

CRANFIELD UNIVERSITY
SILSOE CAMPUS

PhD Thesis

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**Alternative chemical control of anthracnose and crown rot
of banana caused by *Colletotrichum musae***

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**This thesis is submitted in fulfillment of the requirement
for the degree of Doctor of Philosophy**

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ABSTRACT

Chemical alternatives to the fungicides currently used to control *Colletotrichum musae* infections on banana fruit have been investigated. Four isolates of *C. musae* cultured from anthracnose lesions on imported banana fruit were screened against the fungicides thiabendazole (TBZ) and imazalil incorporated into malt extract agar (MEA). Two of the isolates showed some tolerance to TBZ even at 1.24 mM (250 ppm), whereas all four isolates were very sensitive to imazalil at concentrations of ≥ 0.017 mM (5 ppm). One susceptible (CM100) and one tolerant (CM103) isolate (to TBZ) was selected. Eleven chemicals were evaluated over a range of concentrations for their effectiveness in inhibiting the germination and mycelial growth of these isolates *in vitro* (on MEA). The chemicals were the antioxidants ascorbic acid, propionic acid, benzoic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl paraben (PP), propyl gallate (PG), dimethyl sulphoxide (DMSO), thiourea and tannic acid and the natural plant product azadiractin. BHA, azadiractin, benzoic acid, PP and PG gave the highest levels of fungal inhibition *in vitro*. BHA at 5 mM completely inhibited the germination and mycelial growth of CM100 and CM103 at 25°C. BHA (1 mM) in combinations with benzoic acid (1 mM), PP (1 mM) or imazalil (1.68 μ M) completely inhibited germination and mycelial growth of CM103 at 14 and 25°C. The effectiveness of imazalil was not affected by the pH of the medium whereas the effectiveness of BHA was greatest at pH 3.5 and 6.5 and benzoic acid was more effective at lower pH values. *C. musae* was shown to produce nine extracellular enzymes in liquid medium which have not been reported before including lipases, phosphatases and hydrolases. BHA, imazalil and TBZ caused differential inhibition of the production or activity of these enzymes. The toxicity of selected chemicals was tested against anthracnose or crown rot infections of fingers or hands of banana fruits. Inoculations of *C. musae* were made with either mycelium in wounds in the banana peel or with conidia on the intact surface of the fruit or on the cut stalk or crown tissues. Of the alternative chemicals tested on their own, BHA and azadiractin gave the greatest inhibition of anthracnose and crown rot infections with dip treatments at 1 mM giving control levels ranging from 10-39% compared with 17.6-38.3% for imazalil (0.84 mM = 250 ppm). BHA (5 mM) in combinations with imazalil (0.84 mM), PP (10 mM) or benzoic acid (10 mM) suppressed anthracnose lesions (CM103) by 64.7, 54.9 and 35.3 %, and crown rot development by 70.5, 62.3 and 42.6 % at 25°C respectively. At 14°C these same combinations gave complete control of anthracnose lesions and crown rot development. TBZ was less effective at controlling crown rot (CM100 or CM103) than imazalil alone or when in combinations with BHA. Combinations of BHA with imazalil but not with TBZ showed synergism both *in vitro* and *in vivo*. The cost/litre of the best trial treatment (BHA at 5 mM with imazalil at 0.84 mM) was less than that of imazalil at 1.68 mM (500 ppm). This suggests that food grade phenolic antioxidants such as BHA may be useful adjuncts to currently used fungicides such as imazalil for commercial control of banana postharvest diseases.

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*O, God Open our eyes,
to see what is beautified,
Our mind to know what is true,
Our hearts to love what is good,*

*DEDICATED TO
HOLY PROPHET
MUHAMMAD
Peace Be Upon Him
The greatest social reformer*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

It has been claimed that the best way of increasing world food supplies is to reduce post-harvest losses (Eckert & Ogawa, 1985) and that effective technologies for the preservation of harvested crops would be of more benefit than improvements in conventional production techniques (Zentmeyer *et al.*, 1983). Fruits and vegetables are particularly perishable and can be subject to very high losses shortly after harvest if appropriate technology is not applied. For fresh produce, fungal and bacterial pathogens are often a major cause of postharvest loss (Noon, 1984). Considerable reductions in losses from pathogens can be achieved by cooling produce rapidly after harvest and ensuring that physical and physiological injury to the produce is minimal. The use of fungicides is also an important tool in the management of certain postharvest diseases.

The fruits of the banana (*Musa* AAA; Cavendish sub-group) are the most commercially important fruit in the world for fresh consumption. Bananas are widely eaten in the countries of production as well as being transported considerable distances to importing countries in temperate regions. Due to the weight of the fruit, it is not cost effective to transport by air. Sea freight is the normal method between countries. The extended postharvest period required for transportation is made possible by the climacteric nature of banana fruits. They can be harvested unripe and relatively robust and then ripened in the importing country. There are, however, a number of hazards during this process. Fruit may prematurely ripen during transport and if not properly controlled, a number of postharvest diseases can lead to considerable economic losses (Wardlaw, 1972).

The most important postharvest diseases of exported banana fruits are anthracnose, crown rot and pedicel rot all of which can be caused by one fungus *Colletotrichum musae* (Berk. & Curtis) Arx. (Jeffries *et al.*, 1990) although, other pathogens can also cause crown rot symptoms. These diseases are routinely controlled by the application of fungicides shortly after harvest. The most commonly used fungicides are the

benzimidazoles-types, for example, thiabendazole or the ergosterol inhibitor fungicides e.g. imazalil (Eckert, 1990).

Fungicide applications generally work very effectively if applied correctly, however, the need to develop alternative treatments is urgent for two key reasons. Firstly, resistance of fungal pathogens to all the fungicides used on banana has been reported, e.g. *C. musae* to benomyl (Griffie, 1973); *Penicillium digitatum* to imazalil (Eckert, 1988) and *Botrytis cinerea* to thiabendazole (Bollen & Scholten, 1971). Resistance in banana fruit pathogens to commonly used fungicides will probably increase unless they are used very strategically. Secondly, the trend in many of the banana importing countries, is to reduce the number of chemicals that can be applied to food crops. Already in Europe, the maximum residue levels for a number of pesticides routinely used on horticultural crops has been effectively reduced to zero (Aked and Henderson, 1999). This trend is being driven by consumer demands and retailer competition. Consumers have become aware of the damage that some pesticides have caused to the environment and there are also concerns that chemical residues on produce could lead to health problems. On the other hand, consumers are demanding higher and higher quality fruit without blemishes caused by pests and diseases (Jones *et al*, 2000).

To ensure that banana diseases continue to be controlled effectively in the future, research needs to be carried out on a variety of alternative strategies. Possible approaches include looking for alternative chemicals that could replace or be used in conjunction with today's fungicides or developing non-chemical treatments to replace or complement fungicide use. The project described in this thesis investigates some of these possibilities.

1.2 BANANA AS A COMMERCIAL CROP

1.2.1 International importance of banana

Bananas (dessert, cooking and plantain types) are probably the most widely consumed fruit in the world with production in 1999 being estimated at 89 million tonnes (FAO, 2000). Although originally native to South East Asia, bananas are now grown all over the tropics. Leading producer countries are Brazil, India, the Philippines, Ecuador, Indonesia, Thailand, Mexico, Vietnam, Colombia and Honduras (Hallam, 1995). Banana is the staple food for over 400 million people in the developing world being eaten both as a dessert fruit and cooked as a vegetable.

Of the total produced, only a relatively small amount enters world trade. In 1998, for example, only about 15% (13.5 million tonnes) of banana fruit was exported (FAO, 2000). Despite this, banana is the fifth most important agricultural commodity in terms of international trade after cereals, sugar, coffee and cocoa. It is the most important export fruit in the world with trade amounting to more than 4 billion US dollars annually (FAO, 2000). This generates vital hard currency earnings for many developing countries (Lopez-Gomez *et al.*, 1997). Of the bananas which enter the international trade, most come from Ecuador, Colombia, Honduras, Costa, Rica and Panama. There are also substantial exports from the Philippines to Japan, from the West Indies to the U.K., from the Canary Islands to the Spanish mainland, and from west Africa and the French Antilles to France (Hallam, 1995). The great majority of this exported fruit is of the Cavendish dessert type of banana.

1.2.2 Banana taxonomy, botany and production

Banana belongs to the genus *Musa* of the Musaceae family and is derived from the wild species *Musa acuminata* and *Musa balbisiana*. According to Simmonds (1966), the description and classification of cultivated bananas had been by genome nomenclature and is based on ploidy and on the relative genetic contributions of the

two species to each cultivar. Edible banana cultivars may be diploids (AA) triploids (AAA, AAB, ABB) or tetraploids (AAAA, AB BB) where A is from *Musa acuminata* and B is from *Musa balbisiana*. Cultivars under these groups and subgroups, e. g. AAA group and Cavendish subgroup, have different characteristics in terms of shape, size, colour of peel and flavour.

Almost all dessert bananas belong to the triploid group AAA and are eaten when ripe. This includes the 'Cavendish' clones, which are currently the basis of the international banana trade. The groups AAB and ABB are usually cooked before being eaten. The tetraploid cultivars have been bred to try and increase disease resistance, yield and quality although none are in commercial production (Simmonds, 1966; Marriott & Lancaster, 1983).

The banana plant is described as a large perennial herb with a true stem (rhizome) hidden underground, a pseudostem composed of leaf sheaths and a terminal crown of leaves from which the inflorescence emerges (Simmonds & Stover, 1987). At the base of the pseudostem is formed the inflorescence which will develop mature fruits ready for harvesting 90-150 days later after emergence through the leaf crown (Seymour, 1991).

Bananas are tropical crops with growth cessation and physiological problems developing at temperatures below 10°C and above 38 °C. Production is possible in sub-tropical and Mediterranean areas providing sites are carefully selected for frost protection, aspect, wind protection etc. (Robinson, 1995). For optimal commercial production, soils should be deep, well-drained loams with high water holding capacity and high fertility and organic matter. Bananas do not readily tolerate acidic or saline soils. Water supply of about 25mm/week is the minimum for satisfactory growth (Purseglove, 1972).

1.2.3 Postharvest handling and ripening

At harvest the whole bunch is removed by cutting through the pseudostem. The choice of the stage of maturity for cutting depends partly upon the distance from the market and partly upon the clone used. Exported fruit are harvested and shipped green (unripe) (Thompson and Burden, 1995).

In some commercial growing regions e.g. the Windward Islands, bananas may be packaged for export in the field. On most plantations, however the bunches are transported to a packhouse where preliminary postharvest operations are carried out. Bunches are cut into hands or clusters of 5-8 fingers attached to a piece of crown tissue. The hands are placed immediately into a wash tank to remove any dirt and to allow the latex from the cut crown surface to wash away. Active chlorine at about 100 mg/l is added to the wash water to destroy fungal spores and bacteria. Alum (10g/l) may be added to prevent the latex from staining the fruit peel. The hands are then allowed to drain before either dipping in or spraying with a fungicide solution (see section 1.4.2.).

The hands are then packed, usually in telescopic fibreboard cartons holding about 14 or 18 kg each, although some fruit is now being transported in returnable plastic crates. Polyethylene liners are usually wrapped around the fruit to reduce water loss. The boxes have ventilation holes that allow for good ventilation during both refrigeration and ripening.

Packed boxes are usually loaded onto a lorry and taken to a ship where they are may be loaded directly into the refrigerated hold (break bulk) or they may be palletised before loading. An increasing number of banana boxes are being transported to the importing countries in 20 or 40 foot containers (reefers) which are loaded at the packhouse and transported to the ship by road. Transport and storage temperatures must not fall lower than about 12°C or the fruit will suffer from chilling injury, which

reduces fruit quality. If temperatures rise much above 12°C, the incidence of both premature ripening and fruit diseases increases.

In the importing country, the unripe fruit is delivered to special ripening facilities. The boxed/palletised fruit is placed in ripening chambers and the temperature is raised to about 16-18°C. Ripening is triggered by exposing the fruit to the gaseous ripening hormone ethylene at a concentration of between 700-1000 ppm. The ripening process is carefully controlled by monitoring and adjusting the fruit pulp temperature. The degree of ripening at the ripening facilities is determined by market requirements.

During ripening, the fruit undergoes a number of dramatic physical and biochemical changes (Seymour, 1991). The fruits become much more susceptible to physical injury and postharvest pathogens.

1.3 BANANA FRUIT DISEASES

1.3.1 Introduction to banana fruit pathogens

The pathogens of banana fruit can be divided into those that infect pre-harvest and those that infect postharvest, although several pathogens can attack during both periods e.g. *Colletotrichum musae* (Jones *et al.*, 2000). Most pathogens of banana fruits are common as saprophytes on dying banana leaves and other trash in the plantation. The conidia of these fungi are dispersed by air currents or rain splash and are found on the surfaces of banana leaves, stems and fruits. A number of the pathogens infect by invading through cuts and other wounds on the fruit or crown surface, for example, *Fusarium* spp and *Trachysphaeria fructigena*. Other fungi, such as *Deightonella torulosa*, *C. musae* and *Pyricularia grisea* can invade the intact peel of green fruit using appressoria and penetration pegs. The main diseases of banana fruits are described by Wardlaw, (1972); Slabaugh and Grove, (1982); Snowdon (1990) and Jones *et al.*, (2000). These diseases and their causal pathogens are summarised in Table 1.1.

Table 1.1. The main fungal diseases of banana fruit.

Name of diseases	Pathogens	Reference
Anthracnose	<i>Colletotrichum musae</i>	Simmonds, 1963
Black heart	<i>Fusarium moniliforme</i> (Conidial state of <i>Gibberella fujikuroi</i>)	Snowdon, 1990
Black Tip	<i>Deightoniella torulosa</i>	Snowdon, 1990
Brown spot	<i>Cercospora hayi</i>	Kaiser and Lukezik, 1965
Cigar end rot	<i>Trachysphaera fructigena</i>	Bhangale and Patile, 1983
	<i>Verticillium theobromae</i>	
Crown rot	Many, including the following: <i>Acremonium strictum</i> <i>Cephalosporium</i> spp <i>Chelara paradoxa</i> <i>Colletotrichum musae</i> <i>Fusarium</i> spp. <i>Nigrospora</i> spp. <i>Verticillium</i> spp.	Jones <i>et al.</i> , 2000
Diamond spot	<i>Fusarium</i> spp.	Stover, 1972
Dothiorella rot	<i>Botryosphaeria ribis</i>	Snowdon, 1990
Finger rot	<i>C. musae</i> & <i>Fusarium</i> spp. <i>Lasioidiplodia theobromae</i>	Goos <i>et al.</i> , 1961
Freckle	<i>Guignardia musae</i> (Conidial state of <i>Phyllosticta musarum</i>)	(Snowdon, 1990)
Main stalk rot	<i>Chalara paradoxa</i> ; <i>C. musae</i> <i>Lasioidiplodia theobromae</i> ;	Jones <i>et al.</i> , 2000
Phytophthora finger rot	<i>Phytophthora nicotianae</i> var. <i>parasitica</i>	Snowdon, 1990
Pink mould rot	<i>Trichothecium roseum</i>	Snowdon, 1990
Pitting disease	<i>Magnaporthe grisea</i> (Hebert) Barr (Conidial state <i>Pyricularia grisea</i>)	Meredith, 1963
Rhizopus rot	<i>Rhizopus stolonifer</i>	Snowdon, 1990
Sooty mould	<i>Cladosporium cladosporioides</i>	Stover, 1972
Speckle or Swamp spot	<i>Deightoniella torulosa</i>	Meredith, 1961
Squirter disease	<i>Nigrospora sphaerica</i>	Stover, 1972
Watery soft rot	<i>Sclerotinia sclerotiorum</i>	Snowdon, 1990

The relative commercial importance of different postharvest pathogens of banana has changed depending on the postharvest management of the fruit. When bananas were transported as entire bunches, main stalk rot was a serious cause of losses (Stover, 1972). Stem end rots caused by a number of pathogens and squirter disease can be a problem if individual fingers are cut from the bunch and marketed as single fruit. Today, the majority of fruit is exported as boxed clusters of fingers and the most problematic disease is crown rot (see section 1.3.2.).

There is a strong relationship between the physiological state of the fruit and its susceptibility to postharvest diseases. Most disease is kept to a minimum provided that good temperature and humidity control is used during storage and transport and if fruits with physical injuries are discarded. Given good handling procedures fungicide applications are generally effective in minimising losses from postharvest diseases (Jones *et al.* 2000). Future problems are likely to arise from the reduction in the number of postharvest chemicals that are permitted on fruits and the potential for fungal resistance to arise to the limited range of fungicides in use.

1.3.2 *Colletotrichum musae*

Colletotrichum musae (syn *Gloeosporium musarum*: Cooke and Massee) is a deuteromycete in the class Coelomycetes. The fungus exists in the asexual state producing conidia on conidiophores, which arise in acervuli. These acervuli develop on senescing banana plant tissues. They are most common on fruits but may also occur on peduncles, petioles, bracts and occasionally leaves. Conidia are liberated by rainsplash or irrigation water, as well as dispersed by air current and by insects (Snowdon, 1990). The optimum temperature for growth and sporulation of *C. musae* is 27-30°C with little growth below 15°C. A detailed description of the fungus is given by Sutton and Waterston (1970).

Role of *C. musae* in diseases of banana fruit

C. musae can cause a number of banana fruit diseases i.e. anthracnose, crown rot and stem-end rot and has been implicated in a number of others e.g. fungal scald, main-stalk rot, symptoms of speckle etc. Anthracnose disease of fruits can occur both before and after harvest. Pre-harvest infections have caused problems in the Asia-Pacific region. The infections cause lesions initially on the distal end of the fruits. Affected fruits ripen prematurely, rot and eventually shrivel (Jones *et al.*, 2000). Postharvest infection of anthracnose is one of the most important and wide spread postharvest diseases of banana (Snowdon, 1990). It causes significant wastage in domestic markets around the world, especially where damage levels are high and temperatures are not controlled. On the whole, anthracnose is not such a serious problem on exported fruit because wounded fruit is rejected during quality checks and fungicides are generally effective. Currently the main commercial disease of exported bananas is crown rot. Although crown rot can be caused by a number of fungal and bacterial pathogens, *C. musae* has been shown to be one of the more aggressive pathogens involved in this disease complex (Finlay and Brown, 1993). The symptoms and control of anthracnose and crown rot are dealt later in this section and in section 1.4.

Anthracnose

Two types of anthracnose infection can occur, namely; latent and non-latent infections (Simmonds, 1966; Meredith, 1971). Latent infection is often initiated in the field on uninjured green fruit. A fungal spore on the fruit surface germinates and the germ tube produces an appressorium. A hyphal thread from the appressorium penetrates the cuticle and the fungus becomes dormant (quiescent) until the fruit ripens. This dormancy can last for many months (Simmonds, 1941). During fruit ripening, the fungus causes sunken brown to black lesions in the fruit peel, which become covered with salmon-coloured acervuli. Lesions expand as ripening progresses and the pulp may become infected in fruit held at high temperatures or in overripe fruit.

The factors that control appressorial development and quiescence have not been determined, although many hypotheses have been put forward. Conidial germination and appressorial formation are stimulated by leachates from green bananas. The main stimulant appears to be anthracnilic acid, which is converted to a chelating compound, 2,3-dihydroxybenzoic acid by *C. musae* (Harper and Swinburne, 1979). As it is known that iron inhibits conidial germination, it is assumed that the DHBA acts to chelate iron and thus protect the conidia (Brown and Swinburne, 1981). The formation of the appressoria is linked to the physical nature of the underlying substrate (Read *et al.*, 1992). Appressoria will readily initiate on hard surfaces such that on a glass slide (Leu and Chang, 1988). It has also been shown that the presence of ethylene at levels lower than produced during fruit ripening will induce germination and appressorial formation in *C. musae* (Flaishman and Kolattukudy, 1994). Although it has been shown that *C. musae* can produce its own ethylene, (Peacock & Muirhead, 1974) the significance of this in disease development has not yet been demonstrated. Other research has shown that substances found in the cuticle of host plants will stimulate appressorial formation in *Colletotrichum* species (Podila *et al.*, 1993).

It appears that the melanin in the appressoria of *Colletotrichum* spp. is essential to their ability to penetrate intact plant surfaces (Howard and Ferrari, 1989). Albino mutants show no difference in their rates of growth or germination *in vitro* but show a very poor ability to penetrate and form lesions on host plants (Rasmussen and Hanau, 1989) or to penetrate nitrocellulose membranes compared to pigmented wild types (Kubo *et al.*, 1982).

It is thought likely that anti-fungal defence compounds in the green peel may act to prevent the development of the fungal mycelium. These compounds may be pre-formed and possibilities include 3,4-dihydroxybenzaldehyde (Mulvena *et al.*, 1969), 3,4-dimethoxybenzaldehyde (Abdel-Sattar and Nawwar, 1986) and dopamine (Jiang, 1997). It needs to be shown, however, that these compounds are present at the site of infection in sufficient concentrations to be effective (Muirhead and Deverall, 1984). It

has been shown that phytoalexins can form in response to infection by *C. musae*. Five compounds were found to be produced in the necrotic tissue formed when green bananas were challenged by *C. musae* (Brown and Swinburne, 1980). One of these compounds has been identified as 2-(4'-hydroxyphenyl)-naphthalic anhydride (Harai *et al.*, 1994).

Other hypotheses for the control of dormancy include changes in nutrient availability during ripening making it easier for the fungus to develop. It is also possible that the cell walls of green but not yellow banana peel are resistant to fungal enzyme attack (Simmonds, 1963). *C. musae* has been shown to produce a number of extracellular enzymes which may be important in the colonisation process including amylases, cellulases, pectin methyl esterase (PME), pectate lyase (PL), polygalacturonase (Pgal) and xylanase (Shillingford and Sinclair, 1980; Ogundero, 1987; Stanley, 1993).

Non-latent infections usually occur during or after harvest when spores germinate in small wounds and continue to develop without a dormant period (Sepiah *et al.*, 1990). Black, sunken, diamond-shaped lesions with yellow halos develop. As fruit ripen the lesions enlarge and the pulp may be invaded causing a watery rot (Meredith, 1960). When anthracnose develops in wounds on green fruits, the extent of the disease depends on whether the fruit is held for a long period before ripening and under what conditions. The longer the pre-ripening period, the larger the lesion (Jones *et al.*, 2000).

Crown rot

Crown rot is a disease of the part of tissue that is severed when the hand or cluster of fruit is cut from the bunch. It is a firm, dark rot which may spread through the crown during transportation of the fruit. A layer of fluffy white, grey or pink fungal mycelium may cover the cut surface of the crown (Jones *et al.*, 2000). Rotting is most noticeable if transit times exceed seven days and high incidence of the disease can cause premature ripening leading to further commercial losses. During ripening, the

rot, in severe cases, can spread through the pedicel into the pulp, causing fingers to separate from the hands (Greene & Goos, 1963).

Crown rot is a complex disease caused by several fungi and possibly bacteria, which vary both quantitatively and qualitatively. The predominance of different species within the infection is affected by locality, time of year and some other factors (Meredith, 1971). Some the main fungi that have been associated with crown rot are listed in Table 1.1. Tissue isolations done to identify fungi associated with the crown rot complex in the West Indies, found that *C. musae* was the most common fungus associated with crown rot, occurring in 35.8 % of diseased hands (Griffie & Burden 1976). This is supported by many authors who agreed that *C. musae* is the fungus most commonly associated with the crown rot complex (Greene & Goos, 1963; Roth & Loest, 1965; Shillingford, 1970). An exception were reported by Lukezic *et al.* (1967) who found that in South America, species of *Cephalosporium* was dominant and *C. musae* occurred only rarely over a ten-month sampling period.

The fungi associated with the disease in bananas exported to the United Kingdom from the Windward Islands have been well studied (Johanson and Blazquez, 1992). These authors showed that the main pathogens were species of *Fusarium*, principally *F. pallidroseum* and *C. musae*. Other crown-rot causing fungi were also isolated including *Lasiodiplodia theobromae* and *Verticillium theobromae*. When Finlay & Brown (1993) carefully examined the relative importance of the different fungi in the Windward Islands, they concluded that *C. musae* was the most aggressive species. At least 20 times more conidia of *F. pallidroseum* compared to *C. musae* conidia were required to cause comparable levels of crown rot. The relative pathogenicity of the major fungal species involved in development of crown rot was examined. Of the following species, *C. musae*, *F. pallidroseum*, *F. moniliforme*, *F. moniliforme* var. *subglutinans* and *B. theobromae* only *C. musae* was able to rot green tissues. A role has been suggested for enzymes produced by *C. musae* in the transition from the quiescent to the aggressive phase of anthracnose (Shillingford and Sinclair, 1980). Polygalacturonase (Pgal) appears to be particularly important in the colonisation of

banana crown tissues by *C. musae* and has a strong affinity for banana pectin. *C. musae* was shown to produce much higher levels of PGal than five other crown rot fungi (Stanley, 1993) and the presence of five isozymes of PGal has been detected in rotting banana tissues (Stanley and Brown, 1994).

The inoculum for crown rot is ubiquitous in banana plantations and originates mainly from decayed flowers, bracts and normal leaves of the banana plant. Spores of crown rot fungi are either air-dispersed or rain spread (Stover, 1972). The spores are present on the fruit at the time of harvest and are ready to colonise the crown after de-handing at the packing point (ODA, 1971). Fungal spores can be carried from de-handing knives used in separating bunches of fruits from the main stalk, and can enter the vascular tissue of crown and cause rot. However, breaking the stalk near the crown rather than trimming it with a knife resulted in increased disease severity. This suggests that sufficient inoculum was present on the stalk to initiate rot development irrespective of inoculum transfer onto the wound via the de-handing knife (Finlay *et al.*, 1992). Greene & Goos (1963) showed that spores present in the water used to wash the bananas can be drawn into the wounded crown tissues to a depth of 5-7 mm.

A relative humidity of 86% or above is required by the conidial spores of these fungi to germinate. However, under low extremes of humidity and temperature, ungerminated spores can remain viable for several months and could still produce infections in the ripening rooms even after long periods of refrigeration and transport (Meredith, 1971). Lukezic *et al.* (1967) and Stover & Simmonds (1987) considered that short periods of hot dry weather just before fruit are harvested favour crown rot. Jones *et al.*, (2000), however, suggest that in the Windward Islands, crown rot is more severe during humid, wetter and hotter months. The speed of progress of the disease in individual fruits depends on several factors. These factors include the amount of inoculum, the physiological state of the fruit, the length of time before ripening commences and the environmental conditions before and after ripening (Finlay and Brown, 1993; Chillet and de Lapeyre de Bellaire, 1996).

1.4 CONVENTIONAL CONTROL OF BANANA FRUIT DISEASES

1.4.1 Handling and sanitation measures

Most post-harvest diseases of bananas and plantains originate in the field from infected plant trash. Simmonds and Mitchell (1940) found a significant correlation between the number of trash leaves in the field and anthranose incidence of marketed fruit. A regular disease control programme in the field will, therefore, play a major part in reducing the inoculum load in the packhouse (Thompson & Burden, 1995). Any fingers already infected with disease should be removed to avoid contamination of the water in washing and de-latexing tanks. Crown rot severity was reduced by more than 50% in fruit de-handled in the relatively clean environment of a packing shed compared to field packing (Finlay *et al.*, 1992).

The water used in washing and de-latexing tanks is routinely treated with a disinfectant such as chlorine. Although this procedure has been recommended as a control for crown rot, there is evidence that it has a limited effect. Slabaugh and Grove (1982) noted that maintaining the recommended concentration of chlorine in wash water did not reduce crown rot. Shillingford (1977) compared a number of potential disinfectants for use in banana wash water and concluded that the quaternary ammonium chlorides and formalin were superior to chlorine in eradicating conidia of *C. musae*, *B. theobromae* and *F. semitecum*.

In the case of crown rot, it seems that the inoculum penetrates the cut crown at the time of de-handing. The de-handing should be carried out with a sharp tool to reduce the amount of damaged crown tissue. Finlay *et al.* (1992) showed that rough trimming and breaking crowns from the bunch stalk, instead of cutting increased crown rot. The action of breaking the crowns left many fragments of tissue that rapidly dehydrated and senesced, providing ideal conditions for the development of the crown rot fungi. The crowns of broken hands yellowed more quickly, suggesting premature ripening of the tissue, which would also favour fungal growth.

Bananas require extremely careful handling during their post-harvest life. Physical injury accelerates water loss, increases fungal infection and stimulates respiration and ethylene production by the commodity. The latter two factors may cause premature ripening of green banana fruit and increase their susceptibility to injury and direct fungal attack by organisms causing diseases such as anthracnose (Wardlaw, 1972). Slabaugh & Grove (1982) showed that minimising the incidence of cuts and bruises during handling reduced the subsequent effect of disease as the fruit ripened.

1.4.2 Fungicides

Commercially, fungicide application is the main method used in the control of banana fruit diseases. In the Philippines, leaf pruning, spraying the newly emerged bunches with mancozeb and covering them with plastic bags was recommended to control anthracnose (Anon., 1988). Griffiee & Burden (1974) stated that four pre-harvest sprays of benomyl reduced the level of *C. musae* as well as other fungi on unripe bananas and resulted in a lower level of latent infection caused by *C. musae* on ripe fruits. However, reduction of crown rot was not achieved and a post-harvest fungicide treatment was then recommended.

Postharvest applications of fungicides are made after the hands of fruit have been washed. The fruit must first be drained of excess water otherwise the water adhering to the fruit may dilute the fungicide below its effective concentration. For small-scale operations, the hands are dipped in a bowl of fungicide or sprayed with a hand-operated knapsack sprayer. In large-scale packing stations, fungicide is applied mechanically to run-off by passing hands in trays under either a spray or cascade of fungicide (Thompson and Burden, 1995). Application of fungicide by dipping proved to be more effective, especially in the reduction of rot in the banana cushions, than by spraying, but both methods resulted in satisfactory control of fungal rots (Bailey *et al.*, 1970). In the Caribbean, the use of pads impregnated with fungicide placed directly on the cut crown was reported to be effective in controlling crown rots

(Johanson *et al.*, 1989). The method has not been adopted commercially due to retailer concerns about the appearance of dirty pads in the boxes of bananas on display to customers. In addition, pads work better on hands than on clusters, the form of fruit now commonly exported (Jones *et al.*, 2000).

A number of protectant fungicides have been evaluated as postharvest treatments for bananas. Eckert and Sommer (1967) showed that dipping the cut end of bananas in 0.3 % maneb suspension prevented the initiation of crown rot decay. The addition of Triton X-114 as a wetter improved control by increasing fruit coverage by the fungicide (ODA, 1971). Latent infections e.g. anthracnose are, however, hard to control with protectant fungicides because of the relatively resistant appressoria usually formed (Jeger and Plumbley, 1988).

Effective control of both crown rot and anthracnose was first achieved by dipping the fruit in solutions of the systemic benzimidazole fungicides such as benomyl, carbendazim or thiabendazole (Burden, 1967; Frossard and Laville, 1973; Griffie and Burden, 1976; Griffie and Pinegar, 1974; Long, 1971; ODA, 1971; Ogawa *et al.*, 1968; Phillips, 1970; Scott and Roberts, 1967; Shillingford, 1970). These chemicals control both latent and non-latent infections caused by *C. musae* (Jeffries *et al.*, 1990). Today thiabendazole is the main benzimidazole fungicide in use on bananas. Carbendazim have been deregistered by many countries due to concerns about its effect on human health. The agrochemicals company Du Pont has withdrawn benomyl (Benlate®) as a postharvest treatment for fruits and vegetables (Jones, 2000). Thiabendazole is available in the form of wettable powders and emulsifiable concentrates and is typically applied at concentrations of 200-1000 mg a.i./litre).

With the development of resistance problems with the benzimidazole fungicides in the 1970's (see section 1.4.3), alternative systemic chemicals have been evaluated for postharvest use on banana fruit (Eckert, 1990). Dip treatment of fruit in triforine was shown to extend shelf life of banana fruits by reducing rot and deterioration caused by *C. musae* (Vir & Sharma 1985). Prochloraz at 250 ppm has been shown to be more

effective in controlling postharvest pathogens of banana than thiabendazole (Knights, 1986; Sepiah & Nik Mohd, 1987). Jones (1991) investigated eight fungicides for their potential to control crown rot in Queensland (benomyl, flusilazole, hymexazol, imazalil, myclobutanol, prochloraz, propiconazole and triforine). He found that prochloraz and flusilazole gave the best control.

Today, the most commonly used alternative fungicide is imazalil which at 500mg/litre can control *C. musae* and other crown rot pathogens which have become resistant to thiabendazole (Frossard *et al.*, 1976). Other chemicals with the same mode of action as imazalil, such as bitertanol or prochloraz are applied in countries where they have been approved for use. These chemicals are more expensive than thiabendazole and are usually only applied when fruit is intended for long distance transport or when disease pressure is very high in the plantation (Eckert, 1990).

1.4.3 Fungicide resistance

Since the introduction of systemic fungicides in the early 1970's, resistance to certain groups of fungicides by some pathogens has occurred repeatedly where the fungicides are used on a regular basis (Cremlyn 1991). This resistance is recognised when control of the disease is no longer achieved with the recommended rate of fungicide. The development of resistance is due to the following factors: the type of fungicides, frequency of use, target pathogen, the ability of resistant forms to survive, and the programmes of fungicides use (Fletcher *et al.*, 1987).

In the 1970's the benzimidazole fungicide benomyl was used widely in banana plantations to control leaf diseases such as black Sigatoka. This led to a dramatic decline in the control of crown rot using postharvest applications of benzimidazole fungicides during this period (Johanson and Blazquez, 1992; Slabaugh and Grove, 1992). Crown rot pathogens commonly colonise and reproduce on banana debris, so presumably the use of benomyl created suitable conditions for tolerant forms of the fungi to appear and expand their populations. In Guadeloupe, de Lapeyre de Bellaire

and Dubois (1997) reported that an average of 23% of isolates of *C. musae* were tolerant of thiabendazole. Their presence also correlated with the exclusive use of benomyl as an aerial spray to control Sigatoka.

Benzimidazole fungicides specifically interfere with mitosis by binding to the β -tubulin protein and inhibiting microtubule function (Davidse and Flach, 1977). It has been shown that in resistant strains of fungi, there is a reduced affinity between the fungicide and β -tubulin (Davidse, 1986). Resistance develops quite easily in fungal populations because the benzimidazole fungicides act at single sites. At the molecular level, resistance has been associated with point mutations in the β -tubulin genes of phytopathogenic fungi (Koenraadt *et al.*, 1992).

Resistance to benzimidazole fungicides can partly be overcome by the use of more modern systemic fungicides with a different mode of action. For banana, imazalil has been the most frequently used alternative post-harvest treatment (Frossard *et al.*, 1976; Jeffries *et al.*, 1990) usually alternated with the benzimidazole treatment (Cox, 1996).

Imazalil was the first imidazole systemic fungicide used in agriculture and it falls in the class of demethylation inhibitor chemicals, which interfere with ergosterol biosynthesis and hence disrupt fungal membrane biosynthesis (Lye, 1987). Specifically it is believed that these fungicides poison the specific form of microsomal cytochrome P 450 that catalyses the C-14 de-methylation step in ergosterol biosynthesis (Kato, 1986). The mechanism works at several biochemical sites and it is thought that this polygenetic system makes the probability of development of resistant strains much lower than that for the benzimidazole type fungicides (Laville *et al.*, 1977). Despite this, imazalil resistant strains of *Penicillium* spp. have been observed, particularly on Citrus fruits (Eckert, 1988; Eckert *et al.*, 1994). It appears that resistance is not due to a decrease in the affinity of the fungicide to the target enzymes (Guan *et al.*, 1993). For isolates of *P. italicum* showing relatively low levels of resistance (but not medium

or high level resistant biotypes) there is evidence that the uptake of imazalil is less for the wild type sensitive strains (Guan *et al*, 1992).

1.5 ALTERNATIVE TO FUNGICIDES

In a survey of scientists from the United States concerned with chemical control of plant diseases, improved control of post-harvest diseases was identified as an area requiring urgent attention (Tweedy, 1983). A number of highly effective post-harvest chemicals have been withdrawn during the last decade due to links with human disease. The use of the fungicide benomyl in post-harvest chemical treatment of fruits such as apples and peaches was discontinued in 1989 and the alternative chemicals captan and ethylene bis-dithiocarbamate fungicide (EBDC's) were both discontinued in 1991 for post-harvest uses (Agricultural Research, 1990). New, less harmful chemicals alongside non-chemical alternatives for disease control are urgently required by the fresh produce sector. In general, however, the agrochemical industry perceives a very small market for post-harvest treatments and it is unlikely that new compounds, specifically directed to the special features of the post-harvest environment, will be developed.

This section considers some alternative strategies, both non-chemical and chemical and their potential for commercial use to control banana fruit diseases.

1.5.1 Modified atmospheres

The effects of modified atmospheres (reduced levels of oxygen and increased levels of carbon dioxide) on postharvest pathogens of fruits and vegetables was reviewed by El-Goorani and Sommer (1981) and Sommer (1985). There is little direct effect on fungal and bacterial pathogens at the oxygen (O₂) levels typically used in modified atmosphere applications. Levels of about 1% O₂ or less are required to obtain appreciable reduction in growth, spore formation and germination. Storage at such low O₂ levels would cause most fresh produce to rapidly become anaerobic.

The activity of several decay organisms can be reduced by atmospheres containing 10% CO₂ or more. It is well established that growth of *Botrytis cinerea* on fruits and vegetables can be strongly suppressed at levels of 15% CO₂ and above (Thompson, 1998). Al-Zaemey *et al.* (1994) showed that the mycelial growth of *C. musae* colonies, under 5, 10 and 15% CO₂ was significantly reduced by 18%, 25% and 51% respectively after 2 days of incubation and 10%, 20% and 27.7% respectively after 4 days of incubation as compared to control (0% CO₂).

Many commodities, however, cannot tolerate high CO₂ levels so that, in practice, atmospheric control cannot always be relied on to reduce rotting. Nevertheless MA may suppress diseases of produce indirectly by retarding their ripening and senescence, since the natural resistance of the (produce) host to pathogens decreases as it ripens or ages.

The main methods in use for changing or controlling the atmospheric composition around produce are surface coatings, modified atmosphere packaging (MAP) and controlled atmosphere (CA).

Surface coatings

Banks (1981;1984) described the use on bananas of a coating ("Tal Prolong") which consists of sucrose esters of fatty acids and carboxymethyl cellulose (CMC). This coating delayed the ripening of the fruit and reduced infection by postharvest pathogens. Similar formulations under the generic name of "Semperfresh" have been developed to extend the shelf-life of a wide range of fruits and vegetables (Agricoat, 1999).

Some of these coatings appear to have a direct effect on fungal pathogens. Al-Zaemey (1991;1993) reported that Semperfresh formulations (Semper AS and Semper F) at 1% concentration significantly reduced the growth rate of *C. musae in vitro* and

reduced the germination of *C. musae* conidia at three different temperatures as compared to the control, e.g. at 15°C germination was reduced by 63 %. Semper F was shown to restrict the germ tubes of *C. musae*, and also affected the appearance of the mycelium.

Bananas treated with coating materials (Semper F & Semper AS) in the absence of any fungicides, effectively reduced natural crown rot incidence by 39 % (Al-Zaemey, 1991). He also reported that sucrose esters in combination with sodium carboxymethyl cellulose (CMC) reduced crown rot by 57 %. Incorporating fungicides e.g. benomyl or imazalil into the coating materials was found to significantly enhance the effectiveness of the fungicide against anthracnose and crown rot. It has been suggested that coating materials that are hydrophobic might lower water availability, thereby reducing growth of the pathogen (Bancroft, 1989).

Modified atmosphere packaging

Some bananas are shipped commercially in modified atmosphere packaging (MAP) e.g. "Banavac" bags. The effect of MAP on disease levels of bananas is variable. Scott (1971) indicated a higher incidence of crown rot and black end of banana fruits stored in plastic bags and Shillingford (1978) found increased disease levels on bananas wrapped in polyethylene. Sarananda and Wijeratnum (1997) found that bananas stored at 14°C in 1-5% O₂ had lower levels of crown rot than those stored in air but that CO₂ levels of 5-10% increased rotting compared to controls. Wade *et al.*, (1993) found that sealing banana hands in polyethylene bags containing 3-7% O₂ and 10-13% CO₂ suppressed some fungi capable of causing crown rot symptoms (e.g. *Nigrospora sphaerica*) but did not control crown rot infection caused by *C. musae* and *Fusarium moniliforme*.

Controlled atmosphere storage

The commercial application of controlled atmosphere storage (CAS) to bananas is primarily for transportation of the green fruit. A number of banana companies are shipping bananas from some Latin American countries either in CA containers or in reefer ships where equipment has been installed in the refrigerated holds, which enable the level of O₂ and CO₂ to be controlled. Typically the levels used are in the range of 2-5% for both gases at 14°C (Thompson, 1998). The main benefits are a lower incidence of premature ripening and chilling injury. There is also anecdotal evidence that the incidence of crown rot is much lower during CA transportation (Severine Ruel, Pratts Bananas: Personal communication).

