Appendix 4

Recipes

Mango pickle

Mango pieces (from mature sour variety)	1kg
Salt	200g
Turmeric	1 tablespoon
Red chili powder	200g
Fenugreek seed	1 tablespoon
Mustard seed	2 tablespoons
Oil	200g
Asafoetida	1 teaspoon
Red chillies (dried)	5

Mango pulihora

Rice	200g
Grated mango (sour)	50g
Salt	2 tablespoons
Green chillies	30
Black gram dhal	2 tablespoons
Bengal gram dhal	2 tablespoons
Mustard seed	1 tablespoon
Oil	4 tablespoons
Turmeric	1 teaspoon
Coriander and curry leaves	to taste
Sugar	1 teaspoon

Mango icecream

Mango (ripe)	1
Eggs (separated)	4
Caster sugar	100g
Double cream	300ml

Biological Control of *Colletotrichum gloeosporioides*

A thesis submitted to the University of Kent

by

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No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other institute of learning.

Irene Koomen

December 1990

'Science is built up of facts, as a house is built of stones;
but an accumulation of facts is no more science than a heap
of stones is a house'

H. Poincaré

Voor Pa & Moe

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Abstract

Colletotrichum gloeosporioides is the causal agent of anthracnose disease of mangoes. Infection occurs when humidity is high and rain-dispersed spores germinate and form an appressorium on immature mangoes. The infection then becomes quiescent until the fruit is harvested. On ripe fruit infection is visible as black, sunken lesions on the surface. At the pre-harvest stage, the disease is controlled with the application of a range of fungicides, and at the post-harvest stage by hot benomyl treatment. The extensive use of benomyl, both pre- and post-harvest, has resulted in the occurrence of isolates of *C. gloeosporioides* resistant to this fungicide. To devise an alternative strategy of disease control, the potential for biological control of anthracnose has been investigated.

Potential microbial antagonists of *C. gloeosporioides* were isolated from blossom, leaves and fruit of mango, and screened using a series of assay techniques. In total 650 microorganisms, including bacteria, yeasts and filamentous fungi, were isolated and tested for their inhibition of growth of *C. gloeosporioides* on malt extract agar. Of these 650 isolates, 121 inhibited the fungus and were further tested on their ability to inhibit spore germination *in vitro*. Of these, 45 isolates, all bacteria and yeasts, were inoculated onto mangoes, which were artificially inoculated with *C. gloeosporioides*, and assessed for their potential to reduce the development of anthracnose lesions. A further selection was made, and 7 isolates were chosen to be used in a semi-commercial trial in the Philippines. This final screening procedure yielded two potential candidates for field trials, isolate 204 (identified as *Bacillus cereus*) and isolate 558 (identified as *Pseudomonas fluorescens*).

A field trial involving pre-harvest application of the biological control agent, was conducted using isolate 558. This isolate was chosen for this purpose since in *in vitro* experiments it significantly reduced germination of *C. gloeosporioides* spores. In the field trial 558 was applied in combination with nutrients and compared to treatments which had received no treatment or which had received conventional fungicide (benomyl) application. On spraying, high numbers of 558 were recorded on the leaf surface, but no reduction in post-harvest development of disease was observed. Failure of disease control was attributed to rapid death of the bacterium on the phylloplane.

In post-harvest trials, isolates 204 and 558 were both tested in combination with different application methods, including the addition of sticker, peptone, fruit wax or a sucrose polyester. Application of 204 did not reduce disease development. Application of 558, however, did significantly reduce anthracnose development compared to the control fruit. No additional benefit was achieved by incorporating the bacteria in peptone, fruit wax or sucrose polyester.

The mode of action of isolate 558 was investigated in detail. There was no evidence for parasitism taking place, or the production of volatile compounds, in the suppression of disease development. No antibiotic compounds were detected, but isolate 558 did produce a siderophore. A sharp increase in pH was also observed in culture media in which 558 was grown. Disease control may result from a combination of these two factors.

