationships shown in Figs 6.1 – 6.3 allowed the determination of the LC_{50} values shown in Table 6.6.

Table 6.6 LC_{50} values of several extracts and oils on germination of *C. gloeosporioides*.

Extracts/Oils	Critical concentration
Ethanol extract	mg/mL
Galangal rhizome	42.3
Cardamom leaf	357.0
Water extract	mg/mL
Galangal rhizome	53.4
Cardamom leaf	507.9 †
Oils	(%)
Cinnamon bark	0.04
Cinnamon leaf	0.07
Clove bud	0.10
Clove leaf	0.11
Lemon myrtle	0.13
Cardamom	0.47
Lemon grass	0.57
Lesser galangal	0.62

†Extrapolated value: the highest water extract concentration tested was 500 mg/mL

6.3 EFFECT OF PLANT EXTRACTS AND OILS ON *IN VITRO* FUNGAL GROWTH

Inoculation of detached pepper (*Piper nigrum*) leaves did not produce any symptoms of fungal infection, but phytotoxicity was observed especially on application of cinnamon and clove oils (Fig. 6.4; Table 6.7). Given that the chilli (*Capsicum annuum* L.) is well known to be a host sensitive to *C. gloeosporioides* (Roberts *et al*. 2001; Oh *et al*. 1999), chilli fruits and black pepper berries were used in further tests.

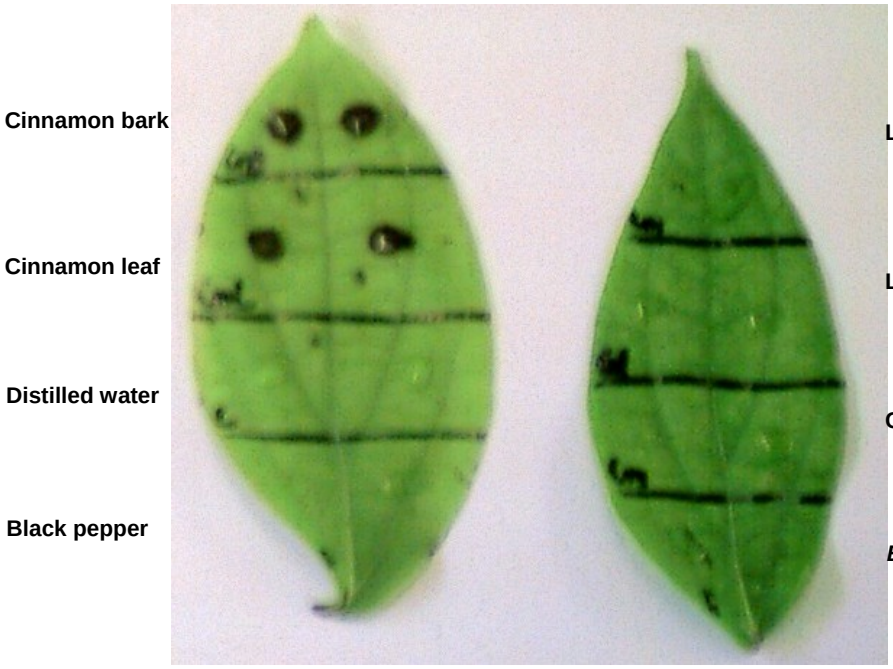

Figure 6.4 Phytotoxicity symptom on pepper leaves when treated with the various oils at 0.5% concentration. Black spots indicate phytotoxic damage to plant tissue on application of the indicated oils.

Table 6.7 Phytotoxicity levels of treatment solutions applied to pepper leaves at concentration of 0.5% (oils) and 500 mg/mL (extracts).

Phytotoxicity level			
Non-toxic	Low toxicity	Medium toxicity	Severe toxicity
Distilled water	Lemon myrtle oil	Clove bud oil	Cinnamon bark oil
Dithane M-45 fungicide	Lesser galangal oil	Clove leaf oil	Cinnamon leaf oil
Amistar fungicide	Lemon grass oil		
Tea-tree oil	Galangal extract		
Cardamom oil			
Eucalyptus oil			
Neem oil			
Pepper oil			
Cardamom extract			

Black pepper berries were inoculated with a spore suspension and mycelial plugs (0.5 cm diameter) of fungi were used to inoculate chillies as described in section 5.2.4.3. The spore suspension (5×10^4 spores/mL) in 0.01% Tween 80 was applied in 1 μL aliquots to berries followed by a 1 μL application of treatment solutions (0.5% oil concentrations or 500 mg/mL

extract concentration). On the chilli fruit test, 10 μL of treatment solution were used per specific site.

The *C. gloeosporioides* inoculations induced anthracnose symptoms on the black pepper berries six days after inoculation with distilled water, lemon grass oil, and clove bud oil treatments. As the incubation time continued, the small black spots increased in number and the fungus sporulated within ten days after inoculation with the distilled water treatment. All extracts and oils treatments were assessed two weeks after inoculation (Table 6.8).

Table 6.8 Disease development (pathogenicity) of *C. gloeosporioides* on pepper berries two weeks after inoculation and the application of 0.5% oils or 500 mg/mL extracts.

Treatments	Percentages of infected population[†]	Disease developments
Untreated	0	No symptoms
DW	83.3	Rot with sporulation
DF	0	No symptoms
AF	33.3	Rot with sporulation
CnBO	0	No symptoms
CnLO	0	No symptoms
CmLe	0	No symptoms
CmO	0	No symptoms
GllesO	0	No symptoms
GlRe	0	No symptoms
GlRw	0	No symptoms
ClBO	16.7	Rot, no sporulation
LmO	16.7	Rot with sporulation
TO	16.7	Black sunken lesion
ClLO	33.3	Rot, no sporulation
CmLw	50	Rot, no sporulation
LgO	50	Rot, no sporulation
PO	66.7	Rot with sporulation

[†] Calculated from three replications with two berries per replication.
Untreated = distilled water with no inoculation, DW = distilled water with inoculation, DF = Dithane M-45 fungicide, AF = Amistar fungicide, TO = tea-tree oil, PO = pepper oil, CmO = cardamom oil, GllesO = lesser galangal oil, LmO = lemon myrtle oil, LgO = lemon grass oil, ClBO = clove bud oil, ClLO = clove leaf oil, CnBO = cinnamon bark oil, CnLO = cinnamon leaf oil, GlRe = ethanol extract of galangal rhizome, GlRw = water extract of galangal rhizome, CmLe = ethanol extract of cardamom leaf, CmLw = water extract of cardamom leaf

Anthracnose development occurred preferentially on the younger or smaller berries rather than on the older or harder berries (Fig. 6.5). Where no symptoms were initially observed on berries, the incubation was continued for up to two months. After this period, the berries were black in colour from shrinkage caused by non-pathogenic factors.

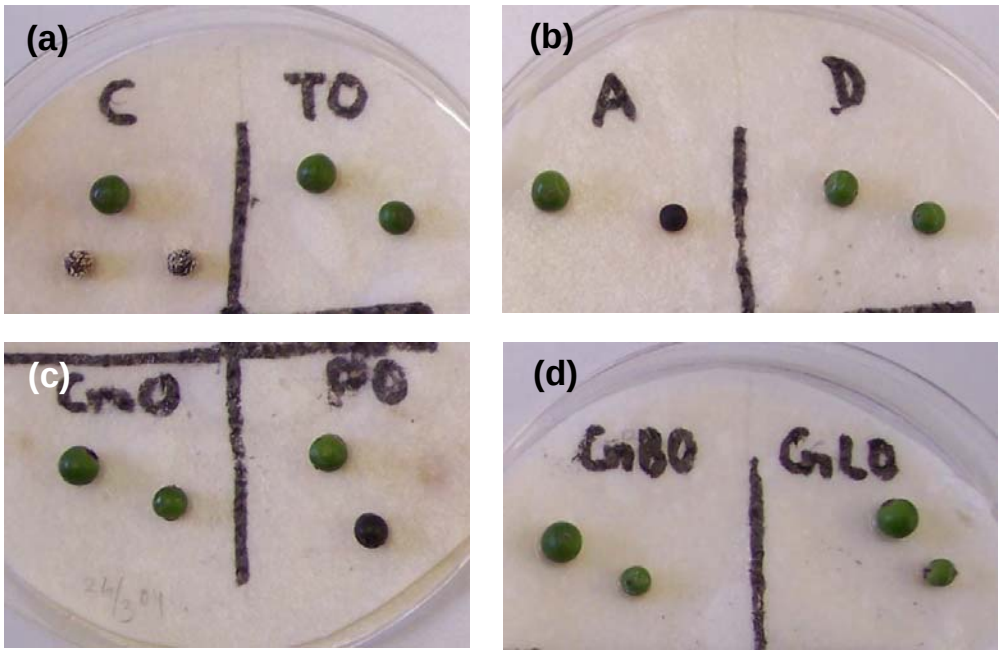

Figure 6.5 Oil treatments on pepper berries at three weeks after inoculation:
(a) distilled water control (C) and tea-tree oil (TO) treatments;
(b) Amistar (A) and Dithane M-45 (D) fungicide treatments;
(c) cardamom (CmO) and black pepper (PO) oil treatments;
(d) cinnamon bark (CnBO) and cinnamon leaf (CnLO) oil treatments.