1.5.2 Biological control

Various research groups have investigated the potential for effective biocontrol of banana fruit diseases. A number of bacterial, yeast and fungal isolates have been shown to be able to inhibit anthracnose lesion expansion on ripe banana fruit. (Chuang and Yang, 1993; Ragazzi and Turco, 1997). Furthermore, several isolates of non-fluorescent *Pseudomonas* sp. were found which were antagonistic *in vitro* to key crown rot pathogens namely; *C. musae*, *Botryodiplodia* spp. and *Ceratocystis paradoxa*. These bacteria were also shown to significantly reduce crown rot of banana when applied as a postharvest dip (Costa *et al.*, 1998). A banana leaf disk system was developed to assist in screening for potential antagonists to *C. musae*. This showed that a number of yeasts are effective antagonists *in vivo* even when they did not show antagonism *in vitro* (Postmaster *et al.*, 1997)

1.5.3 Heat treatments

It has been shown that mycelial growth and spore germination of *C. musae* is suppressed and pathogenicity reduced when subjected to 55°C and 60°C for 20 minutes (XiuJuan *et al.*, 1999). When the fruit crowns were inoculated with *Chalara*

paradoxa and then exposed to hot water at 50°C for 20 min, crown rot symptoms were reduced to less than 3% of the non-heat treated controls (Reyes *et al.*, 1998). There is also some experimental evidence that hot water treatments may also be effective against *Fusarium* and *Colletotrichum* in crown rot infections (Lopez-Cabrera and Marrero-Dominguez, 1998).

The main problem encountered with heat treatments is heat injury to the produce. It has been shown that unripe Brazilian bananas are injured by hot water treatments at 55°C and above, but can tolerate 50°C for 30 minutes without apparent injury (Armstrong, 1982). Reyes *et al.*, (1998) found that hot water treatment of the banana cultivars, ‘Santa catarina Prata’ and ‘Williams’ at 55°C for 10 min or longer caused severe skin scald. At 50°C, scald was only a real problem at 30 min exposure or longer although there were seasonal differences in the severity of fruit injury.

1.5.4 Chemical alternatives to conventional fungicides

Given the current constraints to the development of new fungicides, interest has turned to alternative chemicals for which approval for use on produce may more readily be obtained and which might be perceived as more “natural” or “organic” by the consumer (Wilson and Wisniewski, 1989). Some possibilities are discussed below.

Plant extracts

Plants produce vast numbers of secondary compounds, many of which are believed to play a role in the defence of the plant against browsing animals, pests and pathogens (Heldt, 1997). Some plants have been used for centuries for their antimicrobial properties in the preservation of foods e.g. the addition of bitter herbs to meats and beverages. Scientists are taking a renewed interest in plant extracts as alternatives to synthetic pesticides e.g. Wilson *et al* (1997) screened 49 essential oils and extracts from 345 plants (see below) for their activity against *B. cinerea*. Some extracts have been approved for use on crops in certain countries. A major limiting factor to their

use in crop protection is the difficulty in obtaining sufficient oil at prices which would make their use economic.

Essential oils

Many essential oils from plants show antifungal activity, for example, benzaldehyde, carvone, carvacol, cinnamaldehyde, cuminaldehyde, eugenol, perillaldehyde, salicylaldehyde and thymol. Wilson *et al.*, (1997) found that D-limonene, cineole, β -myrcene, α and β -pinene and camphor were the most frequently occurring constituents in essential oils showing high antifungal activity against *B. cinerea*.

Cinnamaldehyde has low mammalian toxicity and is used as a flavouring in certain foods. Treatment of tomatoes for 30 min with 13 mM cinnamaldehyde delayed visible mould growth by seven days during storage under modified atmospheres at 18°C (Smid *et al.*, 1996). Surface applications of cinnamaldehyde also decreased spoilage by *Penicillium expansum* on apples (Ryu and Holt, 1993). Cinnamaldehyde, salicylaldehyde and thymol all prevented growth of TBZ resistant strains of *Fusarium sambucinum*, a pathogen of potato tubers, when incorporated in media at 0.1% (v/v) levels (Vaughn and Spencer, 1994). Carvacol and eugenol showed antifungal activity against *Rhizopus* spp., *Mucor* spp., *Aspergillus flavus* and *A. parasiticus*. On the other hand, cinnamaldehyde was ineffective against these pathogens in the same experiment (Thompson, 1989).

Hinokitiol

Hinokitiol (β -thujaplicin) is a volatile oil found in the roots of the Japanese cypress (*Hiba arboritae*). It is a strong chelating agent that inhibits many microbial enzymes and is a government-approved food additive in Japan. Hinokitiol has been shown to strongly inhibit the spore germination and mycelial growth of *B. cinerea* and *Alternaria alternata*. When harvested eggplants and red peppers or Galia melons

were dipped in hinokitiol solution ($750\mu\text{l L}^{-1}$), there was a marked reduction in decay and no phytotoxicity was observed (Fallik and Grinberg, 1992; Aharoni *et al.*, 1993). Hinokitiol also prevented decay of commercially harvested peaches (Sholberg and Shimizu, 1992).

Tea tree oils

Essential oils from *Melaleuca alternifolia* (tea tree), a native Australian shrub are used in the pharmaceutical industry for their antimicrobial properties (Lassak and McCarthy, 1983). Bishop and Thornton (1997) tested the oil against 15 common postharvest pathogens and found good antifungal activity both by direct contact and in the vapour phase. The same oil at 3.2% dilution was found to be as effective as three commercially recommended fungicides in decreasing colonies of *Botrytis cinerea* on cabbage leaf disks (Bishop and Reagan, 1998).

Neem tree products

The neem tree *Azadirachta indica* Juss (Family: *Meliaceae*), commonly known as 'margosa', 'neem' 'nim', is native to India and Burma but is widely grown in many tropical countries. The beneficial uses of the neem tree, have been known for centuries in India, but only in recent years have the potential uses been appreciated more widely. There are many reports in the literature on the medicinal and pesticidal uses of neem but neem-based products have only become commercially available in countries such as the U.S.A. for the control of a broad spectrum of insect pests in recent years.

Several active principles from different parts of the neem tree have been reported (Satyavati *et al.*, 1976). Azadirachtin was isolated from the seeds of *Azadirachta indica* by Butterworth & Morgan (1968). Neem seed can contain up to 45% oil which is known to contain a number of biologically active compounds. For some applications the oil can be used directly, e.g., seed treatments or postharvest

applications (Schmutterer, 1995). *In vitro* evaluation of aqueous extracts of neem oil cake demonstrated an inhibitory effect on several test fungi, *Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *lycopersici*, *Helminthosporium nodulosum*, *Alternaria tenuis*, and *Curvularia tuberculata* (Khan *et al.*, 1974b). The inhibitory response was concentration dependent and apparently fungistatic rather than fungicidal. Haberle & Schlosser, (1993) obtained protective and curative effects using various neem seed kernel extracts against *Sphaerotheca fuliginea* on cucumber in green houses. Kazmi *et al.* (1993) observed that the hexane extract of *A. indica* seed was inhibitory to growth of *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus wentii*, fungi commonly causing spoilage of stored grain.

Clarified neem seed oil was evaluated as a postharvest treatment of apple fruits. (Moline & Locke, 1993). The 2% emulsified neem oil treatment was moderately fungicidal to *B. cinerea* and *G. cingulata* in wound-inoculated fruit, but had little activity against *P. expansum*. Ali *et al.* (1992) reported that the growth of isolates of *Penicillium italicum*, *Alternaria alternata* and *Aspergillus niger* from rotted tomato fruits was checked by neem oil *in vitro*. Treatment of infested banana fruits with aqueous leaf extract of *Azadirachta indica* gave good control of disease caused by *B. theobromae*, *F. oxysporum*, *Helminthosporium spiciferum*, *Curvularia lunata*, *Aspergillus flavus* and *Trichothecium roseum* (Sing *et al.*, 1993).

Fruit aroma compounds

A number of the volatile aroma compounds found in fruits e.g. alcohols, aldehydes, ketones and esters been found to have antifungal properties (Archbold *et al.*, 1996; Suprapta *et al.*, 1997; Vaughn *et al.*, 1993). Prasad and Stadelbacher (1973) found that decay by *B. cinerea* on raspberries could be controlled by a 70 min fumigation with acetaldehyde at 0.05%. It has been shown to be inhibitory to *B. cinerea* and *Rhizopus stolonifer* both *in vitro* and *in vivo* on table grapes (Avissar *et al.*, 1990; Avissar and Pesis, 1991) and to *B. cinerea* on strawberries (Pesis and Avissar, 1990). Although acetaldehyde showed some promise for the control of *Penicillium* spp. on

citrus (Yuen *et al.*, 1995) and cherries (Mattheis and Roberts 1993), the latter study showed extensive phytotoxicity on stems and fruits at concentrations needed to prevent conidial germination.

Chitosan

Chitosan, a β -(1,4)-glucosamine polymer is an important structural component of the cell wall of some plant-pathogenic fungi. It has been shown to be fungicidal against a wide range of fungi and to elicit many plant defense responses (El Ghaouth *et al.* 1992a). Chitosan is available commercially from arthropod (e.g. crab) exoskeletons. Due to its polymeric nature, chitosan can form films permeable to gases. It therefore has considerable potential as an edible antifungal coating for fruits and vegetables (El Ghaouth *et al.*, 1992b). A number of studies have shown that application of chitosan can inhibit postharvest pathogens on fruit e.g. strawberries (El Gaouth *et al.*, 1992c); litchi (Zhang and Quantick, 1997); peach, Japanese pear and kiwifruit (Du-JianMing *et al.*, 1997). However, studies by Al Zaemey *et al.*, (1993) did not find any antifungal effect of either chitosan or carboxymethyl chitosan on the growth of *C. musae* *in vitro*. In fact chitosan appeared to stimulate the growth of *C. musae*.

Organic acids

Organic acids such as ascorbic, benzoic, citric, malic, oxalic, salicylic, sorbic, succinic, tannic and tartaric acids are found in certain fruits and other plant materials. Other organic acids such as lactic and propionic acids are formed naturally in certain foods such as cheeses. It has long been known that organic acids possess antimicrobial activities. These properties have been exploited in processed food products to control the growth of spoilage fungi and bacteria (Beuchat and Golden, 1989).

The mode of action of organic acids is attributed primarily to depression of intracellular pH by ionisation of the undissociated acid molecule. Effectiveness is therefore dependent upon the dissociation constant of the acid and the pH of the food

to be preserved. There is also evidence that organic acids may disrupt substrate transport by alteration of cell membrane permeability and also inhibit NADH oxidation (Liewen, 1991).

Benzoic acid and its salts e.g. sodium benzoate is one of the most routinely used organic acids in the food processing industry. It is active against yeasts and moulds, including aflatoxin-forming fungi (Girisham & Reddy, 1986; Pelhate, 1977; Mabrouk & El-Shayeb, 1981; Rusal and Marth, 1988). The minimum inhibitory concentrations of benzoic acid against a range of fungi lie in the range of 20->4000 µg/ml (Chipley, 1983; Davidson and Juneja, 1990).

There has been a limited investigation of the use of benzoic acid to protect fresh produce. Application of benzoic acid at 1% and hydrogen peroxide at 2% to root vegetables before storage in trenches controlled fungal and bacterial pathogens (Gorbunov *et al.*, 1994). Benzoic acid applied either as a dust mixed with kaolin (1:4) or in an alcohol solution at 2.5% was able to reduce stem end rot of pineapples from 34.3% to 5.0 and 9.2% respectively over a four days storage period (Mallikarjunaradhya *et al.*, 1979). Sodium benzoate in the range of 0.1-0.3% controlled banana spoilage in Nigeria (Okonkwo *et al.*, 1990).

Several factors may influence the effectiveness of benzoic acid in the treatment of fresh fruits and vegetables, with perhaps the most pertinent consideration being the pH of the superficial tissues (Eckert, 1967). Rahn & Conn (1944) reported that benzoic acid is approximately 100 times more effective as an antimicrobial agent in acid than neutral solutions due to the undissociated molecular form which is able to pass through the cell membrane more readily than the charged form. Benzoic acid is produced by immature apples infected by pathogens such as *Nectria galligena* and acts as an antifungal agent. As the fruit matures, the benzoic acid is no longer toxic to fungi. It is thought that this is due to increases in the pH of the apple tissues (Brown and Swinburne, 1971).

In the undissociated state, the benzoate ion is soluble in cell membranes and can apparently act as a proton ionophore disrupting normal membrane transport activities (Gould *et al.*, 1983). Bosund (1962) presented evidence showing that benzoic acid interferes with many enzymatic processes in microorganisms. It has been shown that benzoic acid at 0.4 mg ml^{-1} can totally suppress pectic enzyme activity *in vitro* and it is suggested that this may be a primary mechanism in the control of soft rotting fungi e.g. *A. niger*, *B. theobromae*, *Candida* and *Cladosporium* spp. by benzoic acid (0.32 mg l^{-1}) on yam tubers (Obi & Moneke, 1986).

Propionic and acetic acids have been used extensively to control mould growth in grain crops stored at high moisture contents (Dixon and Hamilton, 1981). Beuchat and Golden (1989) showed that the antifungal activity of propionic acid is weak when compared to other organic acids. Therefore, propionates must be used in relatively high concentrations to be effective. The pK_a of propionic acid is 4.87 and at a pH of 4.00, 88% of the compound is undissociated which is the form necessary for its antimicrobial activity (Jay, 1996). There is evidence that propionic acid accumulates in the cells of microorganisms and inhibits various enzymes (Beuchat and Golden, 1989).

Salinas *et al.* (1987) found that dipping asparagus spears in 0.5 or 1.0% propionic acid followed by storing them in perforated polyethylene bags for 28 days at 2°C and 85-90% r.h. reduced the fungal rots caused by *Penicillium* and *Rhizopus* spp.. A post-harvest disease of banana in Delhi markets caused by *Cochliobolus lunatus* was controlled for up to 8 days by propionic acid, salicylic and sodium metabisulphite. (Ved-Ram *et al.*, 1986). Acetic and propionic acid (1%) were found to control *B. theobromae* and *G. musarum* (*Colletotrichum musae*) *in vitro* and also when artificially inoculated fruit were dipped in the acids for 15 min. The dip treatment gave as good a control as a 15 min treatment with 200 or 400 ppm benlate or TBZ (Michail *et al.*, 1988).

Al Zaemey *et al.* (1993) tested eight organic acids and some of their salts *in vitro* against *C. musae* (0-3% w/v). All the acids significantly reduced the growth and complete inhibition of growth was achieved with potassium sorbate and sodium benzoate at 0.125% and maleic, oxalic and propionic acids at 0.05%. All the acids increased the lag period prior to growth initiation.

Tannic acid has been shown to suppress the growth of *Rhizopus*, *Drechslera*, *Trichoderma* spp, and *Diplodia pinea*, fast growing fungi commonly associated with *B. theobromae* on *Pinus elliotti* seeds (Cilliers *et al.*, 1994).

Parabens

The parabens are esters of *p*-hydroxybenzoic acid that have been used as food preservatives for a long time. The main parabens in use are the methyl, ethyl, propyl, butyl and heptyl parabens although not all of these are permitted for use in food in any one country. In the United States, methyl and propyl parabens are permissible in foods up to 0.1%. The pK_a value of the parabens is around 8.5 and unlike the benzoic acid and sodium benzoate, the antimicrobial activity of parabens is not strongly dependent on pH (Jay, 1996). Their effectiveness is, however, increased as the acidity of the surrounding medium increases.

The antimicrobial activity of the propyl paraben has been evaluated against a wide variety of gram-negative and gram-positive food-related bacteria and against some fungi (Aalto *et al.*, 1953). In comparison to bacteria, fungi are much more susceptible to the parabens. Thompson *et al.* (1993) found that 200 $\mu\text{l ml}^{-1}$ propyl paraben at pH 4 completely inhibited conidial germination and mycelial growth of *Fusarium* and *Penicillium* spp.. Thompson (1994) reported that on an individual basis, minimum inhibitory concentration (MIC) of butyl and propyl paraben were most effective, with complete *in vitro* mycelial inhibition at 1.0 to 2.0 mM concentration for the majority of the test fungi (*Aspergillus*, *Fusarium*, and *Penicillium* spp.).

Unlike the organic acids described above, the parabens do not seem to have specific effects against enzymes. Rather they appear to act mainly at the cell membrane eliminating the ΔpH component of the protonmotive force and thus disrupting energy transduction and substrate uptake (Adams and Moss, 1995).

Phenolic antioxidants

Antioxidants are substances capable of delaying or preventing oxidation processes. They are used in foods to reduce the auto-oxidation of lipids that can lead to off-flavours. Some of the most commonly used antioxidants are low molecular weight phenols, for example, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone (TBHQ) and propyl gallate (PG) (Schuler, 1990). A number of phenolic antioxidants which are permitted in food-stuffs have been found to also possess antimicrobial activity (Shih and Harris, 1977; Branen *et al.*, 1980; Raccach, 1984; Fung *et al.*, 1985).

The phenolic antioxidants have been tested against a number of gram negative and gram positive bacteria important in food poisoning, various spoilage yeasts and fungi and a few viruses and protozoa (Fung *et al.*, 1985). In general the antibacterial activity of BHT has been shown to be less effective than either BHA or TBHQ (Sankaran, 1976; Robach & Pierson, 1979; Lahellec *et al.*, 1981).

There is relatively little information available concerning the efficacy of phenolic antioxidants against fungi. Most of the work has been carried out on mycotoxigenic genera of fungi i.e. *Aspergillus*, *Fusarium* and *Penicillium* spp. (Beuchat and Jones, 1978; Lin and Fung, 1983; Thompson, 1992;1997). These authors have shown that BHA was the most effective in reducing the growth of mycotoxigenic fungi while TBHQ was quite effective in controlling *Fusarium* spp.. BHT and propyl gallate were ineffective against these fungi.

Antioxidants (BHA, BHT and TBHQ) at a concentration of 20 mg kg⁻¹ significantly reduced the growth and luteoskyrin production by two toxigenic strains of *Penicillium islandicum* (Tseng and Tseng, 1995). Arnoldi *et al.* (1989) reported that some antioxidants including BHT and propyl gallate controlled *Sphaerotheca fuliginea* on cucumber, *Uromyces appendiculatus* and *Colletotrichum lindemuthianum* on French bean, and *Phytophthora infestans* on potato.

Various possible mechanisms have been proposed for the mycotoxic effects of phenolic antioxidants (Raccach, 1984). There is evidence that they interfere with the membrane lipid structure. BHA causes cellular leakage in bacteria (Degre and Sylvestre, 1983; Davidson and Branen, 1980a). Increased leakage of sugars, amino acids and proteins from fungal mycelia has been detected suggesting disruption of the membranes (Thompson, 1996a;b).

Studies on the protozoan *Tetrahymena pyriformis* showed that BHA at 20 ppm inhibited DNA, RNA and protein synthesis (Surak *et al.*, 1976a). They reported that BHA had no effect on the cell membrane, however Surak *et al.* (1976b) later reported that BHT did disrupt the cell membrane and Surak and Singh (1980) showed that BHA altered the lipid composition of the membranes of *T. pyriformis*. BHT increased the membrane fluidity of liposomes (Singer and Wan, 1977). The antimicrobial activity of BHA was found to be greatly reduced in the presence of lipids and surfactants (Klindworth *et al.*, 1979). It was suggested that the BHA becomes localised in the lipid portion of the medium becoming unavailable to act against the microorganisms.

Other antioxidants

Parida *et al.* (1991) reported the treatment of freshly harvested aubergines, green pepper and tomatoes with carbendazim, thiourea, boric acid and potassium metabisulphate. All of the chemicals controlled storage decay to varying degrees. Thiourea and boric acid were shown to control storage rot of taro (*Colocasia*

antiquorum) *in vivo* and to inhibit mycelial growth of *Geotrichum candidum* *in vitro* (Mishra *et al.*, 1994).

Elad (1992) tested the ability of a broad range of antioxidants (free radical scavengers) at concentrations of 0.1-10 mM to control grey mould (*Botrytis cinerea*) and white mould (*Sclerotinia sclerotiorum*) on various crops and *in vitro*. The chemicals tested included ascorbic acid, benzoic acid, BHA, BHT, TBHQ, *p*-coumaric acid, dimethylsulphoxide (DMSO), ferulic acid, gluconic acid lactone, nordihydroguaiaretic acid, PG, protocatechuic acid, tannic acid, thiopropionic acid, thiopropionic acid lauryl sulphate, thiourea and tocopherol.

When the tested antioxidants were added to potato dextrose agar it was found that the inhibition of the mycelial growth or conidial germination of *B. cinerea* or *S. sclerotiorum* depended on the isolate used. For *B. cinerea*, TBHQ completely inhibited the fungus as 1.0 mM and TBHQ and tannic acid at 0.1 mM significantly inhibited growth of two of the three isolates tested. At 10 mM, most of the chemicals tested gave a significant inhibition of growth for at least one of the isolates. For *S. sclerotiorum*, benzoic acid and thiourea at 10 mM were completely inhibitory.

BHT, tannic acid, ascorbic acid and DMSO at a concentration of 1 mM controlled grey mould of tomato fruit. All these compounds except BHT controlled disease on cucumber fruit (grey mould and white mould). Gluconic acid lactone, thiourea and PG reduced white mould (*Sclerotinia sclerotiorum*) on lettuce by 51-76%. It is suggested that the antioxidants can have a direct toxic effect on the pathogen or may reduce the susceptibility of the host tissues by reducing tissue senescence.

Another potential mechanism for antioxidants in disease control was demonstrated by Prusky (1988). He reported that BHT, tocopherol, tert-butylhydroquinone and epicatechin delayed the appearance of anthracnose and stem end decay in avocado after harvest. These chemicals also delayed the disappearance of endogenous epicatechin and a diene. It has been shown that the diene is a physiologically

important agent which helps maintain resistance to *Colletotrichum gloeosporioides* (*Glomerella cingulata*) (Prusky *et al.*, 1991). It appears that the antioxidants prevent the oxidative breakdown of diene by the enzyme lipoxygenase (Prusky, 1988).

Combining chemicals to improve disease control

No chemical has been shown to be completely effective against all microorganisms. In theory, it should be possible to combine various chemicals which have different modes of action to compensate for this deficiency. As a result of this combination technique it should be possible to achieve a broader spectrum of action or an increased antimicrobial action (Lueck, 1980). Additive, synergistic, and antagonistic effects reported in several studies have been extensively reviewed by Lueck (1980) for benzoic acid, as well as for other preservatives.

Synergistic antimicrobial effects have been shown for combinations of plant extracts, organic acids, antioxidants and fungicides. For example, synergistic effects were found when BHA was combined with the essential oil carvacrol and tested against six toxigenic *Fusarium* spp. (Thompson, 1996b). Narasimhan *et al.* (1989) reported the synergistic interaction of 750 ppm potassium sorbate and 100 ppm of BHA on the keeping quality of butter samples. They observed that synergistic interaction lowered yeast and mould counts, and free fatty acid content. Elad (1992) showed that combinations of antioxidants were more effective than any compound alone when tested on pepper and tomato. Ascorbic acid and citric acid improved the control of *B. cinerea* by BHT, PG, benzoic acid and tert-butylhydroquinone on tomato leaves.

1.6 RATIONALE, AIM AND OBJECTIVES

Important postharvest diseases of banana have been controlled in the past by combining cultural and sanitation practices with the use of benzimidazole fungicides such as thiabendazole. As has been described in the literature review, disease control breaks down when resistant strains of pathogens such as *C. musae* have appeared to

benzimidazole fungicides. Alternative fungicides such as imazalil have so far retained their effectiveness against banana pathogens. Should, however, resistance appear to this type of fungicide, there are few alternatives available.

New fungicides are extremely expensive to develop and there is consumer concern about the use of chemical treatments in general. It seems appropriate therefore to look for ways of extending the use of currently approved postharvest fungicides at the same time as seeking alternatives, both chemical and non-chemical. To date, non-chemical strategies such as the use of MAP, heat treatments and biological control seem unlikely to give sufficient disease control without some use of antimicrobial chemicals.

It is clear from the literature that many food-grade organic acids, antioxidants and natural plant extracts have useful antimicrobial activities that have not been exploited in postharvest disease control. It was decided therefore to investigate the effect of these chemicals on *C. musae* and its associated diseases on the banana fruits (anthracnose and crown rot). The overall aim of this study was therefore to identify chemical treatments which could be used as alternative or adjuncts to fungicides for the control of diseases of banana caused by *C. musae*.

The specific objectives of the research were therefore:

1. *In vitro* studies

- To screen isolates of *C. musae* for their sensitivity / resistance to the fungicides thiabendazole and imazalil.
- To examine the potential of the plant extract, azadirachtin, at different concentrations for the control of germination and mycelial growth of *C. musae*.
- To determine the effect of different antioxidants, alone, or as mixtures, or when combined with fungicides, for control of spore germination, lag time for growth initiation, mycelial growth and extracellular enzyme production of *C. musae*.

2. *In vivo* studies

- To ascertain the effect of azadirachtin and the most promising antioxidants alone or combined with fungicides on the expansion of anthracnose and crown rot lesions. Chemicals were tested in the following ways:
 - a) As protectants before the establishment of infection using conidial suspensions of *C. musae*.
 - b) As eradicants after establishment of infection using conidial suspensions of *C. musae*.
 - c) After infections established by inoculation of wounds on the fruit surface with conidia or mycelium of *C. musae*.
- To estimate the likely cost of any promising new treatments versus conventional fungicides used at recommended rates.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF FRUIT SAMPLES

Fruits (*Musa* AAA Giant Cavendish) used in some experiments were obtained from Wilkinson Banana Ltd, Bedford, UK. Other fruits were organic (no pesticides had been used during their production or post-harvest care) and were obtained from S.H. Pratt & Co. (Bananas) Ltd., Luton, UK. All the fruit was obtained as clusters of 5-7 fingers, approximately $\frac{3}{4}$ fully matured and originating from various countries in the Caribbean and Latin America. They had not been treated commercially with ethylene. All fruit was held at 14°C, 85-90% relative humidity (r.h.) until required for experiments. Clusters of fruits for each experiment were chosen to be of approximately of uniform maturity, size and appearance. Fruits with visible defects were discarded.

2.2 FUNGAL ISOLATION AND STORAGE

The fungal isolates of *Colletotrichum musae* were obtained from anthracnose lesions found on commercially ripened bananas from UK retail stores. The four isolates (CM100, CM101, CM103 and CM104) were maintained on 5% malt extract agar (MEA) at 4°C, and sub-cultures was made prior to use for experiments and grown on MEA at 22°C. Further sub-cultures were made every week or when required for each series of experiments. No obvious changes in the cultural characteristics of the isolates or their virulence on banana fruits were observed over the research period.

2.3 SOURCE OF CHEMICALS

MEA came from Oxoid Ltd, Basingstoke, Hants., UK and mycological peptone came from The Microbiological Supply Company, Toddington, Beds., UK). The chemicals (ascorbic acid, dimethyl sulfoxide (DMSO), propionic acid, thiourea, benzoic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), propyl paraben (PP) and tannic acid) were purchased from Sigma Chemical Co.

Ltd., Poole, Dorset, UK Storate Flowable (thiabendazole, a.i 45%) from MSD Agvet, Hoddesden, Herts., UK, fungazil (imazalil, a.i. 9.26%) from Rhone Poulenc AGR Ltd., Ongar, Essex, UK, ethanol and trypan blue stain were from Merck Ltd., Lutterworth, Leics., UK and azadirachtin from Cardiff Chemical Ltd., Cardiff, UK.

2.4 MEDIA PREPARATION

General medium preparation was carried out by dissolving 50 g of MEA in 1000 ml of distilled water, heating it on a magnetic stirrer for about one hour and autoclaving (Prestige Medical 2075) at 120°C for 20 min. The molten medium was cooled to 50°C before pouring into plastic sterile Petri plates (85 mm internal diameter) in a laminar air flow cabinet. The medium was allowed to cool prior to storage at 6°C in an airtight incubator (Termaks Series 2000 KBP). Chloramphenicol at 0.6189 mM (200 ppm) was dissolved in 94.9% ethanol and added to the agar before sterilisation to suppress bacterial development.

All *in vitro* studies, except for enzymatic studies, were carried out on MEA. Enzymatic studies were carried out in 3% malt extract broth containing 0.5% mycological peptone. Conical flasks containing liquid medium were sterilised by autoclaving at 120°C for 20 min.

2.5 MEASUREMENT OF pH

The pH of MEA with or without chemical amendments was determined with a hand-held pH meter (Jenway 924-001) while the agar was still liquid at 50°C.

The pH of the banana surface was measured before and after the chemical treatment. This was obtained by holding the flat electrode of the pH meter (model 3150) in contact with the surface of the banana peel. The pH of chemical dips used as fruit treatments was also determined with a pH meter.

2.6 PREPARATION OF INOCULUM

2.6.1 Conidia

Twelve days old cultures of *C. musae* grown on Petri plates containing MEA and incubated at 22°C were used as a source of spores (conidia). Plates were flooded with 20 ml of sterile distilled water and the colony surface was agitated with a sterile glass rod to dislodge the conidia. The suspension was filtered through sterile muslin cloth and centrifuged twice at 1200 g using a Sorvall Centrifuge (with an SS-34 rotor) for 5 min and re-suspended in sterile distilled water to remove germination inhibition factors that appear to be present in the conidial matrix (Mondal & Parberry, 1992). Conidial concentration was determined using a haemocytometer (Weber Scientific Ltd.) and was adjusted to the required concentration using sterile distilled water.

2.6.2 Mycelium

Small disks (4 mm diameter) of cold-stored *C. musae* colonies on MEA were sub-cultured onto fresh MEA. Mycelium disks were removed taken from the leading edge of these sub-cultures when they were about 10-12 days old and used for fruit inoculation.

2.7 DETERMINATION OF THE EFFECTS OF CHEMICALS ON *C. MUSAE* GROWTH AND DEVELOPMENT *IN VITRO*

2.7.1 Preparation of chemicals and adjustment of pH

Stock solutions of chemicals were prepared in either distilled water (ascorbic acid, DMSO, propionic acid, thiourea, Storite Flowable (thiabendazole, a.i 45%) and Fungazil (imazalil, a.i. 9.26%) or in 94.9% ethanol (benzoic acid, BHA, BHT, PG, PP, tannic acid and azadirachtin 5%) and added to the autoclaved, molten MEA at

50°C to obtain the required concentrations (Table 2.1). In some experiments, the pH of the medium was adjusted to 3.5, 4.5, 5.5 and 6.5 with citrate phosphate buffer and 7.5 with phosphate buffer prior to autoclaving at 120°C for 20 min.

2.7.2 Conidial germination studies

Fresh conidial suspensions were prepared for each experiment. After vigorous agitation to prevent conidial sedimentation, 0.1 ml of suspension was pipetted onto the surface of each treatment Petri plate. The conidia were spread over the surface of the plate using a surface-sterilised glass spreader. Plates were incubated at 14°C or 22°C. At different time intervals, disks of the agar (18 mm diameter) were removed from random positions on the agar surface using a cork borer. These disks were placed on glass microscope slides and stained with lactophenol / trypan blue (which also kills the spores) and stored within Petri dishes to protect them from desiccating. The disks were covered with a glass cover slip before examination under a light microscope (Labophot, Nikon, Japan). Conidia were considered to have germinated when the germ tube length was equal to or greater than the conidium diameter (Magan, 1988). A total of 200 conidia were examined from each of three disks (= 600 conidia) for each time interval, and for each treatment. Generally, three replicate plates per treatment were used in each experiment.

In some studies of germination, the conidia were given a pre-treatment with BHA or imazalil by suspending them in a solution of the chemical at the required concentration for 30 or 60 min. The suspensions were then centrifuged at 1200 g using a Sorvall Centrifuge (with an SS-34 rotor) for 5 mins and washed by re-suspending the conidia in sterile distilled water. The centrifugation and wash routine was repeated and then the conidia were spread over the surface of Petri plates containing MEA amended with imazalil or BHA. The plates were then incubated at 25°C and the percentage germination was assessed as described above.

Table 2.1. Concentrations of the chemicals tested *in vitro* against conidial germination and mycelial growth of *Colletotrichum musae*.

Antioxidants	Concentration
Ascorbic acid	1 - 15 Mm
Propionic acid	1 - 15 mM
Thiourea	1 - 15 mM
Dimethyl sulphoxide	1 - 15 mM
Tannic acid	1 - 5 mM
Butylated hydroxyanisole	0.03125 - 15 mM
Butylated hydroxytoluene	1 - 15 mM
Propyl gallate	1 - 15 mM
Propyl paraben	1 - 15 mM
Benzoic acid	1 - 15 mM
Fungicides	
Imazalil	0.00168 – 1.6825 mM
Thiabendazole	0.00248 – 2.4851 mM
Antioxidants + Fungicide	
BHA + Imazalil	0.03125 – 0.5 + 0.00168 mM
BHA + Imazalil	0.5 + 0.0034 mM
BHA + Imazalil	0.5 + 0.0084 mM
BHA + Imazalil	1 + 0.5047 mM
BHA + Imazalil	1 + 0.8412 mM
BHA + Imazalil	5 + 0.1682 mM
BHA + Imazalil	5 + 0.5047 mM
BHA + Imazalil	5 + 0.8412 mM
BHA + TBZ	0.03125 – 0.5 + 0.00248 mM
Mixtures	
BHA + PP	0.1 + 5 mM
BHA + PP	0.5 + 5 mM
BHA + PP	1 + 1 mM
BHA + PP	1 + 5 mM
BHA + PP	5 + 5 mM
BHA + PP	5 + 10 mM
BHA + Benzoic acid	0.1 + 5 mM
BHA + Benzoic acid	0.5 + 5 mM
BHA + Benzoic acid	1 + 1 mM
BHA + Benzoic acid	1 + 5 mM
BHA + Benzoic acid	5 + 5 mM
BHA + Benzoic acid	5 + 10 mM
Azadirachtin	0.5 and 1 mM

BHA = butylated hydroxyanisole, TBZ = thiabendazole, PP = propyl paraben

2.7.3 Mycelial growth studies

Petri plates were inoculated centrally with disks (4 mm diameter) from the colony margin of a 9 -11 day old culture of *C. musae*. Four replicates per treatment were sealed with Nescofilm and incubated at either 14°C or 22°C. Temporal growth was determined by measuring the diameter of the colony in two directions at right angles to each other for up to 15 days. The diametric extension rates were plotted and regression lines of the linear phase were used to determine the growth rate (mm day⁻¹) for each treatment. The lag time for mycelial extension to occur from the edge of the inoculum disk was also recorded.

2.7.4 Determination of the best *in vitro* treatments:

Mycelial growth rates were plotted against chemical concentration and the inhibition of diametric growth (LD₅₀ 50% and LD₉₀, 90 % inhibition of growth) determined.

2.7.5 Testing for extracellular enzymes using the API ZYM system

For enzymatic studies, six to seven day-old cultures grown on MEA and incubated at 25°C were used as a source of conidia. Conidial suspensions (10⁵ ml⁻¹) were prepared as described above (Section 2.7.2). After vigorous agitation to prevent conidial sedimentation, 1 ml was pipetted into each conical flask containing 3% MEB amended with 0.5% mycological peptone. The flasks were agitated at 25°C for 10 days on an orbital shaker. Culture filtrates were centrifuged for 5 min and the activity of certain extra-cellular enzymes (Table 2.2) was tested after 3, 6 and 10 days to determine the period when maximum enzyme activities were expressed.

Enzyme activities were determined using the API ZYM system (API system S. A., Montalieu Vercieu, France). This is a semi-quantitative micro-method that allows a rapid and simultaneous evaluation of 19 enzymatic activities using very small amounts

of sample. The substrates are contained in support strips, which allow contact between the enzyme and substrate. Enzymatic activity is revealed by the addition of suitable indicators. The enzymes detected by the API-ZYM system and their respective substrates are listed in Table 2.2.

All chemical treatment tests were subsequently carried out on day six when the fungus was expressing maximum enzyme activity. Each cupule of the API strip was inoculated with 65 μ l of culture filtrate and incubated for 4 hours at 37°C. After incubation, one drop (35 μ l) of ZYM A reagent and an equal amount of ZYM B reagent were added simultaneously. After the colours were visible the strips were placed under a strong light source for 30 seconds to eliminate non-reacted Fast Blue so that negative results become colourless. Results were recorded using the colour chart (Appendix A) supplied with the API ZYM kit.

2.8 DETERMINATION OF THE EFFECTS OF CHEMICALS ON *C. MUSAE* GROWTH AND DEVELOPMENT *IN VIVO*

2.8.1 Fruit preparation

In some experiments, banana fruit clusters were cut into fingers, left for about an hour for de-latexing and then were dipped briefly in 50% ethanol (Muirhead and Deverall, 1981) to make the fruit surface wettable. The fingers were then surface-sterilised by dipping in 1% active sodium hypochlorite solution for 3 min followed by immersion in sterile distilled water. Then the fruits were air-dried. In other experiments on crown rot infection, clusters of hands were kept intact. The surface of the crowns were surface sterilised and then trimmed to give a flat surface before inoculation was carried out.

Table 2.2: Enzymes assayed using API ZYM system.

No	Enzymes assayed	Substrate	pH	Results
1	Control	-		
2	Phosphate alkaline	2-naphthyl phosphate	8.5	Violet
3	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet
4	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet
5	Lipase (C 14)	2-naphthyl myristate	7.5	Violet
6	Leucine arylamidase	L-leucyl-2- naphthylamide	7.5	Orange
7	Valine arylamidase	L-valyl-2- naphthylamide	7.5	Orange
8	Cystine arylamidase	L-cystyl-2- naphthylamide	7.5	Orange
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange
11	Phosphate acid	2-naphthyl phosphate	5.4	Violet
12	Naphthol-AS-BI- phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue
13	α galactosidase	6-Br-2- naphthyl- α D-galactopyranoside	5.4	Violet
14	β galactosidase	2- naphthyl- β D-galactopyranoside	5.4	Violet
15	β glucuronidase	Naphthol-AS-BI- β D-glucuronide	5.4	Blue
16	α glucosidase	2- naphthyl- α D-glucopyranoside	5.4	Violet
17	β glucosidase	6-Br-2- naphthyl- β D- glucopyranoside	5.4	Violet
18	N-acetyl- β glucosaminidase	1-naphthyl-N-acetyl- β D-glucosaminide	5.4	Brown
19	α mannosidase	6-Br-2- naphthyl- α D-mannopyranoside	5.4	Violet
20	α fucosidase	2- naphthyl- α L-fucopyranoside	5.4	Violet

2.8.2 Inoculation of fruit peel with conidia of *C. musae*

A conidial suspension of *C. musae* was prepared as described above (section 2.7) and the concentration was adjusted to 10^6 ml^{-1} . The suspension (10 μl) was gently applied over the marked surface of the fruit for infection by using tube rings made of polyvinyl chloride (PVC). The diameters (external and internal) and thickness of the tube ring were 8 mm, 5 mm and 1.5 mm respectively. The tube rings were held on the surface of fruit using silicon grease. The fruits were then kept at 95% r.h. at 25°C for 48 hours to allow the infection to establish and then the fruits were treated with 1000 ppm ethylene for 24 h to initiate ripening. The fruit was treated with various chemicals (see below) before or after inoculation depending on the experiment.

2.8.3 Wound inoculation of fruit peel with mycelium of *C. musae*

Artificial wounds were made on the surface of the banana fruits using a 4 mm cork borer to remove peel tissue (Plate 2.1).

2.8.4 Inoculation of fruit stalks or crowns with conidia of *C. musae*

Either single fingers or whole banana clusters were used in crown rot experiments. When single fingers were used to simulate crown rot, the cut end of the stalk was inoculated with 10 μl of conidial suspension held in place using a tube ring as described above (section 2.8.2). When clusters were used, the area of each crown was measured using an image analyser (AMS Optomax V image analyser). The volume of conidial suspension applied to the crown was in proportion to this area in order to provide a similar density of conidia over the surface area for each crown. The crowns were then treated with various chemicals (see below) before or after inoculation depending on the experiment.

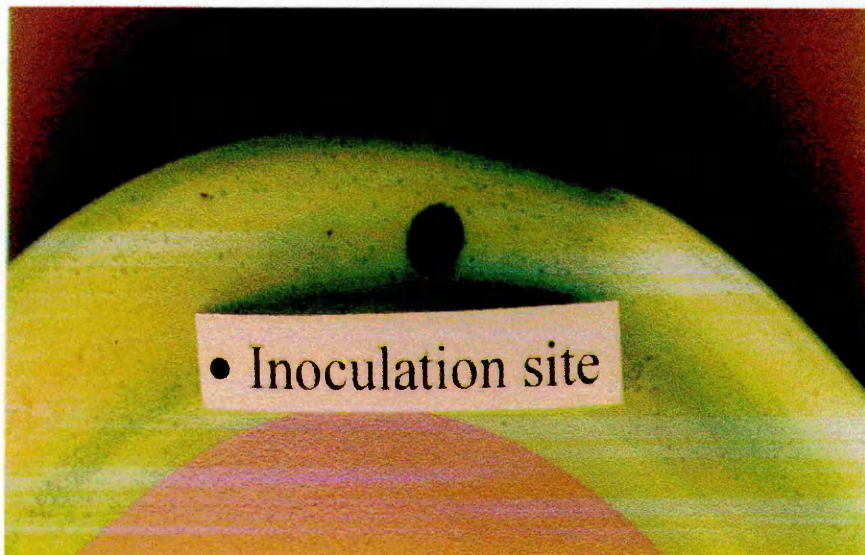


Plate 2.1: Shows the site of inoculation with a disk of *Colletotrichum musae* mycelium on malt extract agar.