Abbreviations

cfu	- colony forming units
CPD	- critical point drier
CzDA	- Czapek Dox agar
CzDL	- Czapek Dox liquid
dH_2O	- distilled water
HWT	- hot water treatment
MEA	- malt extract agar
MEB	- malt extract broth
NA	- nutrient agar
NB	- nutrient broth
PDA	- potato dextrose agar
PHTRC	- Post-harvest Research and Training Center
RH	- relative humidity
S.E.	- standard error of the mean
SEM	- scanning electron microscope
TLC	- thin layer chromatography
TWA	- tap water agar
TWA-suc	- tap water agar with sucrose
UPLB	- University of the Philippines at Los Baños

1 Introduction

1.1 The fungus

1.1.1 *Colletotrichum gloeosporioides* - *C. gloeosporioides* (Penz.) Sacc. is the causal agent of anthracnose in mangoes. The perfect state (telcomorph) of *C. gloeosporioides* is *Glomerella cingulata* (Stonem.) Spauld. & Schrenk (Ascomycotina; Polystigmatales). The fungi included within the species *C. gloeosporioides* are an extremely heterogenous group and many forms were named after their host before von Arx (1957) reorganised the genus. In his monograph there are nearly 600 synonyms mentioned under *C. gloeosporioides*. *C. gloeosporioides* has hyaline, 1 celled, ellipsoid, rounded or slightly pointed spores which are formed in acervuli. The spores appear in lesions as moist, orange masses. The perfect state forms perithecia in which the ascospores are produced (Cook, 1975).

1.1.2 Anthracnose - *C. gloeosporioides* can cause anthracnose in a range of hosts besides mango, avocado, citrus (Baker *et al.*, 1940), papaya (Dickman & Alvarez, 1983), blueberry (Daykin & Milholland, 1984), *Stylosanthes* (Irwin *et al.*, 1984), jute (Purkayatha & Sen Gupta, 1975), cocoa (Chandra Mohanan *et al.*, 1989), rubber (Wastie & Janardhanan, 1970) etc. (see Jeffries *et al.*, 1990). Anthracnose is the common name for the type of lesions caused by most *Colletotrichum* spp. The lesions appear as black spots on the surface which later become sunken and on which the orange spore masses are produced (Agrios, 1988). Except for the typical anthracnose lesions visible on ripe mango fruit (Figure 1.1), *C. gloeosporioides* can also infect blossoms (blossom blight), leaves (shot hole), young fruit (fruit drop of fruit <4cm) and twigs (die back; Quimio & Quimio, 1974; Peterson, 1986). If humidity is high during flowering and fruit set, incidence of anthracnose can be severe and can result in a complete loss of harvest (Fitzell & Peak, 1984; Peterson, 1986; Prior & Ryder, 1987). When fruit are over 4-5 cm no immediate symptoms appear until after harvest, resulting in post-harvest anthracnose (Baker *et al.*, 1940). It has been reported from Australia, that *C. acutatum* also can cause anthracnose in mangoes (Fitzell, 1979).

Inoculum, in the form of spores, is continuously present in the tree, on the bark, twigs, diseased leaves and mummified inflorescenses (Fitzell & Peak, 1984). Rain is necessary to disperse the spores and infection frequency is directly related to rainfall (Fitzell & Peak, 1984; Fitzell *et al.*, 1984) and the tear drop pattern

Figure 1.1 Anthracnose lesions on mango.

of lesions sometimes observed on fruit is due to run off. A relationship between rainfall and disease severity was also observed for *C. gloeosporioides* on citrus (Denham & Waller, 1981), whereas for *C. coffeanum* it was established that there was a direct relationship between the number of spores trapped and the amount of disease present in coffee bushes (Waller, 1972). Recently it has been suggested, that a beetle (*Heterospilus prosopidis*, related to *H. coffeicola*, a potential biocontrol agent of *Hypothenemus hampei*, the coffee berry borer), can collect spores of *C. gloeosporioides* and *C. coffeanum* on its body, and possibly transmit disease that way (Nemeye *et al.*, 1990). Ascospores, which are dispersed by wind and are present in orchards, do not appear to contribute to disease incidence in mango (Fitzell & Peak, 1984; see Figure 1.2 for the disease cycle).

The asexual spores, produced in acervuli, are embedded in a mucilage layer which contains a self-inhibitor which is responsible for a reduction in spore germination at high spore concentrations (10^7 spores/ml; Lax *et al.*, 1985). Spores which have been dispersed adhere firmly onto a surface possibly aided by the mucilage (Emmett & Parbery, 1975). For the rice blast fungus, *Magnaporthe grisea*, it was shown that the spore tip contains mucilage that is discharged upon hydration and makes it possible for the spore to become attached to hydrophobic surfaces (Hamer *et al.*, 1988). This fungus is related to *Colletotrichum* in that they both