C. gloeosporioides caused sunken, anthracnose symptoms on chilli fruits seven days after inoculation (Fig. 6.6). Significant differences in lesion diameter were found between distilled water treatments and the treatment using plant extracts or oils (Tables 6.9 and 6.10). The control treatment (distilled water) supported a greater spore load than did treatments involving extracts or oils.

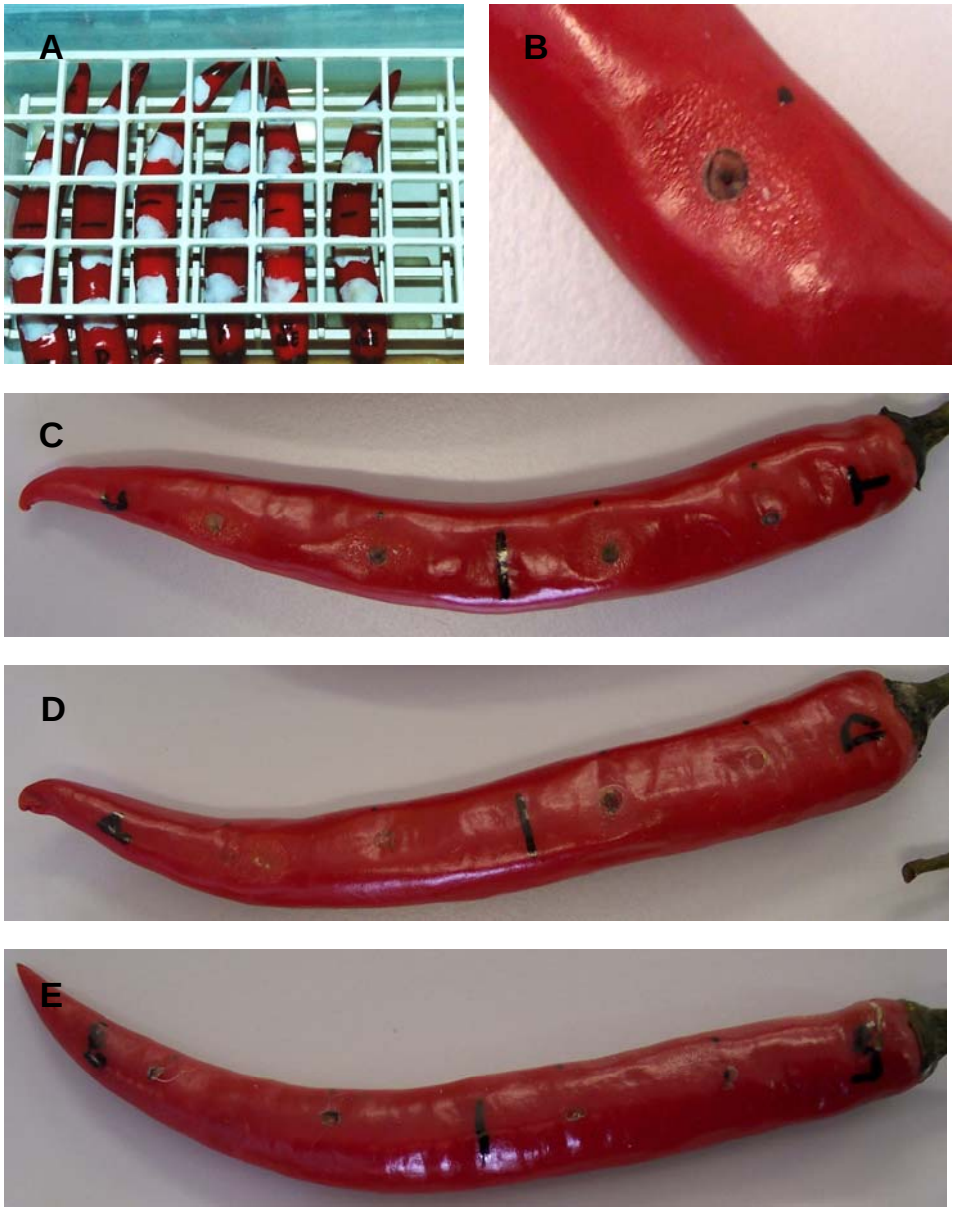

Figure 6.6 Plant extract and oil treatments on chilli fruits: (A) treatment layout for incubation; (B) sunken lesion of anthracnose infection; (C) sunken lesion and sporulation of both inoculated sites in the distilled water treatment (left) and sunken lesion of one site in the tea-tree oil treatment (right); (D) lesion and sporulation of one site of Amistar fungicide treatment (left) and no infection in Dithane M-45 fungicide treatment (right); (E) no infection in cardamom oil (left) and lemon myrtle oil (right) treatments.

The plant extracts or oils reduced fungal infection and a majority of them prevented the development of disease symptoms. Disease development occurred when cinnamon, pepper, tea-tree, and clove oils were

used. From knowledge of the phytotoxicity of these oils on black pepper leaves, phytotoxicity could have rendered the host more susceptible to the infection. Indeed, Oh *et al.* (1999) mentioned that destroyed host tissue on chilli fruits plays a significant role in fungal infection by *C. gloeosporioides*.

Table 6.9 Anthracnose lesion diameter on chilli fruits in oil treatments (0.5% concentrations) at seven days after inoculation.

Treatments	Diameter (mm)	Std Error
DW	16.3	0.9
DF	0*	0
AF	6.0*	2.5
TO	1.8*	1.8
CmO	0*	0
GllesO	0*	0
LmO	0*	0
PO	7.3*	1.0
LgO	0*	0
ClBO	4.8*	1.7
ClLO	0*	0
CnBO	3.0*	1.8
CnLO	2.0*	2.0

* Significant difference from the water treatment ($p < 0.05$, df = 12)
DW = distilled water with inoculation, DF = Dithane M-45 fungicide, AF = Amistar fungicide, TO = tea-tree oil, PO = pepper oil, CmO = cardamom oil, GllesO = lesser galangal oil, LmO = lemon myrtle oil, LgO = lemon grass oil, ClBO = clove bud oil, ClLO = clove leaf oil, CnBO = cinnamon bark oil, CnLO = cinnamon leaf oil

Table 6.10 Anthracnose lesion diameter on chilli fruits in plant extract treatments (500 mg/mL) at seven days after inoculation.

Treatments	Diameter (mm)	Std Error
DW	16.3	0.9
DF	0*	0
AF	6.0*	2.5
GlR ethanol extract	0*	0
GlR water extract	1.5*	1.5
CmL ethanol extract	0*	0
CmL water extract	8.5*	2.2

* Significant difference from the water treatment ($p < 0.05$, df = 6)
DW = distilled water with inoculation, DF = Dithane M-45 fungicide, AF = Amistar fungicide, GlRe = ethanol extractions of galangal rhizome, GlRw = water extractions of galangal rhizome, CmLe = ethanol extractions of cardamom leaf, CmLw = water extractions of cardamom leaf

6.4 EFFECT OF PLANT EXTRACTS AND OILS ON *IN VIVO* FUNGAL GROWTH

In preliminary tests, inoculations of *C. gloeosporioides* produced anthracnose symptoms in papaya seedlings at 72 hours after inoculation compared with the lack of symptoms in non-inoculated plants. Anthracnose was evident as irregular, soaked, brown or transparent lesions in the middle of the leaves, or a blight on the leaf edges (Fig. 6.7).

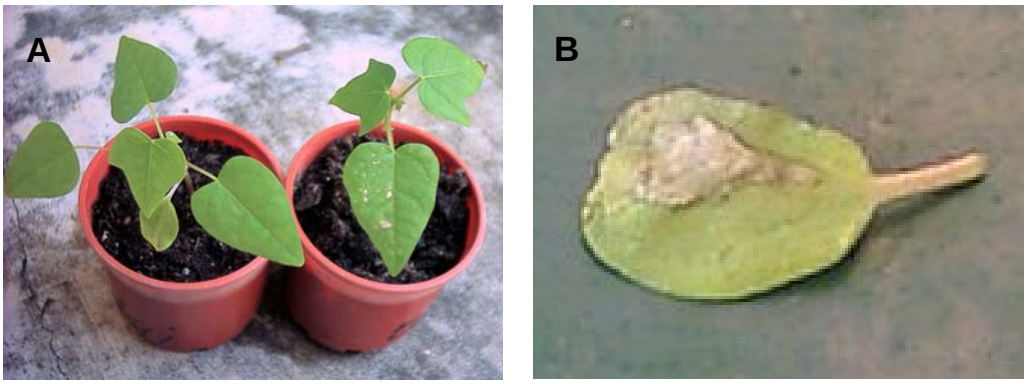

Figure 6.7 Anthracnose symptoms on papaya seedlings at two weeks after inoculation: (A) uninfected and infected (right) plants; (B) anthracnose lesions on the hypocotyl.

Papaya seedlings were used to test the effects of extracts and oils on *in vivo* fungal growth (Fig 6.8). The leaves were treated with suspensions of plant extracts (500 mg/mL) or 0.5% oils. Three days after inoculation, no symptoms were observed in response to the treatments. In contrast, the distilled water-treated leaves were severely infected. However, phytotoxicity was observed after the application of cinnamon oil, clove oil, lemon grass oil, and galangal extract at relatively high concentrations (0.5% or 500 mg/mL; Fig. 6.8).