Plate 2.2: Shows the infection site (IS) for *Colletotrichum musae* spore inoculation on banana crowns.

2.8.5 Chemical treatment of fruit

Chemicals insoluble or only partially soluble in water (BHA, PP, PG, benzoic acid and azadirachtin A 15%) were dissolved in 30% acetone in sterilised distilled water. The water-soluble chemicals (TBZ and imazalil) were mixed in sterilised distilled water. The single and combined antioxidants, antioxidants + fungicide, and azadirachtin treatments used are listed in Tables 2.3 and 2.4.

Fruits were dipped for 30 s in the chemical solutions and allowed to dry. Depending on the experiment, this was either carried out prior to, or after the fruit had been inoculated with the pathogen. Control fruits were dipped in distilled water. A second control was dipped in 30% acetone in distilled water. In post-inoculation studies fruits were treated with the chemicals before inoculation and after one-hour, (when the fruits were dried) inoculation was carried out with a conidial suspension of *C. musae*. All experiments were carried out with four replicate bananas per treatment.

2.8.6 Fruit storage and assessment of disease levels

All fruits were kept in a controlled environment chamber at 14°C or 25°C and 95% r.h. for up to 14 days.

In all the anthracnose experiments carried out in this study, the lesion diameter (mm) of *C. musae* was measured in two directions at right angle to each other on a daily basis for up to 12 days. The crown rot lesions were measured from the inoculation zone towards the fruit centre. In the crown rot experiments with banana hands, lesion extension was measured in a similar way. The incidence of crown rots on banana hands was also evaluated on the basis of healthy and diseased fruits and the percentage infection calculated. The lag time for lesion extension to occur from the inoculation zone down through the pedicel was also measured.

Table 2.3: Treatments used against anthracnose of banana caused by *Colletotrichum musae*.

Treatments	Con.	Pre- inoculation + Pathogen	Post- inoculation + Pathogen	Pre- inoculation + Damaged skin surface
BHA	1 mM	+	-	+
BHA	5 mM	+	-	+
BHA	10 mM	+	+	+
Propyl paraben	5 mM	+	-	+
Propyl paraben	10 mM	+	-	+
Propyl paraben	15 mM	+	+	+
Benzoic acid	5 mM	+	-	+
Benzoic acid	10 mM	+	-	+
Benzoic acid	15 mM	+	+	+
Propyl gallate	5 mM	+	-	+
Propyl gallate	10 mM	+	-	+
Propyl gallate	15 mM	+	+	+
TBZ	2.4850 mM	-	+	-
Imazalil	0.1682 mM	+	-	-
Imazalil	0.5047 mM	+	-	-
Imazalil	0.8412 mM	+	-	+
Imazalil	1.6824 mM	+	+	+
BHA + PP	5 + 10 mM	+	-	+
BHA + BA	5 + 10 mM	+	-	+
BHA + Imazalil	5 + 0.1682 mM	+	-	-
BHA + Imazalil	5 + 0.5047 mM	+	-	-
BHA + Imazalil	5 + 0.8412 mM	+	-	+
Azadirachtin	1 mM	-	+	-

BHA = Butylated hydroxyanisole, TBZ = thiabendazole, PP = propyl paraben, BA = benzoic acid

Table 2.4: Treatments used against crown rot of banana caused by *Colletotrichum musae*.

Treatments	Concentration	Pre-inoculation + Pathogen	Post-inoculation + Pathogen
BHA	1 mM	+	-
BHA	5 mM	+	-
BHA	10 mM	+	+
Propyl paraben	5 mM	+	-
Propyl paraben	10 mM	+	-
Propyl paraben	15 mM	+	+
Benzoic acid	5 mM	+	-
Benzoic acid	10 mM	+	-
Benzoic acid	15 mM	+	+
Propyl gallate	5 mM	+	-
Propyl gallate	10 mM	+	-
Propyl gallate	15 mM	+	+
TBZ	2.4850 mM	+	+
Imazalil	0.1682 mM	+	-
Imazalil	0.5047 mM	+	-
Imazalil	0.8412 mM	+	+
Imazalil	1.6824 mM	+	+
BHA + PP	5 +10 mM	+	+
BHA + BA	5 +10 mM	+	+
BHA +Imazalil	5 + 0.1682 mM	+	-
BHA +Imazalil	5 + 0.5047 mM	+	-
BHA +Imazalil	5 + 0.8412 mM	+	+
Azadirachtin	1 mM	+	+

BHA = butylated hydroxyanisole, TBZ = thiabendazole and PP = propyl paraben, = benzoic acid

2.9 STATISTICAL ANALYSIS

All statistical analyses were carried out using complete randomised design (CRD) in the M Stat-c package. One-way analysis of variance (ANOVA) was carried out on replicate data sets for treatment level for each experiment. The error mean square (*EMS*) was obtained from ANOVA and used to calculate the Least Significant Difference (LSD) at ($p < 0.05$) between treatments according to the equation:

$$LSD = \sqrt{2/n} \cdot EMS \cdot F_{n_1, n_2}$$

Where n was the number of replicate values; n_1 and n_2 were the error degrees of freedom and F was the F value at $P < 0.05$.

In all experiments, lettering of treatments were done to high-light any significant difference between the treatments. This was achieved by using the Range Test in M stat-c package. The parameters used in Range Tests were error mean square (*EMS*) values, degree of freedom, number of replicates and total number of treatments in each experiment.

CHAPTER 3

RESULTS

STUDIES *IN VITRO* (I)

(THE CONTROL OF *C. MUSAE* BY INDIVIDUAL CHEMICALS)

3.1 INTRODUCTION

The investigation of the potential fungal toxicity of chemicals is generally carried out initially *in vitro*. This is a much quicker, cheaper and more convenient way of screening chemicals than using bioassays. Fungi have different tolerances to chemicals at different stages of their life cycle, for example, spores may be more or less tolerant than the actively growing mycelium. Generally, the germinating spore is the most sensitive to chemical treatments. It is therefore common practice to test chemicals against fungi at different stages in their development *in vitro*. When *C. musae* is grown on solid growth medium *in vitro* the main parts of its life cycle are spore germination, mycelial growth and sporulation (production of conidia in acervuli). In the experiments described below, spore germination and mycelial growth were considered but not sporulation.

As has been described in the literature review, anthracnose and crown rot of bananas are commonly controlled by postharvest applications of thiabendazole (Frossard, 1969; Rippon & Glennies-Holmes, 1973) but the treatment has been rendered less effective by the emergence of strains resistant to this fungicide (Hostachy *et al.*, 1990; Johanson & Blasquez, 1992). Imazalil has been the more expensive alternative chemical used in many banana growing areas when TBZ control breaks down. It is possible that isolates tolerant of one chemical might be tolerant of others, depending on the mode of action of the chemicals. In the search for new chemicals that might improve the control of banana diseases, it is therefore important to screen isolates of the relevant pathogens that show different levels of tolerance to TBZ and other fungicides.

3.2 EXPERIMENTAL SET UP

There were three replicate plates per treatment in all spore germination treatments and antioxidants were divided into two groups. Group1 comprised ascorbic acid, dimethyl sulphoxide, thiourea, tannic acid, butylated hydroxytoluene and propyl gallate. Group

2 comprised butylated hydroxyanisole, propyl paraben, propionic acid, and benzoic acid) and analyses have been performed separately for each group and each isolate after different time intervals. Tannic acid was only tested at 1 and 5 mM as at concentrations above this, the chemical crystallised out in the agar. For all mycelial growth experiments each of four replicates per treatment were sealed with Nescofilm and incubated at either 14 or 22 °C. A complete randomised design was used for both studies (conidial germination and mycelial growth). The antioxidants tested against mycelial growth were also divided in two batches (group 1 ascorbic acid, propionic acid, thiourea, dimethyl sulphoxide and tannic acid and group 2 butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, propyl paraben and benzoic acid) and analyses were performed separately for each group and each isolate.

3.3 GROWTH OF *C. MUSAE* ISOLATES ON MALT EXTRACT AGAR (MEA)

The four isolates of *C. musae* isolated for these experiments (CM100, CM101, CM103, CM104) had normal sigmoidal growth curves when grown on MEA in Petri dishes at 22°C (Figure 3.1). CM100 showed the most vigorous growth on MEA at 22°C (20.9 mm day⁻¹) reaching the edge of the Petri plate in 5 days. CM103 showed the lowest growth rate under the same conditions (14.3 mm day⁻¹) reaching the edge of the Petri plate in 8 days.

3.4 SCREENING AGAINST FUNGICIDES

3.4.1 Initial screening

The tolerance of four isolates of *C. musae* (CM100, CM101, CM103, CM104) to a range of concentrations of the fungicides thiabendazole (TBZ) and imazalil was tested (Table 3.1). Both fungicides at all concentrations tested in the range 0.0168 - 1.2425 mM, significantly ($P < 0.05$) reduced the mycelial growth rate (mm day⁻¹) of all the

isolates. Imazalil at 0.0168 to 0.8413 mM completely (100%) inhibited the mycelial growth rate (mm day^{-1}) of all the isolates. TBZ at concentrations of 0.0248 to 1.2425 mM also completely inhibited growth of CM100 and CM104. On the other hand, CM101 and CM103 were found to have some tolerance to TBZ. CM103 was the most tolerant showing some growth on agar containing TBZ up to 1.2425 mM concentration.

3.4.2 Screening against very low concentrations

Table 3.2 shows the effect of low concentrations of both fungicides on growth of all four isolates. Imazalil gave considerable control over the concentration range of 1.7 – 8.4 μM . The sensitivity of CM100 to TBZ was further demonstrated, with 2.5 μM reducing growth by 60%, when compared to the control. On the other hand, at 2.5 μM TBZ, CM103 still grew at 95.8% of the rate of the control although colony formation appeared abnormal (Plate 3.1 a & b). The LD_{50} values for each fungicide were calculated and are compared in Table 3.3. For all isolates, imazalil had a lower LD_{50} than TBZ.

All further work was carried out with one TBZ tolerant isolate (CM103) and in most cases, also with one TBZ sensitive strain (CM100).

3.5 SCREENING C. MUSAE AGAINST ALTERNATIVE COMPOUNDS

3.5.1 Effect on antioxidants on germination

The effect of 10 different antioxidants in the range 1-15 mM concentration on germination of CM100 and CM103 after 6, 24, 48, and 72 hours (hrs) were determined. Generally speaking, the pattern of inhibition with respect to concentration and type of chemical was similar between CM100 (Tables 3.4 and 3.5) and CM103 (Tables 3.6 and 3.7), however, CM100 was more sensitive to the chemicals than CM103. Only five chemicals completely prevented germination after 72 hrs. These were thiourea, propyl gallate (PG) and propyl paraben (PP) at 1 mM and BHA and

Table 3.1: Effect of imazalil and thiabendazole at different concentrations on the mycelial growth of four isolates of *Colletotrichum musae* on malt extract agar at 22 °C (mm growth day⁻¹).

Treatments	Concen. (mM)	Isolates			
		CM100	CM101	CM103	CM104
Imazalil	0.0168	NG ^a	NG ^e	NG ^g	NG ^c
Imazalil	0.0336	NG ^b	NG ^e	NG ^g	NG ^c
Imazalil	0.1683	NG ^b	NG ^e	NG ^g	NG ^c
Imazalil	0.3365	NG ^b	NG ^e	NG ^g	NG ^c
Imazalil	0.6730	NG ^b	NG ^e	NG ^g	NG ^c
Imazalil	0.8412	NG ^b	NG ^e	NG ^g	NG ^c
Thiabendazole	0.0248	NG ^b	2.7 ^b	5.5 ^b	NG ^c
Thiabendazole	0.0497	NG ^b	1.3 ^c	5.2 ^c	NG ^c
Thiabendazole	0.2485	NG ^b	0.3 ^d	3.8 ^d	NG ^c
Thiabendazole	0.4970	NG ^b	NG ^e	2.4 ^e	NG ^c
Thiabendazole	0.9941	NG ^b	NG ^e	1.6 ^f	NG ^c
Thiabendazole	1.2425	NG ^b	NG ^e	1.5 ^f	NG ^c
Control with antibiotic	0	20.8 ^a	17.2 ^a	14.3 ^a	20.1 ^a
Control	0	20.9 ^a	17.2 ^a	14.2 ^a	19.9 ^b
Lsd (p=0.05) =		0.2	0.1	0.1	0.06
CV% =		3.8	3.4	2.6	1.4

Means followed by the same letter do not differ significantly
*NG= no growth

Table 3.2: Effect of imazalil and thiabendazole at different low concentrations on the mycelial growth of four isolates of *Colletotrichum musae* on malt extract agar at 22°C (mm growth day⁻¹).

Treatments	Concen. (µM)	Isolates			
		CM100	CM101	CM103	CM104
Imazalil	1.68	8.5 ^c	11 ^d	11.5 ^d	10.3 ^b
Imazalil	3.40	6.5 ^e	5.6 ^f	5.9 ^e	5.3 ^c
Imazalil	8.40	3.5 ^g	2.4 ^h	4.6 ^g	2.2 ^d
Imazalil	16.80	NG ^{*h}	NG ⁱ	NG ^h	NG ^e
Thiabendazole	2.50	8.7 ^b	13.3 ^b	13.7 ^b	10.4 ^b
Thiabendazole	5.00	6.8 ^d	12.1 ^c	12.3 ^c	5.4 ^c
Thiabendazole	12.40	4.0 ^f	10.1 ^e	11.6 ^d	2.2 ^d
Thiabendazole	24.80	NG ^h	2.6 ^g	5.5 ^f	NG ^e
Control	-	21.7 ^a	17.2 ^a	14.3 ^a	19.9 ^a
Lsd (p=0.05) =		0.2	0.2	0.2	0.1
CV% =		1.7	1.5	1.6	1.5

Means followed by the same letter do not differ significantly

*NG = no growth

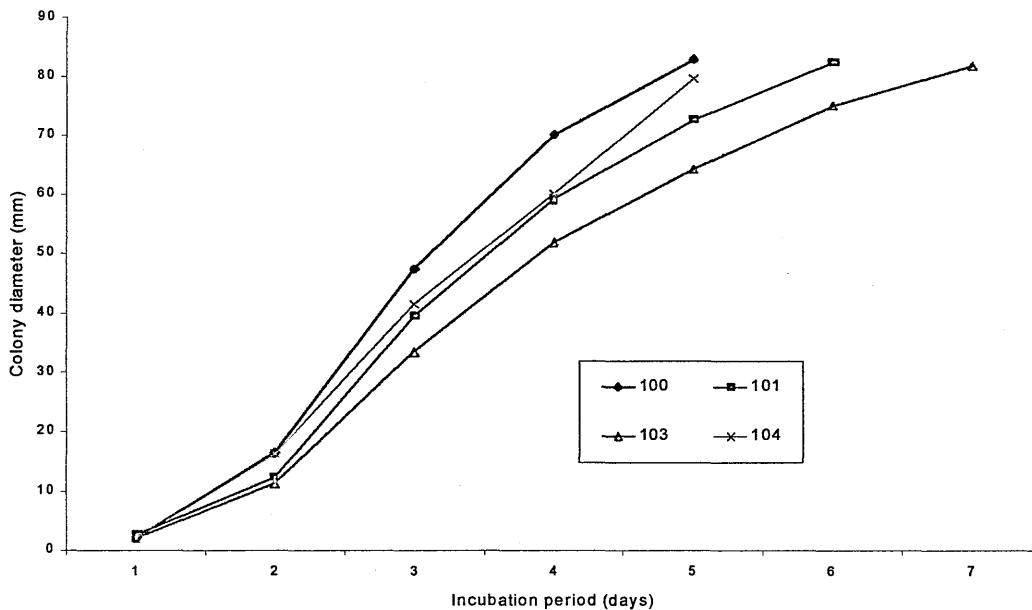


Figure 3.1: Colony diameter of four isolates of *Colletotrichum musae* (CM100, CM101, CM103 & CM104) on malt extract agar in 8.5 cm diameter Petri dishes at 25°C.

Table 3.3: LD₅₀ values for the fungicides imazalil and thiabendazole used against four isolates of *Colletotrichum musae*. LD₅₀ values were determined from the relationship between growth rate (mm day⁻¹) and fungicide concentration.

Isolate	LD ₅₀ values	
	Imazalil	Thiabendazole
CM100	1.58 µM	2.33 µM
CM101	2.25 µM	14.6 µM
CM103	3.16 µM	16.4 µM
CM104	1.95 µM	2.48 µM

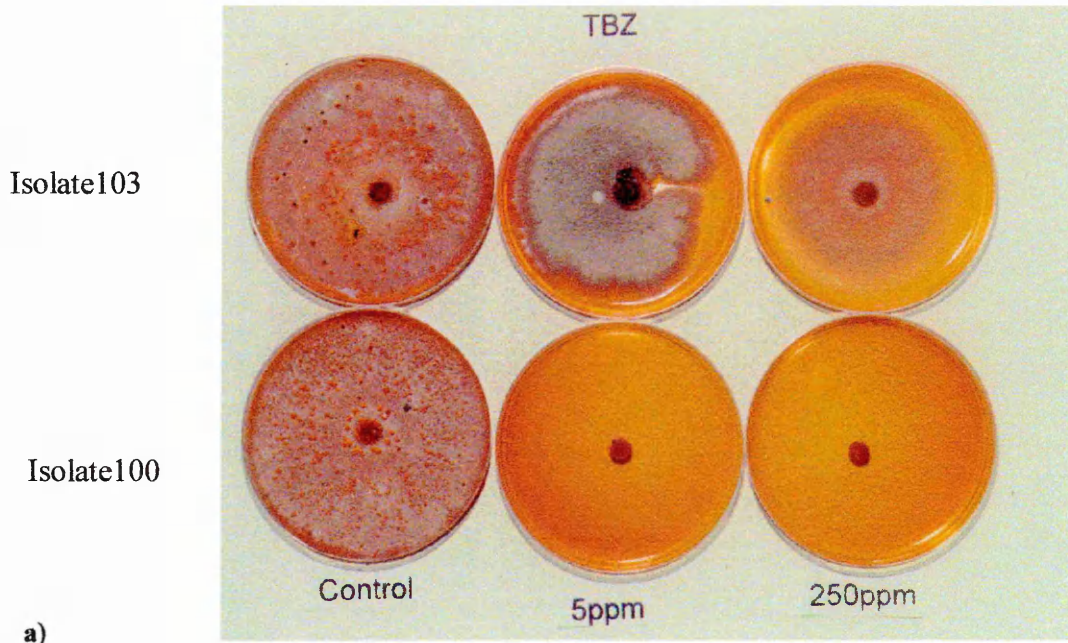
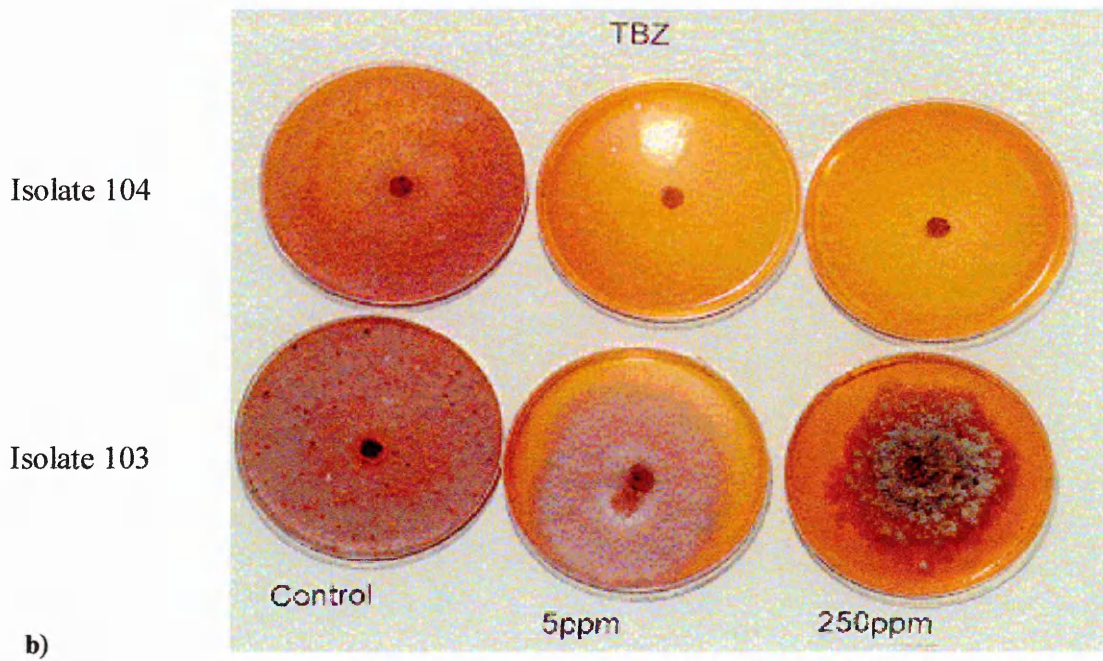


Plate 3.1 a and b: Effect of thiabendazole (at 5 ppm = 0.0248 mM & 250 ppm = 1.2425 mM) on the mycelial growth of *Colletotrichum musae*. (isolate 100, 103 & 104) on malt extract agar 30 days after inoculation at 25 °C.

benzoic acid at ≥ 5 mM. DMSO appeared to be completely ineffective with no control apparent at 6 hrs after germination (1- 15 mM) and ascorbic acid was not much better, with no control at these concentrations seen by 48 hrs after germination. For thiourea and PG, no inhibition of germination was seen after 48 hrs for concentrations up to 10 mM or 5 mM respectively. Tannic acid at 1 mM concentration was not effective at controlling germination after 6 hrs. Propionic acid at 1 to 15 mM delayed the germination rate for a minimum of 24 hrs when compared to untreated controls. The most inhibitory chemicals were therefore benzoic acid, BHA and PP. BHA and PP gave considerable and significant control over the three days of the experiment at all concentrations tested. Benzoic acid was only slightly less effective with poorer control at 1 mM than the other two.

Table 3.4: Effect of antioxidants on temporal conidial germination of *Colletotrichum musae* (isolate CM100) on malt extract agar at 22°C.

Treatments	Conidial germination (%) after different time intervals			
	6 hrs	24 hrs	48 hrs	72 hrs
Ascorbic acid 1 mM	53.7 ^{abc}	100 ^a	100 ^a	100 ^a
Ascorbic acid 5 mM	52.7 ^{bcd}	100 ^a	100 ^a	100 ^a
Ascorbic acid 10 mM	52.3 ^{cd}	100 ^a	100 ^a	100 ^a
Ascorbic acid 15 mM	51.0 ^{de}	88.5 ^b	100 ^a	100 ^a
Dimethyl sulphoxide 1 mM	55.0 ^a	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 5 mM	55.0 ^a	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 10 mM	55.2 ^a	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 15 mM	55.5 ^a	100 ^a	100 ^a	100 ^a
Thiourea 1 mM	54.2 ^{abc}	100 ^a	100 ^a	100 ^a
Thiourea 5 mM	33.8 ^g	88.0 ^b	100 ^a	100 ^a
Thiourea 10 mM	20.5 ^j	63.7 ^d	100 ^a	100 ^a
Thiourea 15 mM	NG ^{*abc}	NG ^f	NG ^c	NG ^c
Tannic acid 1 mM	49.0 ^e	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 1 mM	54.7 ^{abc}	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 5 mM	53.8 ^{abc}	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 10 mM	42.0 ^f	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 15 mM	31.3 ^h	88.7 ^b	100 ^a	100 ^a
Propyl gallate 1 mM	54.5 ^{abc}	100 ^a	100 ^a	100 ^a
Propyl gallate 5 mM	27.2 ⁱ	38.7 ^c	100 ^a	100 ^a
Propyl gallate 10 mM	NG ^k	3.8 ^e	9.0 ^b	13.3 ^b
Propyl gallate 15 mM	NG ^k	NG ^f	NG ^c	NG ^c
Control	55.2 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	2.2	1.9	0.3	0.7
CV% =	5.6	1.4	0.2	0.5

Means followed by the same letter do not differ significantly

*NG = no growth

Tannic acid was tested at only one concentration.

Table 3.5: Effect of antioxidants on temporal conidial germination of *Colletotrichum musae* (isolate CM100) on malt extract agar at 22°C.

Treatments	Conidial germination (%) after different time intervals			
	6 hrs	24 hrs	48 hrs	72 hrs
Butylated hydroxyanisole 1 mM	0.7 ^g	3.5 ^h	8.3 ^f	19.3 ^b
Butylated hydroxyanisole 5 mM	NG ^{*g}	NG ⁱ	NG ^h	NG ^f
Butylated hydroxyanisole 10 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Butylated hydroxyanisole 15 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Propyl paraben 1 mM	5.2 ^c	7.0 ^g	11.2 ^e	18.0 ^c
Propyl paraben 5 mM	1.2 ^d	2.3 ^{hi}	4.5 ^g	9.7 ^d
Propyl paraben 10 mM	0.7 ^d	1.2 ^{hi}	2.7 ^g	5.5 ^e
Propyl paraben 15 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Propionic acid 1 mM	4.2 ^c	96.8 ^b	100 ^a	100 ^a
Propionic acid 5 mM	NG ^g	51.3 ^d	100 ^a	100 ^a
Propionic acid 10 mM	NG ^g	40.8 ^e	85.0 ^b	100 ^a
Propionic acid 15 mM	NG ^g	31.0 ^f	67.7 ^d	100 ^a
Benzoic acid 1 mM	27.3 ^b	64.0 ^c	82.3 ^c	100 ^a
Benzoic acid 5 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Benzoic acid 10 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Benzoic acid 15 mM	NG ^g	NG ⁱ	NG ^h	NG ^f
Control	53.3 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	1.3	2.8	2.6	0.8
CV% =	14.1	7.1	4.8	1.2

Means followed by the same letter do not differ significantly

*NG = no growth

Table 3.6: Effect of antioxidants on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 22°C.

Treatments	Conidial germination (%) after different time intervals			
	6 hrs	24 hrs	48 hrs	72 hrs
Ascorbic acid 1 mM	57.7 ^{def}	100 ^a	100 ^a	100 ^a
Ascorbic acid 5 mM	56.0 ^{ef}	100 ^a	100 ^a	100 ^a
Ascorbic acid 10 mM	55.7 ^{ef}	100 ^a	100 ^a	100 ^a
Ascorbic acid 15 mM	53.8 ^f	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 1 mM	63.3 ^{bcd}	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 5 mM	62.3 ^{bcd}	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 10 mM	61.3 ^{cde}	100 ^a	100 ^a	100 ^a
Dimethyl sulphoxide 15 mM	60.7 ^{cde}	100 ^a	100 ^a	100 ^a
Thiourea 1 mM	62.8 ^{bcd}	100 ^a	100 ^a	100 ^a
Thiourea 5 mM	41.0 ^g	100 ^a	100 ^a	100 ^a
Thiourea 10 mM	25.0 ^h	100 ^a	100 ^a	100 ^a
Thiourea 15 mM	2.5 ⁱ	4.2 ^d	6.5 ^c	11.2 ^c
Tannic acid 1 mM	65.3 ^{abc}	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 1 mM	65.0 ^{abc}	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 5 mM	60.3 ^{cde}	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 10 mM	54.0 ^f	100 ^a	100 ^a	100 ^a
Butylated hydroxytoluene 15 mM	45.7 ^g	100 ^a	100 ^a	100 ^a
Propyl gallate 1 mM	67.3 ^{ab}	100 ^a	100 ^a	100 ^a
Propyl gallate 5 mM	28.7 ^h	78.8 ^b	100 ^a	100 ^a
Propyl gallate 10 mM	4.5 ⁱ	6.8 ^c	12.5 ^b	21.5 ^b
Propyl gallate 15 mM	NG ^{*i}	NG ^e	NG ^d	NG ^d
Control	70.0 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	3.2	0.6	0.5	0.8
CV% =	7.1	0.4	0.3	0.5

Means followed by the same letter do not differ significantly

*NG = no growth

Tannic acid was only tested at 1 concentration.

Table 3.7: Effect of antioxidants on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 22°C.

Treatments	Conidial germination (%) after different time intervals			
	6 hrs	24 hrs	48 hrs	72 hrs
Butylated hydroxyanisole 1 mM	NG ^{*g}	7.8 ^g	13.8 ^c	22.5 ^c
Butylated hydroxyanisole 5 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Butylated hydroxyanisole 10 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Butylated hydroxyanisole 15 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Propyl paraben 1 mM	7.2 ^{cd}	15.0 ^f	22.3 ^d	28.0 ^b
Propyl paraben 5 mM	3.3 ^{ef}	6.0 ^{gh}	7.7 ^f	13.0 ^d
Propyl paraben 10 mM	1.5 ^{fg}	3.0 ^{hi}	5.2 ^f	7.7 ^e
Propyl paraben 15 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Propionic acid 1 mM	8.5 ^c	100 ^a	100 ^a	100 ^a
Propionic acid 5 mM	5.0 ^{de}	59.2 ^c	100 ^a	100 ^a
Propionic acid 10 mM	NG ^g	45.0 ^d	90.0 ^b	100 ^a
Propionic acid 15 mM	NG ^g	37.5 ^e	85.0 ^c	100 ^a
Benzoic acid 1 mM	34.3 ^b	77.3 ^b	100 ^a	100 ^a
Benzoic acid 5 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Benzoic acid 10 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Benzoic acid 15 mM	NG ^g	NG ⁱ	NG ^g	NG ^f
Control	65.0 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	2.3	3.2	3.1	1.5
CV% =	19.3	7.3	5.1	2.3

Means followed by the same letter do not differ significantly

*NG = no growth

3.5.2 Effect of antioxidants on mycelial growth

The effect of 10 different antioxidants in the concentration range of 1-15 mM on the growth of CM100 and CM103 on MEA was tested. BHA (5-15 mM) completely inhibited the growth of both isolates and PG (10-15 mM), PP and benzoic acid (15mM) completely inhibited the growth of CM100 and caused lag times ranging from 2-6 days in the appearance of growth of CM103 (Table 3.8). Other chemicals also caused significant lag periods in the growth of the two isolates e.g. propionic acid (15 mM), BHA (1 mM), PP and benzoic acid (10 mM).

The majority of the antioxidants significantly ($P < 0.05$) reduced the growth rate (mm day^{-1}) of both isolates of *C. musae* (Tables 3.9 and 3.10). Generally, isolate 103 was more tolerant of the chemicals than isolate 100. However, the most and least effective chemicals were the same for both isolates. DMSO at all concentrations and ascorbic acid at 1 mM caused little reduction in mycelial growth of either isolate. CM103 was relatively tolerant (compared to CM100) of ascorbic acid at 5-15 mM e.g. at 15 mM growth rate of CM100 was reduced by about 65%, while that of CM103 was reduced by only about 30%. CM103 was also markedly more tolerant than CM100 of the following chemicals: propionic acid, tannic acid, PG and benzoic acid (1 mM) and thiourea (1-5 mM). Overall, the chemicals that were most effective in controlling the growth of these two isolates of *C. musae* were BHA, PG, PP and benzoic acid. This is indicated by the LD_{50} and LD_{90} value ranges given in Table 3.11.

3.5.3 Effect of antioxidants on the pH of malt extract agar (MEA).

The pH of 5% MEA is 5.4. The addition of five chemicals (ascorbic acid, propionic acid, dimethyl sulphoxide, tannic acid and benzoic acid) reduced the pH of the MEA medium by between 0.4 and 1.4 units depending on the actual concentration (Table 3.12). Thiourea also reduced pH by a maximum of 0.4 units at 15 mM. Butylated hydroxyanisole had no effect on the pH (5.4) of MEA at all, whereas BHT, PP PG

Table 3.8: Effect of a range of antioxidants on the lag period (in days) before initiation of growth of two isolates of *Colletotrichum musae* (100 & 103) on malt extract agar at 22°C.

<i>Treatments</i>	<i>Conc.</i>	<i>Lag time (days)</i>	
		<i>Isolate 100</i>	<i>Isolate 103</i>
Ascorbic acid	1 mM	0	0
Ascorbic acid	5 mM	0	0
Ascorbic acid	10 mM	1	0
Ascorbic acid	15 mM	2	0
Propionic acid	1 mM	0	0
Propionic acid	5 mM	0	0
Propionic acid	10 mM	7	0
Propionic acid	15 mM	9	3
Thiourea	1 mM	0	0
Thiourea	5 mM	1	0
Thiourea	10 mM	1	0
Thiourea	15 mM	2	0
Dimethyl sulphoxide	1 mM	0	0
Dimethyl sulphoxide	5 mM	0	0
Dimethyl sulphoxide	10 mM	0	0
Dimethyl sulphoxide	15 mM	0	0
Butylated hydroxyanisole	1 mM	4	3
Butylated hydroxyanisole	5 mM	NG*	NG
Butylated hydroxyanisole	10 mM	NG	NG
Butylated hydroxyanisole	15 mM	NG	NG
Butylated hydroxytoluene	1 mM	0	0
Butylated hydroxytoluene	5 mM	0	0
Butylated hydroxytoluene	10 mM	0	0
Butylated hydroxytoluene	15 mM	1	0
Propyl gallate	1 mM	0	0
Propyl gallate	5 mM	1	0
Propyl gallate	10 mM	NG	3
Propyl gallate	15 mM	NG	5
Propyl paraben	1 mM	1	0
Propyl paraben	5 mM	0	1
Propyl paraben	10 mM	5	6
Propyl paraben	15 mM	NG	6
Benzoic acid	1 mM	0	0
Benzoic acid	5 mM	5	2
Benzoic acid	10 mM	8	1
Benzoic acid	15 mM	NG	2
Control	n/a	0	0

*NG = no growth after 12 days

Table 3.9: Effect of range of antioxidants on mycelial growth of two isolates of *Colletotrichum musae* on malt extract agar at 22°C. (mm growth day⁻¹)

<i>Treatments</i>	<i>Concentration (mM)</i>	<i>Mycelial growth (mm day⁻¹)</i>	
		<i>Isolate CM100</i>	<i>Isolate CM103</i>
Ascorbic acid	1	19.7 ^b	15.0 ^b
Ascorbic acid	5	13.5 ^c	14.0 ^d
Ascorbic acid	10	9.6 ^g	13.4 ^e
Ascorbic acid	15	7.7 ⁱ	11.1 ^g
Propionic acid	1	12.1 ^d	15.3 ^{ab}
Propionic acid	5	10.1 ^f	12.5 ^f
Propionic acid	10	9.2 ^h	7.6 ⁱ
Propionic acid	15	5.4 ^l	4.3 ^j
Thiourea	1	10.5 ^e	14.1 ^{cd}
Thiourea	5	10.6 ^e	14.0 ^d
Thiourea	10	7.9 ⁱ	12.6 ^f
Thiourea	15	7.1 ^j	9.2 ^h
Dimethyl sulphoxide	1	21.4 ^a	14.5 ^c
Dimethyl sulphoxide	5	21.5 ^a	14.4 ^{cd}
Dimethyl sulphoxide	10	21.4 ^a	14.4 ^{cd}
Dimethyl sulphoxide	15	21.3 ^a	14.2 ^{cd}
Tannic acid	1	10.6 ^e	15.1 ^b
Tannic acid	5	6.2 ^k	9.2 ^h
Control	-	21.3 ^a	15.7 ^a
Lsd (P=0.05) =		0.3	0.4
CV% =		1.7	2.4

Means followed by the same letter do not differ significantly

Tannic acid was only tested at 2 concentrations.

Table 3.10: Effect of range of antioxidants on mycelial growth of two isolates of *Colletotrichum musae* on malt extract agar at 22°C.

<i>Treatments</i>	<i>Concentration (mM)</i>	<i>Mycelial growth (mm day⁻¹)</i>	
		<i>Isolate CM100</i>	<i>Isolate CM103</i>
Butylated hydroxyanisole	1	2.6 ⁿ	4.3 ^j
Butylated hydroxyanisole	5	NG ^{* o}	NG ^m
Butylated hydroxyanisole	10	NG ^o	NG ^m
Butylated hydroxyanisole	15	NG ^o	NG ^m
Butylated hydroxytoluene	1	18.3 ^b	14.8 ^b
Butylated hydroxytoluene	5	13.7 ^d	14.8 ^b
Butylated hydroxytoluene	10	10.3 ^g	11.7 ^d
Butylated hydroxytoluene	15	7.2 ⁱ	10.4 ^e
Propyl gallate	1	12.3 ^e	14.1 ^c
Propyl gallate	5	4.0 ^m	5.3 ⁱ
Propyl gallate	10	NG ^o	2.7 ^k
Propyl gallate	15	NG ^o	2.2 ^l
Propyl paraben	1	10.7 ^f	9.8 ^f
Propyl paraben	5	5.5 ^j	5.2 ⁱ
Propyl paraben	10	4.9 ^k	4.0 ^j
Propyl paraben	15	NG ^o	2.9 ^k
Benzoic acid	1	15.4 ^c	14.2 ^c
Benzoic acid	5	8.0 ^h	7.9 ^g
Benzoic acid	10	4.7 ^l	5.8 ^h
Benzoic acid	15	NG ^o	4.1 ^j
Control	-	21.5 ^a	15.7 ^a
Lsd (P=0.05) =		0.1	0.4
CV% =		1.3	3.9

Means followed by the same letter do not differ significantly

*NG = no growth

Table 3.11 The inhibition of mycelial growth (50% LD₅₀) and (90% LD₉₀) values of each antioxidants alone and combinations with imazalil and thiabendazole against two isolates of *C. musae* (CM100 and CM103). These were determined from the relationship between growth rate (mm day⁻¹) and antioxidant concentration.

Chemicals	LD ₅₀		LD ₉₀	
	CM100	CM103	CM100	CM103
Ascorbic acid	< 10 Mm	> 15 mM	> 15 mM	> 15 mM
Dimethyl sulphoxide	> 15 mM	> 15 mM	> 15 mM	> 15 mM
Thiourea	1 mM	> 15 mM	> 15 mM	> 15 mM
Propionic acid	< 5 mM	< 10 mM	> 15 mM	> 15 mM
Tannic acid	1 mM	> 5 mM	> 5 mM	> 5 mM
BHA	< 1 mM	< 1 mM	> 1 mM	< 5 mM
BHT	10 mM	> 15 mM	> 15 mM	> 15 mM
Propyl gallate	> 1 mM	< 5 mM	> 5 mM	> 15 mM
Propyl paraben	1 mM	< 5 mM	> 10 mM	> 15 mM
Benzoic acid	< 5 mM	> 5 mM	> 10 mM	> 15 mM
BHA + propyl paraben	-	< 0.1 + 5 mM	-	< 0.1 + 5 mM
BHA + benzoic acid	-	< 0.1 + 5 mM	-	< 0.1 + 5 mM
BHA + imazalil	< 0.03125 + 0.00168 mM	< 0.03125 + 0.00168 mM	< 0.25 + 0.00168 mM	< 0.03125 + 0.00168 mM
BHA + TBZ	< 0.03125 + 0.0025 mM	0.125 + 0.0025 mM	> 0.5 + 0.0025 mM	0.25 + 0.0025 mM

BHA = Butylated hydroxyanisole; BHT = Butylated hydroxytoluene; TBZ = Thiabendazole

Table 3.12: Modification of the pH (5.4) of the 5 % malt extract agar by antioxidants at different concentrations. pH_c = pH of the agar incorporated with antioxidant compounds.

Chemical	Conc.	pH _c	Change in pH (pH _c - 5.4)
Ascorbic acid	1 mM	4.8	-0.6
Ascorbic acid	5 mM	4.3	-1.1
Ascorbic acid	10 mM	4.2	-1.2
Ascorbic acid	15 mM	4.1	-1.3
Propionic acid	1 mM	5.0	-0.4
Propionic acid	5 mM	4.7	-0.7
Propionic acid	10 mM	4.6	-0.8
Propionic acid	15 mM	4.5	-0.9
Dimethyl sulphoxide	1 mM	4.4	-1.0
Dimethyl sulphoxide	5 mM	4.2	-1.2
Dimethyl sulphoxide	10 mM	4.2	-1.2
Dimethyl sulphoxide	15 mM	4.15	-1.25
Thiourea	1 mM	5.3	-0.1
Thiourea	5 mM	5.3	-0.1
Thiourea	10 mM	5.2	-0.2
Thiourea	15 mM	5.0	-0.4
Tannic acid	1 mM	5.0	-0.4
Tannic acid	5 mM	4.4	-1.0
Tannic acid	10 mM	4.2	-1.2
Tannic acid	15 mM	4.0	-1.4
Benzoic acid	1 mM	5.3	-0.1
Benzoic acid	5 mM	4.5	-0.9
Benzoic acid	10 mM	4.3	-1.1
Benzoic acid	15 mM	4.2	-1.2
Butylated hydroxyanisole	1 mM	5.4	0.0
Butylated hydroxyanisole	5 mM	5.4	0.0
Butylated hydroxyanisole	10 mM	5.4	0.0
Butylated hydroxyanisole	15 mM	5.4	0.0
Butylated hydroxytoluene	1 mM	5.46	0.06
Butylated hydroxytoluene	5 mM	5.5	0.1
Butylated hydroxytoluene	10 mM	5.6	0.2
Butylated hydroxytoluene	15 mM	5.64	0.24
Propyl gallate	1 mM	5.5	0.1
Propyl gallate	5 mM	5.5	0.1
Propyl gallate	10 mM	5.6	0.2
Propyl gallate	15 mM	5.6	0.2
Propyl paraben	1 mM	5.53	0.13
Propyl paraben	5 mM	5.6	0.2
Propyl paraben	10 mM	5.6	0.2
Propyl paraben	15 mM	5.6	0.2

caused a small increase in the pH of the MEA medium of between 0.06-0.24 depending on the actual concentration.