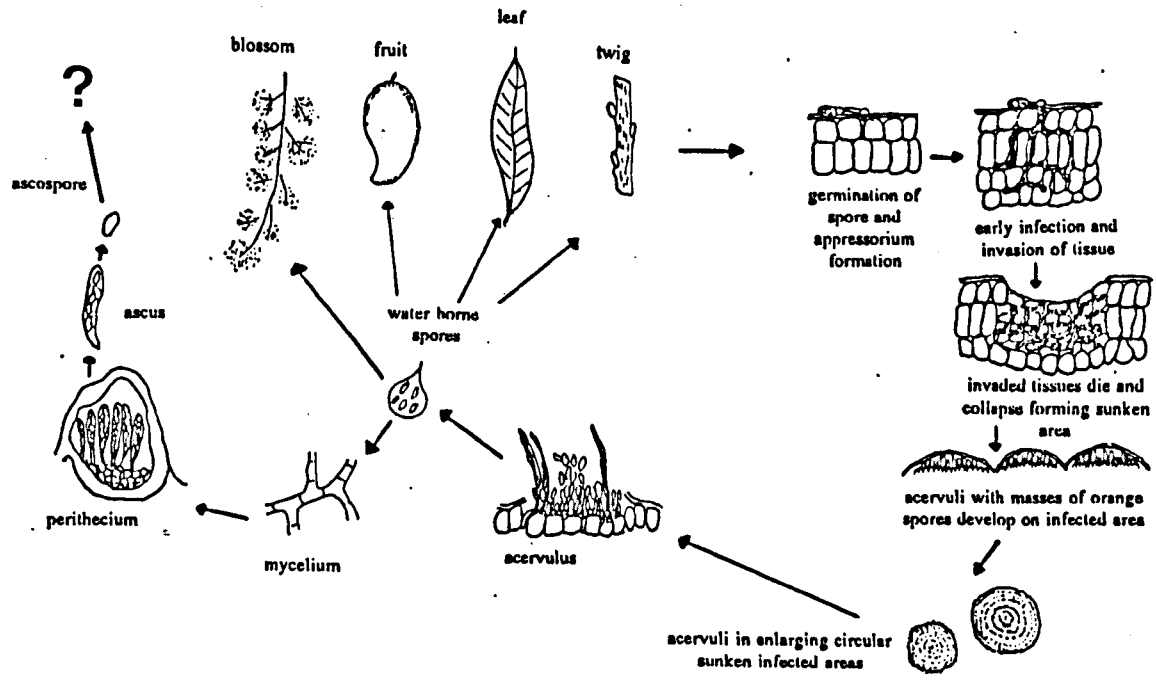


Figure 1.2 Disease cycle of *C. gloeosporioides* on mango (adapted from Agrios, 1988)

belong to the order of the Polystigmatales, this indicates that they may also behave similarly in relation to the function of the mucilage. When relative humidity stays high (>90%) over an extended period the spore germinates (Fitzell *et al.*, 1984) and forms an appressorium which becomes melanized. The timing of the following events is variable, depending whether or not direct penetration takes place, but the same sequence of events usually occurs. Firstly, an infection peg penetrates the cuticle and invasion of the epidermal cells takes place. Hyphae then spread intra-cellularly and necrosis and lesion development follows (Jeffries *et al.*, 1990). Lesions are initially superficial and do not affect lower lying tissues until very late in the infection. In many tropical countries mangoes with a few lesions are preferred by the local population. The lesions are only a cosmetic problem and indicate full ripeness of the fruit. A new generation of spores can be produced on the lesion within six days after the initial spore arriving on the surface, providing relative humidity remains high.

1.1.3 Quiescence - A quiescent or latent infection occurs when a fungus starts to germinate, initiates infection and then the infection halts for an undefined period after which infection is continued, i.e. it represents a dormant phase in the parasitic relationship (Verhoeff, 1974). This phenomenon takes place in