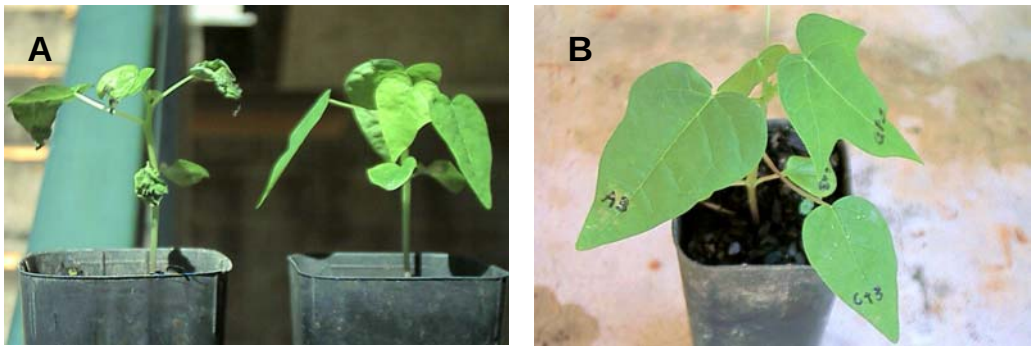

Figure 6.8 Treatments on papaya seedlings at one week after inoculation: (A) phytotoxicity symptoms caused by cinnamon oil (left) compared to untreated plant; (B) anthracnose lesion developed under the distilled water (C+3) and Amistar fungicide treatments (A3); no phytotoxic symptoms evident under the galangal rhizome extract treatment (GE3).

The effects of extracts and oils on fungal infection on papaya seedlings were variable. These effects may have been due to loss of antimicrobial activity on the plant leaves through loss of volatile components from the plant extracts and oils. Alternatively, the injuries resulting from phytotoxicity could also permit pathogens from entering and damaging the plants. The general observation was that the fungal infection symptoms were more severe when the plants showed phytotoxicity responses to the applied plant extracts and oils. However, phytotoxicity symptoms could be distinguished partially from anthracnose symptoms. Phytotoxicity incidence was assessed by 1 – 2 days after the wetness period. The assessment was made by comparing the untreated plants with plants inoculated with distilled water. Phytotoxicity symptoms were expressed as white, dried areas developing on the leaf edges or over the entire leaf area (Fig. 6.9).

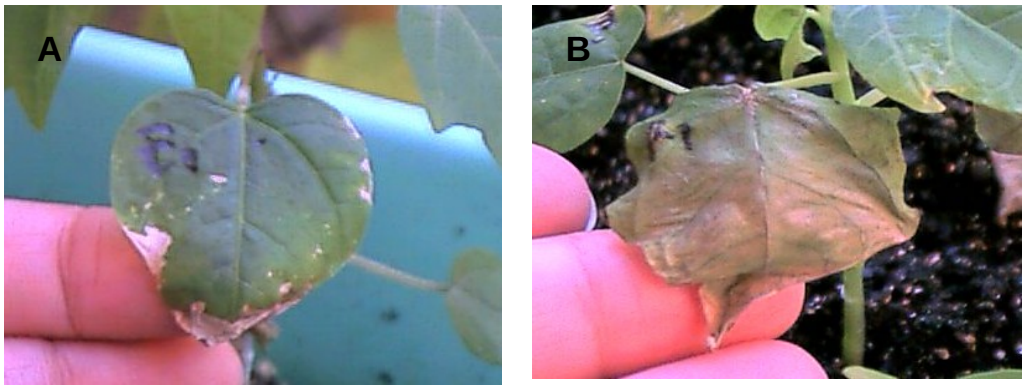

Figure 6.9 Phytotoxicity symptoms on papaya seedlings: (A) on leaf edges; (B) over the entire leaf area.

Anthracnose symptoms were evident as irregular lesions in the middles or edges of the leaves, with the presence of sporulation seen as circular black spots on the lesions. Symptoms first appeared a week after fungal inoculation.

Since no symptoms were observed in untreated leaves, the symptoms that developed were attributed solely to the effect of fungal infection or phytotoxicity. Disease severity was assessed using a 0 – 4 scale with a score for each leaf on each plant, as described by Cowling *et al*. (1999), and with a slight modification as shown in Fig. 6.10. The disease severity scores were averaged scores of three replications taken from leaf samples on different plants; the results are shown in Table 6.11.

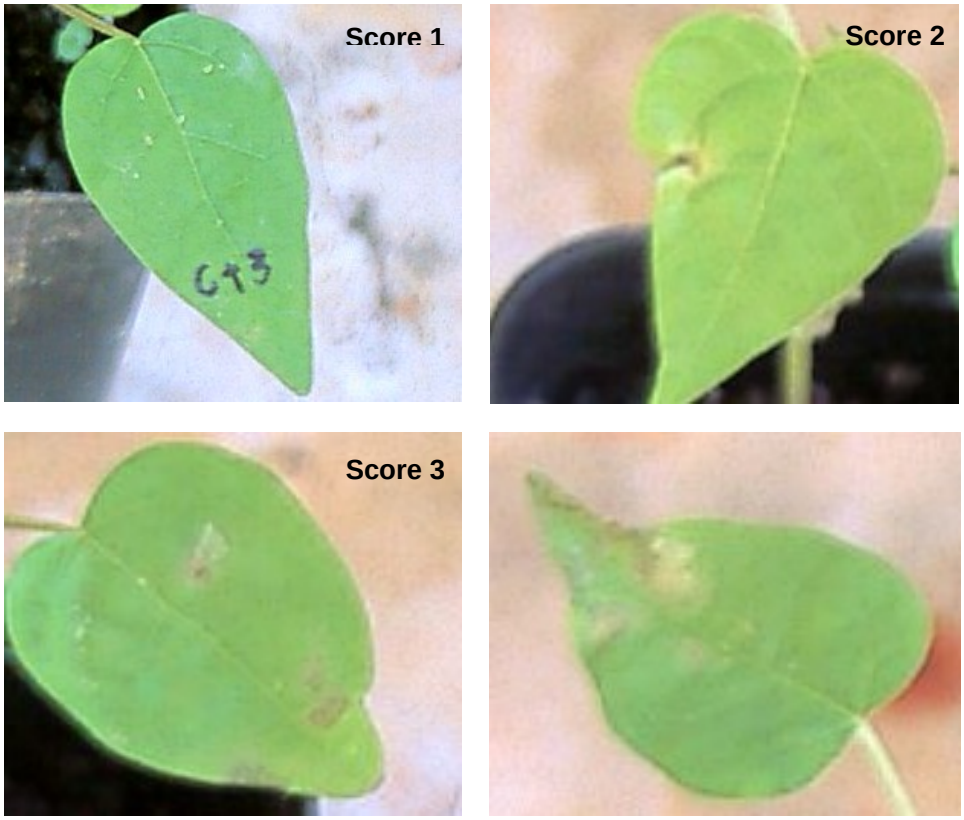

Figure 6.10 Scores for disease severity:

0 = no symptoms;
1 = pin point lesions, no sporulation;
2 = lesions < 3 mm in diameter, no sporulation;
3 = small lesions (3 – 10 mm) with sporulation;
4 = large lesions (>10 mm, > 30% of leaf areas was infected).

Table 6.11 Anthracnose disease severity on papaya seedlings at one week after inoculation after inoculum was treated by several extracts (500 mg/mL) or oils (0.5%).

Treatments	Diseases severity (0 – 4)	Infection rank
Control		
Untreated	0	-
Distilled water	3.7	1
Dithane M-45 fungicide	0	7
Amistar fungicide	2	2
Extracts		
CmL water	0.7	5
GlR ethanol†	0.7	5
LgL ethanol†	0.3	6
LgL water	0.3	6
CnB ethanol	0	7
CnB water	0	7
CmL ethanol	0	7
GlR water	0	7
PL ethanol	0	7
PL water	0	7
Oils		
LgO†	1.3	3
ClBO††	1	4
PO	1	4
CnBO††	0.7	5
ClLO††	0.7	5
GllesO†	0.3	6
CnLO†	0.3	6
LmO	0.3	6
TO	0	7
CmO	0	7
NmO	0	7
EO	0	7

† Indicate phytotoxicity incidence, ††more severe phytotoxicity

6.5 STAINING TECHNIQUES AND SCANNING ELECTRON MICROSCOPY (SEM)

6.5.1 Methods used

Light microscopy and scanning electron microscopy (SEM) observations were made of the infection process of *C. gloeosporioides* on black pepper leaves treated by plant extracts and oils. The aims of this aspect of the present study were to examine the host-pathogen relationships,

the conidial stage of fungal development, and the penetration and infection process of *C. gloeosporioides* on the host leaf.

A whole-leaf clearing and staining technique (Marte and Montalbini 1999; Shipton and Brown 1962) was used on papaya leaves that were treated by extracts or oils. The leaves were collected from the plants (described in Section 5.2.4.4 and Section 5.3.5), decolourised and stained by boiling in alcoholic lactophenol cotton blue, cleared in chloral hydrate, and mounted, unsectioned, in glycerine.