3.5.4 Impact of azadirachtin on the germination and growth of *C. musae*.

Azadirachtin (neem seed compound, azadirachtin A 15%) at two concentrations (0.5 & 1.0 mM) significantly ($P < 0.05$) reduced the germination of *C. musae* conidia (isolate 103) at 22 °C when compared to the untreated control (Table 3.13). At 0.5 mM azadirachtin completely inhibited germination for up to 24 hrs and after 48 and 72 hrs germination was markedly reduced by 89 and 75.2 % respectively. However, there was no germination at 1.0 mM for 48 hrs and only 11 % germination had occurred after 72 hrs. Ethanol (which was used as the solvent) appeared to inhibit germination over the first 6 hrs. By 24 hrs this effect had disappeared.

Table 3.14 and Plate 3.2 shows that azadirachtin also significantly ($P < 0.05$) reduced the growth rate (mm day^{-1}) of isolate 103 of *C. musae*. Azadirachtin at 0.5 and 1.0 mM inhibited the mycelial growth by 80 to 90 % when compared to the control. The effect of ethanol on mycelial growth was not significant.

Table 3.13: The effect of azadirachtin on the temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 22 °C.

Treatments	<u>Conidial germination (%) after different time intervals</u>			
	6 h	24 h	48 h	72 h
Azadirachtin 0.5 mM	NG ^{*b}	NG ^b	11.0 ^b	24.8 ^b
Azadirachtin 1.0 mM	NG ^b	NG ^b	NG ^c	11.2 ^c
Control with ethanol	NG ^b	99.0 ^b	100.0 ^a	100.0 ^a
Control (MEA alone)	67.7 ^a	100.0 ^a	100.0 ^a	100.0 ^a
Lsd (P=0.05) =	6.4	0.9	1.7	2.0
CV% =	20.1	1.0	1.7	1.8

Means followed by the same letter do not differ significantly

*NG = no growth

Table 3.14: Effect of azadirachtin on mycelial growth of isolate CM103 of *Colletotrichum musae* on malt extract agar at 22°C.

Treatments	<i>Mycelial growth (mm day⁻¹)</i>
Azadirachtin 0.5 mM	2.4 ^b
Azadirachtin 1.0 mM	1.5 ^c
Control with ethanol	14.0 ^a
Control (MEA alone)	14.3 ^a
Lsd (P=0.05) =	0.5
CV% =	4.0

Means followed by the same letter do not differ significantly

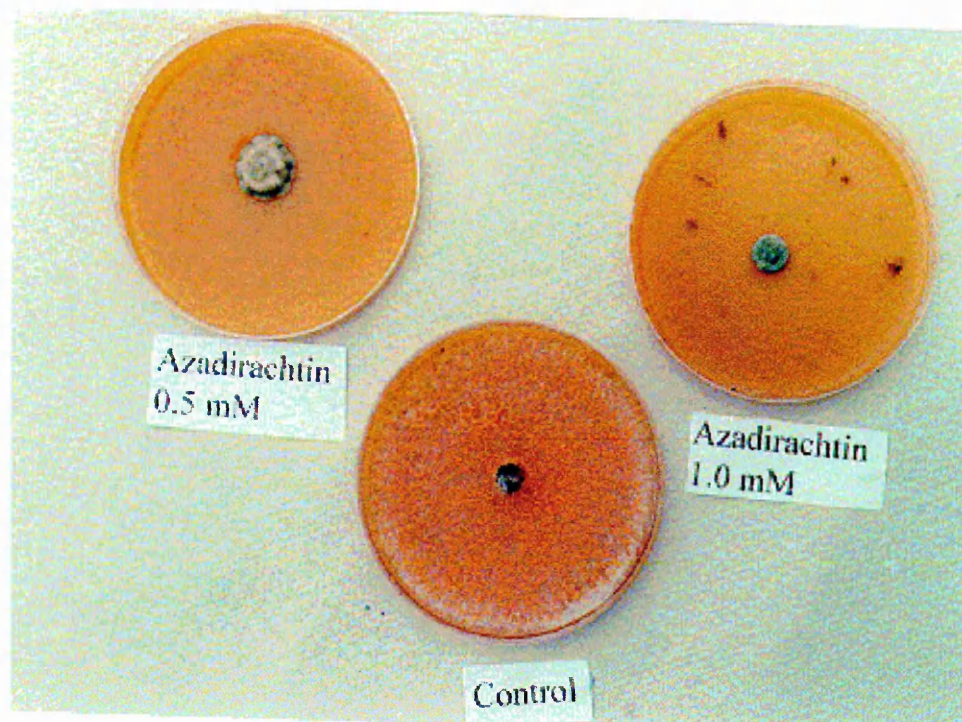


Plate 3.2: The effect of azadirachtin at concentrations of 0.5 and 1 mM on the mycelial growth of *Colletotrichum musae* (isolate 103) on malt extract agar 12 days after inoculation at 25°C.

CHAPTER 4

RESULTS

STUDIES *IN VITRO* (II)

(THE CONTROL OF *C. MUSAE* BY COMBINATIONS OF CHEMICALS)

4.1 INTRODUCTION

A number of chemicals were selected for screening *in vitro* as described in section 1.6. Initial screening involved just one chemical per treatment, however, when chemicals are applied in combination, they can show additive, synergistic or antagonistic effects (Lueck, 1980). It was considered to be useful therefore to also investigate the effect of the chemicals under investigation in different combinations *in vitro*. As it would have been an enormous task to test all possible combinations of chemicals, only selected chemicals were combined on the basis of their effectiveness in inhibiting *C. musae* on their own. These chemicals were the three antioxidants benzoic acid, propyl paraben (PP) and butylated hydroxyanisole (BHA) and the two conventional fungicides, thiabendazole (TBZ) and imazalil (chapter 3). In some cases, the chemicals were tested at both 14 and 25°C to determine the effect of the chemical at temperatures that are sub-optimal and optimal for the growth of the fungus. These temperatures are also typical of those which banana fruits in the postharvest chain might experience.

The activity of some chemicals is strongly dependent on the pH of the medium e.g. benzoic acid (Rahn and Conn, 1944). The pH of the medium changes the proportion of the chemical which is in the undissociated form. It is this form that is most soluble in non-aqueous media and hence is most likely to penetrate the lipid membrane of fungal cells. It is important, therefore to determine the effect of pH on fungal toxicity during the screening process. Lack of activity at higher pH levels might simply mean that the chemical is unable to penetrate the fungal cells.

There are many possible mechanisms of chemicals with fungicidal or fungistatic properties. As described in section 1.4.3. it is well established that the fungicide TBZ inhibits mitosis by binding to β -tubulin in fungal cells and that imazalil inhibits an enzyme that is required for the synthesis of ergosterol (which is vital to fungal membrane synthesis). It is a huge undertaking to try and determine specific mechanisms of inhibition of any one chemical. Some general idea may be obtained by

screening the effect of the chemical in question on the production and activity of various enzymes. Test kits are now available which allow the rapid determination of the activity of a range of enzymes, some of which are relevant to fungal growth and development. Where there are synergistic effects between two chemicals, some indication of the underlying mechanism may also be given by determining the effect of the order of the treatment.

4.2 EXPERIMENTAL SET UP

There were three replicate plates per treatment in all conidial germination treatments. For all mycelial growth experiments each of four replicates per treatment were sealed with Nescofilm and incubated at either 14 or 22°C. A complete randomised design was used for both studies (conidial germination and mycelial growth). There were also three replications of each treatment in API ZYM studies.

4.3 RESULTS OF THE EVALUATION OF ANTIOXIDANTS AND FUNGICIDE COMBINATIONS ON THE GERMINATION AND GROWTH OF *C. MUSAE*.

4.3.1 The effect of benzoic acid, PP, BHA and imazalil combinations on the germination of *C. musae* at 14 and 25°C.

The effect of these chemicals on conidial germination at 14 and 25°C of isolate CM103 are shown in Table 4.1 and for lower concentrations in Table 4.2. The germination of conidia of *C. musae* was quite sensitive to temperature so that after 6 hrs the number of germinated conidia at 14°C was only about 3% of the number germinated at 25°C in the control plates. However, after 2 days all the conidia had germinated at both temperatures.

All chemicals tested on their own gave some control of conidial germination with a relatively greater effect at 14°C than 25°C. BHA on its own at 0.1, 0.5 and 1.0 mM did not prevent complete conidial germination, however, all three concentrations

significantly slowed down the rate of germination. By day three, on average only 14.3% and 24.7% germination had occurred on treatments containing 1 mM BHA at 14 and 25°C respectively. Imazalil at concentrations of 1.68, 3.4 and 8.4 μ M did not prevent full conidial germination by day 3 at either temperature but slowed down the germination rate. Imazalil at 1.6825 mM completely inhibited germination over the 3 days. Neither PP or benzoic acid at 1.0 mM prevented germination at either temperature but at 14°C, 5 and 10 mM prevented germination by day 3.

Complete inhibition (100 %) of germination over the 3 day period of the experiment was achieved by combining BHA at 0.1 to 5 mM with propyl paraben and benzoic acid at 1 to 10 mM. Similarly there was no germination in treatments where BHA at 0.5 to 5 mM was combined with imazalil at 1.68 μ M to 0.8413 mM for 72 hrs at 14 and 25°C (Plates 4.1 & 4.2) respectively.

4.3.2 The effect of benzoic acid, PP, BHA and imazalil combinations on the mycelial growth of *C. musae* at 14 and 25°C.

The growth rate of *C. musae* isolate CM103 on MEA at 14°C was half that at 25°C, with measurable growth delayed by 4 days at the lower temperature (Table 4.3).

All the chemicals tested gave a significant reduction in mycelial growth at the concentrations tested (Tables 4.4 and 4.5). BHA on its own at 5 mM completely inhibited mycelial growth, but at 0.1, 0.5 and 1.0 mM did not prevent the mycelial growth at either temperature (Plate 4.4 to 4.6). However, these concentrations significantly increased the lag period for 7, 8 and 10 days, respectively at 14°C and 2, 3 and 4 days respectively at 25°C (Table 4.3). PP (10 mM) completely suppressed growth at 14°C but not at 25°C. The effect of temperature on the fungal growth rate in response to 1mM benzoic acid was particularly marked (Table 4.5). At 14°C the growth rate was half the control rate, whereas at 25°C, the growth rate was almost the same as the control rate. Imazalil alone at 1.68, 3.4 and 8.4 μ M reduced the

mycelial growth rate by 42, 62 and 72% at 14°C and 21, 58 and 67% at 25°C respectively.

Complete inhibition of growth was achieved at both temperatures by all the combinations of chemicals tested i.e. by combining BHA (0.5 – 5.0 mM) with benzoic acid and propyl paraben (1 and 5 mM) or imazalil (1.68 μ M to 0.8413 mM).

Table 4.1: The effect of three antioxidants alone and combined with imazalil on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 14 and 25°C.

Treatments	Conidial germination (%) after different time intervals at 2 temperatures									
	14°C					25°C				
	6 h	24 h	48 h	72 h		6 h	24 h	48 h	72 h	
BHA 1 mM + propyl paraben 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + propyl paraben 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + propyl paraben 10 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 1 mM + benzoic acid 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + benzoic acid 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + benzoic acid 10 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 1 mM + imazalil 0.5047 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 1 mM + imazalil 0.8413 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + imazalil 0.5047 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 5 mM + imazalil 0.8413 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
BHA 1 mM	0 ^b	3 ^b	8.7 ^b	16.7 ^b		0.7 ^c	8.5 ^b	18.3 ^b	27.3 ^b	
BHA 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
Propyl paraben 5 mM	0 ^b	0 ^c	2.7 ^c	5.5 ^c		3.5 ^b	6.8 ^c	9.7 ^c	13.8 ^c	
Propyl paraben 10 mM	0 ^b	0 ^c	0 ^d	0 ^d		1.8 ^{bc}	3.3 ^d	5 ^d	8 ^d	
Benzoic acid 5 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
Benzoic acid 10 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
Imazalil 1.6825 mM	0 ^b	0 ^c	0 ^d	0 ^d		0 ^c	0 ^e	0 ^e	0 ^e	
Control	2.2 ^a	58.2 ^a	100 ^a	100 ^a		64.7 ^a	100 ^a	100 ^a	100 ^a	

Lsd (p = 0.05)

CV % =

1.9

13.9

Means followed by the same letter do not differ significantly

Table 4.2 The effect of three antioxidants alone and combined with imazalil on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 14 and 25°C.

Treatments	Conidial germination (%) after different time intervals at 2 temperatures							
	14 °C				25 °C			
	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
BHA 0.1 mM + propyl paraben 5 mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.5 mM + propyl paraben 5 mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 1mM + propyl paraben 1mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.1 mM + benzoic acid 5 mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.5 mM + benzoic acid 5 mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 1mM + benzoic acid 1 mM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.5 mM + imazalil 1.68 µM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.5 mM + imazalil 3.4 µM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.5 mM + imazalil 8.4 µM	0 ^b	0 ^g	0 ^h	0 ^g	0 ^f	0 ^h	0 ^e	0 ^d
BHA 0.1 mM	0 ^b	14.2 ^e	40.8 ^d	80 ^b	25.7 ^d	62.5 ^d	100 ^a	100 ^a
BHA 0.5 mM	0 ^b	3.8 ^f	35.3 ^e	70.7 ^c	10 ^e	24.2 ^e	59.2 ^b	100 ^a
BHA 1 mM	0 ^b	0 ^g	8.3 ^g	11.8 ^f	1.3 ^f	8.3 ^g	14.7 ^d	22 ^c
Benzoic acid 1 mM	0 ^b	0 ^g	11.7 ^f	58 ^d	34 ^c	79 ^c	100 ^a	100 ^a
Propyl paraben 1 mM	0 ^b	0 ^g	13 ^f	17.7 ^e	7.2 ^e	16.3 ^f	23.3 ^c	31.3 ^b
Imazalil 1.68 µM	0 ^b	55 ^b	100 ^a	100 ^a	65 ^a	100 ^a	100 ^a	100 ^a
Imazalil 3.4 µM	0 ^b	32.7 ^c	100 ^a	100 ^a	45.7 ^b	100 ^a	100 ^a	100 ^a
Imazalil 8.4 µM	0 ^b	18 ^d	51.7 ^c	100 ^a	34.3 ^c	90.7 ^b	100 ^a	100 ^a
Control	2.2 ^a	96 ^a	100 ^a	100 ^a	69 ^a	100 ^a	100 ^a	100 ^a

Lsd (p = 0.05)

CV % = 0.1 2.9 2.7 3.1 4.2 3.1 1.9 1.7

Means followed by the same letter do not differ significant

Table 4.3: The effect of three antioxidants and the fungicide imazalil alone and in combination on the lag time (in days) before growth was observed of isolate CM103 of *Colletotrichum musae* on malt extract agar at two temperatures (14 and 25°C).

Treatments	Lag time (days)	
	14°C	25°C
BHA 0.1 mM + propyl paraben 5 mM	NG*	NG
BHA 0.5 mM + propyl paraben 5 mM	NG	NG
BHA 1mM + propyl paraben 1mM	NG	NG
BHA 0.1 mM + benzoic acid 5 mM	NG	NG
BHA 0.5 mM + benzoic acid 5 mM	NG	NG
BHA 1mM + benzoic acid 1 mM	NG	NG
BHA 0.5 mM + imazalil 1.68 µM	NG	NG
BHA 0.5 mM + imazalil 3.4 µM	NG	NG
BHA 0.5 mM + imazalil 8.4 µM	NG	NG
BHA 0.1 mM	>7	2
BHA 0.5 mM	8	3
BHA 1 mM	10	4
Benzoic acid 1 mM	4	0
Propyl paraben 1 mM	8	0
Imazalil 1.68 µM	6	0
Imazalil 3.4 µM	6	0
Imazalil 8.4 µM	9	3
Control	4	0

*NG = no growth

Table 4.4: The effect of three antioxidants alone and in combination with imazalil on the mycelial growth of *Colletotrichum musae* (isolate CM103) on malt extract agar at 14 and 25°C.

<i>Treatments</i>	<i>Mycelial growth (mm day⁻¹)</i>	
	<i>14 °C</i>	<i>25 °C</i>
BHA 1 mM + propyl paraben 5 mM	NG ^{*e}	NG ^g
BHA 5 mM + propyl paraben 5 mM	NG ^e	NG ^g
BHA 5 mM + propyl paraben 10 mM	NG ^e	NG ^g
BHA 1 mM + benzoic acid 5 mM	NG ^e	NG ^g
BHA 5 mM + benzoic acid 5 mM	NG ^e	NG ^g
BHA 5 mM + benzoic acid 10 mM	NG ^e	NG ^g
BHA 1 mM + imazalil 0.5047 mM	NG ^e	NG ^g
BHA 1 mM + imazalil 0.8413 mM	NG ^e	NG ^g
BHA 5 mM + imazalil 0.5047 mM	NG ^e	NG ^g
BHA 5 mM + imazalil 0.8413 mM	NG ^e	NG ^g
BHA 1 mM	3.0 ^c	4.6 ^e
BHA 5 mM	NG ^e	NG ^g
Propyl paraben 5 mM	3.9 ^b	5.1 ^d
Propyl paraben 10 mM	NG ^e	4.2 ^f
Benzoic acid 5 mM	2.9 ^c	7.8 ^b
Benzoic acid 10 mM	2.5 ^d	5.8 ^c
Imazalil 1.6825 mM	NG ^e	NG ^g
Control	7.2 ^a	16 ^a
Lsd (p = 0.05)	0.2	0.3
CV % =	15.6	7.8

*NG = no growth

Means followed by the same letter do not differ significantly

BHA = Butylated hydroxyanisole

Table 4.5: The effect of three antioxidants alone and in combination with imazalil on the mycelial growth of *Colletotrichum musae* (isolate CM103) on malt extract agar at 14 and 25°C.

<i>Treatments</i>	<i>Mycelial growth (mm day⁻¹)</i>	
	<i>14°C</i>	<i>25°C</i>
BHA 0.1 mM + propyl paraben 5 mM	NG ^{*g}	NG ⁱ
BHA 0.5 mM + propyl paraben 5 mM	NG ^g	NG ⁱ
BHA 1mM + propyl paraben 1mM	NG ^g	NG ⁱ
BHA 0.1 mM + benzoic acid 5 mM	NG ^g	NG ⁱ
BHA 0.5 mM + benzoic acid 5 mM	NG ^g	NG ⁱ
BHA 1mM + benzoic acid 1 mM	NG ^g	NG ⁱ
BHA 0.5 mM + imazalil 1.68 µM	NG ^g	NG ⁱ
BHA 0.5 mM + imazalil 3.4 µM	NG ^g	NG ⁱ
BHA 0.5 mM + imazalil 8.4 µM	NG ^g	NG ⁱ
BHA 0.1 mM	3.7 ^d	8.5 ^e
BHA 0.5 mM	2.7 ^e	7.7 ^f
BHA 1 mM	2.0 ^f	4.7 ^h
Benzoic acid 1 mM	3.6 ^d	14.1 ^b
Propyl paraben 1 mM	5.1 ^b	9.1 ^d
Imazalil 1.68 µM	4.1 ^c	11.4 ^c
Imazalil 3.4 µM	2.8 ^e	6.0 ^g
Imazalil 8.4 µM	2.0 ^f	4.7 ^h
Control	7.2 ^a	14.4 ^a
Lsd (p = 0.05)	0.3	0.1
CV % =	12.2	2.2

*NG = no growth

Means followed by the same letter do not differ significantly

BHA = Butylated hydroxyanisole

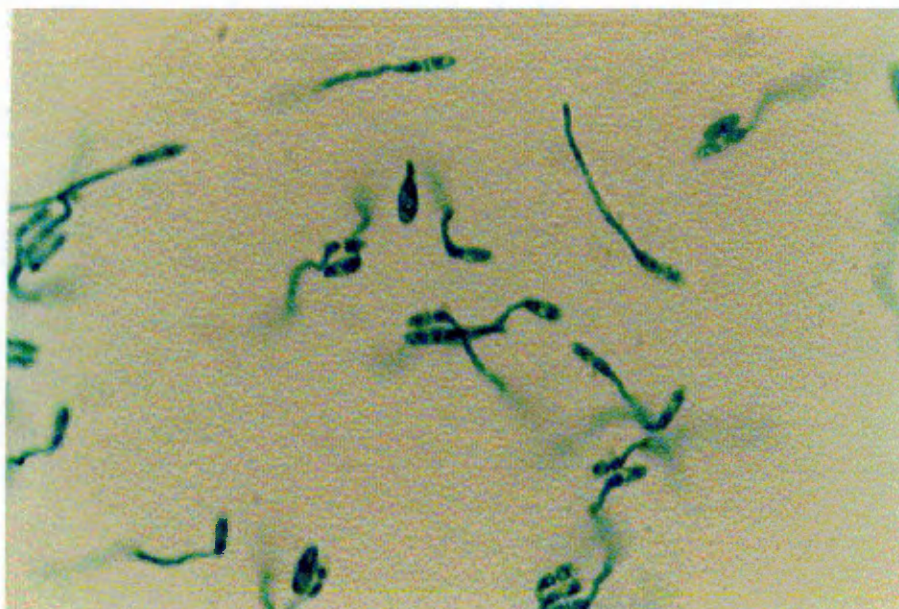


Plate 4.1: Conidial germination of *Colletotrichum musae* CM103 on malt extract agar at 25°C after 6 hrs.

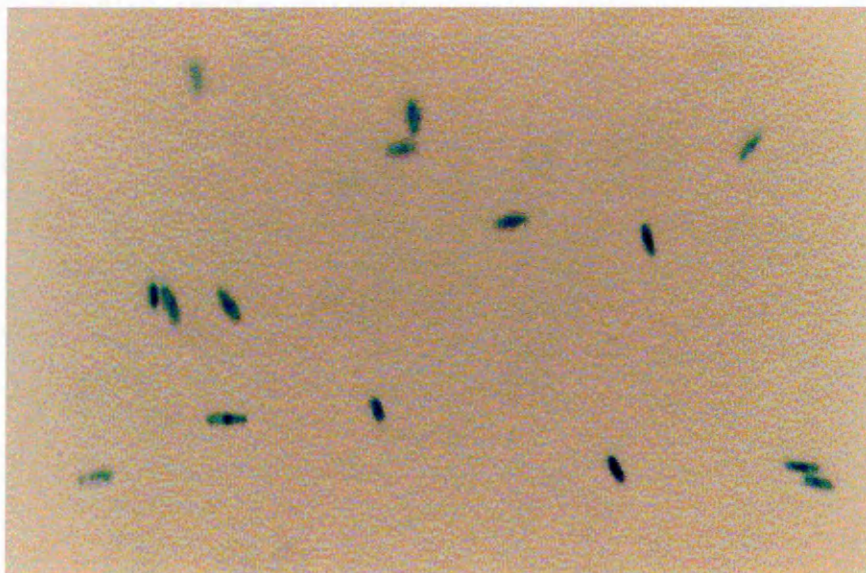


Plate 4.2: Effect of butylated hydroxyanisole (0.5 mM) + imazalil (1.68 µM) on the conidial germination of *Colletotrichum musae* CM103 on malt extract agar at 25°C after 72 hrs

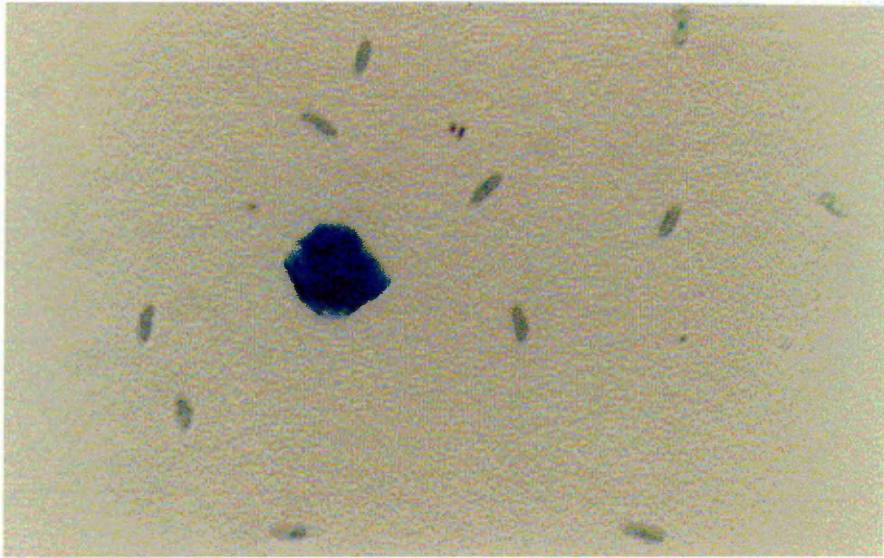


Plate 4.3: Effect of butylated hydroxyanisole (0.5 mM) + propyl paraben (1 mM) on the conidial germination of *Colletotrichum musae* (CM103) on malt extract agar at 25 °C after 72 hrs.

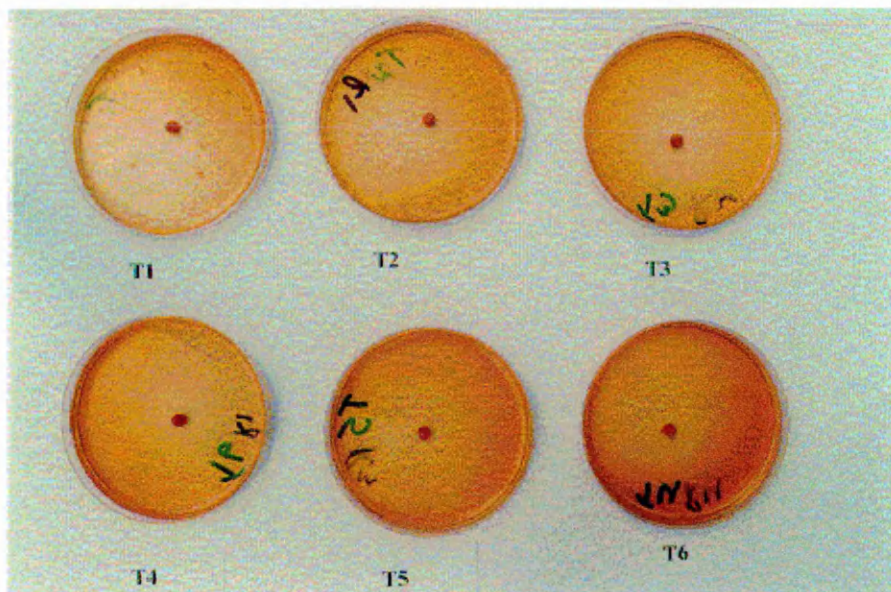


Plate 4.4: The effect of anti-oxidants in combination with imazalil on the mycelial growth of *Colletotrichum musae* (CM 103) on malt extract agar after 12 days inoculation at 25 °C.

T1= Butylated hydroxyanisole (0.1 mM) + propyl paraben (5 mM); T2= Butylated hydroxyanisole (0.5 mM) + propyl paraben (5 mM); T3 = Butylated hydroxyanisole (1 mM) + propyl paraben (1 mM), T4 = Butylated hydroxyanisole (0.1 mM) + benzoic acid (5 mM), T5 = Butylated hydroxyanisole (0.5 mM) + benzoic acid (5 mM), T6 = Butylated hydroxyanisole (1 mM) + benzoic acid (1 mM).

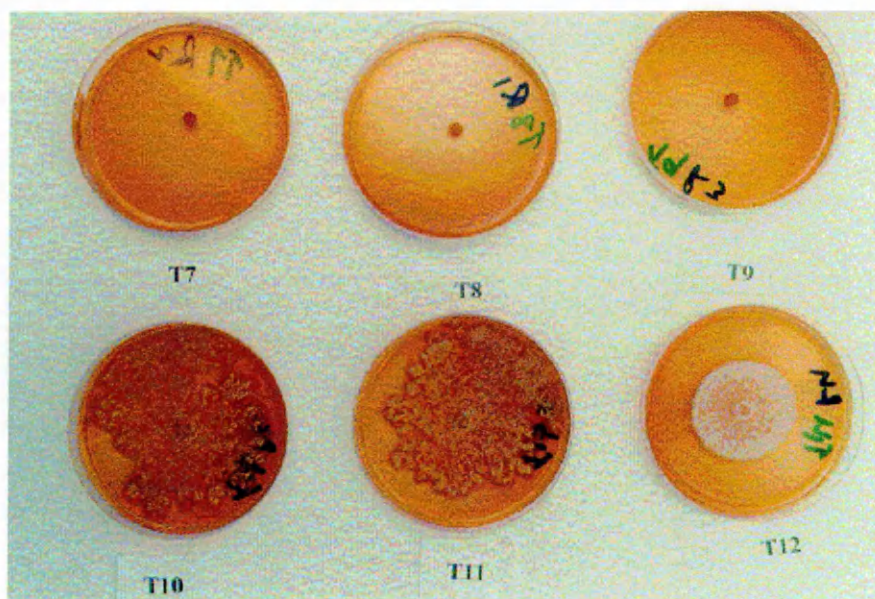


Plate 4.5: The effect of butylated hydroxyanisole in combinations with imazalil and alone on the mycelial growth of *Colletotrichum musae* isolate CM 103 on malt extract agar 12 days after inoculation at 25°C.

T7 = Butylated hydroxyanisole (0.5 mM) + imazalil (1.68 μ M); **T8** = Butylated hydroxyanisole (0.5 mM) + imazalil (3.4 μ M); **T9** = Butylated hydroxyanisole (0.5 mM) + imazalil (8.4 μ M); **T10** = Butylated hydroxyanisole (0.1 mM); **T11** = Butylated hydroxyanisole (0.5 mM) **T12** = Butylated hydroxyanisole (1 mM).

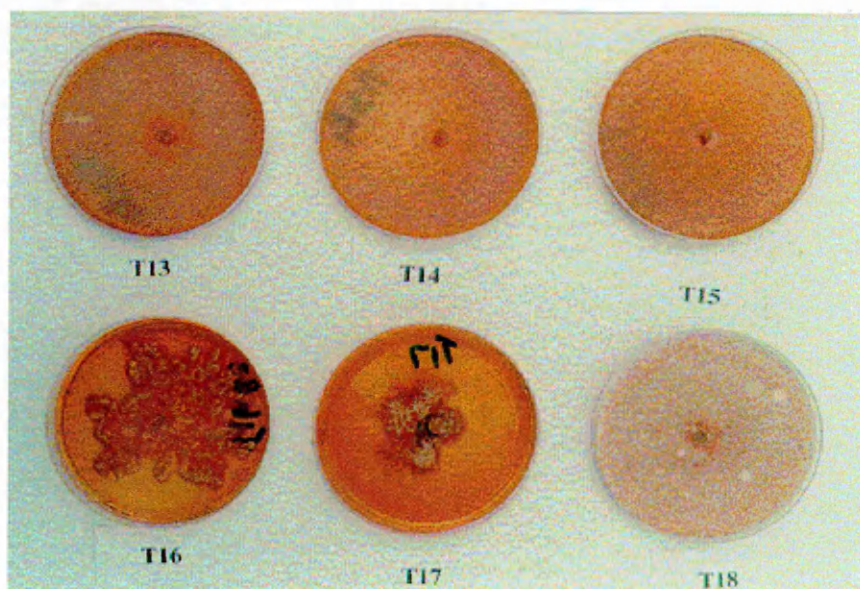


Plate 4.6: The effect of anti-oxidants and imazalil on the mycelial growth of *Colletotrichum musae* (isolate CM103) on malt extract agar after incubation at 25°C for 12 days.

T13 = Benzoic acid (1mM); **T14** = Propyl paraben (1 mM); **T15** = Imazalil (1.68 μ M); **T16** = Imazalil (3.4 μ M); **T17** = Imazalil (8.4 μ M); **T18** = Control.

4.3.3: The effect of BHA alone and in combination with imazalil on the mycelial growth of *C. musae* at different pH levels.

At 25°C, the mycelium of *C. musae* grew over a wide range of pH levels tested (Table 4.6). The optimum pH for growth was around pH 6.5. The slowest growth rate occurred at pH 3.5. i.e. only 51.3% the growth rate obtained at pH 6.5.

The effect of pH was also tested for its effect on the inhibition of fungal growth by low concentrations of BHA and imazalil alone and in combination. It appears that pH has little if no effect on the inhibitory effect of imazalil (Table 4.7) and strangely, the growth on imazalil at pH 3.5 was much higher than the control. In contrast, the effect of BHA on its own at 31.25 µM and 0.5 mM was strongly effected by pH, with the greatest inhibition of growth at pH values of 3.5 and 6.5 and the least at pH values of 5.5 and 7.5. When BHA (0.5 mM) was combined with imazalil (1.68 µM) there was no mycelial growth by day 14 of the experiment. When the concentration of BHA was reduced to 31.25 µM and combined with imazalil (1.68 µM), total inhibition of growth was seen at pH levels of 3.5 and 6.5. The greatest growth was seen at pH 5.5.

4.3.4 The effect on the germination of *C. musae* of low concentrations of BHA and imazalil applied together over six days.

Complete inhibition (100 %) of germination was observed by combining BHA at 1.0 to 5 mM with imazalil 1.68 µM up to 6 days (Table 4.8). At lower concentrations of BHA (0.1 and 0.5 mM) with 1.68 µM imazalil, germination was slowed down but not prevented. There was some indication that there was a synergistic response to the two chemicals when applied together.

Table 4.6: Growth (mm day⁻¹) of *Colletotrichum musae* (isolate CM103) at different pH values inoculated on 5 % malt extract agar at 25 °C.

<i>pH values</i>	<i>Growth (mm day⁻¹)</i>
3.5	7.8 ^e
4.5	11.8 ^c
5.5	14.3 ^b
6.5	15.2 ^a
7.5	11.3 ^d
Lsd	0.3
CV% =	1.9

Results followed by a common letter do not differ significantly

Table 4.7: Growth rate (mm day⁻¹) of *Colletotrichum musae* (isolate CM103) at different pH values in malt extract agar containing butylated hydroxyanisole (BHA) alone and combined with imazalil at 25°C.

<i>Treatments (Concen. mM)</i>	<i>Growth (mm day⁻¹)</i>				
	<i>pH values</i>				
	3.5	4.5	5.5	6.5	7.5
BHA (0.03125) + imazalil (0.00168)	NG ^{* e}	4.1 ^e	6.0 ^e	NG ^f	3.9 ^e
BHA (0.5) + imazalil (0.00168)	NG ^e	NG ^f	NG ^f	NG ^f	NG ^f
Imazalil (0.00168)	11.5 ^b	11.5 ^c	11.5 ^c	11.0 ^c	11.5 ^b
BHA (0.03125)	4.1 ^d	8.6 ^d	12.0 ^b	4.3 ^d	9.8 ^c
BHA (0.5)	NG ^e	4.1 ^e	7.2 ^d	2.3 ^e	5.5 ^d
Control (buffered)	7.8 ^c	11.8 ^b	14.3 ^a	15.2 ^a	11.2 ^b
Control (pH 5.5)	14.3 ^a	14.3 ^a	14.3 ^a	14.3 ^a	14.3 ^a
Lsd (p = 0.05) =	0.2	0.2	0.2	0.3	0.4
CV % =	2.2	1.9	1.6	3.3	3.8

Results followed by a common letter do not differ significantly

*NG = no growth

Table 4.8: The effect of butylated hydroxyanisole (BHA) alone and combined with imazalil on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 25°C.

Treatment concentrations	% germination after different intervals		
	2 days	4 days	6 days
BHA 0.1 mM + imazalil 1.68 µM	72.7 ^b	100 ^a	100 ^a
BHA 0.5 mM + imazalil 1.68 µM	NG ^{*c}	NG ^c	5.7 ^d
BHA 1.0 mM + imazalil 1.68 µM	NG ^c	NG ^c	NG ^c
BHA 2.5 mM + imazalil 1.68 µM	NG ^c	NG ^c	NG ^c
BHA 5.0 mM + imazalil 1.68 µM	NG ^c	NG ^c	NG ^c
Imazalil 1.68 µM	100 ^a	100 ^a	100 ^a
BHA 0.1 mM	100 ^a	100 ^a	100 ^a
BHA 0.5 mM	62.3 ^c	100 ^a	100 ^a
BHA 1.0 mM	14.7 ^d	31.3 ^b	62.3 ^b
BHA 2.5 mM	NG ^c	NG ^c	10.0 ^c
BHA 5.0 mM	NG ^c	NG ^c	NG ^c
Control	100 ^a	100 ^a	100 ^a
Lsd (P = 0.05) =	1.7	0.7	1.3
CV % =	2.8	1.0	1.7

*NG = no growth

Results followed by a common letter do not differ significantly

4.3.5 The effect of very low concentrations of BHA in combination with imazalil and thiabendazole on the germination of *C. musae* at 25°C.

The effect of BHA concentrations as geometric and logarithmic series in combination with imazalil and thiabendazole on conidial germination of *C. musae* (isolate CM 103) at 25°C is shown in tables 4.9 and 4.10 respectively. The only concentration combination which totally prevented germination over 48 hrs was BHA (0.5 mM) with imazalil (1.68 µM). All concentrations of BHA tested (0.03125 – 0.5 mM) with either imazalil (1.68 µM) or TBZ (2.5 µM) significantly reduced germination rate over 24 hrs and in some cases over 48 hrs. It appears that combinations of TBZ and BHA may have a very slight synergistic effect but BHA with imazalil gives a much more dramatic synergistic effect. For example, 100% conidial germination occurred on imazalil at 1.68 µM after 24 hrs and 54% of conidia germinated after 24 hrs on 0.125 mM BHA. When imazalil (1.68 µM) was combined with 0.125 mM BHA, there was no germination after 24 hrs and only 45.7% germination after 48 hrs.

4.3.6 Effect of BHA in combination with imazalil and thiabendazole on the mycelial growth of *C. musae* at 25°C.

The same range of chemical treatments as described above (section 4.3.5) were also tested for their effect on mycelial growth of *C. musae*. Isolate CM100 was only tested using the logarithmic series while CM103 was tested on both series. BHA (geometric and logarithmic concentration series) with imazalil (1.68 µM) or TBZ (2.5 µM) significantly ($P = 0.05$) reduced the growth rate (mm day^{-1}) of both isolates (Table 4.11 to 4.13). Complete inhibition (100%) of growth of CM100 was achieved by combining BHA at concentrations above 0.0625 mM with imazalil (1.68 µM). CM103 was less sensitive to the chemicals and growth was completely inhibited at concentrations of BHA of 0.2 mM and above combined with imazalil (1.68 µM). None of the combinations tested between BHA and TBZ, completely inhibited growth

of CM103 but CM100 was completely inhibited by combinations of BHA (0.25 mM and above) with TBZ (2.5 μ M).

There is strong evidence that combinations of BHA and imazalil have a synergistic effect with respect to control of mycelial growth of *C. musae*. For example, on its own, BHA (0.2 mM) reduced the mycelial growth of CM103 by 43.4 % and imazalil (1.68 μ M) by 19.6 %, but in combination inhibition was 100 % (Table 4.12). On the other hand, combinations of BHA with thiabendazole appeared to give more of an additive effect than a synergistic effect. For example, on its own BHA (0.2 mM) inhibited mycelial growth of CM103 by 42.7% and TBZ (2.5 μ M) inhibited growth by 3.5%. In combination, inhibition was 49% (Table 4.12). Although CM100 is much more sensitive to TBZ than CM103, there was only a slight indication of a synergistic effect when the fungus was treated with combinations of BHA and TBZ (Table 4.13).

Table 4.9: The effect of butylated hydroxyanisole (BHA) alone and combined with imazalil and thiabendazole (TBZ) on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 25°C.