several genera, including *Alternaria alternata* (Prusky *et al.*, 1981), *Rhabdocline parkeri* (Stone, 1987, 1988), *Botrytis cinerea*, *Gloeosporium* spp. and *Colletotrichum* spp. (Verhoeff, 1974). Bacteria, such as *Erwinia* spp., *Acetobacter* spp. and *Enterobacter cloacae*, can also be the cause of diseases with a latent period (Rohrbach, 1989). In *C. gloeosporioides* on mango it is uncertain whether the appressorium lying on or embedded in the cuticle comprises the latent state or whether latency occurs after an infection peg has penetrated the cuticle. Simmonds (1963) reported that in the latent infection on mango subcuticular hyphae were present, however this was disputed by Daquioag & Quimio (1979) who found no evidence for this. Differences in infection patterns between *Colletotrichum* spp., start to appear once the appressorium has been formed and infection takes place. Extensive ultrastructural work has been carried out on *C. lindemuthianum* on *Phaseolus vulgaris* (Mercer *et al.*, 1975; O'Connell *et al.*, 1985) and *C. graminicola* on maize (Politis & Wheeler, 1973) and oats (Politis, 1976). These infections result in a direct penetration without a latent period (except in oat varieties which are resistant to *C. graminicola*). Direct infections similar to these take place in mango on young leaves, blossom and very young fruit, but infection of immature fruit results in a latent period. Generally it has now been accepted that subcuticular hyphae are formed, this being the mode in which *Colletotrichum* survives the latent period (Simmonds, 1963; Muirhead, 1981). On banana, however, two types of appressoria can be formed, hyaline and melanized. The hyaline appressoria immediately form an infection peg which makes limited subcuticular growth accompanied by a hypersensitive reaction by the host (Muirhead & Deverall, 1981). At first this was thought to be the latent infection (Chakravarty, 1957), but this type of infection never resulted in lesions (Muirhead & Deverall, 1981). The melanized appressoria, on the other hand, remain on the surface of the fruit and only form an infection peg after the fruit has ripened. This mode of infection does result in the formation of a lesion (Muirhead & Deverall, 1981). On immature avocado fruit, inoculated on the tree, it was observed that the germ tube of *C. gloeosporioides* enters the wax layer and forms a dark appressorium embedded within it (Binyamini & Schiffmann-Nadel, 1972a). The formation of infection pegs and penetration into the cuticle and epidermis only took place after the fruit had been harvested and started to soften. Similar observations were made for *Glomerella cingulata* and *C. capsici* on pepper (Adikaram *et al.*, 1983) and for *C. gloeosporioides* on citrus (Brown, 1975). In several studies of latent infections on fruit (*C. gloeosporioides* on papaya, Chau & Alvarez, 1983a; *C. musae* on banana, Chang *et al.*, 1987) it has been difficult to interpret results, since experiments were carried out on detached mature fruit. In mango, it was noted that

immature fruit, which are resistant to anthracnose while still on the tree, do show lesions within a few days if they are detached (Daquioag & Quimio, 1979). This implies that a factor responsible for inducing latency disappears when fruit is detached, resulting in lesion formation. Studies on detached fruit do therefore not represent the conditions *in situ* (Stanghellini & Aragaki, 1966).

The question how quiescence is induced still remains largely unanswered. Verhoeff (1974) suggested three possible reasons : 1 - toxic compounds are present in unripe, but not in ripening fruit; 2 - unripe and ripening fruit are different nutritionally; 3 - the enzyme potential of the fungus is insufficient to invade unripe fruit. Muirhead (1981) questioned how these factors could influence dormancy if the appressorium represents the latent phase, and considered whether the appressorium attempted penetration of the cuticle before becoming quiescent. This seems unlikely. An alternative suggestion was that germination inhibitors, either volatile or non-volatile, are released and cross the cuticle where they induced latency (Muirhead, 1981). Volatile compounds, produced by peach and plum, are known to inhibit spore germination and mycelial growth of *Monilinia fructicola* (Wilson *et al.*, 1987a). More recently Swinburne (1983) suggested a scheme similar to that of Verhoeff (1974), except that he divided the toxic compounds in pre-formed compounds and phytoalexins. The possible explanations were as follows : 1 - preformed toxic compounds that inhibit the pathogen are present in unripe but not in ripe fruit; 2 - phytoalexins are produced in unripe fruit; 3 - unripe fruit does not provide a suitable substrate to fulfil the nutritional and energy requirements of the pathogen; 4 - the enzyme potential of the fungus is inadequate to colonize unripe fruit. Examples of each of these possibilities are given by Jeffries *et al.* (1990). The presence or production of toxic compounds is most strongly supported by recent research. The nutrient theory seems to be unlikely since *Colletotrichum* spp. can grow *in vitro* on media made from unripe fruit (Simmonds, 1963). Enzymes (exo-pectin lyase and protease) are produced in the presence of, and are capable of degrading, unripe avocado cells (Sivanathan & Adikaram, 1989), suggesting that inadequate enzyme potential of the fungus is not the cause.