SEM observations were made on treated pepper and papaya leaves. The leaves were inoculated with conidia of *C. gloeosporioides*: that had been harvested from a 7-day-old fungal culture from which a suspension of spores (1×10^5 spores/mL) had been prepared in sterile distilled water. The plant extracts and oils tested were from galangal rhizomes and cinnamon bark. Concentrations used were based on *in vitro* tests that gave 50% and 0% spore germination and other treatments were incorporated as follows: untreated leaves, distilled water, 100% concentration of Amistar and Dithane M-45 fungicides, 50 mg/mL and 100 mg/mL concentrations of galangal rhizome extract (Section 3.2), and 0.05% and 0.1% concentrations of commercial cinnamon bark oil.

The young leaves of pepper and papaya were washed with sterile water, and dried between filter papers. The leaf samples were prepared as leaf discs for *in vitro* tests since *in vivo* tests on pepper plants were difficult to establish as the leaves were found to be difficult to infect. The treatment solutions were applied by dropper and allowed to run-off on both leaf surfaces. Both surfaces of the leaves were inoculated with the conidial

suspension within 5 – 10 minutes of the treatment solutions being applied. Inoculated leaves were incubated in humid boxes. Leaf samples were collected at 24 hours and 7 days after inoculation to study conidial germination, the infection process, and various developmental stages of the fungus. The leaf samples were cut into 7 mm^2 pieces and fixed in FAA (formaldehyde-acetic acid-alcohol) at 10: 5: 85 v/v/v for 48 hours, and then transferred to 70% ethanol. The samples then were dehydrated in a graded ethanol series (70%, 80%, 90%, and three times at 100%) for a period of 30 minutes in each series (Fiori *et al*. 2000; Kumar *et al*. 2001). Afterwards, the leaf material was mounted on stubs using double-sided carbon tape, was dried and then coated in a sputter-coater (JCU – 5000). The SEM observations were made at 10 KV under a JEOL JSM-540LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

6.5.2 Light microscopy: results

Observations made one week after inoculation showed abundant hyphal growth over papaya leaves in the distilled water treatment and in the Amistar fungicide treatment, but rarely in the ethanol extractions of galangal rhizome extract, or in the water extractions of cardamom leaf extract, cinnamon oils, clove oils, pepper black oil, and lemon grass oil. The fungus appeared to infect the plant by entry, into plant, through the stomates (Fig. 6.11A).

The fungus was abundant and sporulated on leaves with anthracnose symptoms in the distilled water treatment developing within one month of inoculation (Fig. 6.11D). The fungus also was found on most extract or oil treatments that showed any disease symptoms (refer to Table 6.11),

especially on senescent or necrotic leaves resulting from phytotoxicity influences. It is possible that the fungus was retained on the plant debris produced by destruction of tissues by phytotoxicity of the extracts or oils. Infection proceeds readily on such partially destroyed tissues (Oh *et al*. 1999).

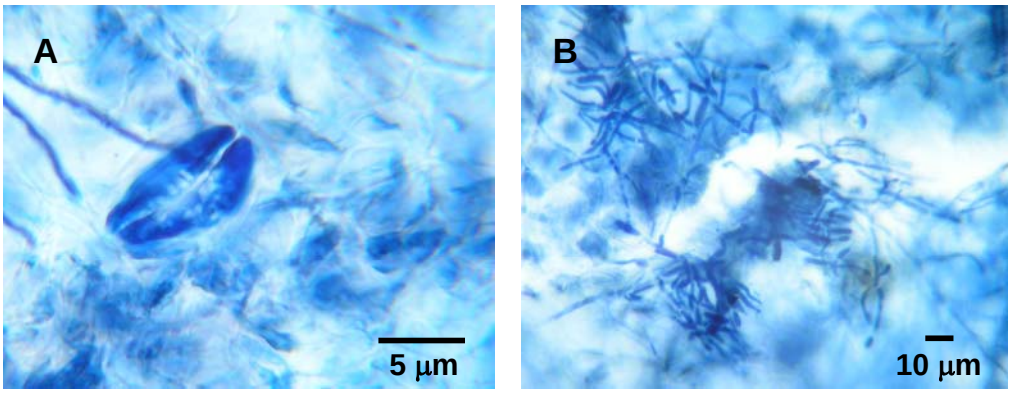

Figure 6.11 Photomicrograph of anthracnose infection on cleared and stained papaya leaves: (A) growth of hyphae oriented towards an open stomate; (B) sporulating fungi on leaf surface.

6.5.3 Scanning electron microscopy: results

Observations made by scanning electron microscopy identified germinated fungal spores and the growth of fungal germ tubes in all of the treatments, although there was less evidence of fungal growth 24 hours after inoculation on leaves exposed to the extract and oil treatments compared to those under the distilled water treatment (Fig 6.12A – C). No germinated spores or germ tubes were found in any of the synthetic fungicide treatments (Figs. 6.12D). The infection process of the fungus was not directly confirmed although several images show that germ tubes grew away from closed

stomata (Fig. 6.12E). The stomates were frequently found closed in the treated leaf samples in the present study (Fig. 6.12F).

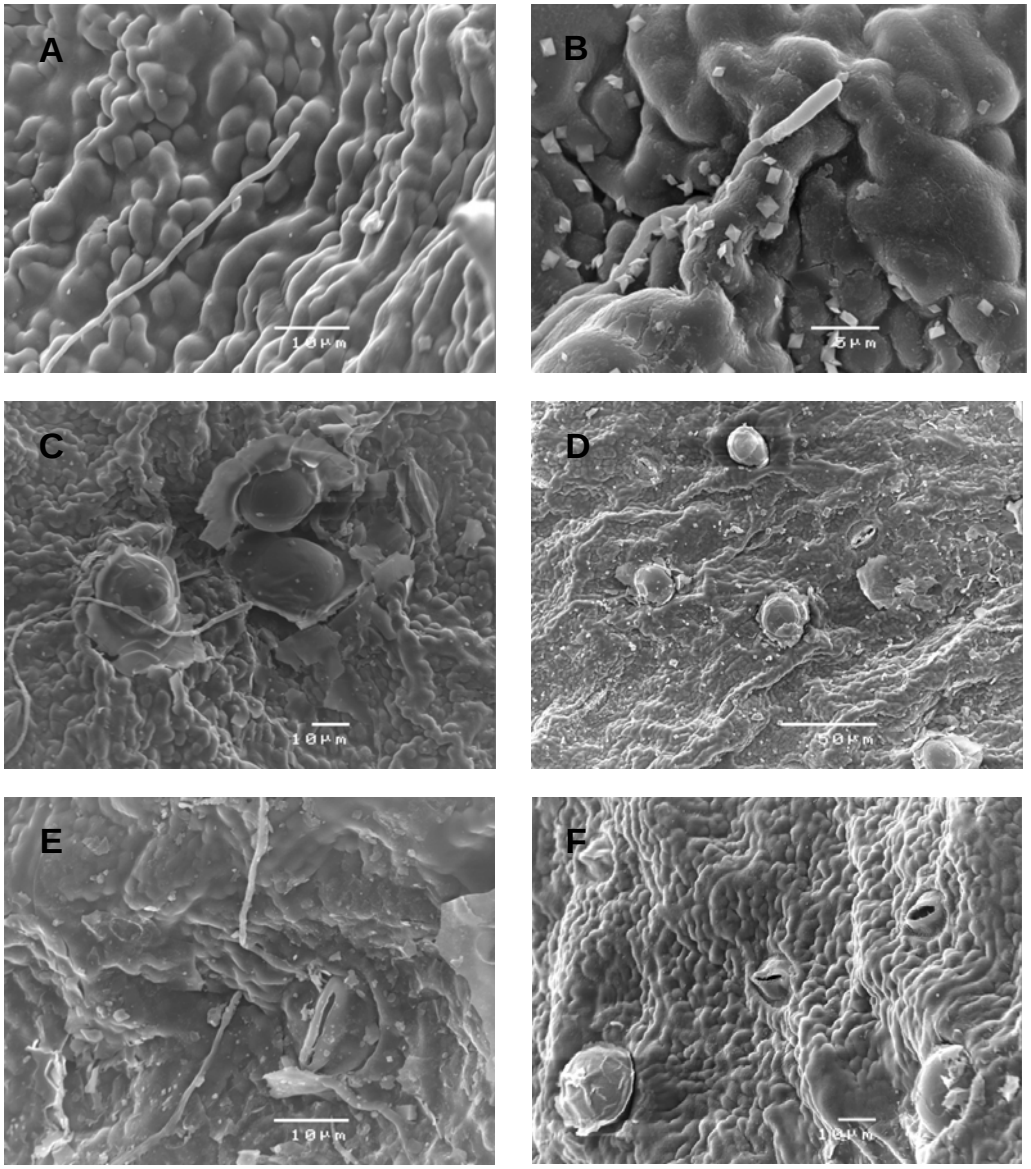

Figure 6.12 Scanning electron micrographs of hyphae and spores of *C. gloeosporioides* on pepper leaves at 24 hours after inoculation: (A, B, and C) germinated spores in distilled water, cinnamon bark oil, and galangal rhizome extract, respectively; (D) clean surface on Dithane M-45 fungicide treatment; (E) germ tube by-passing a closed stomata (F) predominantly closed stomates on leaves treated with cinnamon bark oil.