Treatments	Conidial germination (%) after different time intervals			
	BHA + Imazalil		BHA + Thiabendazole	
	24 h	48 h	24 h	48 h
BHA 0.1 mM + imazalil 1.68 µM or TBZ 2.5 µM	22.7 ^f	73.7 ^b	60 ^b	100 ^a
BHA 0.2 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^{*g}	64 ^d	48 ^c	100 ^a
BHA 0.3 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^g	37.7 ^f	35 ^e	93.7 ^b
BHA 0.4 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^g	20.0 ^g	25 ^f	60 ^d
BHA 0.5 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^g	NG ^{*h}	18 ^g	45.7 ^e
Imazalil 1.68 µM	100 ^a	100 ^a	-	-
TBZ 2.5 µM	-	-	100 ^a	100 ^a
BHA 0.1 Mm	63.3 ^b	100 ^a	64.7 ^b	100 ^a
BHA 0.2 mM	52.3 ^c	100 ^a	52.3 ^c	100 ^a
BHA 0.3 mM	41.7 ^d	100 ^a	41.3 ^d	100 ^a
BHA 0.4 mM	34.0 ^{e,c}	70.0 ^c	33 ^e	69.7 ^c
BHA 0.5 mM	22.3 ^f	59.7 ^e	23.3 ^{fg}	60.7 ^d
Control	100 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	2.9	2.6	5.4	4.1
CV% =	4.7	2.2	6.4	2.9

Means followed by the same letter do not differ significantly
*NG = no growth

Table 4.10: The effect of butylated hydroxyanisole (BHA) alone and combined with imazalil and thiabendazole (TBZ) on temporal conidial germination of *Colletotrichum musae* (isolate CM103) on malt extract agar at 25°C.

Treatments	Conidial germination (%) after different time intervals			
	BHA + Imazalil		BHA + Thiabendazole	
	24 h	48 h	24 h	48 h
BHA 0.03125 mM + imazalil 1.68 µM or TBZ 2.5 µM	72 ^d	100 ^a	85 ^c	100 ^a
BHA 0.0625 mM + imazalil 1.68 µM or TBZ 2.5 µM	46.7 ^f	100 ^a	72 ^e	100 ^a
BHA 0.125 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^h	68 ^b	44.3 ^g	100 ^a
BHA 0.25 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^h	45.7 ^d	33.5 ^h	96.3 ^a
BHA 0.5 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^h	NG ^e	18.3 ^j	46.2 ^c
Imazalil 1.68 µM	100 ^a	100 ^a	-	-
TBZ 2.5 µM	-	-	100 ^a	100 ^a
BHA 0.03125 mM	90 ^b	100 ^a	91.3 ^b	100 ^a
BHA 0.0625 mM	80 ^c	100 ^a	79.8 ^d	100 ^a
BHA 0.125 mM	54 ^e	100 ^a	53.7 ^f	100 ^a
BHA 0.25 mM	45 ^f	100 ^a	45 ^g	100 ^a
BHA 0.5 mM	24.3 ^g	61 ^c	24.3 ⁱ	60.7 ^b
Control	100 ^a	100 ^a	100 ^a	100 ^a
Lsd (P=0.05) =	6.9	1.9	4.1	3.7
CV% =	8.0	1.4	3.9	2.5

Means followed by the same letter do not differ significantly

*NG = no growth

Table 4.11: The effect of Butylated hydroxyanisole (BHA) in combination with imazalil and thiabendazole (TBZ) on the mycelial growth of *Colletotrichum musae* (isolate CM103) on malt extract agar at 25°C. (growth mm day⁻¹)

Treatment concentrations	Mycelial growth (mm day ⁻¹)	
	<u>BHA + imazalil</u>	<u>BHA + TBZ</u>
BHA 0.03125 mM + imazalil 1.68 µM or TBZ 2.5 µM	6 ^g	11 ^e
BHA 0.0625 mM + imazalil 1.68 µM or TBZ 2.5 µM	5.4 ^h	10.1 ^f
BHA 0.125 mM + imazalil 1.68 µM or TBZ 2.5 µM	2.5 ⁱ	7.5 ⁱ
BHA 0.25 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^{*j}	7.2 ^j
BHA 0.5 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^j	6 ^k
Imazalil 0.00168 µM	11.5 ^c	-
TBZ 2.5 µM	-	13.8 ^b
BHA 0.03125 mM	12 ^b	11.9 ^c
BHA 0.0625 mM	11.4 ^c	11.4 ^d
BHA 0.125 mM	8.5 ^d	8.6 ^g
BHA 0.25 mM	8 ^e	8 ^h
BHA 0.5 mM	7.2 ^f	7.2 ^j
Control	14.3 ^a	14.3 ^a
Lsd (P = 0.05) =	0.2	0.2
CV % =	1.7	1.61

Results followed by a common letter do not differ significantly

*NG = no growth

Table 4.12: The effect of butylated hydroxyanisole (BHA) in combination with imazalil and thiabendazole (TBZ) on the mycelial growth of *Colletotrichum musae* (isolate CM103) on malt extract agar at 25°C. (growth mm day⁻¹)

Treatment concentrations	Mycelial growth (mm day ⁻¹)	
	<u>BHA + imazalil</u>	<u>BHA + TBZ</u>
BHA 0.1 mM + imazalil 1.68 µM or TBZ 2.5 µM	2.8 ^h	7.8 ^e
BHA 0.2 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^{*i}	7.3 ^g
BHA 0.3 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	6.9 ^h
BHA 0.4 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	6.6 ⁱ
BHA 0.5 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	6.0 ^j
Imazalil 1.68 µM	11.5 ^b	-
TBZ 2.5 µM	-	13.8 ^b
BHA 0.1 mM	8.5 ^c	8.5 ^c
BHA 0.2 mM	8.1 ^d	8.2 ^d
BHA 0.3 mM	7.6 ^e	7.6 ^f
BHA 0.4 mM	7.3 ^f	7.3 ^g
BHA 0.5 mM	7.1 ^g	7.2 ^g
Control	14.3 ^a	14.3 ^a
Lsd (P = 0.05) =	0.2	0.2
CV % =	2.0	1.8

Results followed by a common letter do not differ significantly

*NG = no growth

Table 4.13: The effect of butylated hydroxyanisole (BHA) in combination with imazalil and thiabendazole (TBZ) on the mycelial growth of *Colletotrichum musae* (isolate CM100) on malt extract agar at 25°C. (growth mm day⁻¹)

Treatment concentrations	Mycelial growth (mm day ⁻¹)	
	<u>BHA + imazalil</u>	<u>BHA + TBZ</u>
BHA 0.03125 mM+ imazalil 1.68 µM or TBZ 2.5 µM	1.2 ^h	5.7 ^h
BHA 0.0625 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ^{*i}	3.9 ⁱ
BHA 0.125 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	1.3 ^j
BHA 0.25 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	NG ^k
BHA 0.5 mM + imazalil 1.68 µM or TBZ 2.5 µM	NG ⁱ	NG ^k
Imazalil 1.68 µM	8.6 ^g	-
TBZ 2.5 µM	-	9.7 ^g
BHA 0.03125 mM	18.2 ^b	18.3 ^b
BHA 0.0625 mM	16.5 ^c	16.4 ^c
BHA 0.125 mM	13.0 ^d	13.1 ^d
BHA 0.25 mM	12.0 ^e	12.0 ^e
BHA 0.5 mM	10.1 ^f	10.1 ^f
Control	21.6 ^a	21.6 ^a
Lsd (P = 0.05) =	0.2	1.5
CV % =	0.2	1.9

Results followed by a common letter do not differ significantly

*NG = no growth

4.3.7 The effect on the germination of *C. musae* of low concentrations of BHA and imazalil applied sequentially.

The results of pre-treatment of conidia (for 30 and 60 minutes) with either BHA (0.1 and 0.5 mM) or imazalil (1.68 μ M) followed by plating out on agar containing the other chemical i.e. imazalil (1.68 μ M) or BHA (0.1 and 0.5 mM) are shown in Table 4.14. Germination was completely inhibited when conidia were pre-treated (for 30 and 60 minutes) with BHA (0.5 mM) then plated onto imazalil (1.68 μ M) or vice versa. There was also no germination when these two chemicals were applied together in the agar medium. At the lower concentration of BHA (0.1 mM), the order and length of time of the pre-treatment did not seem to make any difference to the levels of inhibition that occurred. For example, after 48 hrs, there was between 73.3-76.7% germination of the conidia, irrespective of whether the BHA or the imazalil was applied first or whether they were applied together in the agar medium.

Table 4.14: The effect of pre-treatment with butylated hydroxyanisole (BHA) or imazalil on the sensitivity of conidial germination of *C. musae* (isolate CM103) to imazalil and butylated hydroxyanisole respectively, at 25°C.

(Concentrations of chemical treatments)	<u>Conidial germination (%) after different time intervals</u>			
	<u>Treatment time 30</u>		<u>Treatment time 60</u>	
	<u>minutes</u>		<u>minutes</u>	
	<u>24 hrs</u>	<u>48 hrs</u>	<u>24 hrs</u>	<u>48 hrs</u>
BHA 0.1 mM → Imazalil 1.68 µM ⁺	23.3 ^c	73.3 ^c	20.3 ^d	72.8 ^b
BHA 0.5 mM → Imazalil 1.68 µM ⁺	NG ^{*d}	NG ^e	NG ^e	NG ^e
BHA 0.1 mM → Imazalil 0.0 µM ⁺	65 ^b	100 ^a	63.3 ^b	100 ^a
BHA 0.5 mM → Imazalil 0.0 µM ⁺	25.3 ^c	65 ^d	24.3 ^c	63.3 ^d
Imazalil 1.68 µM → BHA 0.1 mM ⁺	24 ^c	76.7 ^b	23 ^{cd}	75.7 ^b
Imazalil 1.68 µM → BHA 0.5 mM ⁺	NG ^d	NG ^e	NG ^e	NG ^e
Imazalil 1.68 µM → BHA 0.0 mM	100 ^a	100 ^a	100 ^a	100 ^a
BHA 0.1 mM + Imazalil 1.68 µM	23 ^c	75 ^{bc}	23.3 ^{cd}	75.3 ^{bc}
BHA 0.5 mM + Imazalil 1.68 µM	NG ^d	NG ^e	NG ^e	NG ^e
Control	100 ^a	100 ^a	100 ^a	100 ^a
Lsd (P = 0.05) =	2.7	2.3	3.0	2.6
CV % =	4.3	2.2	5.0	2.6

Results followed by a common letter do not differ significantly

⁺Conidia are treated in solution for 30 or 60 minutes and then centrifuged and washed and plated onto MEA plates with or without chemical treatments.

^{*}NG = no growth

4.3.8 Effect of BHA alone and in combination with imazalil and thiabendazole on extracellular enzymes produced by *C. musae*.

The results of extracellular enzymes produced by *C. musae* (isolates CM100 and CM103) by using API test are summarised in Table 4.15. Both isolates produced identical enzymes activity patterns on the API ZYM strips. The reactions of extracellular enzymes were very strong allowing further classification of the results using the colour chart supplied by the manufacturer. Reactions were classified on scale of 0 to 5 with increasing strength of reaction. Some enzymes such as leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β glucuronidase, α mannosidase and α fucosidase were not produced by either isolate.

BHA in combination with imazalil and TBZ significantly ($P = 0.05$) reduced the production of enzymes produced by isolate CM103 (Table 4.16). The complete inhibition (100 %) of all enzymes produced by CM103 was achieved by combination of BHA (0.5 mM) + imazalil (1.68 μ M). Only three enzymes were produced when the fungus was grown in a medium containing a combination of BHA (0.5 mM) + TBZ (2.5 μ M). These were alkaline phosphatase, lipase (C14) and N-acetyl- β glucosaminidase. The inhibition percentage by BHA (31.25 μ M) + imazalil (1.68 μ M) was greater than that caused by BHA (31.25 μ M) + TBZ (2.5 μ M). The inhibition rate by imazalil alone (1.68 μ M) was greater than by TBZ (2.5 μ M) but the results were not statistically significant. BHA its own (0.125 & 0.5 mM) significantly inhibited enzymes production as compared to the control.

Table 4.15: Comparison of API ZYM reactions with extracellular enzymes of *Colletotrichum musae* (isolates CM100 and CM103) after different incubation periods (days).

<i>Enzymes assayed</i>	<u><i>CM100</i></u>			<u><i>CM103</i></u>		
	<i>3 d</i>	<i>6 d</i>	<i>10 d</i>	<i>3 d</i>	<i>6 d</i>	<i>10 d</i>
Control	0	0	0	0	0	0
Alkaline phosphatase	2*	4.3	4.3	2	4	4.7
Esterase (C 4)	2	3.7	3.7	1.7	3.7	3.7
Esterase Lipase (C 8)	2.3	3.7	3.7	2.7	4	4
Lipase (C 14)	2	3.7	4.7	2.7	4	4.7
Leucine arylamidase	0	0	0	0	0	0
Valine arylamidase	0	0	0	0	0	0
Cystine arylamidase	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0
Acid phosphatase	2.3	4.3	4.3	2.3	4	4
Naphthol-AS-BI- phosphohydrolase	2.3	4	4.7	2.3	3.7	4.7
α galactosidase	2.3	4	4	2	4	4.3
β galactosidase	2	4.3	4.3	2.3	4	4.3
β glucuronidase	0	0	0	0	0	0
α glucosidase	2	4.3	4.3	2	4	4.7
β glucosidase	2.7	4.7	4.7	3	4.7	4.3
N-acetyl- β glucosaminidase	2	4.3	4.3	2.3	4.7	4.3
α mannosidase	0	0	0	0	0	0
α fucosidase	0	0	0	0	0	0

* Each value is mean of 3 replicates

4.16: Effect of butylated hydroxyanisole (BHA) alone and in combination with imazalil and thiabendazole (TBZ) on extracellular enzyme production by *Colletotrichum musae* (isolate CM103).

Treatments		Enzymes										
(Concentrations mM)	A	B	C	D	E	F	G	H	I	J	K	
BHA (0.03125) + IMZL (0.00168)	1.3 ^d	1 ^c	1.5 ^d	2 ^d	2 ^d	2 ^d	2.3 ^{de}	2 ^d	1.3 ^d	1.7 ^d	1.7 ^{ef}	
BHA (0.5) + IMZL (0.00168)	0 ^e	0 ^d	0 ^e	0 ^e	0 ^e	0 ^e	0 ^f	0 ^e	0 ^e	0 ^e	0 ^g	
BHA (0.03125) + TBZ (0.0025)	2.7 ^b	1.7 ^{bc}	2 ^{cd}	2.7 ^c	2.7 ^c	2.7 ^c	2.7 ^{cd}	2.7 ^c	2.3 ^c	2 ^d	2.3 ^{de}	
BHA (0.5) + TBZ (0.0025)	1.3 ^d	0 ^d	0 ^e	0.3 ^e	0 ^e	0 ^e	0 ^f	0 ^e	0 ^e	0 ^e	1 ^f	
Imazalil (0.00168)	2.7 ^b	1.7 ^{bc}	2 ^{cd}	2.5 ^{cd}	2.5 ^{cd}	2.5 ^{cd}	3 ^c	2.5 ^{cd}	2.3 ^c	2 ^d	2 ^{de}	
TBZ (0.0025)	3 ^b	2 ^b	2.3 ^c	3 ^c	3 ^c	3 ^c	3 ^c	3 ^c	2.7 ^c	2.7 ^c	2.7 ^{cd}	
BHA (0.03125)	4 ^a	3.3 ^a	4 ^{ab}	4.5 ^{ab}	4.5 ^{ab}	4.5 ^{ab}	4.7 ^{ab}	4.5 ^{ab}	4.3 ^{ab}	4 ^a	4 ^{ab}	
BHA (0.125)	2.3 ^{bc}	1.7 ^{bc}	3.3 ^b	4 ^b	4 ^b	4 ^b	4.3 ^b	4 ^b	3.7 ^b	3.3 ^b	3.3 ^{bc}	
BHA (0.5)	1.7 ^{cd}	1.3 ^{bc}	2 ^{cd}	2 ^d	2 ^d	2 ^d	2 ^e	2 ^d	2 ^{cd}	2 ^d	2 ^{de}	
Control	4.7 ^a	3.7 ^a	4.3 ^a	4.7 ^a	4.7 ^a	4.7 ^a	5 ^a	4.7 ^a	4.7 ^a	4.3 ^a	4.3 ^a	
Lsd(P = 0.05) =	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.8	0.6	0.7	
CV % =	20.4	27.4	19.5	15.1	15.1	13.5	13.5	13.5	20.7	16.6	17.5	

A = Alkaline Phosphatase, **B** = Esterase Lipase (C 4), **C** = Esterase Lipase (C 8), **D** = Lipase (C 14), **E** = Acid phosphatase, **F** = Naphthol-AS-BI- phosphohydrolase, **G** = α galactosidase, **H** = β galactosidase, **I** = α glucosidase, **J** = β glucosidase and **K** = N-acetyl- β glucosaminidase
Results followed by a common letter do not differ significantly

CHAPTER 5

CONTROL OF

ANTHRACNOSE

OF BANANAS

5.1 INTRODUCTION

Many fungicides that show strong toxic effects against micro-organisms *in vitro* are also phytotoxic. In the development of new fungicidal treatments, it is therefore essential for the chemicals to also be tested on the host plant (*in vivo*) to determine if phytotoxicity will be a problem (Cremlyn, 1991). It is also important to determine whether the chemicals will retain their fungi-toxicity *in vivo*.

The previous two chapters (3 and 4) describe research that shows that certain antioxidants (butylated hydroxyanisole, propyl paraben and benzoic acid) and the natural plant extract azadiractin, have strong antifungal properties *in vitro*. Furthermore, combinations of some of these chemicals together and in combination with the fungicides imazalil or thiabendazole gave excellent control of *C. musae* at relatively low doses. The next step required was to test these chemicals alone and in combination for their ability to control actual diseases of bananas caused by *C. musae*.

Colletotrichum musae (Berk. & Curts) Arx is the causal agent of two main diseases important in exported banana fruit i.e. anthracnose (this chapter) and crown rot (chapter 6). The fungus most commonly infects through its conidia which may germinate in wounds on the fruit or can germinate and penetrate directly through the banana skin (section 1.3.2). Where anthracnose infects pre-harvest, the disease is usually latent and not visible on the fruit at the time of harvest. The only really sure way to control anthracnose disease is to apply postharvest fungicides that can penetrate the skin to destroy appressoria and other latent infection structures (eradicant effect).

Conidia of *C. musae* are considered to be ubiquitous in banana plantations, so the surfaces of harvested fruit are likely to carry numerous conidia. If these spores are not removed or killed during the washing and postharvest treatment process, it is also possible that they may still be able to infect wounds that occur on the fruit during subsequent handling. It is therefore desirable that fungicide treatments are able to

sustain their activity against *C. musae* throughout the postharvest chain (protectant effect).

5.2 EXPERIMENTAL SET-UP

Four replicates (consisting of three bananas each replicate) were used in all anthracnose experiments (wound and spore inoculation study), except in Experiment, where the pH values of 60 bananas were measured and the mean was calculated. Two experiments (5.4 & 5.5) were repeated. Citrate-phosphate buffer was used to stabilise the pH value (4.6) of different treatments. All treatments in each experiment were randomised and a complete randomised design was used for analysis.

5.3 RESULTS

5.3.1 The effect of antioxidants and imazalil on the control of anthracnose wound infections of banana fruit

When mycelium of *C. musae* (isolate CM103) was inoculated into wounds on the peel of banana fruits, the resulting anthracnose lesion showed an initial slower growth period for the first couple of days and then grew in a diametrically linear fashion (Figure 5.1). Treatment of the fruit after inoculation with butylated hydroxyanisole (BHA), propyl paraben (PP) and benzoic acid (1-15 mM) caused a significant reduction in the lesion growth (Figure 5.1; Table 5.1). BHA was the most effective chemical with a concentration of 10 mM reducing the growth rate by 32 % compared to the control. Propyl gallate was the least effective with concentrations of 5 and 10 mM not giving any control and 15 mM concentration giving a reduction in growth of only 9.1%. Only slight increases in the lag period (1-2 days) were seen with any of the chemical treatments.

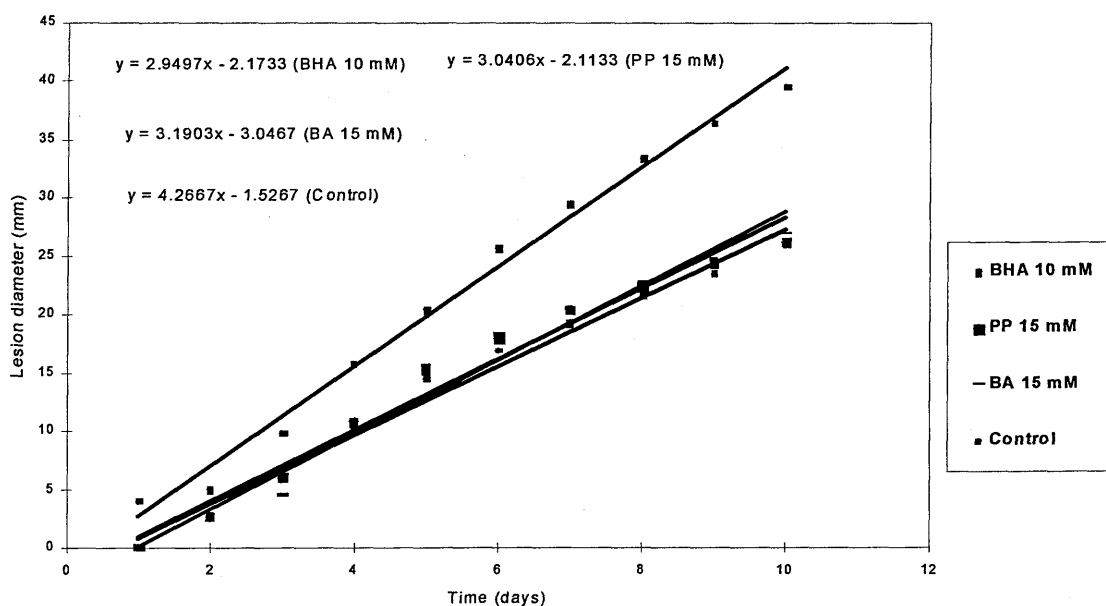


Figure 5.1: Effect of butylated hydroxyanisole (BHA), propyl paraben (PP) and benzoic acid (BA) on the temporal growth of anthracnose lesions after wound inoculation of banana fruit with mycelial plugs of *Colletotrichum musae* (isolate CM103) at 25°C. Regression lines, used to calculate the rate of growth are presented for each treatment data set.

Imazalil alone or BHA in combinations with benzoic acid, PP or imazalil significantly ($P < 0.05$) reduced the expansion of anthracnose lesions after wound inoculation compared to control (Table 5.2; Plate 5.1). At 14°C, the lesions in the control fruit grew at about half the rate that they did at 25°C. Also at 14°C, there was a lag period of 4 days before growth was observed compared with no lag period at 25°C. The percentage reduction in growth rate caused by the chemical treatments was much greater than at 14°C compared to that at 25°C. Imazalil on its own at 0.8412 mM and 1.6824 mM caused a reduction in growth at 25°C of 17.7% and 31.4% respectively. At 14°C, the respective inhibition levels were 25% and 66.7%.

At either temperature, the most effective treatments were BHA (5 mM) + imazalil (0.8412 mM) which reduced the growth rate by 75% (14°C) and 37.3 % (25°C) and BHA (5 mM) + PP (10 mM) which reduced the growth rate by 70.8% (14°C) and 33 % (25°C). All the treatments gave rise to considerable lag periods ranging from 6-10 days at 14°C and 3-8 days at 25°C.

5.3.2 The effect of antioxidants, imazalil and thiabendazole on banana fruit anthracnose induced by spore inoculation

In this study the skin of the banana was not damaged and *C. musae* conidia were used as the source of infection. Fruits were either inoculated and then incubated for three days before treatment (Tables 5.3 and 5.5) or treated first and then inoculated after 1 hr (Table 5.4). In control fruit, inoculation gave rise to brown lesions which grew slower for about three days, before achieving a faster, linear growth rate (Figure 5.2).

Overall, all treatments significantly ($P < 0.05$) reduced lesion size (mm day^{-1}) of *C. musae* when compared to the control. When single chemical treatments was carried out after establishment of latent infections, the most effective chemical was found to be BHA which at 10 mM inhibited growth at 25°C by 41.2%. The least effective chemical was propyl gallate (Table 5.3). The same pattern was seen when treatment

was carried out before inoculation, however, inhibition levels were slightly higher e.g. BHA (10 mM) inhibited growth by 48.2% (Table 5.4). In this experiment, both thiabendazole (TBZ) and imazalil gave equal levels of inhibition (52.7%) at 2.485 mM and 1.6824 mM (both 500 ppm) respectively. The lag time in days was increased by some individual treatments (1-4 days). BHA at higher concentration (15 mM) was found to cause injury to the banana peel in the form of tissue blackening.

The rate of lesion growth at 14°C was half that at 25°C and the lower temperature caused a lag of 5 days in observable growth (Table 5.5). The effect of chemical treatments on growth at 14°C was much greater than that which might be predicted from the effects at 25°C. In fact several treatments at 14°C completely prevented growth including imazalil (0.5047-1.6824 mM) and combinations of BHA (5 mM) with PP or benzoic acid (10 mM) or imazalil (0.5047 and 0.8412 mM) (Table 5.5). At 25°C, the most effective treatments was BHA (5 mM) + imazalil (0.8412 mM) giving 66.7% inhibition compared to control. This was better than imazalil on its own at 1.6824 mM) which gave 58.8% control. At 25°C, some combinations of chemicals gave less control than might have been expected from their individual efficacy, however, the two best combination treatments actually gave slightly higher inhibition than might have been expected (possible synergistic effect).

The lag time prior to growth was markedly increased by chemical treatments i.e. 2-10 days at 25°C and 7 days to no growth at 14°C (Table 5.5). Plate 5.2 shows some examples of the efficacy of treatments at 25°C.

Table 5.1: Effect of anti-oxidants on the expansion of anthracnose lesions (mm day⁻¹) after wound inoculation of banana fruits with mycelial plugs of *Colletotrichum musae* (isolate CM103) at 25°C.

<i>Treatments</i>	<i>Growth rate (mm day⁻¹)</i>	<i>Lag Time (days)</i>
Butylated hydroxyanisole 1 mM	3.6 ^c	1
Butylated hydroxyanisole 5 mM	3.4 ^{cd}	2
Butylated hydroxyanisole 10 mM	3.0 ^e	2
Propyl paraben 5 mM	3.6 ^c	0
Propyl paraben 10 mM	3.5 ^c	1
Propyl paraben 15 mM	3.0 ^e	1
Benzoic acid 5 mM	3.6 ^c	0
Benzoic acid 10 mM	3.2 ^{de}	1
Benzoic acid 15 mM	3.2 ^{de}	1
Propyl gallate 5 mM	4.4 ^a	0
Propyl gallate 10 mM	4.4 ^a	1
Propyl gallate 15 mM	4.0 ^b	1
Control with 50 % acetone	4.4 ^a	0
Control with distilled water	4.4 ^a	0
Lsd (P = 0.05) =	0.3	
CV % =	5.7	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

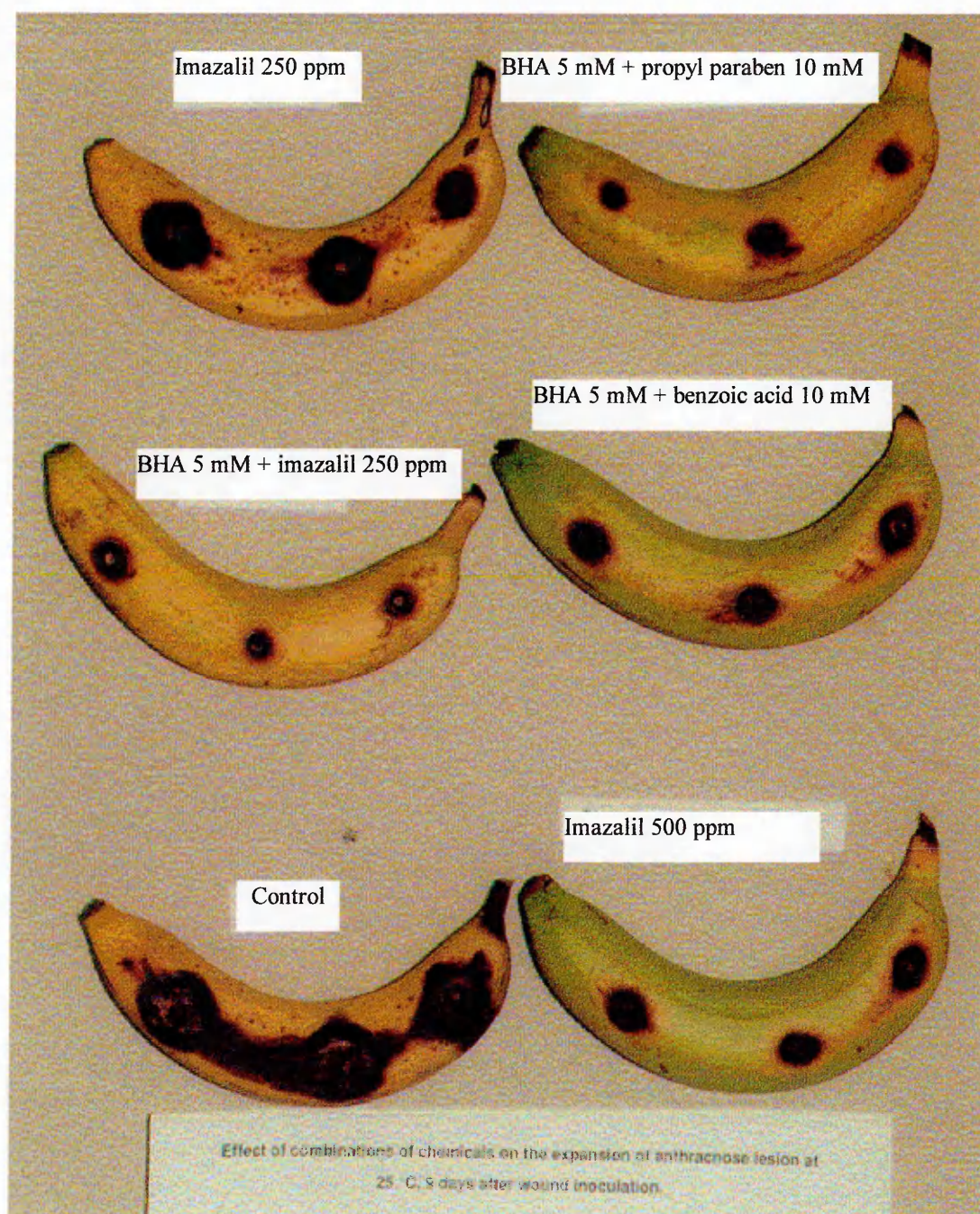


Plate 5.1: Effect of imazalil (0.8412 mM and 1.6824 mM = 250 and 500 ppm) and butylated hydroxyanisole (5 mM) in combinations with propyl paraben (10 mM), benzoic acid (10 mM) and imazalil (0.8412 mM = 250 ppm) on the expansion of anthracnose lesions. Photograph taken 9 days after wound inoculation of banana fruits with mycelial plugs of *Colletotrichum musae* at 25 °C.

Table 5.2: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben, benzoic acid and imazalil on the expansion of anthracnose lesions (mm day^{-1}) after wound inoculation of banana fruits with a mycelial plugs of *Colletotrichum musae* (isolate CM103) at 25 and 14 °C.

<i>Treatments</i>	<i>Growth rate (mm day^{-1})</i>		<i>Lag Time (days)</i>	
	14 °C	25 °C	14 °C	25 °C
BHA 5 + propyl paraben 10 mM	0.7 ^{de}	3.4 ^d	8	6
BHA 5 + benzoic acid 10 mM	1.3 ^c	3.6 ^c	6	5
BHA 5 + imazalil 0.8412 mM	0.6 ^e	3.2 ^e	10	8
Imazalil 0.8412 mM	1.8 ^b	4.2 ^b	6	3
Imazalil 1.6824 mM	0.8 ^d	3.5 ^{cd}	9	7
Control	2.4 ^a	5.1 ^a	4	0
Lsd ($P = 0.05$) =	0.2	0.1		
CV % =	9.9	2.3		

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

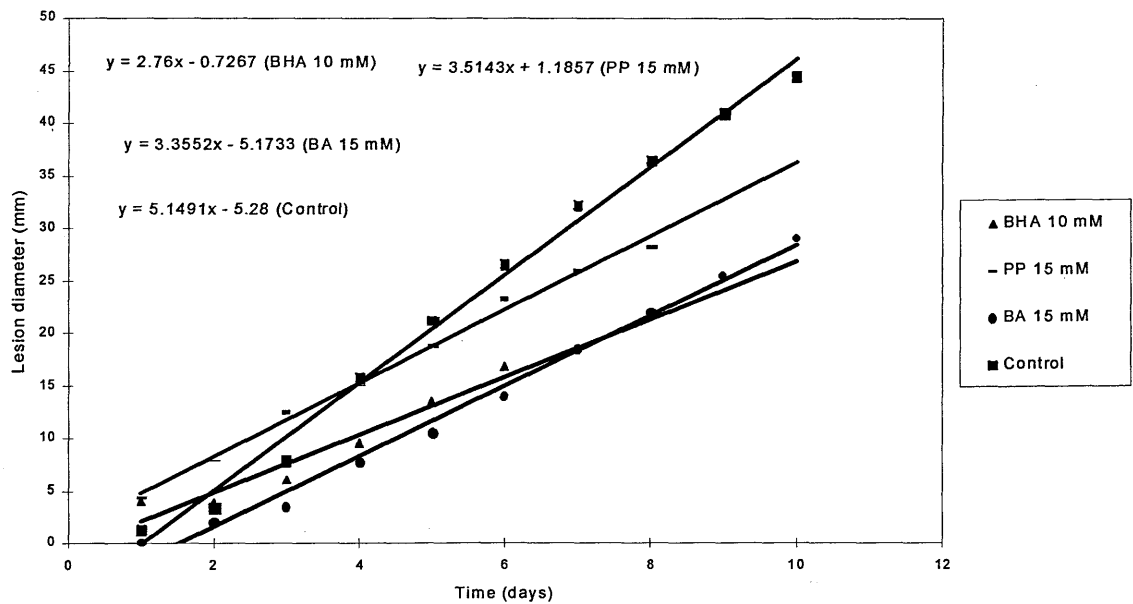


Figure 5.2: Effect of butylated hydroxyanisole (BHA), propyl paraben (PP) and benzoic acid (BA) on the temporal expansion of anthracnose lesions. Treatment was carried out after establishment of infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C. Regression lines, used to calculate the rate of growth are presented for each treatment data set.

Table 5.3: Effect of antioxidants on the expansion of anthracnose lesions (mm day^{-1}). Treatment was carried out after establishment of the infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

Treatments	Growth rate (mm day^{-1})	Lag Time (days)
Butylated hydroxyanisole 1 mM	4.6 ^{bc}	1
Butylated hydroxyanisole 5 mM	4.0 ^d	3
Butylated hydroxyanisole 10 mM	3.0 ^f	4
Propyl paraben 5 mM	4.8 ^b	1
Propyl paraben 10 mM	4.0 ^d	2
Propyl paraben 15 mM	3.5 ^e	2
Benzoic acid 5 mM	4.5 ^c	0
Benzoic acid 10 mM	3.9 ^d	1
Benzoic acid 15 mM	3.4 ^e	1
Propyl gallate 5 mM	4.6 ^{bc}	0
Propyl gallate 10 mM	4.7 ^{bc}	0
Propyl gallate 15 mM	4.7 ^{bc}	0
Control with 50 % acetone	5.1 ^a	0
Control with distilled water	5.2 ^a	0
Lsd (P = 0.05) =	0.2	
CV % =	3.4	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 5.4: Effect of anti-oxidants and fungicides on the expansion of anthracnose lesions (mm day⁻¹). Treatment was carried out prior to establishment of infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

<i>Treatments</i>	<i>Growth rate (mm day⁻¹)</i>	<i>Lag Time (days)</i>
Butylated hydroxyanisole 10 mM	2.8 ^e	2
Propyl paraben 15 mM	3.3 ^d	2
Benzoic acid 15 mM	3.7 ^c	2
Propyl gallate 15 mM	4.2 ^b	1
Thiabendazole 2.4850 mM	2.6 ^f	4
Imazalil 1.6824 mM	2.6 ^f	4
Control with distilled water	5.5 ^a	0
Control with 50% acetone	5.4 ^a	0
Lsd (P = 0.05) =	0.2	
CV % =	3.5	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 5.5: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben, benzoic acid and imazalil on the expansion of anthracnose lesions (mm day⁻¹). Treatment was carried out after establishment of infection by intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 14 and 25°C.

<i>Treatments</i>	<i>Growth rate (mm day⁻¹)</i>		<i>Lag Time (days)</i>	
	14 °C	25 °C	14 °C	25 °C
BHA 5 + propyl paraben 10 mM	NG ^e	2.3 ^g	NG*	9
BHA 5 + benzoic acid 10 mM	NG ^e	3.3 ^f	NG	7
BHA 5 + imazalil 0.1682 mM	1.4 ^d	3.9 ^{cd}	10	4
BHA 5 + imazalil 0.5047 mM	NG ^e	3.5 ^{ef}	NG	5
BHA 5 + imazalil 0.8412 mM	NG ^e	1.7 ^h	NG	10
BHA 5 mM	1.8 ^c	3.9 ^{cd}	9	3
Propyl paraben 10 mM	1.8 ^c	3.7 ^{de}	9	3
Benzoic acid 10 mM	2.0 ^b	4.1 ^c	8	2
Imazalil 0.1682 mM	2.0 ^b	4.5 ^b	7	2
Imazalil 0.5047 mM	NG ^e	4.2 ^{bc}	NG	2
Imazalil 0.8412 mM	NG ^e	3.2 ^f	NG	3
Imazalil 1.6824 mM	NG ^e	2.1 ^g	NG	10
Control ¹	2.5 ^a	5.1 ^a	5	0
Lsd (P = 0.05) =	0.2	0.4		
CV % =	14.2	7.5		

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed



Plate 5.2: Effect of imazalil alone and of butylated hydroxyanisole in combinations with propyl paraben, benzoic acid and imazalil on the expansion of anthracnose lesions (photograph taken 9 days after inoculation). Treatment was carried out after establishment of infection by intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate 103) at 25 °C.

T1 = Control; **T2** = BHA (5 mM) + benzoic acid (10 mM); **T3** = BHA (5 mM) + propyl paraben (10 mM); **T4** = Imazalil (1.6824 mM); **T5** = BHA (5 mM) + imazalil (0.8412 mM).

5.3.3 pH studies

(a) pH of banana surface

The pH values at the peel surface of 60 fruits were measured. The mean value was 4.7 with a range of 4.3-5.1.

(b) pH of treatment solutions

The pH value of each treatment used in *in vivo* studies against anthracnose and crown rot of bananas (chapter 6) was measured and is presented in Table 5.6. The maximum pH (6.6) was recorded in the BHA (10 mM) solution. The minimum pH value was 3.2 and 3.3 for benzoic acid (10 and 15 mM), and BHA (5 mM) + benzoic acid (10 mM) solution respectively.

(c) Effect of treatments (buffered at 4.6 or unbuffered) on the pH of the banana surface and on the expansion of anthracnose lesions

Fruit was inoculated with *C. musae* conidia and incubated for 3 days before treatments. The pH of the peel surface of individual banana fruits were determined before and after treatment with a number of antioxidants, imazalil and TBZ (Table 5.7). In some treatments, the chemicals were buffered to pH 4.6. The buffer solution citrate-phosphate buffer itself had very little effect on disease development (about 2%)

The maximum reduction of pH was caused by benzoic acid (10 mM) both in unbuffered treatments (0.6 units) and in the treatments buffered to pH 4.6 (0.4 units). BHA at 10 mM (buffer pH 4.6) reduced the pH by 0.2 units, and at the same concentration without buffer reduced pH by 0.4 units. PP (10 mM) under both conditions (buffered and unbuffered) increased pH value by 0.2 units. Imazalil (1.6824

mM) on the other hand reduced pH by 0.2 units in both buffered and unbuffered treatments.

Overall, all treatments (buffered and unbuffered) significantly ($P < 0.05$) reduced lesion size (mm day^{-1}) of *C. musae* (Table 5.7). With buffer (pH 4.6), BHA (10 mM) reduced the lesion size by 33.3 %, PP (10 mM) by 26 %, benzoic acid (10 mM) by 25.5 %, TBZ (2.4850 mM) by 38% and imazalil (1.6824 mM) by 56 %. On the other hand, the same chemicals at the same concentrations without being buffered, reduced the lesion size by 29.4, 25.5, 31.4, 39.2 and 52.9 % respectively. Propy gallate at 10 mM was hardly effective at controlling lesion growth, giving inhibition levels of just 7.9 and 3.9% in buffered and unbuffered solutions respectively.