Quiescence is broken when the fruit ripens and might be triggered by changes in levels of ethylene (Brown, 1975; Biles *et al.*, 1990), nutrient content of the fruit or more likely a decrease in the amount of inhibitory compounds present in the peel (Muirhead, 1981).

1.1.4 The appressorium - The appressorium is an integral part of the infection process and is generally essential for infection (Chau & Alvarez, 1983a), even though it has been reported that direct penetration by hyphae of *C. gloeosporioides* on mango and avocado can occasionally take place (Daquioag & Quimio,

1979; Chau & Alvarez, 1983a). Appressoria can be formed without the need for a direct stimulus, although exogenous factors can also exert an effect (Parbery, 1981). The most important exogenous factor is the nutrient status and the presence of relatively high levels of nutrients results in saprophytic growth of the fungus, rather than in appressorium formation (see Emmett & Parbery, 1975). The reverse is observed when spores are germinated in the presence of bacteria, and an increase in the number of appressoria is found (Lenné & Parbery, 1976; Blakeman & Parbery, 1977). It is thought that bacteria compete with the fungus for nutrients and when nutrients are not sufficient for further germ tube elongation, an appressorium is formed (Parbery, 1981). Other factors, such as the texture of the surface that the germ tubes are in contact with, seem to play a minor role since most *Colletotrichum* spp., except *C. lindemuthianum*, are capable of forming appressoria in distilled water (dH_2O), both on glass slides and cellophane (Lenné, 1978). The events associated with appressorium formation are as follows: the germ tube swells and forms the so-called proto-appressorium (Emmett & Parbery, 1975); a septum is formed between the proto-appressorium and the germ tube; melanization of the cell wall of the appressorium occurs; and a germ pore is visible where the appressorium touches the surface (Griffiths & Campbell, 1973; Emmett & Parbery, 1975; Kozar & Netolitzky, 1978). The appressorium adheres firmly to the surface through a layer of mucilage, a hemicellulose, underneath the appressorium (Lapp & Skoropad, 1978). The function of melanin could be to protect the appressorium from desiccation, UV-radiation and lysis by other micro organisms (Lockwood, 1960; Potgieter & Alexander, 1966; Bell & Wheeler, 1986). This is a useful function as it has been shown that appressoria might have to survive the severe conditions as experienced on the plant surface for a period as long as 3 months (Binyamini & Schiffmann-Nadel, 1972a).

The melanized layer is also thought to be necessary for penetration (Kubo *et al.*, 1982 a, b; Rasmussen & Hanau, 1989), it gives the appressorium strength and directs penetration towards the germ pore (Kubo *et al.*, 1982a; Wolkow *et al.*, 1983). Howard & Ferari (1989) argued very strongly for a different function of the melanized wall, using observations of *Magnaporthe grisea*. They proposed that the appressorial wall was semipermeable, only allowing influx of water which resulted in an increased osmotic pressure generated by the strength of the melanin, and through sheer mechanical force the penetration peg is able to penetrate the cuticle. However *Colletotrichum* spp. produce cutinases (Baker & Bateman, 1978; Dickman *et al.*, 1982; Bonnen & Hammerschmidt, 1989) and cellulases (Suzuki *et al.*, 1981, 1982, 1983). Cutinase deficient mutants of *C. gloeosporioides* were unable to infect papayas (Dickman & Patil, 1986) and pesticides, mainly

organophosphates, which block cutinase activity, prevented infection by *C. gloeosporioides* (Dickman *et al.*, 1983). Mutants of *C. lagenarium* which lacked the production of P-95, a protein of 95 KDaltons which resembles cellulase, but had kept their pigmentation, failed to penetrate nitrocellulose membranes (Katoh *et al.*, 1988). Suzuki *et al.* (1983) suggested that P-95, which is capable of dissolving nitrocellulose membranes, supplies nutrients necessary for the formation of penetration hyphae rather than being required for penetration *per se*. Most results indicate however, that a combined action of enzymes and mechanical force is necessary to allow successful infection to take place (Staples & Hoch, 1987).