In the distilled water treatment, the fungal infection became apparent seven days after inoculation as black spots on the leaf, when conidiophores

emerged on the leaf surface. This was seen under the electron microscope in the production of abundant conidiophores with developed conidia arising from fruiting bodies (acervuli) on the adaxial surface of the leaves (Figs. 613A and 6.13B). Long setae were also formed (Fig. 6.13B). No evidence of sporulation was found on inoculated pepper leaves exposed to the galangal rhizome extract and cinnamon bark oil treatments compared to the leaves under the distilled water treatment at seven days after inoculation. Clean surfaces were frequently found, although black spots and mycelia were sometimes evident on the leaves.

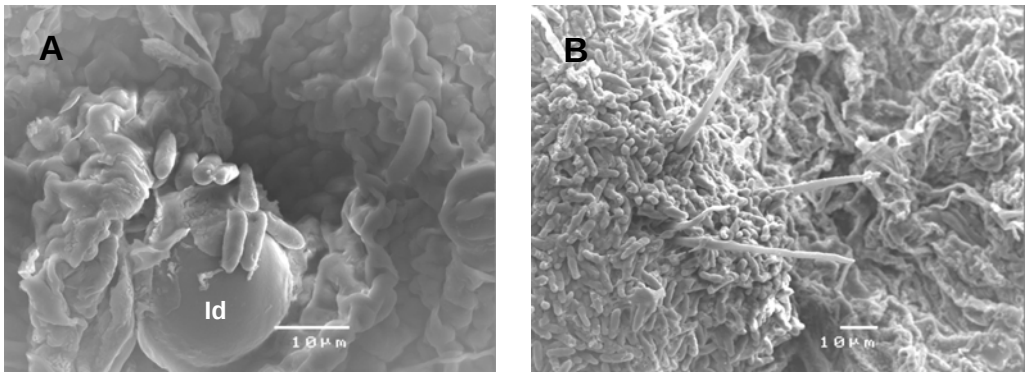

Figure 6.13 Scanning electron micrographs of spores and sporulation of *C. gloeosporioides* on pepper leaves at seven days after inoculation: (A) sporulation in distilled water, release of spores from acervuli (fruiting bodies) formed near idioblast cells (Id); (B) an acervulus with long setae.

Damaged spores or alterations to the hyphae were associated with extract and oil treatments. There was evidence of degeneration of hyphae and spore damage in the presence of galangal rhizome extract or cinnamon bark oil. Putative lysis in the germ tubes or hyphal walls was seen (Fig. 6.14A and 6.14E) and, in older mycelia, emptying of the cytoplasmic content was observed (Fig. 6.14B and 6.14F) in the presence of galangal extract or

cinnamon bark oil. Some of the treated spores were malformed when compared with normal spores found in the distilled water treatment (Figs. 6.14C and 6.14D).

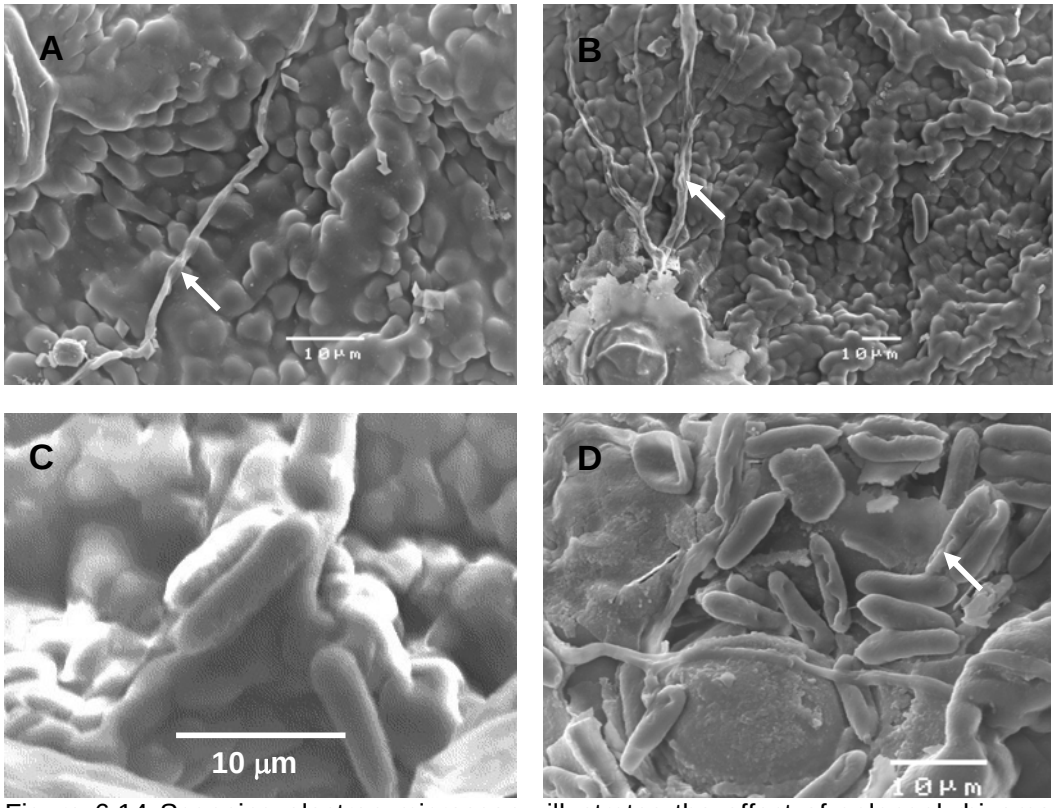

Figure 6.14 Scanning electron microscopy illustrates the effect of galangal rhizome extract and cinnamon bark oil on *C. gloeosporioides* inoculation on pepper leaves: (A) putative lysis (arrow) in germ tubes on oil treatment at 24 hours after inoculation; (B) putative lysis (arrow) in hypha wall on extract treatment at seven days after inoculation; (C) normal spores on distilled water treatment; (D) damaged spores (arrow) on oil treatment.

6.6 SUMMARY AND CONCLUSIONS

The effects of plant extracts or oils in inhibiting spore germination of *Colletotrichum gloeosporioides* were discussed in Section 5.3.1 and Section 5.3.1.1.

Extract and oil treatments essentially gave unchanged results upon further incubation on spore germination of *C. gloeosporioides*. The spores of this fungus did not germinate in galangal rhizome extracts (both solvents), cardamom leaf ethanol extract, and most oils after prolonged incubation. Germination in oils after 48 hours incubation was unchanged over that shown after 24 hours. Spore germinations of 10 – 25% after 48 hours incubation were noticed after application of cinnamon leaf, clove leaf, and lesser galangal oils and this compared with up to 100% germination in Amistar fungicide after 48 hours (Section 6.2.1.1, Table 6.1).

The lowest lethal concentrations (LC_{50} value), at which 50 per cent spores failed to germinate, were produced by the cinnamon oils at a concentration of 0.04% for cinnamon bark and 0.07% for cinnamon leaf (Section 6.2.3).

Phytotoxicity was observed especially on application of cinnamon and clove oils at a high concentration (0.5%), up to 10 times higher than the corresponding LC_{50} concentrations (Section 6.3 and Section 6.4). Phytotoxic effects were more severe on pepper leaves for a range of plant extracts or oils than on papaya seedlings. However, this was not found in the application of plant extracts or oils on pepper berries and chilli fruits. Phytotoxicity problems in the study, especially with cinnamon oils, was considered high, but the oil concentration tested (0.5%) was 7 – 12 times greater than their LC_{50} values.

In contrast to the *in vitro* results, the results of the *in vivo* trial on papaya leaves were variable and inconclusive (Section 6.4). The problem of

phytotoxicity and the possible loss of antimicrobial activity through the escape of volatile components might have been responsible.

From the SEM results, it was evident that several extracts and oils affected the fungal pathogens by damaging their spores, germ tubes, or hyphae. Sometimes, shorter germ tubes, and lysis of germ tubes or hyphal walls was seen in extract and oil treatments compared to distilled water controls (Section 6.5).

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

7.1 CONCLUSIONS

Several plant extracts and oils have shown remarkable biological activity when tested against bacteria and fungi (Agrios 1997; Bhatm 2001; Cohen *et al*. 2003; Dorman and Deans 2000; Hay and Waterman 1993; Jenett-Siems *et al*. 1999; Kamalakannan *et al*. 2001; Kim *et al*. 1999; Letessier *et al*. 2001; Mahmoud 1999; Meena and Muthusamy 2002; Muda *et al*. 2001; Northover and Scheider 1996; Parveen and Kumar 2000; Saleem *et al*. 2001; Suganda and Yulia 1998; Yusuf *et al*. 2001). Cinnamon oil and clove oil have been reported as fungicidal against *C. musae*, *L. theobromae*, and *F. proliferatum* by Ranasinghe *et al*. (2002), while eucalyptus oil and lemon grass oil were reported to inhibit mycelial growth of *Didymella bryoniae* (Fiori *et al.* 2000).

In the present study, it is clear that several plant extracts and oils were active against the germination of spores of fungi isolated from pepper, cinnamon, and turmeric (Section 5.3). The results revealed that ethanol extractions were more efficient in inhibiting spore germination of fungi than water extractions (Section 5.3.2). The lower inhibition effect of water extractions was also shown by Cohen *et al*. (2003) who suggested that water extracts showed a poor yield of active antimicrobial compounds, and suggested that the fungicidal materials are mostly lipophilic.