Table 5.6: pH of different treatments used *in vivo* studies against anthracnose and crown rot of bananas caused by *Colletotrichum musae*

<i>Treatment</i>	<i>pH</i>
BHA 1 mM	6.6 ^a
BHA 5 mM	6.6 ^a
BHA 10 mM	6.6 ^a
Propyl paraben 5 mM	5.6 ^b
Propyl paraben 10 mM	5.2 ^d
Propyl paraben 15 mM	4.8 ^e
Benzoic acid 5 mM	3.4 ^j
Benzoic acid 10 mM	3.2 ^{jk}
Benzoic acid 15 mM	3.2 ^k
Propyl gallate 5 mM	3.9 ⁱ
Propyl gallate 10 mM	4.2 ^{gh}
Propyl gallate 15 mM	4.5 ^f
TBZ 2.4850 mM	4.4 ^f
Imazalil 0.1685 mM	4.4 ^f
Imazalil 0.5047 mM	4.1 ^h
Imazalil 0.8412 mM	4.2 ^{gh}
Imazalil 1.6824 mM	4.2 ^{gh}
BHA 5 + propyl paraben 10 mM	5.5 ^{bc}
BHA 5 + benzoic acid 10 mM	3.3 ^{jk}
BHA 5 + imazalil 0.1685 mM	4.1 ^h
BHA 5 + imazalil 0.5047 mM	4.2 ^{gh}
BHA 5 + imazalil 0.8412 mM	4.3 ^{fg}
Acetone (30 %)	5.4 ^{cd}
Distilled water	5.0 ^e
Lsd (P = 0.05) =	0.2
CV % =	2.6

Results followed by a common letter do not differ significantly

Table 5.7: The effect of antioxidants and fungicides (buffer 4.6 and without buffer) on the pH of banana surface and on the expansion of anthracnose lesion. Treatment was carried out after establishment of infection by inoculation of intact banana fruits with conidial suspension of *Colletotrichum musae* (isolate CM 103) at 25 °C. pH_a = pH of banana surface before treatment. pH_b = pH of banana surface after treatment.

<i>Treatments</i>	pH_a	pH_b	<i>Change in pH</i> = ($pH_a - pH_b$)	<i>Growth rate</i> (mm day ⁻¹)
BHA 10 mM*	4.9	4.7	- 0.2	3.4 ^{bc}
BHA 10 mM	4.9	4.5	- 0.4	3.6 ^b
Propyl paraben 10 mM*	5.0	5.2	+ 0.2	3.7 ^b
Propyl paraben 10 mM	4.6	4.8	+ 0.2	3.8 ^b
Benzoic acid 10 mM*	5.0	4.6	- 0.4	3.8 ^b
Benzoic acid 10 mM	4.9	4.3	- 0.6	3.5 ^{bc}
Propyl gallate 10 mM*	4.5	4.7	+ 0.2	4.8 ^a
Propyl gallate 10 mM	4.8	5.2	+ 0.4	4.9 ^a
TBZ 2.4850 mM*	4.9	5.0	+ 0.1	3.1 ^c
TBZ 2.4850 mM	4.5	4.4	- 0.1	3.1 ^c
Imazalil 1.6824 mM*	4.4	4.2	- 0.2	2.2 ^d
Imazalil 1.6824 mM	4.6	4.4	- 0.2	2.4 ^d
Acetone (30 %)*	5.2	5.1	- 0.1	5.1 ^a
Acetone (30 %)	5.1	5.2	+ 0.1	5.1 ^a
Buffer*	4.4	4.4	0.0	5.0 ^a
Distilled water	4.9	5.0	+ 0.1	5.1 ^a
Lsd (p = 0.05) =				0.4
CV % =				7.9

Results followed by a common letter do not differ significantly

* pH (4.6) of the chemicals were maintained by using a citrate-phosphate buffer.

BHA = butylated hydroxyanisole TBZ = thiabendazole

5.3.4: The effect of azadiractin on the control of wound anthracnose on banana fruits

The effect of 15% azadiractin A (neem seed compound) was tested on the growth of anthracnose lesions from wounds inoculated with mycelium of *C. musae*. At concentrations of 0.5 and 1 mM only a slight inhibition of lesion expansion (18 and 27% respectively) was observed when compared to the control (Table 5.8).

5.3.5: The effect of azadiractin on anthracnose of banana fruits induced by spore inoculation

Azadiractin was also tested against anthracnose lesions induced by fruit inoculation with conidial suspensions. When treatment was carried out after inoculation, concentrations of 0.5 and 1 mM reduced the lesion size by 27.5 and 39.2 % respectively at 25°C (Table 5.9).

When treatment was carried out before inoculation, azadirachtin at a concentration of 1 mM reduced the growth rate of *C. musae* by 27.3 %. TBZ (2.4850 mM) and imazalil (1.6824 mM) concentration inhibited the growth rate by 51.9 % when compared to the control (Table 5.10).

Table 5.8: Effect of azadirachtin on the expansion of anthracnose lesions (mm day^{-1}) after wound inoculation with a mycelial plug of *Colletotrichum musae* (isolate CM103) at 25°C.

Treatments	Growth rate (mm day^{-1})
Azadirachtin 0.5 mM	3.6 ^b
Azadirachtin 1.0 mM	3.1 ^c
Control with 30 % acetone	4.4 ^a
Control with distilled water	4.4 ^a
Lsd ($P = 0.05$) =	0.2
CV % =	3.0
Means followed by the same letter do not differ significantly	

Table 5.9: Effect of azadirachtin on the expansion of anthracnose lesions (mm day^{-1}). Treatment was carried out after establishment of latent infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

Treatments	Growth rate (mm day^{-1})
Azadirachtin 0.5 mM	3.7 ^b
Azadirachtin 1.0 mM	3.1 ^c
Control with 30 % acetone	5.1 ^a
Control with distilled water	5.2 ^a
Lsd ($P = 0.05$) =	0.3
CV % =	4.4
Means followed by the same letter do not differ significantly	

Table 5.10: Effect of azadirachtin on the expansion of anthracnose lesions (mm day^{-1}). Treatment was carried out prior to establishment of infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

Treatments	Growth rate (mm day^{-1})
Azadirachtin 1.0 mM	4.0 ^b
Thiabendazole 2.4850 mM	2.6 ^c
Imazalil 1.6824 mM	2.6 ^c
Control with 30 % acetone	5.5 ^a
Control with distilled water	5.4 ^a
Lsd (P = 0.05) =	0.1
CV % =	2.6
Means followed by the same letter do not differ significantly	

CHAPTER 6

CONTROL OF

CROWN ROT OF

OF BANANAS

6.1 INTRODUCTION

Today, the most serious export disease of banana fruits is crown rot. Although this can be caused by a complex of pathogens, as described in section 1.3.2, *C. musae* is the principal fungus implicated in the disease in many countries. Infection occurs shortly after harvest when hands or clusters of fruit are cut from the bunch. Control of crown rot is difficult owing to the range of micro-organisms that may be involved and the physical barrier created by the conidia being drawn up deep (5-7 mm) into the crown vascular tissues (Greene & Goos, 1963). Postharvest treatments using fungicides such as imazalil or thiabendazole are not always successful in controlling crown rot.

This chapter describes the *in vivo* testing of a small number of antioxidants (butylated hydroxyanisole, propyl paraben and benzoic acid) and the natural plant extract azadirachtin alone and in combination with the fungicides imazalil or thiabendazole against crown rot.

6.2 EXPERIMENTAL SET-UP

In some experiments, single banana fingers were used to simulate crown rot (Tables 6.1, 6.2, 6.3, 6.6, 6.9 and 6.10). The individual fingers were cut from the crown to give a similar length of stalk in each replicate. The cut end of the stalk was inoculated with conidia to induce stalk rot. In other experiments, whole banana clusters were used and the cut crown was inoculated (Tables 6.4, 6.5, 6.7 and 6.8). In all experiments except one (Table 6.3), just one isolate, CM103 was used.

In normal postharvest practice, infection is likely to occur before treatment is applied. Experiments were therefore carried out by establishing an infection first and then treating with the chemicals (section 6.3.1 *a*). It was also considered to be of interest to see whether the presence of the chemical in the crown tissues would prevent the conidia from establishing an infection should the crown tissue become contaminated

after treatment. Some experiments were therefore also carried out where the fruit crowns were treated first and then inoculated (section 6.3.1 *b*)).

Four replicates were used in all crown rot experiments (pre-inoculation and post-inoculation study). Three experiments (6.2, 6.4 and 6.8) were repeated. All treatments in each experiment were randomised and a complete randomised design was used for analysis.

6.3 RESULTS

In all the experiments, the expansion of the stalk or crown rot lesions was discernible from the blackening of the tissues and later from the appearance of salmon coloured acervuli on the surface of the infected tissues.

Growth rates (mm day^{-1}) of each treatment was determined by plotting the lesion expansion against time and calculating the slope of the regression line for the linear growth phase. An example is shown in figure 6.1.

6.3.1 The effect of antioxidants and imazalil in controlling stalk and crown rot infections

a) Pre-inoculation studies

In these experiments, the banana tissues were first inoculated and then treated after 48 hours incubation at 25 °C. Lesion expansion in the control fruits was fairly linear over the measurement period (Figure 6.1).

In a series of studies with antioxidants alone, and in combination with each other and with imazalil, it was found that there were significant ($P < 0.05$) reductions in crown rot infections between control and treated fruits. When applied as individual chemicals to the stalks of single fingers, the most effective treatments were butylated

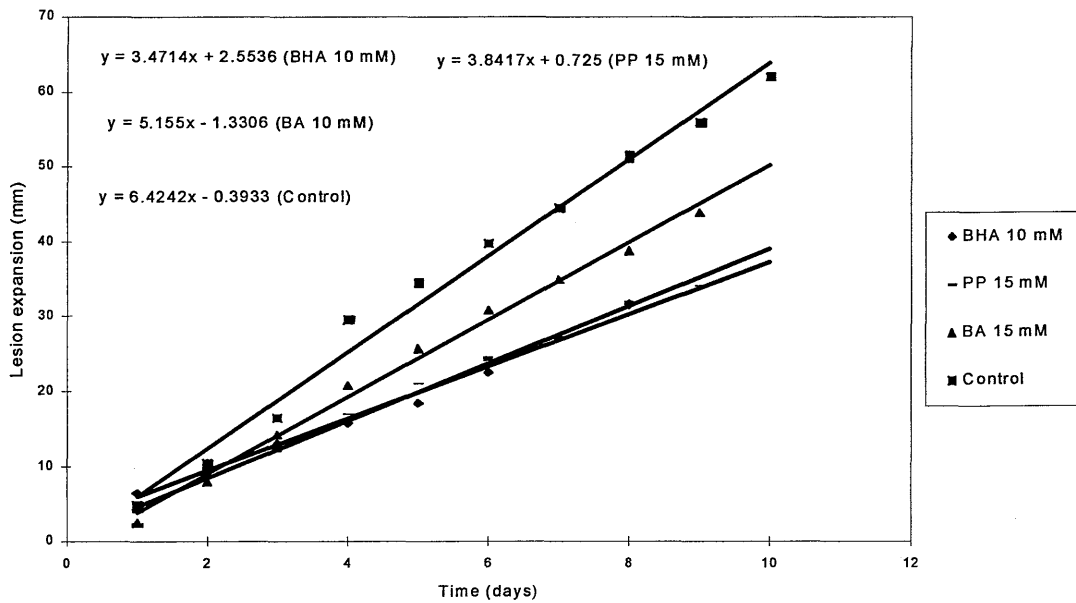


Figure 6.1: Effect of butylated hydroxyanisole (BHA), propyl paraben (PP) and benzoic acid (BA) on the temporal expansion of crown rot lesions. Treatment was carried out after establishment of infection by inoculation of intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

hydroxyanisole (BHA) and imazalil. BHA (1 and 5 mM) inhibited the rate of lesion expansion at 25°C by between 35-39% when compared to the control (Tables 6.1 and 6.2). At 14°C, inhibition by 5 mM was 48% and this concentration of BHA increased the lag period by 1-2 days at 25°C and 9 days at 14°C. At 25°C, imazalil (0.8412 and 2.485 mM) inhibited the rate of lesion expansion by 38 and 52% respectively and caused a lag period of 3 and 8 days respectively. There was no growth of the fungus at either of these concentrations of imazalil at 14°C (Table 5.2). The least effective chemical tried was propyl gallate (PG), which at 15 mM, only inhibited the rate of lesion expansion by 13% at 25°C.

When some of the chemicals were applied to the fruit stalks in combination, the most effective treatments were BHA (5 mM) with imazalil (0.8412 mM) or propyl paraben (PP) (10 mM). These treatments gave a reduction in the rate of lesion expansion at 25°C of 72 and 62% respectively and caused a lag period of 10 and 9 days respectively

Table 6.1: Effect of anti-oxidants on the expansion of crown rot lesions (mm day⁻¹) after establishment of infection by inoculation on crown surface of the banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

<i>Treatments</i>	<i>Growth rate (mm day⁻¹)</i>	<i>Lag Time (days)</i>
Butylated hydroxyanisole 1 mM	4.0 ^g	0
Butylated hydroxyanisole 5 mM	3.8 ^h	1
Butylated hydroxyanisole 10 mM	3.6 ⁱ	2
Propyl paraben 5 mM	4.9 ^e	0
Propyl paraben 10 mM	4.5 ^f	1
Propyl paraben 15 mM	3.9 ^{gh}	1
Benzoic acid 5 mM	5.2 ^d	0
Benzoic acid 10 mM	5.3 ^c	1
Benzoic acid 15 mM	4.8 ^e	1
Propyl gallate 5 mM	5.9 ^b	0
Propyl gallate 10 mM	5.4 ^c	0
Propyl gallate 15 mM	5.4 ^c	1
Control with 50 % acetone	6.2 ^a	0
Control with distilled water	6.1 ^a	0
Lsd (P = 0.05) =	0.2	
CV % =	2.8	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 6.2: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben, benzoic acid and imazalil on the expansion of crown rot lesions (mm day^{-1}). Treatment was carried out after establishment of infection by intact banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 14 and 25 °C.

Treatments	<u>Growth rate (mm day^{-1})</u>		<u>Lag Time (days)</u>	
	14 °C	25 °C	14 °C	25 °C
BHA 5 + propyl paraben 10 mM	NG ^e	2.3 ^g	NG*	9
BHA 5 + benzoic acid 10 mM	NG ^e	3.5 ^e	NG	7
BHA 5 + imazalil 0.1685 mM	NG ^e	3.9 ^d	NG	2
BHA 5 + imazalil 0.5047 mM	NG ^e	3.4 ^e	NG	4
BHA 5 + imazalil 0.8412 mM	NG ^e	1.7 ^h	NG	10
BHA 5 mM	2.6 ^c	3.9 ^d	9	2
Propyl paraben 10 mM	2.8 ^c	4.3 ^c	9	2
Benzoic acid 10 mM	2.8 ^c	5.1 ^b	8	2
Imazalil 0.1685 mM	3.2 ^b	4.6 ^c	7	2
Imazalil 0.5047 mM	1.9 ^d	4.4 ^c	10	2
Imazalil 0.8412 mM	NG ^e	3.7 ^{de}	NG	3
Imazalil 1.6824 mM	NG ^e	2.9 ^f	NG	8
Control	5.0 ^a	6.0 ^a	4	0
Lsd (P = 0.05) =	0.3	0.3		
CV % =	12.6	5.7		

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

*NG = no growth

Table 6.3: The effect of butylated hydroxyanisole (BHA) alone and in combinations with imazalil and thiabendazole (TBZ) on the expansion of crown rot lesions (mm day^{-1}). Treatment was carried out after establishment of infection by intact organic banana fruits with conidial suspension of *Colletotrichum musae* (CM 100 and CM 103) at 25 °C.

Treatments	<u>Growth rate (mm day^{-1})</u>	
	CM 100	CM 103
BHA (0.5 mM) + imazalil (0.22 mM)	5.0 ^d	5.0 ^c
BHA (0.5 mM) + imazalil (0.45 mM)	4.4 ^e	4.4 ^d
BHA (5 mM) + imazalil (0.22 mM)	2.0 ^j	2.7 ^h
BHA (5 mM) + imazalil (0.45 mM)	1.4 ^k	2.2 ⁱ
BHA (0.5 mM) + TBZ (0.31 mM)	5.7 ^b	5.5 ^b
BHA (0.5 mM) + TBZ (0.62 mM)	4.5 ^e	5.2 ^c
BHA (5 mM) + TBZ (0.31 mM)	3.0 ^h	3.5 ^f
BHA (5 mM) + TBZ (0.62 mM)	2.3 ⁱ	3.2 ^g
BHA (0.5 mM)	7.1 ^a	6.0 ^a
BHA (5 mM)	4.2 ^{ef}	3.9 ^e
Imazalil (0.22 mM)	5.4 ^c	5.0 ^c
Imazalil (0.45 mM)	4.5 ^e	4.4 ^d
Imazalil (0.90 mM)	4.0 ^f	3.9 ^e
Imazalil (1.78 mM)	3.0 ^h	2.8 ^h
Thiabendazole (0.31 mM)	5.7 ^b	5.5 ^b
Thiabendazole (0.62 mM)	5.1 ^{cd}	5.2 ^c
Thiabendazole (1.24 mM)	4.5 ^e	4.6 ^d
Thiabendazole (2.48 mM)	3.3 ^g	3.4 ^{fg}
Control	7.1 ^a	6.0 ^a
Lsd (P = 0.05) =	0.3	0.3
CV % =	5.3	5.1

BHA = butylated hydroxyanisole

TBZ = thiabendazole

(Table 6.2). At 14°C, all the combinations of chemicals evaluated completely prevented lesion growth. In these experiments, it was unusual that at 14°C, lesion expansion in the control fruit (5 mm day⁻¹) was not much slower than at 25 °C (6.0 mm day⁻¹). The most effective treatments at 25 °C were BHA (5 mM) + imazalil (0.45 mM) or TBZ (0.62 mM), which gave a reduction in the rate of lesion expansion of CM 100 by 80 and 67.6 % and the same treatments gave a reduction in the rate of lesion expansion of CM 103 by 63 and 46.7 % respectively (Table 6.3).

Based on the results described above, five treatments were evaluated for their effects on lesion expansion of *C. musae* in the crown tissues of whole banana clusters. All treatments significantly inhibited lesion expansion (Plate 6.1). The most effective treatments at 25°C were BHA (5 mM) combined with imazalil (0.8412 mM) or PP (10 mM), which gave a reduction in the rate of lesion expansion of 66 and 61% respectively (Table 6.4). Lag times were 10 and 9 days respectively. Imazalil on its own (0.8412 and 1.6824 mM) gave an inhibition of growth of 36 and 49% respectively with lag times of 3 and 8 days respectively.

At 14°C, complete inhibition (100 %) of crown rot was observed on banana clusters treated with BHA (5 mM) + propyl paraben (10 mM), BHA (5 mM) + benzoic acid (10 mM) and BHA (5 mM) + imazalil (0.8412 mM) (Table 6.4). Imazalil on its own (0.8412 and 1.6824 mM) reduced growth of the fungus by 26 and 36% respectively and gave lag periods of 6 and 9 days respectively.

Table 6.5 shows the disease incidence in individual fingers by day 12 of the experiment. At both 14 and 25 °C, the highest disease incidence was for imazalil (0.8412 mM), which was 80 and 100% (all fingers infected) respectively. The lowest incidence was for the treatments imazalil (1.6824 mM) and the combination of BHA (5 mM) + imazalil (0.8412 mM) which were 50 and 40% respectively at 25 °C and 35 and 20% respectively at 14 °C.

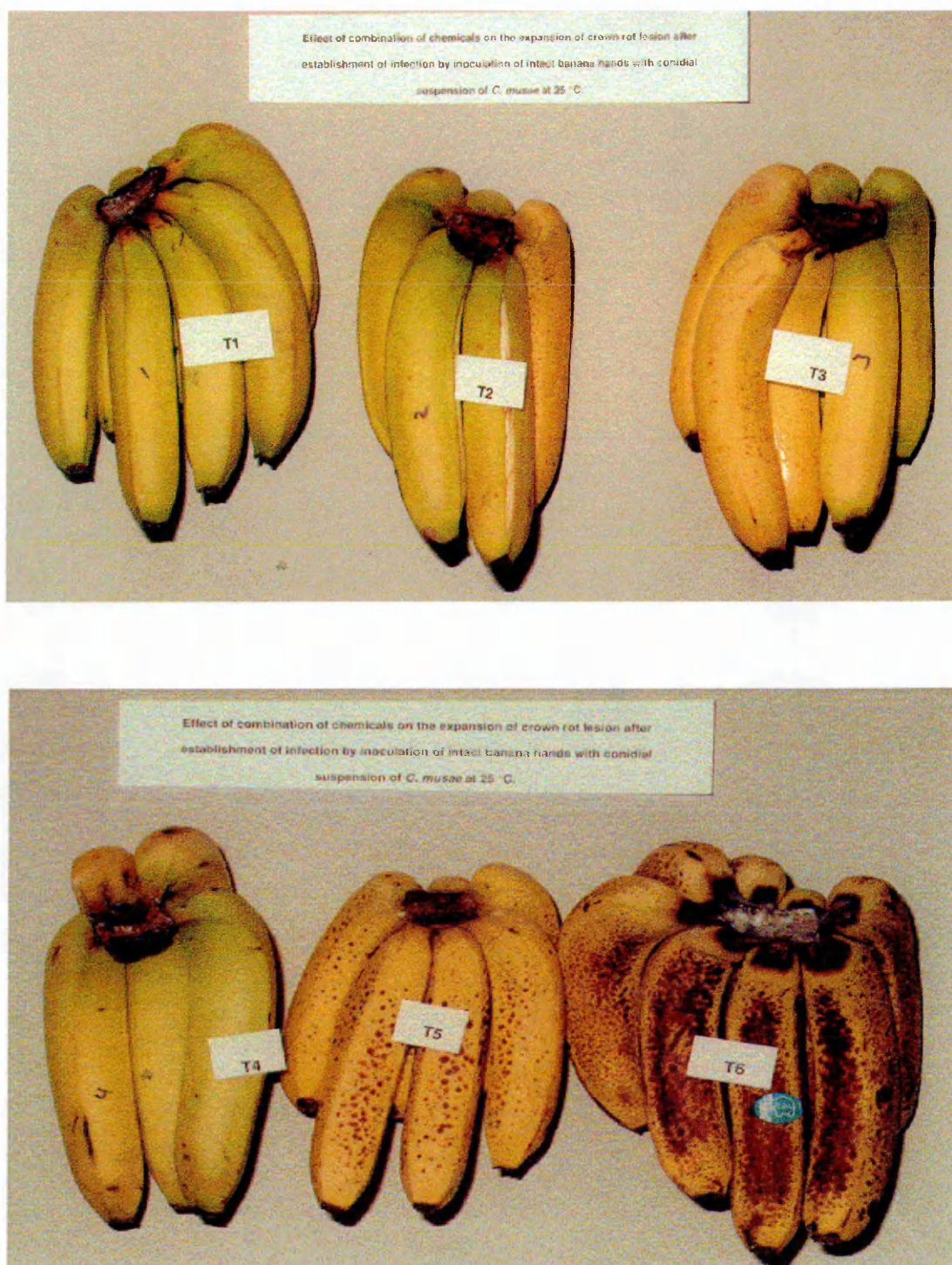


Plate 6.1: Effect of butylated hydroxyanisole in combinations with propyl paraben, benzoic acid and imazalil on the expansion of crown rot lesions (photograph taken 9 days after inoculation). Treatment was carried out after establishment of infection by intact banana hands with conidial suspension of *Colletotrichum musae* at 25 °C.

T1 = BHA (5 mM) + propyl paraben 10 mM; **T2** = BHA (5 mM) + benzoic acid (10 mM); **T3** = BHA (5 mM) + imazalil (0.8412 mM); **T4** = Imazalil (0.8412 mM); **T5** = Imazalil (1.6824 mM); **T6** = Control

Table 6.4: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben, benzoic acid and imazalil on the expansion of crown rot lesions (mm day^{-1}). Treatment was carried out after establishment of infection on the crown surface of banana hands with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 14 and 25 °C.

<i>Treatments</i>	<i>Growth rate (mm day^{-1})</i>		<i>Lag Time (days)</i>	
	14 °C	25 °C	14 °C	25 °C
BHA 5 + propyl paraben 10 mM	NG ^d	2.3 ^d	NG*	9
BHA 5 + benzoic acid 10 mM	NG ^d	3.5 ^{bc}	NG	7
BHA 5 + imazalil 0.8412 mM	NG ^d	2.0 ^d	NG	10
Imazalil 0.8412 mM	2.3 ^b	3.8 ^b	6	3
Imazalil 1.6824 mM	2.0 ^c	3.0 ^c	9	8
Control	3.1 ^a	5.9 ^a	4	0
Lsd (P = 0.05) =	0.3	0.6		
CV % =	14.7	11.9		

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

NG* = no growth

Table 6.5: Effect of butylated hydroxyanisole in combination with propyl paraben, benzoic acid and imazalil on crown rot incidence. Treatment was carried out after establishment of infection on the crown surface of the banana hands with a conidial suspension of *Colletotrichum musae* after 12 days storage period (isolate CM103) at 14 and 25 °C.

Treatments	<u>% disease incidence</u>	
	14 °C	25 °C
Butylated hydroxyanisole 5 + propyl paraben 10 mM	35.0 ^{bc}	55.0 ^{bc}
Butylated hydroxyanisole 5 + benzoic acid 10 mM	55.0 ^b	65.0 ^b
Butylated hydroxyanisole 5 + imazalil 0.8412 mM	20.0 ^c	40.0 ^d
Imazalil 0.8412 Mm	80.0 ^a	100.0 ^a
Imazalil 1.6824 mM	35.0 ^{bc}	50.0 ^{cd}
Control	100.0 ^a	100.0 ^a
Lsd (P = 0.05) =	20.1	11.1
CV % =	25.0	10.9
Means followed by the same letter do not differ significantly		

b) Post-inoculation studies

In these experiments, the stalk ends of banana fingers or the crowns of banana clusters were treated with chemicals before inoculation with *C. musae*. In other respects the experiments were similar to those described above.

When individual chemicals were applied to stalk ends at 25°C, the most effective treatments were imazalil (1.6824 mM), TBZ (2.4850 mM) and BHA (10 mM), which inhibited crown rot infection by 50 %, 47 % and 37 % respectively (Table 6.6). Lag times for these treatments were 4, 3 and 3 days respectively. Propyl gallate (PG) (15 mM) was the least effective giving only 7% control.

All the combination treatments tested on the cut crowns of banana clusters at 25 °C gave significant control of lesion expansion (Plate 6.2). The most effective treatments were BHA (5 mM) + imazalil (0.8412 mM) and BHA (5 mM) + propyl paraben (10 mM) which gave a 72 and 68% inhibition of the rate of lesion expansion respectively (Table 6.7). The respective lag periods were 10 and 9 days. This compared well to imazalil (1.6824 mM) on its own which only inhibited lesion expansion by 48 % although the lag period was 8 days.

At 14°C, no growth was observed in the following treatments: BHA (5 mM) + imazalil (0.8412 mM); BHA (5 mM) + PP (10 mM) and imazalil (1.6824 mM) alone (Table 6.7). The other two treatments tested also gave highly significant levels of control at this temperature i.e. BHA (5 mM) + benzoic acid (10 mM) which inhibited lesion growth by 67% and imazalil (0.8412 mM) which inhibited lesion growth by 54%. The lag periods for these two treatments were 10 and 7 days respectively.

Table 6.8 shows the disease incidence in individual fingers by day 12 of the experiment. At both 14 and 25°C, the highest disease incidence was for imazalil (0.8412 mM), which was 90 and 100% (all fingers infected) respectively. The lowest



Plate 6.2: Effect of butylated hydroxyanisole in combinations with propyl paraben, benzoic acid and imazalil on the expansion of crown rot lesions (photograph taken 9 days after inoculation). Treatment was carried out before establishment of infection by intact banana hands with conidial suspension of *Colletotrichum musae* at 25 °C.

T1 = BHA (5 mM) + propyl paraben (10 mM); **T2** = BHA (5 mM) + benzoic acid (10 mM); **T3** = BHA (5 mM) + imazalil (0.8412 mM); **T4** = Imazalil (0.8412 mM); **T5** = Imazalil (1.6824 mM); **T6** = Control.

Table 6.6: Effect of anti-oxidants and fungicides on the expansion of crown rot lesion (mm day^{-1}). Treatment was carried out before establishment of infection by inoculation on crown surface of the banana fruits with a conidial suspension of *Colletotrichum musae* (isolate CM103) at 25°C.

<i>Treatments</i>	<i>Growth rate (mm day^{-1})</i>	<i>Lag Time (days)</i>
Butylated hydroxyanisole 10 mM	3.7 ^e	3
Propyl paraben 10 mM	4.1 ^d	2
Benzoic acid 15 mM	4.5 ^c	1
Propyl gallate 15 mM	5.5 ^b	1
Thiabendazole 2.4850 mM	3.1 ^f	3
Imazalil 1.6824 mM	3.0 ^f	4
Control with 30 % acetone	5.9 ^a	0
Control with distilled water	6.0 ^a	0
Lsd ($P = 0.05$) =	0.2	
CV % =	3.7	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 6.7: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben, benzoic acid and imazalil on the expansion of crown rot lesions (mm day^{-1}). Treatment was carried out before establishment of infection on the crown surface of banana hands with a conidial suspension of *Colletotrichum musae* after 12 days storage period (isolate CM103) at 14 and 25 °C.

Treatments	<u>Growth rate (mm day^{-1})</u>		<u>Lag Time (days)</u>	
	14 °C	25 °C	14 °C	25 °C
BHA 5 + propyl paraben 10 mM	NG ^d	1.9 ^d	NG*	9
BHA 5 + benzoic acid 10 mM	1.6 ^c	3.5 ^{bc}	10	7
BHA 5 + imazalil 0.8412 mM	NG ^d	1.7 ^d	NG	10
Imazalil 0.8412 mM	2.2 ^b	4.0 ^b	7	5
Imazalil 1.6824 mM	NG ^d	3.0 ^c	NG	8
Control	4.8 ^a	6.0 ^a	4	0
Lsd (P = 0.05) =	0.4	0.9		
CV % =	18.1	17.4		

Means followed by the same letter do not differ significantly

BHA = Butylated hydroxyanisole

Lag time: The period after inoculation at which growth is first observed

NG* = no growth

Table 6.8: Effect of butylated hydroxyanisole in combination with propyl paraben, benzoic acid and imazalil on crown rot incidence. Treatment was carried out before establishment of infection on the crown surface of banana hands with a conidial suspension of *Colletotrichum musae* after 12 days storage period (isolate CM103) at 14 and 25°C.

Treatments	<u>% disease incidence</u>	
	14 °C	25 °C
Butylated hydroxyanisole 5 + propyl paraben 10 mM	0.0 ^d	45.0 ^{bc}
Butylated hydroxyanisole 5 + benzoic acid 10 mM	40.0 ^c	55.0 ^b
Butylated hydroxyanisole 5 + imazalil 0.8412 mM	0.0 ^d	25.0 ^d
Imazalil 0.8412 mM	90.0 ^b	100.0 ^a
Imazalil 1.6824 mM	0.0 ^d	40.0 ^c
Control	100.0 ^a	100.0 ^a
Lsd (P = 0.05) =	7.0	14.4
CV % =	12.3	16.0

Means followed by the same letter do not differ significantly

incidence was for the treatments imazalil (1.6824 mM) and the combinations of BHA (5 mM) + imazalil (0.8412 mM) and BHA (5 mM) + PP (10 mM) which were 40, 25 and 45% respectively at 25°C. At 14°C, there was no growth of the fungus at all so the disease incidence was 0%.

6.3.2 The effect of azadirachtin in controlling crown rot infection

Azadirachtin A (neem seed extract) at concentration of 1 mM, significantly ($P = 0.05$) inhibited crown rot lesion expansion by 27% when treatment was carried out after infection had been established (Table 6.9). This compared to levels of inhibition by imazalil (1.6824 mM) and TBZ (2.485 mM) of 49 and 46% respectively. When the treatment was applied before inoculation with *C. musae*, the results were almost identical to that those described above (Table 6.10). Acetone (30%), which was used as a solvent for azadirachtin, had no significant effect on crown rot infection (Tables 6.9 and 6.10).

6.4 TREATMENT COST-BENEFIT ANALYSIS

The prices of the best chemicals (imazalil, BHA, PP and benzoic acid) used against anthracnose and crown of bananas are given in Table 6.11. The prices (per litre) of the best treatments (imazalil [1.6824 mM]; BHA 5 mM + PP 10 mM; BHA 5 mM + benzoic acid 10 mM; BHA 5 mM + imazalil 0.8412 mM) were calculated and are presented in Table 6.12. The minimum and maximum cost per litre was £0.13 and £0.30 containing BHA 5mM + benzoic acid 10 mM and imazalil 1.6824 mM respectively. This was comparable to the cost of imazalil.

Table 6.9: Effect of azadirachtin (1 mM) and fungicides (thiabendazole and imazalil) on the expansion of crown rot lesion (mm day⁻¹). Treatment was carried out after establishment of infection on the crown surface of banana fruits with a conidial suspension of *Colletotrichum musae* at 25°C.

Treatments	Growth rate (mm day⁻¹)	Lag Time (days)
Azadirachtin 1.0 mM	4.5 ^b	3
Thiabendazole 2.4850 mM	3.3 ^c	3
Imazalil 1.6824 mM	3.1 ^c	4
Control with 30 % acetone	6.2 ^a	0
Control with distilled water	6.1 ^a	0
Lsd (P = 0.05) =	0.2	
CV % =	3.0	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 6.10: Effect of azadirachtin and fungicides (thiabendazole and imazalil) on the expansion of crown rot lesion (mm day⁻¹). Treatment was carried out before establishment of infection on the crown surface of the banana fruits with a conidial suspension of *Colletotrichum musae* at 25°C.

Treatments	Growth rate (mm day⁻¹)	Lag Time (days)
Azadirachtin 1.0 mM	4.3 ^b	3
Thiabendazole 2.4850 mM	3.1 ^c	3
Imazalil 1.6824 mM	3.0 ^c	4
Control with 30 % acetone	5.9 ^a	0
Control with distilled water	6.0 ^a	0
Lsd (P = 0.05) =	0.2	
CV % =	3.7	

Means followed by the same letter do not differ significantly

Lag time: The period after inoculation at which growth is first observed

Table 6.11: Chemicals and their prices used *in vivo* studies against anthracnose and crown rot of bananas caused by *Colletotrichum musae*.

<i>Name of chemicals</i>	<i>Prices</i>
Imazalil (10 % a.i.)	£ 53.50 / litre
Butylated hydroxyanisole	£ 12.80 / 100 g
Propyl paraben	£ 8.40. / 100 g
Benzoic acid	£ 3.60 / 100 g

Table 6.12: Prices of best treatments used *in vivo* studies against anthracnose and crown rot of bananas caused by *Colletotrichum musae*.

<i>Treatments</i>	<i>Price / litre</i>
BHA 5 mM + PP 10 mM	£ 0.20
BHA 5 mM + benzoic acid 10 mM	£ 0.13
BHA 5 mM + imazalil 0.8412 mM	£ 0.25
Imazalil 1.6824 mM	£ 0.30

BHA = butylated hydroxyanisole and PP = propyl paraben

CHAPTER 7

DISCUSSION

AND

CONCLUSIONS

7.1 INTRODUCTION

The previous four chapters described the results of investigations into chemicals which might be used in the control of an important pathogen of banana fruits, *Colletotrichum musae*. A diverse range of chemicals has been screened, although the majority have common anti-oxidant properties. The effectiveness of these chemicals against *C. musae*, the mechanisms which may underlie their activity, and the possible development of alternative post-harvest treatments of banana are discussed below.

7.2 FUNGICIDE RESISTANCE

As was described earlier the two fungicides, thiabendazole (TBZ) and imazalil, have both been widely used on export banana fruits to control diseases such as anthracnose, finger rot and crown rot (Slabaugh and Grove, 1982). In the current research, TBZ and imazalil were found to be inhibitors of mycelial growth of *C. musae in vitro*. However, there was a range of sensitivities to both fungicides across the four isolates tested (CM100, CM101, CM103 and CM104).

CM100 was the most sensitive being completely inhibited by both fungicides at 5 ppm although it showed some slight growth when grown on both fungicides at 2.5 ppm. CM103 was the least sensitive to both fungicides and can be considered to be resistant to TBZ given that it showed some growth at 1.2425 mM (250 ppm). This falls in the range applied commercially for postharvest disease control (200 - 600 ppm). ED₅₀ (TBZ) values found for the four isolates in this study were slightly higher than those given for sensitive and resistant isolates of *C. musae* found in the Windward islands by Cox (1996) which ranged from 0.0006 – 0.0013 mM (0.13 – 0.25 ppm) to 0.005 – 0.01 mM (1.0 - 2.0 ppm) respectively.

Resistance to benzimidazole fungicides is very common in a wide range of fungi and resistant isolates of *C. musae* against methyl-thiophanate, TBZ and benomyl have been described (Griffiee, 1973). Several studies have noted resistance to benomyl and

benomyl related fungicides in the Windward Islands, a major supplier of bananas to the UK (Griffie, 1973; Johanson and Blaquez, 1992). Resistance of *C. musae* to benzimidazole fungicides is wide spread in banana plantations of Guadeloupe. De Lapeyre deBellaire and Dubois (1997), reported that an average of 23 % of all isolates evaluated were resistant to TBZ at concentrations of at least 1 µg/ml (1 ppm). This is sufficient to result in failure of postharvest treatments with this fungicide to control fruit diseases. The same authors used regression-tree analysis of the distribution of resistance to show that aerial sprays of benomyl for Sigatoka disease control are the primary factor associated with thiabendazole resistance in *C. musae* populations.

It is not surprising, therefore, to find TBZ resistant isolates of *C. musae* on imported banana fruit as has been shown in the current study. The fruits from which the *C. musae* was isolated had almost certainly been treated with fungicide, so in fact these four isolates effectively had already been “screened” for fungicide tolerance. For a wide range of fungi, the mechanism for tolerance to benzimidazole fungicides has been shown to be an altered form of tubulin which has a reduced binding affinity for the fungicide (Davidse and Flach, 1977; Cooley *et al.*, 1991; Osmani and Oakley, 1991; Koenraadt *et al.*, (1992); Yarden and Katan (1993). There is no reason to believe that the same mechanism is not functioning in the TBZ tolerant forms of *C. musae*.

CM103 showed an abnormal colony appearance and was slower growing than the more fungicide sensitive strains when grown on fungicide free malt extract agar (MEA). It is often the case that fungicide tolerance is linked with reduced vigour. Bartles and MacNeill (1970), for example, showed that in *Fusarium oxysporum* the mechanism of resistance appears to be conferring a disadvantage upon the tolerant strains. Quimio (1976) investigated variability in *C. musae* in the Luzon area of the Philippines. The author found that four out of ten isolates were resistant to benomyl at 1000 ppm, and of these four, two showed reduced growth on various media without the fungicide.

All of the isolates were controlled *in vitro* by concentrations of imazalil as low as 0.0168 mM (5 ppm) which is well below the levels recommended for post-harvest application for disease control on bananas (200 - 500 ppm). At present, imazalil tolerant strains of *C. musae* have not been reported in the literature, however, the potential for resistance to arise is ever present as has been shown in *Penicillium* spp on citrus fruit (Eckert, 1988; Eckert *et al.*, 1994). It is vital that appropriate anti-resistance strategies should be developed and adopted by banana producers. Such strategies are nowadays well defined and understood by the plant protection community but are not always implemented in practice (Staub, 1991).

7.3 THE *IN VITRO* ACTIVITY OF CHEMICALS TESTED ON THEIR OWN AGAINST *C. MUSAE*

Initial screening of ten chemicals which have antioxidant properties, showed a wide range of *in vitro* activity against *C. musae*. Dimethyl sulphoxide (DMSO) was virtually inactive against either germination or mycelial growth of *C. musae* whereas some of the phenolic antioxidants gave very strong inhibition of germination and mycelial growth (see below). This variation in activity suggests that the antioxidant property of the chemicals was not necessarily of significance in their anti-fungal effects. In fact, due to the diverse nature of these chemicals (Appendix B for the formulae/structures of the compounds used), it is likely that the mechanisms underlying any anti-fungal activity may vary in each case.

7.3.1 Ascorbic acid, butylated hydroxytoluene and propionic acid

At 22°C some of the antioxidants tested slowed the rate of conidial germination but did not actually prevent 100% germination within a three days period of exposure to the chemical. These were ascorbic acid, butylated hydroxytoluene and propionic acid. These chemicals therefore have a fungistatic effect rather than a fungitoxic effect at the concentrations tested. This effect was also seen in mycelial growth experiments in which these chemicals caused a lag in growth in one or both of the isolates tested.

Propionic acid was particularly effective with concentrations of 15 mM giving a lag period of nine and three days respectively for isolates CM100 and CM103. This concentration suppressed mycelial growth by 75% in CM100 and 73 % in CM103 when compared to the controls. Similar levels of control were found by Al Zaemey *et al.* (1993) in which propionic acid at 0.1% (i.e. about 13.5 mM) caused a lag of two days in growth and reduced the growth rate of *C. musae* on MEA by 79% when compared to the control. They found that at 0.5%, propionic acid completely suppressed mycelial growth.

In the same paper it was noted that only concentrations of ascorbic acid above about 60 mM (1%) were able to completely inhibit *C. musae*. Propionic acid has been used widely since the 1960's to control mould growth in grain crops stored at high moisture contents. Concentrations used in practice range from 0.3-1.2% (Sauer *et al.*, 1992). In order for a fungistatic effect to be useful for controlling postharvest diseases of banana, it would have to last for the postharvest period typically experienced by bananas (in many cases over two weeks). In conclusion, it is likely that fairly high concentrations of these organic acids or BHT would be needed to be useful in disease control.

7.3.2 Thiourea, propyl gallate, benzoic acid and propyl paraben

The next most effective chemicals tested were thiourea and propyl gallate (PG) which prevented 100% germination by day three at the higher concentrations tested. Both chemicals substantially slowed mycelial growth of CM100 even at the lower concentrations (1 and 5 mM) and at 10 and 15 mM, PG completely prevented growth of CM100. CM103 on the other hand was relatively resistant to both chemicals.

Benzoic acid and propyl paraben (PP) were not much more effective than thiourea and PG at slowing mycelial growth, however, they were much better at suppressing germination in both isolates. Al Zaemey (1991) reported that 0.125 % (8.9 mM) benzoic acid completely inhibited the growth of *C. musae* whereas in the present

study, the concentration of benzoic acid had to be as high as 15 mM to completely inhibited the growth of two *C. musae* isolates.

Benzoic acid, PG and thiourea were found to significantly inhibit the mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum in vitro* at 10 mM and benzoic acid at this concentration inhibited germination of *B. cinerea* (Elad, 1992). PP was not tested in those experiments, however, it was shown to completely inhibit the growth of *Penicillium digitatum*, *Aspergillus niger* and *Rhizopus nigricans* in peptone broth at 0.0063% (0.35 mM), 0.02% (1.1 mM) and 0.0125% (0.7 mM) respectively (Aalto *et al.*, 1953). Other studies have shown that concentrations of PP ranging from 0.7 –2 mM could strongly inhibit the growth of various species of *Alternaria*, *Aspergillus*, *Penicillium* and *Fusarium* ((Jurd *et al.*, 1971; Thompson *et al.*, 1993; Thompson, 1994).

It appears, therefore, that these four chemicals could have some potential as anti-fungals across a broad range of plant pathogens important on fruits and vegetables. Benzoic acid and propyl paraben are already widely used in the food industry to reduce the development of spoilage micro-organisms, however, PG and thiourea are not currently exploited for their anti-fungal activity.

7.3.3 Azadiractin

Azadiractin (extracted from neem seed) at 0.5 and 1.0 mM was able to substantially delay germination of *C. musae* over a three day period and caused a strong inhibition of mycelial growth at 22 °C. This chemical has been tested for its fungicidal properties on a wide range of plant pathogens. Neem seed product (oil) had a significant effect against *Alternaria* spp at concentrations ranging from 0.025-10% (Kazmi *et al.*, 1993; Bhowmic and Chaudhary, 1982, Vir and Sharma, 1985a). Vir and Sharma (1985b) also reported that 10% neem oil concentration gave 100% inhibition of *Aspergillus niger*, *Drechslera rostrata* and *Macrophomina phaseolina*. Singh *et al.* (1980) noted

the fungicidal effect of neem extracts on *Fusarium oxysporum*, *F. solani*, *Sclerotium rolfsi* and *Sclerotinia sclerotiorum*.

The attraction of neem extracts is that the neem tree (*Azadiracta indica*) grows very widely in the tropics. It would be very advantageous in tropical developing countries, if simple neem extracts could be used for disease control instead of expensive imported fungicides. Unfortunately for the current studies, which were carried out in the UK, azadiractin was relatively expensive compared with the other compounds tested. As a consequence only a couple of concentrations were evaluated against *C. musae*.

7.3.4 Butylated hydroxyanisole

By far the best chemical against both germination and mycelial growth of *C. musae* was butylated hydroxyanisole (BHA). It was equally effective against both isolates of the fungus tested and completely inhibited germination and mycelial growth at concentrations of 5 mM and above. A number of studies have shown a direct inhibition by BHA of a number of toxigenic fungi, including species of *Aspergillus*, *Fusarium* and *Penicillium* (Chang and Branen, 1975; Fung *et al.*, 1977; Ahmad, 1979; Lin and Fung, 1983; Thompson, 1991; Thompson, 1992; Thompson *et al.*, 1993).

Thompson (1996b) showed that BHA (0.6-1.2 mM) caused a leakage of amino acids, glucose, and protein in culture filtrates of *Fusarium* spp. Although the specific mode of action by which BHA inhibits fungi is still unknown, it appears that it may cause some distortion in the mycelial membrane, possibly leading to loss of its integrity.

Despite these observations, BHA has been commonly used to prevent the auto-oxidation of lipids in a variety of food products (Davidson and Branen, 1980) but its anti-fungal properties do not appear to have been deliberately utilised by the food industry.

7.3.5. Isolate factors

Of the two isolates tested, CM103 showed a greater tolerance of the antioxidants although the relative effect of the chemicals was the same as for CM100. This is interesting in that it suggests that CM103, which is also more tolerant of imazalil and much more tolerant to TBZ than CM100, has some property that protects it against chemicals in general. Possibly it has a defective membrane uptake system. This might also explain why it shows slower growth on malt extract agar, as nutrient uptake might also be slower. The differences in isolate sensitivity to a wide range of chemical types, indicates the importance of not just testing one isolate in screening trials.

7.3.6 pH effects on anti-fungal activity

The effectiveness of some chemicals (in particular, weak acids) is dependent on the pH of the medium surrounding the micro-organism because it determines the extent of dissociation of the molecule. Generally only the undissociated form of a weak acid penetrates the cell membrane (Ingram *et al.*, 1956; Corlett and Brown, 1980). Rahn and Conn (1944) had established that with benzoic acid, the undissociated fraction was the inhibitory principle, not the acid ions. At pH 5.1, only about 0.1% of benzoic acid is undissociated whereas at pH 4.1 about 56% is undissociated (Bosound, 1962).

The pH of MEA is 5.4. It was found in the current study that the addition of benzoic acid at 1 mM reduced the pH only slightly to 5.3 whereas at 5, 10 and 15 mM, the pH was lowered to 4.5, 4.3 and 4.2 respectively. At pH 5.3 very little of the acid would be undissociated compared to the lower pH values. This may well explain why benzoic acid at 1 mM was relatively ineffective compared to the higher concentrations.

Similarly, propionic acid has a pK_a of 4.87 and at pH 4.0, 88% of the compound is undissociated i.e. in the form necessary for its anti-microbial activity (Jay, 1996). In

these studies, propionic acid (1-15 mM) reduced the acidity of the medium to pH 5-4.5. The higher pH values may also have contributed to the reduced inhibitory effect at the lower concentrations of this acid.

The reduced pH of the growing medium could conceivably have made some direct contribution to the inhibition of *C. musae*. The optimal pH at 25 °C for mycelial growth of isolate CM103 was found to be between pH 5.5 and 6.5. This finding supports that of Al Zaemey *et al.*, (1994) who found that the optimal pH for *C. musae* growth at the same temperature was 6.0. The experimental evidence, however, suggests otherwise as DMSO (15 mM) lowered the pH of the medium by 1.25 units but the chemical apparently had no effect on conidial germination and mycelial growth of *C. musae*.

Thiourea decreased the pH of the medium by maximum of 0.4 units (15 mM). The other chemicals used caused little (approximately 0.1 units) change to the pH of the growth medium. It is unlikely that pH had much effect on the effectiveness of PP as a large proportion of a paraben is in the undissociated form even at a pH level as high as 8.5. Parabens are thus effective over a wide pH range from 4 to 8 (Aalto *et al.*, 1953). Despite this, it has been reported, however, that there can be a strong interaction between the pH of the growth medium and the effectiveness of BHA and PP in controlling mycelial growth of several fungal species (Thompson *et al.*, 1993).

7.3.7 The effect of temperature on chemical inhibition

The growth rate of *C. musae* on MEA at 14 °C was half that at 25 °C which would be expected from the normal effect of reduced temperature on metabolic processes. The rate of germination at 14°C was also halved compared to that at 25°C. The effect of BHA, benzoic acid and imazalil at low concentrations appeared to have a slightly greater inhibitory effect at 14 °C relative to that at 25 °C (Table 4.5). These temperature effects are consistent with results obtained by Al Zaemey *et al.*, (1994) in a study of the environmental growth parameters of *C. musae*. They observed that

combinations of low temperature (15 °C) and low pH (<3.0) markedly delay both germination (for 72 h) and growth (32 days) of *C. musae*. In other words, negative environmental factors (temperature, pH or the presence of toxic chemicals) can have a synergistic effect when experienced together by an organism. This property is frequently exploited in food preservation and is known as the hurdles concept (Scott, 1989).

7.4 EFFECT OF COMBINATION OF SELECTED CHEMICALS ON *C. MUSAE* GROWTH IN VITRO

It is clear that of the ten chemicals tested in the current study, three were particularly inhibitory to *C. musae* i.e. benzoic acid, propyl paraben and in particular, BHA. Thus these three were selected to test at even lower concentrations and in combinations with each other and with low concentrations of fungicides. Only one isolate of *C. musae* was used i.e. CM103 which appeared to be more tolerant of the chemicals being evaluated than CM100.

7.4.1 Synergistic and additive effects from combining chemicals

a) Treatments applied together

The lowest concentrations of antioxidants tested together in combination were BHA (0.1 mM) combined with PP or benzoic acid (5 mM) or BHA (1.0 mM) combined with PP or benzoic acid (1 mM). All of these combinations completely controlled germination and mycelial growth of *C. musae*. From these results, it was difficult, however, to determine if there was an additive effect or a synergistic interaction. It would have been necessary to test much lower concentrations together. Instead, the focus was turned to the combination of BHA and the fungicides imazalil and TBZ. Concentration series of BHA were tested in combination with a fixed low concentration of TBZ at 2.5 µM and imazalil at 1.68 µM. These fungicide

concentrations (equivalent to 0.5 ppm) are about 1000 times lower than is applied on bananas in practice.

Of the concentrations tested, the only one to completely inhibit both germination and mycelial growth was BHA (0.5 mM) with imazalil (1.68 μ M). Significant inhibition of germination after 48 hrs was seen with imazalil when BHA concentration was lowered to 0.1 mM and with TBZ when BHA concentration was lowered to 0.25 mM. Significant inhibition of mycelial growth was seen with both fungicides when BHA concentration was as low as 0.03125 mM. In these experiments, it was clear that there was a strong synergistic effect between BHA and imazalil and an additive or possibly a slight synergistic effect between BHA and TBZ.

One of the possible antimicrobial mechanisms of BHA is that it damages cell membranes. This could in part explain the synergistic effect with imazalil. It is well established that imazalil inhibits the synthesis of ergosterol which is important in the synthesis of new cell membranes in fungi. Thus imazalil would inhibit the fungus from repairing damage caused by the BHA. Alternatively, the fact that the cells are becoming more leaky could simply allow more fungicide into the cells. If this were the case, however, one might expect a stronger synergistic effect with TBZ.

b) Treatments applied one after the other

Due to the synergism seen between BHA and imazalil, an experiment was carried out to determine the effect of these two chemicals on *C. musae* if they were applied sequentially to the fungus. It was expected that the order of application might have a considerable effect on the degree of fungal inhibition. The effect of the chemicals on germination rather than mycelial growth was tested as it was easier to separate the conidia from the initial chemical treatment and the results could be determined more quickly.

In fact, the order of treatment with imazalil or BHA did not appear to make any difference. The percentage inhibition of germination was the same whether the conidia were exposed to imazalil or BHA first or to the two chemicals together in the agar medium. It seems unlikely therefore that the mechanism of synergism was due to the BHA damage allowing more fungicide to enter the cell. If this were the case then one would expect less inhibition when the imazalil was applied first, compared to the situation when the BHA was applied first or together with the imazalil.

7.4.2 The effect of pH on chemical inhibition

The pH of the agar medium did not have any effect on inhibition of *C. musae* by imazalil but did influence the effectiveness of BHA alone and in combination with imazalil (Table 4.7). It appears that the strongest inhibition of growth occurred at pH 6.5 and 3.5 and the least inhibition occurred at pH 7.5 and 5.5.

It is possible that this outcome is due to two effects. There is clearly a direct effect of the pH on the fungus with the fastest growth at pH 6.5 and the slowest at pH 3.5. It might be expected that chemicals would have more impact at non-optimal pH levels, for example at pH 3.5. On the other hand, the greatest membrane penetration by BHA may occur at more acidic pH values. Ahmed (1979) found that the activity of BHA against *Aspergillus flavus* decreased as the pH was increased from 7 to 9.

As however, there was no growth at any pH when BHA (0.5 mM) was combined with imazalil (1.68 μ M), it appears that pH may not be critical in the development of an effective treatment with these two chemicals.

7.4.3 The effect of chemicals on extracellular enzyme production

Plant-pathogenic fungi produce a range of extracellular enzymes. These enzymes can have a number of roles. Some are thought to be important in the penetration of intact host surfaces, for example, cutinases (Koller *et al.*, 1995). Cutinase deficient mutants

of *C. gloeosporioides* have been shown to be non-pathogenic to papaya fruit (Dickman and Patil, 1986). Other enzymes modify cells walls of plant tissues (Annis and Goodwin, 1997). Enzymes that are associated with cell wall degradation include pectin methyl esterase (PME), pectin lyase (PL) polygalacturonase (PGal), cellulases, hemicellulases and ligninases. Phospholipases catalyse the degradation of cell membranes (Dean and Timberlake, 1989). Yet other enzymes enable the pathogen to detoxify inhibitors produced by the host plant, for example, beta-galactosidases can cleave toxic glucosides (Osbourn *et al*, 1995).

In the present study, the production of extracellular enzymes by *C. musae* was investigated using a commercial test kit. This kit detects 19 extracellular enzymes produced by a wide range of organisms in a semi-quantitative fashion. Although many of these enzymes (e.g. trypsin and β -glucuronidase) are not relevant to fungal biology, some of those detected are known to be produced by fungal pathogens. For example, (α -galactosidase and β -galactosidase from *Aspergillus niger*; (Manzanares *et al.*, 1998), β -glucosidase from *Scelerotinia sclerotiorum* (Waksman, 1988) and *B. cinerea* (Staples and Mayer, 1995); acid-phosphatases from *B. cinerea* (Weber and Pitt, 1997) and N-acetyl-B-glucosaminidase from *Fusarium moniliforme* (Marin *et al.*, 1998).

API ZYM reactions proved to be a quick and reproducible method of screening *C. musae* for the production of various extracellular enzymes. Isolates CM100 and CM103 of *C. musae* produced identical extracellular enzymes which included several lipases (esterase (C 4), esterase lipase (C 8), lipase (C 14)) which might be important in the digestion of cell membranes, phosphatases (alkaline and acid and naphthol-AS-BI- phosphohydrolase) and a range of hydrolases that can breakdown various poly- and oligo- and di-saccharides (α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase and N-acetyl- β glucosaminidase). The production of a number of these enzymes has been noted for other *Colletotrichum* spp. for example, *C. lindemuthianum* has been shown to secrete α -galactosidase and , β -glucosidase

(English *et al.*, 1971). It appears, however, that only two of these enzymes, have been reported in the literature for *C. musae* i.e. esterases (Yang and Chung, 1991) and β -glucosidase (Ravise and Chopin, 1981).

Isolate CM103 was challenged with various fungicides and combinations of fungicides with BHA. No enzyme activity was detected when BHA (0.5 mM) + imazalil (0.00168 mM) was used and it had been shown earlier, that this combination completely inhibits conidial germination and mycelial growth of *C. musae* on solid MEA. The activities of five of the enzymes (lipase, acid phosphatase; naphthol phosphohydrolase; α -galactosidase, β -galactosidase) were decreased about the same amount in each of the other treatments compared to the control. This suggests that their decrease was a function of an overall decrease in fungal growth and not a specific effect on the enzymes in question.

BHA appeared to inhibit two enzymes to a greater extent than the other enzymes. These were alkaline phosphatase and esterase. It is likely either that there is a direct effect of the treatments on the activity of the enzymes or on their biosynthesis. There is evidence that alkaline phosphatase is a membrane-bound enzyme and may play a role in pH regulation (Hipkiss *et al.*, 1987) and the uptake of nutrients (Ajayi *et al.*, 1985). It also may help to anchor specific fatty acids in the membrane (Ikezawa *et al.*, 1989). As described above, BHA has been shown to affect membrane function. BHA causes cellular leakage in bacteria (Degre and Sylvestre, 1983; Davidson and Branen, 1980a). Increased leakage of sugars, amino acids and proteins from fungal mycelia has been detected suggesting disruption of the membranes (Thompson, 1996a;b). If BHA disrupts the cell membrane in some way, then it is not surprising that activity of bound enzymes is also affected.

Imazalil and TBZ appeared to inhibit four and five enzymes respectively to a greater extent than the other enzymes. In the presence of both fungicides, esterase, esterase lipase, α -glucosidase and β -glucosidase were affected and N-acetyl- β

glucosaminidase activity was also decreased by imazalil relative to other enzymes. Imazalil inhibits a form of microsomal enzyme P 450 which normally catalyses the C-14 demethylation of 24-methylenedihydrolanosterol to ergosterol (Kato, 1986). TBZ on the other hand binds to β -tubulin and prevents the formation of microtubules and thus mitosis and cell division. Although these are the specific actions associated with these two fungicides, it has been noted that benzimidazole fungicides can differentially affect a number of fungal enzymes. In *Cladosporium cucumerinum*, sublethal doses of benomyl caused a marked decrease in the production of β -glucosidase but an increase in acid phosphatase activity (Monistrol *et al.*, 1988). The authors suggest that microtubules are important in the secretory process for certain extracellular enzymes. There is some evidence that EBI fungicides such as imazalil may damage membranes directly in some fungi (Dahmen *et al* 1988; Lyr, 1988). This might also influence the secretion of extracellular enzymes.

7.5 THE EFFECT OF CHEMICALS AGAINST ANTHRACNOSE AND CROWN ROT INFECTIONS OF BANANAS

Seven chemicals which were found to strongly inhibit *C. musae in vitro* were tested against the fungus when it was inoculated onto banana fruits. These chemicals were imazalil, TBZ, BHA, PP, PG, benzoic acid and azadiractin.

7.5.1 Effect of chemicals on their own at 25 °C

Propyl gallate (PG) was the least effective of the chemicals tested. Levels of control were generally no higher than 10% apart from treatment at 15 mM prior to infection of the peel with conidia which gave better control (22%). Propyl paraben and benzoic acid gave similar levels of control at the concentration range of (5-15 mM) although PP was generally slightly more effective giving at 15 mM, control levels of 32-39% against mycelial and conidial infections of the peel and conidial infections of stalk and crown tissues.

BHA gave good levels of control of anthracnose and crown rot at 10 mM (32-48%). At 1 mM BHA, control ranged from 10-36%. Azadiractin (1 mM) gave fairly consistent control levels against both diseases (27 – 39%). This compared with imazalil (0.84 and 1.68 mM) giving typical levels of control ranging from 36-38% and 49-59% respectively for conidial infections but only 18% and 31% respectively for mycelial infection of wounded peel. These concentrations of imazalil fall in the range used commercially on bananas (200-500 ppm). On a concentration basis, it appears that of all the alternative chemicals tested, BHA and azadiractin are the closest in activity to imazalil *in vivo*.

There was not a large difference between levels of control when the chemicals were applied before inoculation or afterwards. It appeared as if BHA, PP and PG were more effective when applied to the fruit before rather than after peel inoculation with conidia. On the other hand these chemicals were apparently less effective when applied to the crown before rather than after inoculation with conidia. Azadiractin and benzoic acid on the other hand gave very similar levels of control in both cases. As the different timings of applications were tested in separate experiments, it is not possible to say whether any differences were statistically significant. It is reasonable to conclude, however, that all the chemicals tested in this way, were capable of some persistent effect in the fruit tissues and were thus able to offer protectant as well as eradicant benefits against *C. musae*.

It is possible that the differing antifungal activities of the four phenolic compounds, BHA, PP, PG and benzoic acid, relate in part to their hydrophobicity. Based on their chemical structures and their relative solubility in water, it would be expected that the relative partitioning of these chemicals across cell membranes would be in the order BHA > PP > PG > benzoic acid. So it might be the case that PG, for example, is less able to disrupt cellular activity than BHA because it is repelled from the cell membrane, whereas BHA rapidly dissolves in the membrane.

The concentrations of chemicals needed to give reasonable levels of control of banana diseases *in vivo* are much higher than those needed to control *C. musae in vitro*. For example, BHA (5 mM) completely inhibited conidial germination and mycelial growth of *C. musae in vitro* at 22 °C. In contrast, the same concentration only gave a control of wound anthracnose of 23 % and crown rot of 39 % at 25 °C. This effect was also true of conventional fungicides such as TBZ and imazalil. Clearly, there is a problem in getting enough active ingredient to the fungus when it is growing in the host tissues. Failure to penetrate the infected tissues and interactions with the host tissues (binding or detoxification) may be factors in this situation.

7.5.2 Effect of chemicals in combination

Five chemicals were tested in combination with one another on their effect on anthracnose and crown rot infections. These were BHA, PP, benzoic acid, imazalil and TBZ. Azadirachtin was not included in these experiments due to the cost of the chemical.

No treatment at 25 °C, completely controlled any of the infections. The combinations that were tested which gave the strongest disease control were BHA (5 mM) with either PP (10 mM) or imazalil (0.84 mM). At 25 °C, these combinations were most effective against stalk and crown rot infections (61-68 % and 66-72 % inhibition respectively). This compared with the effect of imazalil at 1.68 mM (500 ppm) which inhibited these infections by 49-52 % compared to the control. BHA (5 mM) with imazalil (0.45 mM) or thiabendazole (0.62 mM) was also very effective against crown rot infections with both isolates (CM 100 and CM 103) of *C. musae* at 25 °C (80 - 67.6 % and 63 - 46.7 % inhibition respectively). There was a very slight synergistic effect for the BHA + imazalil combinations but not for BHA + TBZ. None of these chemicals was as effective against anthracnose when it had been established on the peel, particularly when mycelium was inoculated into wounds. In this latter case, the effects of all three chemical treatments were not statistically different from one another. Inhibition of lesion growth ranged from 31-37 % compared to the control.

At 25 °C, the fungus is growing at a temperature close to optimal (27–30 °C) (Goos and Tschirsch, 1962). It was of interest to see how the chemical treatments performed at 14 °C which is close to the normal transport and storage temperature for green bananas. This may be of relevance for infections that might occur later in the postharvest chain, after the initial postharvest treatment. In fact, at 14 °C, the effect of the chemicals was much greater than at 25 °C, even taking into account the slower growth of the fungus at the lower temperature. The control of mycelial induced anthracnose by the treatments BHA (5 mM) + PP (10 mM); BHA (5 mM) + imazalil (0.84 mM) and imazalil (0.84 mM) was increased to 75, 71 and 67% respectively. Further more these treatments and some other treatments tested, gave complete control of conidial infections of the peel, stalk or crown tissues over the 14 days of the experiments.

7.5.3 Effect of pH on the effectiveness of treatments

The pH of some of the treatment solutions was as low as 3.2 (benzoic acid at 10 and 15 mM) and as high as 6.6 (BHA at 1-10 mM). When these solutions were applied to the peel of the banana, there was clearly a buffering effect on the banana surface and the actual change in pH was no more than 0.6 units (benzoic acid at 10 mM). A selected number of treatments were carried out with and without buffering the solutions to the approximate normal pH of the banana peel (4.6). This had no significant effect on the effectiveness of the chemicals. It is probably reasonable, therefore, to conclude that differences between treatments were not due, in the main part, to pH changes caused by the treatment.

7.5.4 Possible alternative mechanisms of disease control by antioxidants

Based on the *in vitro* studies, It seems likely that the control of banana disease by the chemicals described above, is primarily due to their toxicity to *C. musae*. It is possible, however, that the antioxidant properties of the chemicals might give them a different role in the host-pathogen relationship.

There is increasing evidence that suggests that the generation of free radicals plays an important part in the success or failure of a plant-pathogen interaction. Free-radical peroxidation is an important mechanism of membrane deterioration and ethylene production during the senescence of tissues (Baker *et al.*, 1978; Apelbaum *et al.*, 1981; Dhindsa *et al.*, 1981; Droillard *et al.*, 1987). Anything that speeds up this process, will make plant tissues more susceptible to pathogen attack. Edlich *et al.* (1989) showed that active oxygen radicals assisted extracellular enzymes of *B. cinerea* in plant infection. Antioxidants, therefore, might actually slow down plant tissue senescence. Propyl gallate and sodium benzoate have been shown, for example, to inhibit ethylene production in fruit slices, flowers and vegetative tissue. It is well established that controlling ethylene production can reduce the susceptibility of plant tissues to necrotrophic pathogens such as *B. cinerea* (Elad, 1988; 1990).

On the other hand, there is also evidence, that the generation of free-radicals is important in the synthesis of phytoalexins and other aspects of plant defence systems. Therefore, antioxidants could actually block host defence systems and increase sensitivity to pathogens and in particular, those that invade directly such as *C. musae* in anthracnose disease.

BHA has been developed by an Israeli research group as a pre-harvest spray in combination with the fungicide prochloraz to control anthracnose caused by *C. gloeosporioides* on avocado fruits (Prusky *et al.*, 1995). The concentration of BHA found to be effective in semi-commercial trials was around 6.7 mM. The authors

believe from earlier work that the benefits of the BHA are due to the antioxidant delaying the oxidative breakdown of natural antifungal compounds (dienes) in the fruit tissues (Prusky *et al.*, 1983; Prusky, 1988; Prusky *et al.*, 1991). From the present research on *C. musae*, it seems likely that at 6.7 mM, the BHA would also be having a direct toxic effect on *C. gleosporioides*.

From the research reported here, it is not possible to say whether antioxidants such as BHA have a positive or negative effect on host resistance. It is worth noting, however, that at higher concentrations (greater than 10 mM) BHA was found to cause injury to the banana peel in the form of tissue blackening. This is further evidence that the toxic effect of BHA functions by damaging cell membranes. The potential benefits of BHA as a fungicide will depend on being able to apply the chemical at a concentration that kills fungal cells without damaging host cells.

7.6 COST BENEFIT ANALYSIS

The cost benefit analysis revealed that the cost of the best treatment (BHA + imazalil) was £0.25 / litre. The cost of imazalil alone (1.6824 mM = 500 ppm) was £0.30 / litre. The cost of second good treatment (BHA 5 mM + propyl paraben 10 mM) was £0.20 / litre. The best treatment (BHA + imazalil) was cheaper than the imazalil alone.

7.7 CONCLUSIONS

The major findings of this study can be summarised as following:

- A number of chemicals have been identified which show significant antifungal activity against the mycelial growth and conidial germination of two isolates of *C. musae* *in vitro*.

- Five chemicals, butylated hydroxyanisole (BHA), propyl paraben (PP), propyl gallate, benzoic acid and azadiractin have been shown for the first time to inhibit the growth of *C. musae* on banana fruits.
- The pH of the growth medium or the banana surface following treatment, did not appear to account for the difference in fungitoxicity between the *in vitro* or *in vivo* treatments.
- Much better control of anthracnose and crown rot (as a percentage of lesion development in controls) was obtained on fruit held at 14 °C compared to those held at 25 °C.
- The most consistently active chemicals against *C. musae* were BHA, PP, benzoic acid and azadiractin.
- Different isolates of *C. musae* showed differential sensitivity to chemicals with fungicidal activity although the relative response to different concentrations and type of chemicals, was similar.
- When BHA, PP or benzoic acid were combined together or with various concentrations of imazalil or TBZ, significant additive or synergistic effects were seen in antifungal activity both *in vitro* and *in vivo*.
- For the first time, *C. musae* has been shown to produce the following extracellular enzymes: Esterase lipase (C 8), lipase (C 14), alkaline and acid phosphatases, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase and N-acetyl- β glucosaminidase. Reports of the production of esterase and β -glucosidase by other researchers has been confirmed.

- BHA, imazalil and TBZ caused a differential effect on the production of a number of extracellular enzymes produced by isolate CM103 of *C. musae*.
- The best treatment tested was BHA (0.5 mM) + imazalil (0.84 mM) is cheaper than imazalil at typical concentrations applied in practice (1.68 mM).

Based on these research findings, it seems to be reasonable to suggest that combinations of BHA with PP, benzoic acid or imazalil might be successfully developed for post-harvest application on bananas. It is clear that such treatments could be at least as effective as current fungicide applications and that the cost of the active ingredients would be in the same range as for TBZ and imazalil treatments. The apparent synergy of BHA with imazalil *in vitro* suggests that the site of action is different for the two chemicals. This would make it highly unlikely that *C. musae* would become resistant to the treatment. Complete control of anthracnose or crown rot infections caused by *C. musae* would be dependent, however, on good postharvest control of temperature. Banana fruit would need to be cooled rapidly after harvest to 13-14 °C and maintained at this temperature throughout transportation and storage.

Suitable formulations would need to be developed. This might add to the cost of the final product (s). BHA and PP are insoluble in water, so an oil-based emulsion system might be appropriate for them. As BHA has been used as a spray on avocado trees, clearly suitable formulations do exist. Furthermore, recent research at Cranfield University have shown that the oil-based solvent HastenTM can be used to dissolve BHA and that this can be dissolved in water without precipitation at concentrations as high as 1.8 mM (Chan Ho Tong, 1999). Benzoic acid on the other hand has a solubility in water of 21.0 g l⁻¹ at 25 °C (Smith, 1991) and so could be formulated directly in an aqueous medium.

The development of safer treatments (for example, using common food additives to replace or reduce commercial fungicides) to protect the postharvest life of banana fruits should be an attractive proposition for both producers and consumers alike.

Currently BHA, PP and benzoic acid are permitted for use as antioxidants and / or antimicrobials in a number of foods (E numbers E320, E216 and E210 respectively) and are on the generally regarded as safe (GRAS) list of the US Food and Drug Administration. There have, however, been questions raised over the safety of all these three chemicals. Both PP and benzoic acid may cause allergic reactions in sensitive individuals (Hanssen, 1987). Some studies have implicated BHA in cancer (Reddy and Maeura, 1984), however, more recent studies have suggested the reverse and claim that the antioxidant effect of BHA prevents the free radical damage of cells which lead to cancers (Hocman, 1988).

It is almost certain that however effective these phenolic food additives are as antifungal treatments, their use as fungicides or in combination with fungicides will come under close scrutiny by government departments responsible for food safety and by consumer interest groups. It is hoped, however, that in future more attention will be focused on the potential for food additives such as BHA, to be a relatively cheap means of controlling postharvest diseases of fruits and vegetables. In the present context, it is not suggested that BHA or PP could of themselves replace existing disease control programmes. Rather that these chemicals may serve a useful purpose in an integrated system of postharvest management which would employ a number of different methods to ensure the protection of fresh produce prior to consumption.

7.8 SUGGESTIONS FOR FUTURE WORK

This study suggests a number of new lines for research.

- An investigation into the potential of food additives such as BHA, PP and benzoic acid to control other fungal pathogens of importance on banana fruits (in particular, others commonly associated with crown rot infection) and on other fruits and vegetables.

- It is vital that a detailed investigation is carried out into the effect of any potential new treatments (for example, those suggested in the current study) on the quality of banana fruits.
- Experimental and ultimately, simulated-commercial trials of any new treatments must be carried out using freshly harvest fruits in producer countries.
- Studies should be undertaken to determine the role of the extracellular enzymes identified as being produced by *C. musae* in this current study.
- Further investigations would be of interest to determine the mechanism/s of the direct antifungal activity of BHA and related compounds.
- A study of pre-harvest sprays of antioxidants on post-harvest disease control is suggested. It would be of interest to see if these chemicals can influence changes of natural disease inhibitors in banana fruits as is seen in avocado fruits (Prusky, 1988).

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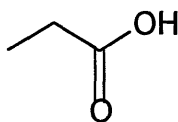
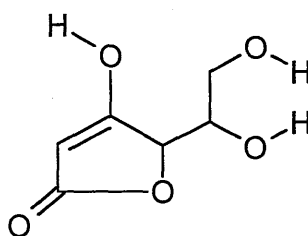
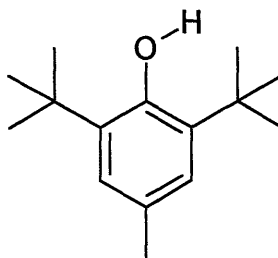
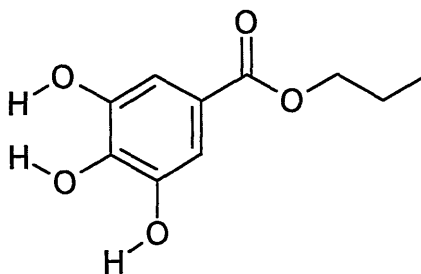
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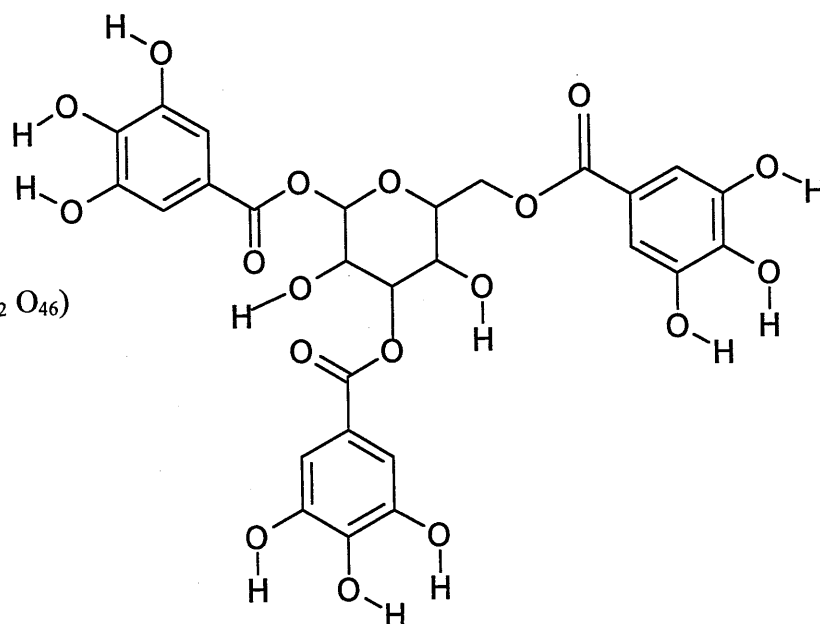
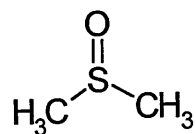
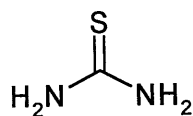
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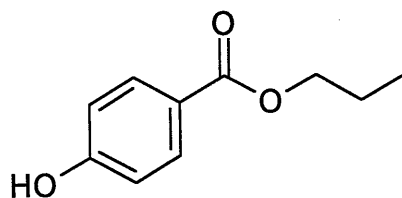
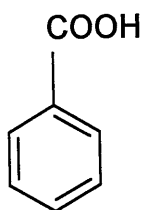
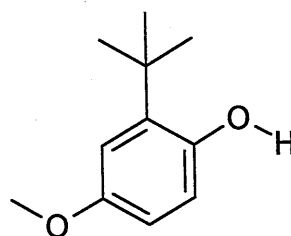
APPENDICES

Appendix A: The colour chart (supplied by API ZYM kit) used to determine extracellular enzymes of *Colletotrichum musae*

Quantity of hydrolysed substrate Quantité de substrat hydrolysé		0 nanomole	5 nanomoles	10 nanomoles	20 nanomoles	30 nanomoles	> 40 nanomoles
Activity mark Activité chiffrée		0	1	2	3	4	5
Control — Témoins	1						
2 - naphthyl - phosphate	2						
2 - naphthyl - butyrate	3						
2 - naphthyl - caprylate	4						
2 - naphthyl - myristate	5						
L - leucyl - 2 - naphthylamide	6						
L - valyl - 2 - naphthylamide	7						
L - cystyl - 2 - naphthylamide	8						
N-benzoyl-DL-arginine-2-naphthylamide	9						
N-glutaryl-phénylalanine-2-naphthylamide	10						
2 - naphthyl - phosphate	11						
Naphtol-AS-BI-phosphate	12						
6-Br-2-naphthyl- α D-galactopyranoside	13						
2-naphthyl- β D-galactopyranoside	14						
Naphtol-AS-BI- β D-glucuronide	15						
2-naphthyl- α D-glucopyranoside	16						
6-Br-2-naphthyl- β D-glucopyranoside	17						
1-naphthyl-N-acétyl- β D-glucosaminide	18						
6-Br-2-naphthyl- α D-mannopyranoside	19						
2-naphthyl- α L-fucopyranoside	20						

Appendix B: Formulae and structures of the compounds used in this study.Propionic acid ($C_3 H_6 O_2$)Ascorbic acid ($C_6 H_8 O_6$)Butylated hydroxytoluene ($C_{15} H_{24} O$)Propyl gallate ($C_{10} H_{12} O_5$)

Tannic acid ($C_{76} H_{52} O_{46}$)Dimethyl sulphoxide ($C_2 H_6 S O$)Thiourea ($C H_4 N_2 S$)

Propyl paraben ($C_8 H_{16} O_2$)Benzoic acid ($C_7 H_6 O_2$)Butylated hydroxyanisole ($C_{11} H_{16} O_2$)

PUBLICATION

THE POTENTIAL FOR ANTI-OXIDANT CHEMICALS TO CONTROL *COLLETOTRICHUM MUSAE* ON BANANA FRUIT

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SUMMARY

The fruit disease, crown rot is a major cause of fruit losses in export bananas. Infection leads to blackening of the crown, weakened fruit pedicels and subsequent finger drop. Crown rot is caused by a complex of fungal pathogens of which *Colletotrichum musae* is one of the most prevalent. This pathogen also causes unsightly blemishes on the peel of the ripe fruit called anthracnose disease. With intensive use of fungicides pre-harvest, many fruit pathogens such as *C. musae* are becoming resistant to the same fungicides when applied postharvest to control fruit disease. Alternative postharvest chemicals will be needed in the future. In this study, four isolates of *Colletotrichum musae* cultured from anthracnose lesions on imported banana fruit were screened against the fungicides thiabendazole (TBZ) and imazalil. Two of the isolates showed some resistance to TBZ even at 250 ppm, whereas all 4 isolates were very sensitive to imazalil at concentrations of ≥ 5 ppm. One susceptible and one tolerant isolate (to TBZ) was selected, and nine antioxidants were evaluated over a range of concentrations for their effectiveness in inhibiting the germination and growth of these isolates *in vitro*. These chemicals were ascorbic acid, propionic acid, benzoic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene, propyl paraben (PP), propyl gallate (PG), dimethyl sulphoxide and thiourea. BHA, benzoic acid, PP and PG showed the most promise as control agents *in vitro* at 1 mM. These four chemicals were evaluated for their effectiveness alone and in combination in retarding the growth of *Colletotrichum musae* on banana fruit both on the fruit peel and the cut crown. Different concentrations of antioxidant compounds or thiabendazole or imazalil were applied either before inoculation or 24 hours after inoculation to test their protectant and eradicator potential respectively. BHA showed the most promise as a control agent *in vivo* with low concentrations causing strong suppression of anthracnose lesions and crown rot development in combination with low concentrations of imazalil, benzoic acid and PP.

INTRODUCTION

Anthracnose and crown rot of bananas severely affect fruit quality and result in significant economic losses in many parts of the tropics. The fungal pathogen *Colletotrichum musae* (Berk. & Curtis) Arx is the causal agent of anthracnose and is commonly present in the complex of micro-organisms that cause crown rot (Jeffries *et al.*, 1990). There is evidence that *C. musae* has developed resistance to benomyl, thiabendazole and related fungicides (Griffie, 1973) and that there is a likelihood of resistance developing against other fungicides in use on bananas such as imazalil and bitertanol (based, for example, on the experience of the citrus industry (Eckert, 1990).

The range of fungicides with clearance for use on fresh produce is limited to about 20 chemicals (Eckert, 1990) and there is little incentive for the agro-chemical industry to develop new products specifically for this niche application

sector. There is, therefore, a growing need to find alternative or complementary methods for postharvest disease control.

There is evidence that a number of anti-oxidant compounds have considerable anti-microbial activity, including a number of food grade additives (Adams and Moss, 1995). Their effectiveness against postharvest fungal diseases has not been evaluated although Elad (1992) showed significant control of grey mould in various crops by a range of anti-oxidants. This paper describes an investigation into the effect of 9 different anti-oxidants on *C. musae*. The research described in this paper had the following objectives:

- To screen 4 isolates of *C. musae* for sensitivity to the fungicides thiabendazole & imazalil.
- To determine the effect of different anti-oxidants for control of *C. musae* *in vitro*.
- To evaluate the efficacy selected individual, and combined treatments to control anthracnose lesions after establishment of latent infection by inoculation of intact banana fruits with conidial suspension of *C. musae*.
- To assess the efficacy of the best individual and combinations of chemicals against crown rot lesions after establishment of infection by inoculation of intact banana fruits with conidial suspension of *C. musae*.

MATERIALS AND METHODS

The fungal isolates of *Colletotrichum musae* were obtained from anthracnose lesions found on commercially ripened bananas from UK retail stores. The four isolates (100, 101 103 and 104) were maintained on 5% malt extract agar (MEA) containing chloramphenicol (200 ppm) to suppress bacterial development at 22 °C. Colonies were sub-cultured every week or when necessary. Conidial suspensions were prepared by flooding 12 day old colonies with sterile water, agitating with a glass rod and filtering the resulting suspension through muslin cloth. The suspension was then centrifuged and the pellet resuspended in sterile water to give the required concentration of conidia as determined using a haemocytometer.