The highest inhibition of spore germination was provided by cinnamon and clove oils in a range concentration of 0.1 – 3%. These results accord

with those on Ranasinghe *et al.* (2002) who showed that these two oils were fungicidal against anthracnose and crown rot pathogens on bananas. However, the concentration of the oils used in the present study was higher. Many researchers have reported cinnamon and clove oils as good sources of antifungal compounds (Ranasinghe *et al*. 2002).

The concentration of oils that produced significant inhibition of spore germination compared to a distilled water treatment was varied across oils and fungal species (Section 5.3.1). However, many of the oils inhibited spore germination at even the lowest concentrations tested (0.1%). Cinnamon and clove oils, along with other oils such as lemon grass oil, cardamom oil, and lemon myrtle oil in certain concentrations, were more efficient than the synthetic fungicide Amistar at inhibiting spore germination (Section 5.3.1).

Some plant extracts (500 mg/mL) were also efficient at inhibiting fungal spore germination. Galangal rhizome and cardamom leaf extracts produced strong reductions in spore germination of most fungi. Water and ethanol extracts of the galangal rhizome inhibited the germination of all fungal species. Cardamom leaf extract was a better inhibitor of putative pathogenic fungi (*C. gloeosporioides*, *Fusarium* spp., *Pestalotiopsis* sp., *C. orbiculare*, and *Phoma* sp.) than putative saprophytic fungi (*Cladosporium* sp., *Curvularia* sp., *Bipolaris* sp., and *Helminthosporium* sp. isolated from pepper, cinnamon and turmeric). However, the cardamom leaf extract in both solvents permitted 15.6% and 37.2% germination of *Exserohilum* sp. isolated from turmeric.

The study also indicated that ethanol extracts of cinnamon bark were efficient in inhibiting spore germination of putative saprophytic fungi (*Curvularia* sp., *Bipolaris* sp. and *Helminthosporium* sp. isolated from all plant samples). The extract completely inhibited germination of these fungi and permitted up to only 21.1% germination of *Cladosporium* sp. Germination of putative pathogenic fungi (*C. gloeosporioides*, *C. orbiculare*, *Fusarium* spp., and *Pestalotiopsis* spp.) was not effectively controlled. Water preparations of cinnamon bark extract were less effective than the ethanol extracts with all of the fungi tested.

Several plants extracts (500 mg/mL) were efficient at inhibiting spore germination. Galangal rhizome and cardamom leaf extracts showed the greatest antifungal activity (Section 5.3.2). The galangal extract (both solvents) had an LC_{50} on inhibition of germination of the fungus *C. gloeosporioides* of around 50 mg/mL, whereas the cardamom leaf extract (ethanol solvent) had an LC_{50} of around 350 mg/mL (Section 6.2.3). The results suggest that reasonably low concentrations of these plants extracts may be applied for effective fungicidal effects.

The poor performance of other potential fungicidal extracts used in the present study, such as lemon grass, was verified by Fiori *et al*. (2000), who also found that a crude extract of *C. citratus* and *Eucalyptus citriodora* leaves were not so efficient at inhibiting spore germination of the fungus *Didymella bryoniae* (Auersw.). Extracts derived from other species of the Zingiberaceae family were not efficient in inhibiting spore germination of most fungi in the present study, with the exception of ethanol extractions from galangal stem and the lesser galangal rhizome (Section 5.3.2).

Several plant extracts had no impact on fungal spore germination, or even promoted rapid germination (e.g., galangal leaf, ginger rhizome, lemon grass, and turmeric; Section 5.3.2). These extracts do not appear to have antifungal properties.

Volatility of treatment components (Letessier *et al*. 2001) may explain some of the difference between the *in vitro* and *in vivo* results of the present study (Section 6.4). The volatile compounds responsible for antifungal activity were confined in the *in vitro* experiments, whereas they were able to diffuse away from the plants in the *in vivo* experiments. Even though the same concentrations of oil or extract were used in the two experiments, the effective concentration was likely to be different in the different situations. However, the application of higher oil concentrations in the *in vivo* experiments was unsafe due to the phytotoxicity effects of the oils. The phytotoxicity, in turn, may have injured the cuticle of the leaf and facilitated penetration by the pathogen (Section 6.3 and 6.4).

It is essential to make sure that the symptoms attributed to a pathogen are not caused by non-infectious disorders. Symptoms closely resembling those caused by a range of pathogens may have originated from non-pathogenic actions (Fox 1993). Efforts to determine the existence of the pathogen on diseased tissue were made in several ways. Data obtained by scanning electron microscopy was used to give some idea of the possible processes by which the extracts or oils function as fungicides. However, the results obtained in the present study were of limited use in this respect. They confirmed that some extracts reduced or slowed fungal development (Section 6.5). Galangal rhizome and cinnamon bark oil caused damage to

spores and induced putative lysis in the hyphal walls, thereby emptying the cytoplasmic content of *C. gloeosporioides* (Section 6.5.2).

The following plant extracts and oils tested in the present study were shown to contain antifungal compounds and could produce significant inhibition of germination of fungal spores: cinnamon bark oil, cinnamon leaf oil, clove bud oil, clove leaf oil, water and ethanol extracts of galangal rhizome, cardamom leaf ethanol extract, lesser galangal ethanol extracts, water and ethanol extracts of cinnamon bark.

7.2 POSSIBLE IMPROVEMENTS TO THE RESEARCH APPROACH

In order to improve the operation of similar projects in the future, several suggestions can be made as follows:

- Spray application of extracts or oils tested should be tried in order to verify whether this might reduce phytotoxicity in the host plant.
- Since several extracts stimulated fungal development, spore germination assessments should be commenced earlier than at the peak germination times.
- Future research should focus on possible phytotoxic effects of alternative fungicides when applied in the field at LC_{50} rates.

7.3 FURTHER STUDY AND RECOMMENDATIONS

Field experiments should be considered in order to gain information about the effectiveness of the extracts or oils tested for fungal control in spice crops. The results will be very important since the field studies will reveal efficacy of extracts or oils in controlling fungi as they interact with

environmental factors. Extracts or oils should be applied to the desirable plant hosts rather than to sensitive hosts, as there are many variations among plants that can influence the results. The tests could be conducted also on a wide range of pathogens, so that *in vivo* assessments can be broadened.

Plant extracts prepared through maceration by blending provides unwanted nutrients for spore germination. Other methods should be investigated in order to isolate the bioactive compounds. Previous research has indicated that the plant materials, when dried and ground before solvent extraction, gave good results (Cohen *et al*. 2003; Jenett-Siems *et al*. 1999; Kim *et al*. 1999; Saleem *et al*. 2001; Yusuf *et al*. 2001).

Spore germination testing on an agar medium rather than in a water medium can be considered as a superior method, since spore germination percentages were higher than in the water medium. In the same way, application of extracts or oils on agar plugs clearly worked well and significantly influenced the spore germination.

The oil and plant extract tests involving chilli suggested that it might be included in post-harvest treatment systems for assessing the efficacy of natural products to protect against post-harvest damage by *C. gloeosporioides*. Previous research has shown that cinnamon and clove oils protect against post-harvest fungal diseases on bananas (Ranasinghe *et al*. 2002).

CHAPTER 8

REFERENCES

Agrios, G.N. 1997. *Plant Pathology*. 4th ed. San Diego, California: Academic Press.

Anonymous. 1943. The slide germination method of evaluating protectant fungicides. *Phytopathology* 33:627-632.

———. 2000. *Crop information.* Agriculture Research Center, 2000 [cited March 20 2003]. Available from http://www.indiaagronet.com.

———. 2001. *Medicinal plants: Herbs and Aromas,* 2001 [cited April 14 2003]. Available from http://world.std.com/~krahe/index.html.

———. 2003a. *Spice gallery.* Indian Institute of Spices Research, 2003 [cited March 21 2003]. Available from http://www.iisr.org.

———. 2003b. *Center for new crops and plant products.* Horticulture and Landscape Architecture Department, Purdue University, 2003 [cited March 26 2003]. Available from http://www.hort.purdue.edu.

———. 2003c. *Products and Services - Fungicides.* Syngenta, 2003 [cited April 8 2003]. Available from http://www.syngenta.com.

———. 2003d. *Labels and safety data sheets - Dithane M45.* Dow AgroSciences, 2003 [cited April 9 2003]. Available from http://www.dowagro.com.

———. 2004a. *Agar technical (Agar No.3), LP 0013.* Oxoid, 2004 [cited 24 May 2004]. Available from http://www.oxoid.com/uk/index.asp.

———. 2004b. *Leaf Spots.* Urban Integrated Pest Management - Oklahama Cooperative Extension Service, 2004 [cited 26 May 2004]. Available from http:cipm.ncsu.edu/ent/Southern_Region/.

———. 2005. *Botanical and Tropical Seed*. Tropilab® Inc., 2005 [cited 9 May 2005]. Available from http://www.tropilab.com.

Arx, J.A., von. 1981. *The Genera of Fungi Sporulating in Pure Culture*. Hirschberg, Germany: J. Cramer.

Aziz, N.H., Y.A. Youssef, M.Z. El-Fouly, and L.A. Moussa. 1998. Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. *Botanical Bulletin of Academia Sinica* 39:279-285.