Stock solutions of all anti-oxidants or fungicides were prepared in either distilled water (ascorbic acid, dimethyl sulfoxide (DMSO), propionic acid, thiourea, storite flowable (thiabendazole, a.i 45%) and fungazil (imazalil, i.a. 9.26%) or in 94.9% ethanol (benzoic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and propyl paraben (PP) and added to the autoclaved, molten MEA at 50°C to obtain the required concentrations before pouring into treatment Petri plates.

Treatment plates were inoculated centrally with 4-mm diameter discs from the colony margin of a 9-11 day old culture of *C. musae*. Each of four replicates per treatments were sealed with Nescofilm and incubated at 22°C. Temporal growth was determined by measuring the diameter of the colony in two directions at right angles to each other for up to 15 days. The diametric extension rates were plotted and regression lines of the linear phase were used to determine the growth rate (mm day⁻¹) for each treatment.

For the *in vivo* experiments, banana fruits (*Musa* AAA Giant Cavendish) were obtained from local fruit importers. Only fruit which was green, $\frac{3}{4}$ fully matured and were free of damage or disease was selected for experimental use. Intact fruits were inoculated by placing 10 μ l of spore suspension (10^6 spores cm^{-3}) from 10 to 14 day old cultures on their skin (anthracnose simulation) or on the cut crown of a fruit cluster (crown rot simulation). The fruit were then kept at 95% r.h. and 25°C for 48 hours to allow the infection to establish. Fruits were subsequently dipped for 30s in the chemical solutions and allowed to dry. Control fruits were dipped in distilled water. All fruit was then exposed to 1000 ppm ethylene for 24 hours to initiate ripening. The fruit was before storage in a controlled environment room at 25°C and 95% r.h. for up to 14 days. Anthracnose lesion diameters were measured on daily basis for up to 12 days. The depth of crown rot lesions were measured from the inoculated crown surface towards the finger stalks. Results are presented as the rate of increase in lesion diameter or depth (mm day^{-1}). All experiments were carried out with 4 replicate bananas per treatment.

RESULTS

Figure 1 shows the effect of a range of concentrations of imazalil and TBZ on growth of all 4 isolates (100, 101, 103 and 104) of *C. musae*. Imazalil gave considerable control even at 0.5, 1.0 and 2.5 ppm. The sensitivity of isolate 100 to TBZ was demonstrated, with 0.5 ppm reducing growth by 60%, when compared to control. On the other hand, isolate 103 (R) still grew at 250 ppm TBZ, which is within the concentration range typically applied to bananas to control post-harvest disease. All further work was carried out with TBZ resistant isolate (103).

Most of the anti-oxidants tested reduced the growth rate (mm day^{-1}) of isolate 103 of *C. musae* (Figure 2). The four most effective anti-oxidants were butylated hydroxyanisole (BHA), propyl paraben (PP), benzoic acid and propyl gallate (PG). At 1 mM, BHA reduced the growth rate of isolate 103 by 70% and completely suppressed mycelial growth at concentrations greater than 5 mM. At 15 mM concentration, the growth of isolate 103 was reduced by 81.5% (PP), 74% (benzoic acid) and 85.3% (PG).

Figure 3 shows some results of various treatments on the development of anthracnose lesions after inoculation of banana fruit with *C. musae* conidia. All treatments reduced lesion size (mm day^{-1}) when compared to the control, although none actually completely inhibited lesion development, including imazalil at 500 ppm. The best treatment was the combination of BHA (5 mM) + imazalil (250 ppm), reducing lesion growth rate by 65% at 25°C. Combinations of BHA (5 mM) and PP or benzoic acid (10 mM) gave lesion reductions of 55% and 35% respectively.

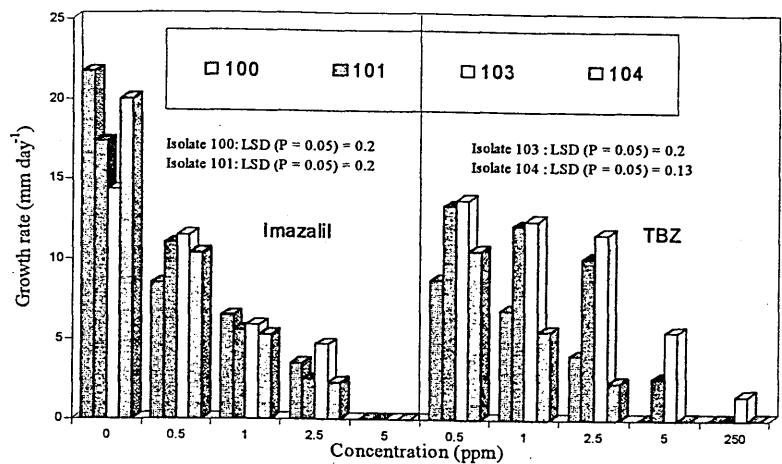


Figure 1: Effect of imazalil & thiabendazole (TBZ) at different concentrations on the mycelial growth of *Colletotrichum musae* (isolates 100, 101, 103 & 104) on malt extract agar at 22°C.

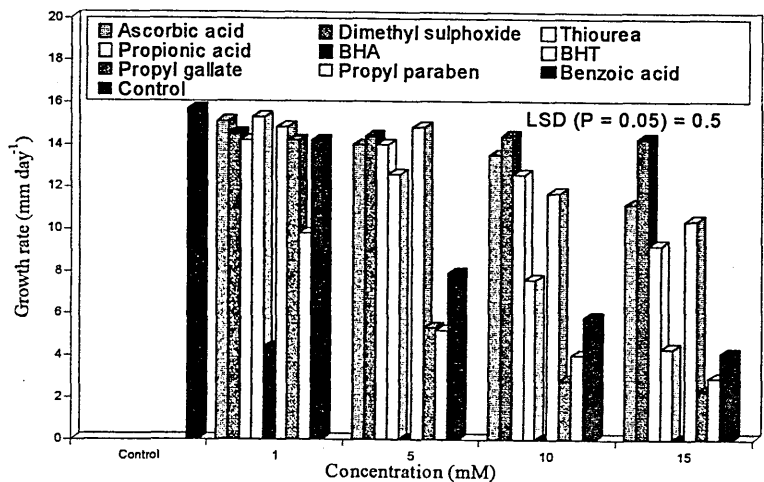


Figure 2: Effect of a range of antioxidants on mycelial growth of isolate 103 of *Colletotrichum musae* on malt extract agar at 22°C. BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene.

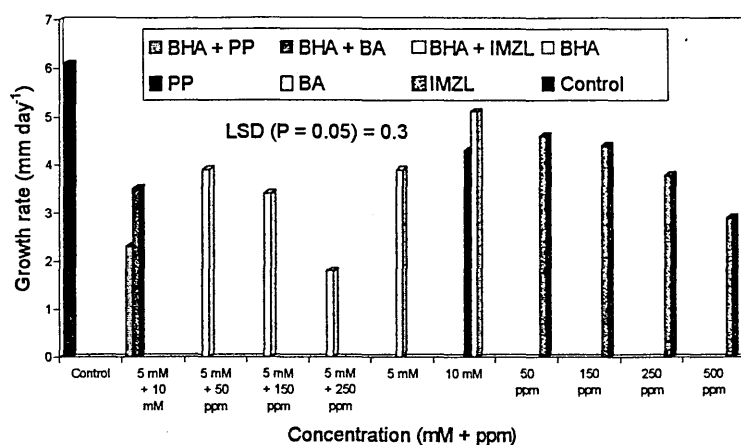


Figure 3: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben (PP), benzoic acid (BA) and imazalil (IMZL) on the expansion of latent lesions of *Colletotrichum musae* on banana fruits at 25°C.

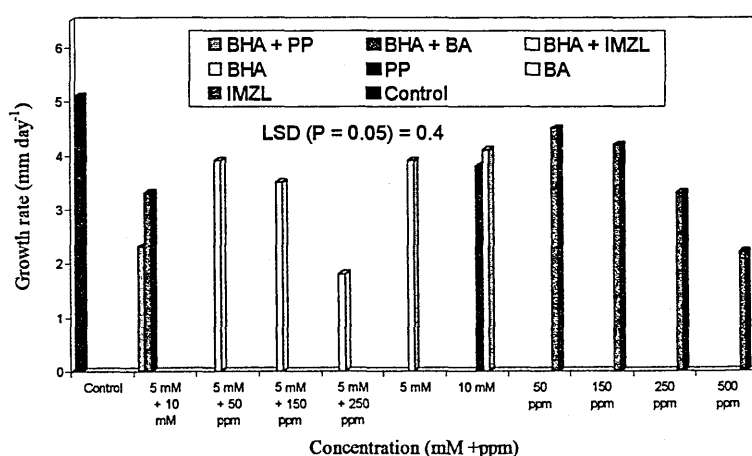


Figure 4: Effect of butylated hydroxyanisole (BHA) in combination with propyl paraben (PP), benzoic acid (BA) and imazalil (IMZL) on the penetration of crown rot into the crowns of banana clusters inoculated with conidia of *Colletotrichum musae* at 25°C.

A very similar pattern of results was obtained when these treatments were used to try and control simulated crown rot (Figure 4). All treatments reduced the penetration of crown rot into the inoculated crown compared to the control. The most effective treatment was BHA (5 mM) combined with imazalil (250 ppm) which reduced crown rot by 71%.

DISCUSSION

TBZ is a benzimidazole derivative with anti-mitotic activity which causes inhibition of spore germination and hyphal growth. It is well established that resistance to benzimidazole fungicides by fungal pathogens of banana fruit is common, and may have been encouraged by the use of fungicides with same mode of action for control of foliar disease in the plantations (Slabaugh & Grove, 1982). It is not surprising therefore, that TBZ tolerance was detected in some of the isolates from banana fruits imported into the UK. In this study, imazalil was, however, an effective biocide for control of the *C. musae* isolates. The imazalil works at several biochemical sites and it is thought that this polygenetic system makes the probability of development of resistant strains much lower than that for the benzimidazole type fungicides (Laville *et al*, 1977). All the compounds except dimethyl sulfoxide (DMSO) tested showed some inhibitory activity against TBZ tolerant isolates of *C. musae in vitro* at 15 mM. The mechanisms behind this activity may be quite varied. Organic acids such as propionic, benzoic and sorbic acids have long been used to control fungal spoilage of food and the mode of action has been attributed to two factors; depression of intracellular pH by ionisation of the undissociated acid molecule or disruption of substrate transport by alteration of cell membrane permeability (Liewen, 1991). Benzoic acid has also been shown to inhibit cell wall degrading enzymic activity in bacteria (Lyon and McGill, 1989). Propyl paraben and BHA appears to work mainly at the cell membrane, eliminating the pH component of the protomotive force and affecting energy transduction and substrate transport (Adams & Moss, 1995). BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration (Aldunate *et al*, 1992).

Generally, anti-oxidants with or without imazalil were found to be effective in delaying the development of *C. musae* infection on banana fruit in simulated trials of both anthracnose and crown rot. There is, therefore, scope to develop the use of anti-oxidants as either replacements for currently used fungicides or in combination with reduced doses of fungicides. Before the commercial potential of these treatments can be fully determined, the most promising anti-oxidants need to be tested on freshly harvested fruit under simulated commercial handling conditions. Treated fruit will need to be carefully evaluated for any deleterious effects on fruit quality.

ACKNOWLEDGEMENTS

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The potential for antioxidant chemicals to control *Colletotrichum musae* - *In Vitro* studies

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Abstract

Four isolates of *Colletotrichum musae* cultured from anthracnose lesions on imported banana fruit were screened against the fungicides thiabendazole (TBZ) and imazalil. Two of the isolates showed some resistance to TBZ even at 250 ppm, whereas all 4 isolates were very sensitive to imazalil at concentrations of ≥ 5 ppm. One susceptible and one tolerant isolate (to TBZ) was selected, and nine antioxidants were evaluated over a range of concentrations for their effectiveness in inhibiting the germination and growth of these isolates *in vitro*. The compounds showing the most anti-fungal activity were tested alone, in combination with each other, and with imazalil. Butylated hydroxyanisole, benzoic acid and propyl paraben showed the most promise as control agents *in vitro* at 1 mM and may be useful in combinations with reduced concentrations of imazalil to enhance control of the anthracnose pathogen.

Key words: antioxidant, *Colletotrichum musae*, thiabendazole, imazalil

Introduction

Fungal infections are a frequent cause of loss in harvested fruits. Infection may lead to rotting and physical loss or may cause unsightly blemishes which lead to rejection or down-grading of the produce. Control of most fungal infections is through the application, shortly after harvest, of fungicides as a dip or spray or sometimes incorporated into waxes (Eckert and Ogawa, 1985; 1988). Only a small number of post-harvest chemicals are approved for post-harvest use and this number is diminishing. Furthermore, the market share for post-harvest fungicides compared to pre-harvest fungicides is very small. Few chemical companies, if any, are prepared to invest in the search for new products particularly suited for postharvest use. Some of the most frequently used post-harvest fungicides are benzimidazole derivatives, and many fungi are

showing resistance to these compounds, particularly where they are used both pre- and post-harvest (Fletcher *et al.*, 1987).

The most important disease problem of export bananas (Cavendish cultivars) is crown rot. This disease is a complex which may involve several species of which one of the more common is *Colletotrichum musae*. This organism also causes anthracnose blemishes on the fruit peel. These lens shaped lesions tend to appear on the fruit most often in the retail sector after ripening (Griffie and Burden, 1974). There is evidence that *C. musae* has developed some resistance to benomyl, thiabendazole and related fungicides which were the most common post-harvest fungicides used to control banana fruit diseases (Griffie, 1973). Increasingly, the ergosterol biosynthesis inhibitor, imazalil is replacing these benzimidazole based fungicides in fruit fungicide dips (Eckert, 1990). Currently no resistant isolates of *C. musae* have been found against imazalil. However, as the selection pressure increases, this may happen in the future. Imazalil tolerant or resistant isolates have been found in other fungi, for example, *Penicillium digitatum* (Eckert, 1990).

This paper describes the results of testing a number of compounds with anti-oxidant properties for their effectiveness in inhibiting growth of *C. musae in vitro*. Some of these chemicals, for example, benzoic and propionic acids, are used in food products for their anti-microbial properties against bacteria and yeast contamination. Others, such as BHA and BHT, are used in fat-containing foods primarily as antioxidants. There are relatively few studies on the antimicrobial activity of these chemicals, particularly on fungal pathogens of fresh produce (Adams and Moss, 1995). There are two main objectives to this study; firstly to determine if any of the anti-oxidant compounds investigated have significant anti-fungal properties; secondly, to see whether any of these chemicals can enhance the effectiveness of fungicides currently used to control diseases of banana fruit caused by *C. musae*.

Materials and Methods

Chemical preparations

Malt extract agar (MEA) was prepared at a 5% concentration according to the manufacturers instructions (Oxoid, Hants., UK). The media was autoclaved at 120°C for 20 minutes before cooling to 50°C and pouring into plastic sterile Petri dishes (86mm internal diameter from Sterilin,). Chloramphenicol (Sigma Chemical Co., Dorset, UK) at 200ppm was dissolved in 95% ethanol and added to the agar medium to suppress bacterial development.

The fungicide Storite Flowable™ (thiabendazole wettable powder, a.i.45%) from MSD Agvet, Herts., UK was made up by diluting the product with sterile distilled water and adding to the cooled liquid agar with constant agitation to prevent settling of the suspension. The fungicide Fungazil™ (imazalil, a.i.-9.26%) from Rhone Poulenc, Essex, UK was prepared by diluting the liquid product with sterile distilled water before adding to the cooled agar.

All the anti-oxidant chemicals listed below were obtained from Sigma Chemical Co., Dorset, UK. The water soluble chemicals were dissolved in distilled water and used to prepare the MEA. These were ascorbic acid, dimethyl sulphoxide, propionic acid and thiourea. Chemicals which are either insoluble or poorly soluble in water were firstly dissolved in ethanol before adding to the cooled liquid agar. These were benzoic acid, 2(3)-*tert*-butyl-4-hydroxyanisole (butylated hydroxyanisole - BHA); 2,6-di (*tert*-butyl)-*p*-cresol (butylated hydroxytoluene - BHT), 3,4,5-trihydroxybenzoic acid (propyl gallate - PG) and *n*-propyl *p*-hydroxybenzoate (propyl paraben - PP). The pH values of the various inoculation media were determined while the agar was still liquid using a Jenway 3020 pH meter with a Jenway 924-001 probe (Jenway Ltd., Essex, UK).

Four strains of *C. musae* were isolated from anthracnose lesions found on commercially ripened bananas from UK retail stores. These strains were maintained on 5% malt extract agar (MEA) at 22°C..

Germination and growth experiments

Conidial suspensions were obtained by flooding 12 day old colonies of *C. musae* with sterile distilled water and agitating the surface with a glass rod. The suspension was filtered through sterile muslin cloth and centrifuged at 4000 RPM for 5 min using a Sorvall centrifuge with an SS-34 rotor. The resulting pellet was re-suspended in sterile distilled water to remove germination inhibition factors that appear to be present in the spore matrix (Mondal and Parberry, 1992) to give a final concentration of 10^4 spores cm^{-3} . A sample of this suspension (0.1 cm^3) was spread over the surface of a test agar plate and incubated at 22°C for three days. After different time intervals, 15mm diameter disks of the agar were removed aseptically, placed on glass microscope slides and stained with lactophenol trypan blue (Merck Ltd., Leics., UK). 200 spores per disk were examined microscopically for germination which was considered to have taken place if the germ tube was equal to or longer than the spore diameter (Magan, 1988). Percentage germination could then be calculated.

For tests of mycelial growth, a 4mm diameter disk was removed with a cork borer from the growing margin of a 10 day old *C. musae* colony and transferred to the centre of a new MEA plate into which the chemical to be tested was incorporated. Test plates were incubated at 22°C . Colony diameter was measured in two directions on a daily basis for a maximum of 14 days. Growth rates (mm day^{-1}) were determined by plotting colony diameter against time and calculating the slope of the regression line for the linear growth phase. The lag period was defined as the number of days which elapsed before measurable radial growth was observed from a particular mycelial inoculum disk.

Replication and statistics

In all mycelial growth experiments, 3 replicate plates were used. In all germination experiments, 3 replicate plates were used and 3 disks per plate were removed for staining and conidial germination counts. Analysis of variance was carried out on the data. Treatment means were compared with the least significant difference at a probability of 5%. (For spore germination, analysis was carried out on the actual spore numbers but the mean (transformed) percentage germination is presented for clarity??) .

Results

The effect of TBZ and imazalil on 4 isolates of *C. musae*

The four isolates of *C. musae* had similar growth curves with linear growth rate being achieved after day 2 (data not shown). Isolate 100 showed the most vigorous growth on MEA at 22°C (21 mm day⁻¹) and isolate 103 showed the slowest growth rate under these conditions (14.2 mm day⁻¹) (Figure 1). The tolerance of these isolates of *C. musae* to 9 different concentrations (0.5 - 250 ppm) of the fungicides thiabendazole (TBZ) and imazalil was tested. The growth of the fungal isolates on TBZ (Figure 1) suggested that two of the isolates (101 and 103) were relatively resistant to this fungicide showing some growth on agar containing up to 50 and 250 ppm TBZ respectively. The other two strains (100 and 104) were completely suppressed by TBZ at concentrations of ≥ 5 mM. Imazalil completely suppressed mycelial growth of all 4 strains at ≥ 5 ppm and gave considerable control even at 0.5, 1.0 and 2.5 ppm.

All further work was carried out with one TBZ tolerant isolate (103) and one TBZ sensitive strain (100). The growth curves of isolates 100 and 103 on TBZ at 0, 0.5, 1.0 and 5 ppm and 0, 0.5, 1.0, 5, 50 and 250 ppm respectively are shown in Figure 3. The sensitivity of isolate 100 to TBZ is clear, with 0.5 ppm reducing growth rate by 60% when compared to the control and 1 ppm causing a lag in growth by 2 days. There was no growth at 5 ppm. On the other hand, isolate 103 grew (albeit slowly) at 250 ppm which is within the concentration range typically applied to bananas to control postharvest disease (i.e. 150 - 300 ppm) although there was a considerable lag time (6 days) before growth commenced on this concentration of TBZ (Figure 1).

Efficacy of antioxidants against germination of *C. musae* isolates

Table 1. shows the degree of inhibition of germination of conidia from isolates 100 and 103 over a 72 hour period by 9 different antioxidants at concentrations of 1-15 mM. Only 5 chemicals prevented 100% germination after 72h. These were benzoic acid at ≥ 5 mM, BHA and propyl paraben at ≥ 1 mM, propyl gallate at ≥ 10 mM and thiourea at 15 mM. Concentrations of ≤ 15 mM ascorbic acid, DMSO, BHT or propionic acid were ineffective although propionic acid at ≥ 1 mM delayed germination when compared to the control. At 10

mM, thiourea slowed the germination rate during the first 24 hours. At 1 mM benzoic acid slowed germination in the first 24 h period compared with the control. Interestingly, isolate 100 was more sensitive than 103 to the chemicals although the relative response to different concentrations and types of chemicals was similar.

Suppression of mycelial growth by antioxidants

The growth inhibition and lag period induced by the presence of antioxidants are shown in Table 2. Generally, isolate 103 was more tolerant of the range of chemicals than isolate 100, however, the most and least effective chemicals were the same for both isolates. DMSO was virtually ineffective at slowing down mycelial growth up to 15 mM concentration whereas all the other chemicals tested caused some reduction in growth rate of both isolates and in some cases delayed the start of growth of the inoculum by a number of days. At 1 mM and 15 mM, propionic acid reduced the growth rate of isolate 100 by 43.2% and 74.6% respectively and the higher concentration (15 mM) inhibited growth by 9 days. Isolate 103, however, was more tolerant of this chemical showing faster growth rates and a lag of only 3 days on 15 mM propionic acid. Propyl gallate at 10 and 15 mM prevented growth of isolate 100 and reduced the growth rate of 103 by 82.2% and 85.3% respectively. Propyl paraben prevented growth of isolate 100 at 15 mM and the same concentration reduced the growth rate of isolate 103 by 81.5%. BHA completely suppressed mycelial growth of both isolates at concentrations > 5 mM and even at 1 mM, BHA reduced the growth rate of isolate 100 by 87.8% and isolate 103 by 72.6% and prevented growth of both isolates for the first 4 days after inoculation.

The effect of combinations of BHA, PP and benzoic acid on *C. musae* germination and growth at 14°C and 25°C

It is clear that of the nine chemicals tested in the current study, three were particularly inhibitory to *C. musae* i.e. benzoic acid, propyl paraben and in particular, BHA. These three were tested in a selective number of combinations with each other and with low concentrations of imazalil. These experiments were carried out at two temperatures, 14°C which is close to the normal transport and storage temperature for green bananas and 25°C which is closer to the optimal growth temperature for *C. musae* which is 27-30°C (Goos and Tschirsch, 1962). Only one isolate of *C. musae* was used, i.e. 103, which appeared to be more tolerant of the

chemicals being evaluated than isolate 100. The effect of these chemicals on conidial germination, mycelial growth and lag period at 14°C and 25°C are shown in Table 3 and 4.

The growth rate of *C. musae* on MEA at 14°C was half that at 25°C which would be expected from the normal effect of reduced temperature on metabolic processes. Measurable growth was delayed by 4 days at the lower temperature (Table 4). On the other hand, initiation of germination appeared to be more sensitive to temperature than mycelial growth so that after 6h the number of germinated conidia at 14°C was only 32% of the number germinated at 25°C in the control plates (Table 3). These temperature effects are consistent with results obtained by Al Zaemey *et al.*, (1994) in a study of the environmental growth parameters of *C. musae*. It is clear from the latter study that adverse environmental factors can have a synergistic effect on one another. It is therefore not surprising that at a temperature considerably lower than optimal (14°C), the fungus is less tolerant of antifungal chemicals than at a more optimal temperature (25°C).

BHA on its own at 0.1 and 0.5 mM did not prevent full conidial germination by day 3. However, both concentrations slowed down the rate of germination and mycelial growth and significantly increased the lag period over this 72h period. The effect was relatively greater at 14°C than at 25°C. A similar story is apparent for imazalil at concentrations of 0.5, 1.0 and 5 ppm i.e. some reduction in germination and mycelial growth rate and a more marked affect at the lower temperature. The effect of temperature on the fungal growth and germination rate in response to 1mM benzoic acid was particularly marked. At 14°C the growth rate was half the control rate, whereas at 25°C, the growth rate was almost the same as the control rate. After 24h, there was no germination on 1 mM benzoic acid (96% in control) at 14°C compared to 79% at 25°C (100% in control). Complete inhibition of growth and germination was achieved by combining BHA with benzoic acid or propyl paraben, each at 1 mM and BHA with imazalil both at 0.5 ppm at both temperatures.

Discussion

Fungicide resistance

TBZ is a benzimidazole derivative introduced as a systemic fungicide in the 1960's. Its fungitoxicity is associated with the benzimidazole nucleus which binds to tubulin dimers and hinders their polymersiation to form microtubules. TBZ thus has an anti-mitotic activity which causes inhibition of spore germination and hyphal growth. It is not surprising to find TBZ resistance isolates of *C. musae* on imported banana fruit. It is well established that resistance to benzimidazole fungicides by fungal pathogens of banana fruit is common, and may have been encouraged by the use of fungicides with the same mode of action for control of foliar disease in the plantations (Slabaugh and Grove, 1982). Several studies have noted resistance to benomyl and benomyl related fungicides in the Windward Islands, a major supplier of bananas to the UK (Griffie, 1973; Johanson and Blaquez, 1992). Imazalil was, however, an effective biocide for control of the *C. musae* isolates used in this study. Imazalil was the first imidazole systemic fungicide used in agriculture and it falls in the class of demethylation inhibitor chemicals which interferes with ergosterol biosynthesis and hence disrupts fungal membrane biosynthesis (Lye, 1987). The mechanism works at several biochemical sites and it is thought that this polygenetic system makes the probability of development of resistant strains much lower than that for the benzimidazole type fungicides (Laville *et al*, 1977). Despite this, imazalil resistant strains of *Penicillium* spp. have been observed, particularly on *Citrus* fruits (Eckert *et al.*, 1994).

Efficacy of antioxidants against germination of *C. musae* isolates

Apart from DMSO, all the compounds tested showed some inhibitory activity against *C. musae* at 15 mM. The mechanisms behind this activity may be quite varied. Organic acids such as propionic, benzoic and sorbic acids have long been used to control fungal spoilage of food and the mode of action has been attributed to two factors; depression of intracellular pH by ionisation of the undissociated acid molecule or disruption of substrate transport by alteration of cell membrane permeability (Liewen, 1991). The effectiveness of some acids is dependent on the pH of the medium surrounding the micro-organism because it determines the extent of dissociation of the molecule. Generally only the undissociated form of an acid penetrates the

cell membrane. In the case of benzoic acid, at pH 5.1, only about 0.1% of the compound is undissociated whereas at pH 4.1 about 56% is undissociated (Bosund, 1962). The pH of MEA is 5.4 and the addition of organic acids altered the pH of this medium. Ascorbic acid, propionic acid, dimethyl sulphoxide and benzoic acid at 15 mM lowered the pH by about 0.9-1.2 units. These acidifications are likely to have made some direct contribution to the inhibition of *C. musae* conidial germination and mycelial growth for which the optimal pH is 6.0 (Al Zaemey *et al*, 1994). They will also have enhanced the uptake of these antioxidants.

Some chemicals may have more than one antifungal mechanism. Benzoic acid, for example, was reported to inhibit *in vitro* activity of polygalacturonase and polygalacturonic acid lyase from *Erwinia caratovora* (Lyon and McGill, 1989). It has also been shown, however, that the undissociated molecule interferes with membrane energetics and function. Propyl paraben, another organic acid appears to work mainly at the cell membrane, eliminating the ΔpH component of the protomotive force and affecting energy transduction and substrate transport and there is little evidence that parabens interfere directly with specific enzyme activities (Adams and Moss, 1995). BHT and its analogues probably act via a non-specific mechanism involving the perturbation of membrane function, for example, BHT appears to cause a reduction in the permeability of vesicles of phospholipid bilayer membranes (Singer and Wan, 1977). BHA on the other hand has been shown to increase leakage of sugars, amino acids and proteins from *Fusarium* spp. (Thompson, 1996). It has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration (Aldunate *et al*, 1992).

There are only a small number of reports in the literature of antioxidants being tested against postharvest fungal pathogens of fresh produce. The effect of various organic acids on the growth of *C. musae in vitro* was examined by Al Zaemey *et al.*, (1993). Potassium and sodium benzoate and propionic acid were the most effective of the acids tested. At 0.5% propionic acid completely suppressed mycelial growth on MEA and at 0.1% (i.e. about 13.5 mM) reduced the growth rate by 79% compared to the control. This is consistent with results shown in Table 2 where 15 mM propionic acid suppressed mycelial growth by 75% in one isolate (100) and 72.6 % in another (103) compared to the controls. In the same paper it was noted that only concentrations of ascorbic acid above about 60 mM (1%) were able to completely inhibit *C. musae*.

The effect of 17 different free radical scavenging chemicals on the mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum* on potato dextrose agar were examined by Elad (1992). This included all the chemicals investigated in the current study except propyl paraben. The results were variable depending on the fungal isolate used, however, the most effective chemical against *B. cinerea* was *tert*-butylhydroquinone, which suppressed growth completely at 1 and 10mM and significantly even at 0.1 mM. At 1.0 and 10 mM BHA caused significant growth inhibition of *B. cinerea* as did thiourea and BHT at 10 mM. None of the chemicals inhibited the growth of *S. sclerotiorum* at 0.1mM, whereas PG and benzoic acid at 1 mM significantly inhibited its growth by 95% and 65% respectively and significant inhibition was observed with 10 mM benzoic acid, thiourea, PG, BHT and ascorbic acid.

A number of studies have been published of the effect of the parabens and other phenolic antioxidants on mycotoxigenic fungi in the genera *Penicillium*, *Aspergillus*, and *Fusarium*. Esters of *p*-hydroxybenzoic acid (parabens) have been shown to be effective against these fungi but tend to be fungistatic rather than fungicidal. (Aalto *et al.*, 1953; Thompson, 1992; 1994; Chang and Branen (1975)). It appears that there is a wide range of tolerance to the parabens in genera of fungi known to be pathogenic to stored crops and fresh produce, with *Aspergillus* spp. being relatively resistant compared to *Penicillium* spp.. From the current study it appears that *C. musae* seems to fall within this range of tolerance.

Many food preservatives and fungicides are used in combination. Some combinations are more effective than a comparable level of the individual components alone e.g. benzoic and sorbic acids together are better at inhibiting yeast and moulds than either alone (Liewen, 1991). BHA and BHT have been shown to enhance the action of sorbic acid (Scott, 1989). In the presence of 0.6 µg/ml (about 0.0033 mM) BHA which is sub-inhibitory to two pathogenic *Candida* spp., weakly fungistatic levels of amphotericin B became highly lethal. It was thought that the BHA may have prevented auto-oxidation of the amphotericin B, thus stabilising its activity (Beggs, *et al.*, 1978).

The use of combinations of chemicals with different sites of action can help to prevent the development of resistance in the fungal population. Three anti-oxidants were found to be most effective in this study, that is, BHA, PP and benzoic acid. All three are effective in controlling

the germination and mycelial growth of *C. musae*. The preliminary experiments described in this paper also suggest that these chemicals may be useful used in combination with the fungicide imazalil. The basis of resistance to imazalil is not clear. It does not seem to be due to a decrease in the affinity of the fungicide for the target enzyme (Guan *et al*, 1993). At present, imazalil tolerant strains of *C. musae* have not been reported, however, the potential for resistance to arise is ever present and appropriate anti-resistance strategies should be developed. Such strategies are nowadays well defined and understood by the plant protection community (Staub, 1991). It would therefore be interesting to investigate combinations of BHA and other phenolic antioxidants with imazalil on imazalil resistant biotypes of *Penicillium*.

Resistance to TBZ and related fungicides is considered to result from altered amino acid sites on the tubulin with a reduced binding affinity for the fungicides (Martin *et al.*, 1992). The mechanism of resistance is such that it seems unlikely that there would be cross resistance to other antifungal chemicals which function by disrupting membrane integrity. Further research is required to determine the minimum effective combination concentrations *in vitro* and the effectiveness of these chemicals in controlling the diseases caused by *C. musae* on banana fruits.

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Table 1: Effect of a range of antioxidants on conidial germination of 2 isolates of *Colletotrichum musae* (100 & 103) on malt extract agar at 22°C.

Treatment	Concentration ^a	% germination of conidia after different time intervals							
		6 h		24 h		72 h		Isolate 100	Isolate 103
Control	n/a	Isolate 100	Isolate 103	Isolate 100	Isolate 103	Isolate 100	Isolate 103		
Ascorbic acid	1 mM	55.2	65.0	100.0	100.0	100.0	100.0	100.0	100.0
	15 mM	53.8	57.7	100.0	100.0	100.0	100.0	100.0	100.0
Propionic acid	1 mM	51.0	53.8	100.0	100.0	100.0	100.0	100.0	100.0
	15 mM	4.2	8.5	51.3	100.0	100.0	100.0	100.0	100.0
Benzoic acid	1 mM	0.0	0.0	31.0	37.5	100.0	100.0	100.0	100.0
	5 mM	27.0	34.4	64.0	77.4	100.0	100.0	100.0	100.0
BHA	1 mM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5 mM	0.7	0.0	3.5	7.8	19.4	0.0	22.5	0.0
BHT	1 mM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	15 mM	54.7	65.0	100.0	100.0	100.0	100.0	100.0	100.0
Propyl paraben	1 mM	31.4	45.7	88.7	100.0	100.0	100.0	100.0	100.0
	15 mM	5.2	7.2	7.0	10.9	18.0	28.0	0.0	0.0
Propyl gallate	1 mM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5 mM	54.5	67.4	100.0	100.0	100.0	100.0	100.0	100.0
DMSO	1 mM	27.2	28.7	78.7	78.8	100.0	100.0	100.0	100.0
	15 mM	0.0	4.5	3.8	6.8	13.4	21.5	0.0	0.0
Thiourea	1 mM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5 mM	55.0	63.4	100.0	100.0	100.0	100.0	100.0	100.0
	10 mM	55.2	60.7	100.0	100.0	100.0	100.0	100.0	100.0
	15 mM	54.2	62.7	100.0	100.0	100.0	100.0	100.0	100.0
	1 mM	33.8	41.0	88.0	100.0	100.0	100.0	100.0	100.0
	5 mM	20.5	25.0	63.7	100.0	100.0	100.0	100.0	100.0
LSD (t=0.05)	15mM	0.0	2.5	0.0	4.2	0.0	11.2	0.0	0.7
		1.9	4.7	2.6	6.2	0.9	0.9	0.9	0.7

^aAll chemicals listed here were tested at 4 concentrations (1, 5, 10 and 15 mM). Only selective results have been presented, where there were significant differences between treatments and control (p = 0.05)

Table 2: Effect of a range of antioxidants on mycelial growth and lag period of 2 isolates of *Colletotrichum musae* (100 & 103) on malt extract agar at 22°C

Treatment	Concentration ^a	Mycelial growth rate (mm h ⁻¹)		Lag period (days)	
		Isolate 100	Isolate 103	Isolate 100	Isolate 103
Control		21.3	15.7	0	0
Ascorbic acid	1 mM	19.7	15.0	0	0
	15 mM	7.7	11.1	2	0
Propionic acid	1 mM	12.1	15.3	0	0
	15 mM	5.4	4.3	9	3
Benzoic acid	1 mM	15.4	14.2	0	0
	5 mM	8.1	7.9	5	2
Butylated hydroxyanisole	1 mM	2.6	4.3	4	4
	5 mM	0.0	0.0	NG*	NG*
Butylated hydroxytoluene	1 mM	18.3	14.8	0	0
	15 mM	7.2	7.8	1	0
Propyl paraben	1 mM	5.6	9.8	1	0
	15 mM	0.0	2.9	NG*	6
Propyl gallate	1 mM	12.3	14.1	0	0
	5 mM	4.0	5.3	1	0
	10 mM	0.0	2.8	NG*	3
	15 mM	0.0	2.3	NG*	5
DMSO	1 mM	21.4	14.5	0	0
	15 mM	21.4	14.2	0	0
Thiourea	1 mM	10.5	14.2	0	0
	5 mM	10.6	14.0	1	0
	10 mM	7.9	12.6	1	0
	15mM	7.1	9.2	2	0
LSD (t=0.05)		0.3	0.4	0.0	0.1

^aAll chemicals listed here were tested at 4 concentrations (1, 5, 10 and 15 mM). Only selective results have been presented, where there were significant differences between treatments (p = 0.05)
*NG - no growth of colony by 14 days after inoculation of agar

Table 3: The effect of 3 antioxidants and the fungicide imazalil alone and in combination on the conidial germination of *Colletotrichum musae* (isolate 103) on malt extract agar at two temperatures (14°C and 25°C).

Treatment	Conidial germination (%) after different time intervals at 2 temperatures					
	6h	14°C 24h	72h	6h	25°C 24h	72h
Control	2.2	96.0	100.0	69.0	100.0	100.0
BHA (0.1 mM)	0.0	14.2	80.0	25.7	62.5	100.0
BHA (0.5 mM)	0.0	3.8	71.0	10.0	24.0	100.0
BHA (1.0 mM)	0.0	0.0	11.8	1.3	8.3	22.0
Benzoic acid (1 mM)	0.0	0.0	58.0	34.0	79.0	100.0
Propyl paraben (1 mM)	0.0	0.0	17.7	7.2	16.3	31.3
Imazalil (0.5 ppm)	0.0	55.0	100.0	65.0	100.0	100.0
Imazalil (1.0 ppm)	0.0	32.7	100.0	45.7	100.0	100.0
Imazalil (2.5 ppm)	0.0	18.0	100.0	34.3	90.7	100.0
BHA (1 mM) + benzoic acid (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
BHA (1 mM) + propyl paraben (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
BHA (0.5 mM) + imazalil (0.5 ppm)	0.0	0.0	0.0	0.0	0.0	0.0
LSD (t=0.05)	0.1	3.6	3.9	5.5	3.9	2.1

Table 4: The effect of 3 antioxidants and the fungicide imazalil alone and in combination on the mycelial growth and lag period of *Colletotrichum musae* (isolate 103) on malt extract agar at two temperatures (14°C and 25°C).

Treatment	Mycelial growth (mm day ⁻¹)		Lag period (days)	
	14°C	25°C	14°C	25°C
Control	7.2	14.4	4	0
BHA (0.1 mM)	3.6	8.5	7.5	2
BHA (0.5 mM)	2.7	7.7	8	3
BHA (1.0 mM)	2.0	4.7	10	5.5
Benzoic acid (1 mM)	3.6	14.1	4	0
Propyl paraben (1 mM)	6.3	9.2	6	0
Imazalil (0.5 ppm)	4.1	11.5	6	0
Imazalil (1.0 ppm)	2.8	5.9	6	0
Imazalil (2.5 ppm)	2.0	4.7	9	3
BHA (1 mM) + benzoic acid (1 mM)	0.0	0.0	NG*	NG*
BHA (1 mM) + propyl paraben (1 mM)	0.0	0.0	NG*	NG*
BHA (0.5 mM) + imazalil (0.5 ppm)	0.0	0.0	NG*	NG*
LSD (t=0.05)	0.9	0.7	1.7	1.2

*NG - no growth of colony by day 14 after inoculation of agar

Table 5: The effect of dipping treatments with butylated hydroxyanisole (BHA) alone or in combination with imazalil and thiabendazole (TBZ) on the lesion expansion down the stalk of banana fruits inoculated with *Colletotrichum musae*. Fruit was incubated after treatment at 25°C, relative humidity >97%. Two different fungal isolates were used (CM100 and CM103).

Treatment	Growth rate (mm day ⁻¹)	
	CM100	CM103
Control	7.1	6.0
BHA (0.5 mM)	7.1	6.0
BHA (5.0 mM)	4.2	3.9
Imazalil (0.22 mM $\equiv$ 62.5 ppm)	5.4	5.0
Imazalil (0.45 mM $\equiv$ 125 ppm)	4.5	4.4
Imazalil (1.78 mM $\equiv$ 500 ppm)	3.0	2.8
TBZ (0.31 mM $\equiv$ 62.5 ppm)	5.7	5.5
TBZ (0.62 mM $\equiv$ 125 ppm)	5.1	5.2
TBZ (2.46 mM $\equiv$ 500 ppm)	3.3	3.4
BHA (0.5 mM) + Imazalil (0.22 mM)	5.0	5.0
BHA (0.5 mM) + Imazalil (0.45 mM)	4.4	4.4
BHA (5.0 mM) + Imazalil (0.22 mM)	2.0	2.7
BHA (5.0 mM) + Imazalil (0.45 mM)	1.4	2.2
BHA (0.5 mM) + TBZ (0.31 mM)	5.7	5.5
BHA (0.5 mM) + TBZ (0.62 mM)	4.5	5.2
BHA (5.0 mM) + TBZ (0.31 mM)	3.0	3.5
BHA (5.0 mM) + TBZ (0.62 mM)	2.3	3.2
Lsd (P = 0.05)	0.3	0.3