Bailey, J.A, and M.J. Jeger. 1992. *Colletotrichum - Biology, Pathology and Control*. Wallingford, United Kingdom: CAB International.

Baird, R (Ed). 2005. *Colletotrichum*. The University of Georgia - Plant Pathology Department, 2005 [cited 20 May 2005]. Available from http://www.plant.uga.edu.

Barnett, H.L., and B.B. Hunter. 1972. *Illustrated Genera of Imperfect Fungi*. Minnesota: Burgess Publishing Company.

Barron, G.L. 1972. *The Genera of Hyphomycetes from Soil*. Huntington, New York: Robert E. Krieger Publishing Company.

Bhatm, N. 2001. *In vitro* evaluation of some leaf extracts against *Fusarium* spp. causing yellows of ginger in Sikkim . [*Plant Disease Research* 16 (2), 259-262]. 2001 [cited. Available from CABI Abstract: 20023033539.

Black, J.G. 1999. *Microbiology - Principles and Explorations*. 4th ed. New Jersey, USA: Prentice-Hall, Inc.

Brophy, J.J., and J.C. Doran. 1996. Essential oils of tropical *Asteromyrtus, Callistemon*, and *Melaleuca* species. In *ACIAR monograph;* Vol. 40. Canberra: Australian Centre for International Agriculture Research.

Caldas, T. 1998. New dimensions in cultivating medicinal plants. Part 1. [Colloqium Transcripts]. International Trust for Traditional Medicine, 1998 [cited 10 September 2003]. Available from http://www.kreisels.com/ittm/publications.

Carlisle, D.J., L.R. Cooke, S. Watson, and A.E. Brown. 2002. Foliar aggressiveness of Northern Ireland isolates of *Phytophthora infestans* on detached leaflets of three potato cultivars. *Plant Pathology* 51 (4):424-434.

Carmichael, J.W., W.B. Kendrick, I.L. Conners, and L. Sigler. 1980. *Genera of Hyphomycetes*. Alberta, Canada: The University of Alberto Press.

Carson, C.F, and T.V. Riley. 1998. *Antimicrobial activity of tea tree oil*. University of Western Australia - Rural Industries Research and Development Corporation, UWA 24A - 98170.

Chilosi, G., C. Caruso, C. Caporale, L. Leonardi, L. Bertini, A. Buzi, M. Nobile, P. Magro, and V. Buonocore. 2000. Antifungal activity of a Bowman-Birk-type trypsin inhibitor from wheat kernel. *Journal of Phytopathology* 148 (7-8):477-481.

Clark, S, and D. Gilrein. 1992. Horticultural oils, 1992 [cited December 4 2002]. Available from http:www.cce.cornell.edu/Suffolk.

Cochrane, V.W. 1963. *Physiology of fungi*. New York: John Wiley & Sons, Inc.

Cohen, Y, A. Baider, B. Ben-Daniel, and Y. Ben-Daniel. 2003. Fungicidal preparations from *Inula viscose* 2003 [cited September 9 2003]. Available from http://www.infodienst-mlr.bwl.de/la/lvwo/ecofruvit.

Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* 12 (4): 564-582.

Cowling, W.A., B.J. Buirchell, I. Frencel, S. Koch, J.M. Neves Martins, and P. Romer. 1999. International evaluation of resistance to anthracnose in lupin. In *Proceedings of the 9th International Lupin Conference*, edited by E. Van Santen, M. Wink, S. Weissmann, P. Roemer. Canterbury: International Lupin Association. Pp. 16-22.

Dhingra, O.D., and J.B. Sinclair. 1985. *Basic Plant Pathology Methods*. Florida: CRC Press, Inc.

Dickman, M.B. 1993. *Plant disease pathogen - Colletotrichum gloeosporioides.* Crop knowledge master, 1993 [cited 22 April 2004]. Available from http://www.extento.hawaii.edu/kbase/crop.

Didier, C., and M. Doumbia. 2003. Technical advice for papaya growing. Europe PIP (Pesticides Initiative Program) - ACP, 2003 [cited 19 May 2004]. Available from http://www.coleacp.org.

Dorman, H.J.D., and S.G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* 88 (2):308-316.

Edman, M., M. Gustafsson, J. Stenlid, and L. Ericson. 2004. Abundance and viability of fungal spores along a forestry gradient - responses to habitat loss and isolation? *OIKOS* 104:35 - 42.

Elliott, M.L., and G.W. Simone. 2004. *Helminthosporium* Leaf Spot. University of Florida - Institute of Food and Agricultural Science, 2004 [cited 26 March 2004]. Available from http://edis.ifas.ufl.edu/.

Ellis, M.B. 1971. *Dematiaceous Hyphomycetes*. Kew, Surrey, England: Commonwealth Mycological Institute.

Ellis, M.B. 1976. *More Dematiaceous Hyphomycetes*. Kew, Surrey, England: Commonwealth Mycological Institute.

Fiori, A.C.G., K.R.F. Schwan-Estrada, J.R. Stangarlin, J.B. Vida, C.A. Scapim, M.E.S. Cruz, and S.F. Pascholati. 2000. Antifungal activity of leaf extracts and essential oils of some medicinal plants against *Didymella bryoniae*. *Journal of Phytopathology* 148 (7-8):483-487.

Flors, V., M.C. Miralles, E. Varas, P. Company, C. Gonzalez-Bosch, and P. Garcia-Agustin. 2004. Effect of analogues of plant growth regulators on *in vitro* growth of eukaryotic plant pathogens. *Plant Pathology* 53 (1):58-64.

Fluck, H., and R.J. Schib. 1976. *Medicinal Plants and Their Uses*. London: W. Foulsham & Co. Ltd.

Fokkema, N.J., and J. Van Den Heuvel. 1986. *Microbiology of the Phyllosphere*. London: Cambridge University Press.

Fox, R.T.V. 1993. *Principles of Diagnostic Techniques in Plant Pathology*. Oxon: CAB International.

Fry, W.E. 1982. *Principles of Plant Disease Management*. San Diego, California: Academic Press.

Hammer, K.A., C.F. Carson, and T.V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology* 86 (6):985-990.

Hay, R.K.M, and P.G. Waterman. 1993. *Volatile Oil Crops: Their Biology, Biochemistry and Production*. Edited by R. K. M. Hay and P. G. Waterman. Essex: Longman Scientific & Technical.

Hong, J.K., and B.K. Hwang. 1998. Influence of inoculum density, wetness duration, plant age, inoculation method, and cultivar resistance on infection of pepper plants by *Colletotrichum coccodes*. *Plant Disease* 82 (10):1079-1083.

Hughes, I. 2002. Herb in Africa Part 3 - Extraction of herbal material. Science in Africa, 2002 [cited September 9 2003]. Available from http://www.scienceinafrica.co.za.

Ingold, C.T., and H.J. Hudson. 1993. *The Biology of Fungi*. 6th ed. London: Chapman & Hall.

Isaac, S. 1992. *Fungal-Plant Interactions*. London: Chapman & Hall.

Jenett-Siems, K., F.P. Mockenhaupt, U. Bienzle, M.P. Gupta, and E. Eich. 1999. *In vitro antiplasmodial activity of Central American medicinal plants* [Published Journal]. Blackwell Science Ltd., 1999 [cited 10 September 2003]. Available from http://www.znaturforsch.com.

Jhorar, O.P., D.R. Butler, and S.S. Mathauda. 1998. Effects of leaf wetness duration, relative humidity, light and dark on infection and sporulation by *Didymella rabiei* on chickpea. *Plant Pathology* 47:586 - 594.

Kamalakannan, A., V. Shanmugam, M. Surendran, and R. Srinivasan. Antifungal properties of plant extracts against *Pyricularia grisea*, the rice blast pathogen [*Indian Phytopathology* 54 (4), 490-492]. 2001 [cited. Available from CABI Abstract: 20023029446.

Katzer, G. 1998. Gernot Katzer's Spice Pages, 1998 [cited March 26 2003]. Available from http://www-ang.kfunigraz.ac.at/~katzer.

Kaufman, P.B., L.J. Cseke, S. Warber, J.A. Duke, and H.L. Brielmann. 1999. *Natural Products from Plants*. Washington, D.C.: CRC Press Ltd.

Kim, Y., Y.H. Choi, Y.W. Chin, Y.P. Jang, Y.C. Kim, J. Kim, J.Y. Kim, S.N. Jot, M.J. Noh, and K.P. Yoo. 2003. Effect of plant matrix and fluid ethanol concentration on supercritical fluid extraction efficiency of schisandrin derivates [*J Chromatogr Sci.* 37 (12), 457-461]. 1999 [cited September 16 2003]. Available from PubMed: 10615592.

Kirk, P.M., P.F. Cannon, J.C. David, and J.A. Stalpers. 2001. *Ainsworth & Bisby's - Dictionary of The Fungi*. 9th Ed. Wallingford, United Kingdom: CAB International.

Kumar, V., V.P. Gupta, A.M. Babu, R.K. Mishra, V. Thiagarajan, and R. K. Datta. 2001. Surface ultrastructural studies on penetration and infection process of *Colletotrichum gloeosporioides* on mulberry leaf causing black spot disease. *Journal of Phytopathology* 149:629-633.

Kumari, P.S., and B.R. Gopalan. 2002. *Status of fungal foliar diseases of pepper in Kerala* [Abstract of book chapter, pp.274-275]. Coconut Research Station, India, 2000 [cited 2002]. Available from CABI Abstract: 20013114377.

Latunde-Dada, A.O. 2001. *Colletotrichum*: tales of forcible entry, stealth, transient confinement and breakout. *Molecular Plant Pathology* 2 (4):187-198.

Letessier, M.P., K.P. Svoboda, and D.R. Walters. 2001. Antifungal activity of the essential oil of hyssop (*Hyssopus officinalis*). *Journal of Phytopathology* 149 (11-12):673.

Lindow, S.E., and V. Elliott. 2002. Phyllosphere microbiology, 2000 [cited August 10 2002]. Available from http://www.micro.iastate.edu.

Madigan, M.T., J.M. Martinko, and J. Parker. 2000. *Biology of Microorganism*. 9th ed. New Jersey, USA: Prentice-Hall, Inc.

Mahmoud, A.L.E. 1999. Inhibition of growth and aflatoxin biosynthesis of *Aspergillus flavus* by extracts of some Egyptian plants. *Letters in Applied Microbiology* 29 (5):334-336.

Marte, M., and P. Montalbini. 1999. Histological observations on *Uromyces phaseoli* and *Puccinia recondita* infection in allopurinol-treated susceptible plants. *Journal of Phytopathology* 147:163-168.

Mather, J.P., and P.E. Roberts. 1998. *Introduction to Cell and Tissue Culture: Theory and Technique*. New York: Plenum Press.

Meena, B., and M. Muthusamy. 2002. Fungitoxic properties of plant extracts against *Sclerotium rolfsii* in jasmine [*Journal of Ornamental Horticulture* (New Series) 5 (1), 82-83]. 2002 [cited 2002]. Available from CABI Abstract: 20023080802.

Meyer, B. 1999. *The King of Spices*. Selangor: Behn Meyer & Co.

Meyer, L. 2001. Tropical Medicinal Plants, 2001 [cited 7 February 2004]. Available from http://jrscience.wcp.muohio.edu.

Migvar, L. 1967. *Peppers in the Caroline Islands*. Sydney: Bridge Printery Pty. Ltd.

Moore, E. 1996. *Fundamentals of the Fungi*. 4th ed. New Jersey: Prentice Hall.

Muda, M.A., H. Ibrahim, and N. Khalid. 2001. Micropropagation study and genetic analysis with RAPD markers on selected medicinal gingers [NSF Workshop, Kuala Lumpur]. 2001 [cited March 24 2003]. Available from http://www.moste.gov.my/kstas/NSFWorkshop.

Nishijima, W.T. 1999. Common names of plant diseases, The American Phytopathology Society, 1999 [cited 14 April 2004]. Available from http://www.apsnet.org.

Northover, J., and K.E. Scheider. 1993. Activity of plant oils on diseases caused by *Podosphaera leucotricha*, *Venturia inaequalis*, and *Albugo occidentalis*. *Plant Disease* 77:152-156.

———. 1996. Physical modes of action of petroleum and plant oils on powdery and downy mildews of grapevines. *Plant Disease* 80:544-549.

Oh, B.J., K.D. Kim, and Y.S. Kim. 1999. Effect of cuticular wax layers of green and red pepper fruits on infection by *Colletotrichum gloeosporioides*. *Journal of Phytopathology* 147:547-552.

Parveen, S., and V. R. Kumar. 2002. Effect of extracts of some medicinal plants on the growth of *Alternaria triticina* [*Journal of Phytological Research* Vol.13, No.2, pp.195-196, 2000]. 2000 [cited 2002]. Available from CABI Abstract: 20013102241.

Persley, D. (Ed.). 1993. *Diseases of Fruit Crops*. Brisbane: Department of Primary Industries.

Pitt, J.I., and A.D. Hocking. 1991. Significance of fungi in stored products. In *Fungi and Mycotoxins in Stored Products*, edited by B. R. Champ, E. Highley, A. D. Hocking and J. I. Pitt. Canberra: Australian Centre for International Agricultural Research. Pp. 16-21.

Plotkin, L.I., L. Squiquera, I. Mathov, R. Galimberti, and J. Leoni. 1996. Characterization of the lipase activity of *Malassezeia furfur*. *Journal of Medical and Veterinary Mycology* 34:43-48.

Purseglove, J.W. 1974. *Tropical Crops Dicotyledons*. Vol. 1 & 2 combined. Essex: Longman Group Ltd.

Purseglove, J.W., E.G. Brown, C.L. Green, and S.R.J. Robbins. 1981. *Spices*. Vol. 1 & 2 combined. Essex: Longman Group Ltd.

Ranasinghe, L., B. Jayawardena, and K. Abeywickrama. 2002. Fungicidal activity of essential oils of *Cinnamon zeylanicum* (L.) and *Syzygium aromaticum* (L.) Merr et L.M. Perry against crown rot and anthracnose pathogens isolated from banana. *Letters in Applied Microbiology* 35 (3):208-211.

Rayner, R.W. 1970. *A Mycological Colour Chart*. London: Commonwealth Mycological Institute Kew, Surrey & British Mycological Society.

Roberts, P.D., K. L. Pernezny, and T.A. Kucharek. 2001. *Anthracnose caused by Colletotrichum sp. on pepper*. University of Florida - Institute of Food and Agricultural Science, 2005 [cited 20 May 2005]. Available from http://edis.ifas.ufl.edu.

Saleem, A., M. Engstrom, S. Wurster, J. Savola, and K. Pihlaja. 2001. Interaction of folk medicinal plant extracts with human α_2-adrenoceptor subtypes. Verlag der Zeitschrift fur Naturforschung, 2001 [cited 9 September 2003]. Available from http://www.znaturforsch.com.

Sarma, Y.R., M. Anandaraj, M.N. Venugopal, and M.K. Dasgupta (Eds). 2002. Present status and future strategies of disease management of pepper, cardamom and large cardamom [Book Chapter]. Indian Institute of Spices Research, 2000 [cited 2002]. Available from CABI Abstract: 20013028294.

Satish, S., K.A. Raveesha, and G.R. Janardhana. 1999. Antibacterial activity of plant extracts on phytopathogenic *Xanthomonas campestris* pathovars. *Letters in Applied Microbiology* 28 (2):145-147.

Setzer, M.C, W.N. Setzer, B.R. Jackes, G.A. Gentry, and D.M. Moriarity. 2001. The Medicinal Value of Tropical Rainforest Plants from Paluma, north Queensland, Australia. *Pharmaceutical Biology* 39 (1):67-68.

Shipton, W.A., and J.F. Brown. 1962. A whole-leaf clearing and staining technique to demonstrate host-pathogen relationships of wheat stem rust. *Phytopathology* 52 (12):1313-1315.

Shipton, W.A., R.L. MCCown, and W.T. Williams. 1981. Influence of weather on mouldiness and the mycoflora of legume pasture during the dry season in tropical Australia. *Australian Journal of Botany* 29:59-69.

Soylu, E.M., and S. Soylu. 2003. Light and electron microscopy of the compatible interaction between *Arabidopsis* and the downy mildew pathogen *Peronospora parasitica*. *Journal of Phytopathology* 151:300-306.

Stevens, L.H., G.M. Stoopen, I.J.W. Elbers, J.W. Molthoff, H.A.C. Bakker, A. Lommen, D. Bosch, and W. Jordi. 2000. Effect of climate conditions and plant developmental stage on the stability of antibodies expressed in transgenic tobacco. *Plant Physiology* 124 (1):173-182.

Strange, R.N. 1993. *Plant Disease Control Towards Environmentally Acceptable Methods*. London: Chapman & Hall.

Suganda, T, and E. Yulia. 1998. Effect of crude water extract of cogon grass (*Imperata cylindrica* Beauv.) rhizome against Fusarium wilt disease of tomato. *International Pest Control* 40 (3):79-80.

Vallejo, I., L. Rebordinos, I. G. Collado, and J. M. Cantoral Fernandez. 2001. Differential behaviour of mycelial growth of several *Botrytis cinerea* strains on either patchoulol- or globulol-amended media. *Journal of Phytopathology* 149 (2):113-118.

Vaughan, J.G., and C. Geissler. 1998. *The New Oxford Book of Food Plants*. New York: Oxford University Press Inc.

Vloutoglou, I., and S.N. Kalogerakis. 2000. Effects of inoculum concentration, wetness duration and plant age on development of early blight (*Alternaria solani*) and on shedding of leaves in tomato plants. *Plant Pathology* 49:339-345.

Warcup, J.H. 1955. Isolation of fungi from hyphae present in soil. *Nature* 175:953-954.

Weiss, E.A. 1997. *Essential Oil Crops*. London: CAB International.

Yusuf, N.A., H. Ibrahim, and N. Khalid. 2001. Antibacterial evaluation and tissue culture studies of selected medicinal *Curcuma* species. [Workshop Transcripts]. NSF, 2001 [cited March 24 2003]. Available from http://www.moste.gov.my/kstas/NSFWorkshop/pembentang.htm.

Zar, J.H. 1999. *Biostatistical Analysis*. 4th ed. New Jersey: Prentice Hall International, Inc.