1.2 The host

1.2.1 Mango - Mango, *Mangifera indica* L., belongs to the family *Anacardiaceae*, together with the cashew and pistachio nut (Heywood, 1978). The centre of origin for the mango tree is probably the Indo-Burma region (Tjiptono *et al.*, 1984), but nowadays the mango is grown all over the tropical world. There are several hundred varieties (Samson, 1986) and every country prides itself in producing the best mango. There are in general two different types of mango, the yellow mangoes, which are drupe shaped and are produced in Asia and Australia, with well known varieties such as Nam Dok Mai (Thailand), Carabao (Philippines) and Kensington Pride (Australia). The other type is also known as the apple mango, green-red in colour and mainly produced in the Americas with as best known varieties Tommy Atkins and Haden. Mangoes are best known as a sweet dessert fruit, but in many asian countries green mangoes are eaten raw, used in spicy dishes and made into mango pickle (see Appendix 4). With the increase in world travel and communications, demand for exotic produce has increased in Europe, North America and Japan, and for many tropical countries the export of tropical fruit is a good source of hard currency. Until recently mangoes were grown in many countries as a backyard crop, but much effort has been put into the promotion of orchard establishment to produce export quality mangoes (Tjiptono *et al.*, 1984; Anon., 1988) and increased attention has been given to post-harvest care (Prinsley, 1987). The total world production of mangoes was estimated at 15 million tonnes in 1989 (FAO yearbook for 1989, 1990) and production ranks fourth among tropical fruit after citrus, banana and plantain. The major producer of mangoes is India which produces over 60% of the total, followed by Pakistan, Mexico, Brazil, Indonesia and the Philippines.

1.2.2 Post-harvest problems - Post-harvest losses are much larger in the developing countries than they are in the developed countries (Harvey, 1978), due to lack of resources (Salunkhe & Desai, 1984). For all fruit and vegetables post-harvest losses in tropical countries are estimated at 30% (Harvey, 1978; Salunkhe & Desai, 1984; Eckert & Ogawa, 1985). Post-harvest losses of mango due to anthracnose have been estimated at 25% in Mexico (Noon, 1984), 40% in the Philippines (Pordesimo *et al.*, 1984) and over 65% of 717 shipments arriving between 1972 and 1985 to the New York market were affected by anthracnose (Cappellini *et al.*, 1988). Once the mango has been harvested it becomes a high-value commodity, but still only a small percentage of mangoes is suitable for the export market (30% of export graded mangoes were actually suitable for export from Thailand; Anon., 1988), the remainder goes to the local market for which a lower quality is acceptable and storage time is shorter. For export, mangoes have to be transported from the farm, often first to a collecting point, and onwards to a central packing house where they are given post-harvest treatments and sent to their destinations (Torres *et al.*, 1984). For the Philippines all exports are organised from Manila and even mangoes produced in Cebu (see map, Figure 5.1) first have to be transported to Manila. Especially at the first stages of transport, cooled containers are often not available. The recommendation for mangoes is storage at 10-12°C for up to 4 weeks to slow down ripening (Salunkhe & Desai, 1984), which commences again as soon as the fruit are returned to ambient temperatures. Storage at temperatures which are too cold or keeping mangoes too long a period at 10°C can result in the fruit suffering from chilling injury (Pordesimo *et al.*, 1984; Bagshaw, 1989). As well as being treated for fungal diseases (see 1.2.3), mangoes in the Philippines intended for the market in Japan are hot vapour treated (HVT) to eradicate fruit flies. During HVT the fruit are heated to a pulp temperature of 46°C for 10 min, and in 1989 this resulted in great losses due to breakdown of the fruit pulp (C. Lizada, pers. comm.). The last steps of packing and transport could result in physical damage of the fruit if no consideration is allowed for the delicate consistency of the fruit, and the heat produced during ripening (Snowdon, 1990).

The major cause of post-harvest losses are, nevertheless, fungal diseases of which anthracnose, on a global scale, is the most important, with stem end rot caused by the *Lasiodiplodia/Dothiorella* complex as the next most important. Minor diseases are caused by *Aspergillus niger*, *Rhizopus stolonifer* and *Alternaria alternata* (Cook, 1975; Pordesimo *et al.*, 1984; Bagshaw, 1989; Snowdon, 1990). Bacterial blackspot caused by *Xanthomonas campestris* pv. *magniferaeindicae*, is also a major problem in some mango growing areas

(Snowdon, 1990; Pruvost *et al.*, 1990).

1.2.3 Control of anthracnose disease - Strategies for post-harvest control of fruit diseases include : 1 - inoculum reduction and prevention of field infections; 2 - suppression of disease development and spread; 3 - inactivation of wound infections (Eckert & Ogawa, 1985). This latter strategy does not apply for anthracnose, since *C. gloeosporioides* does not require wounds to be able to cause disease. The first strategy emphasises the fact that pre-harvest control measures are necessary to reduce post-harvest losses (Conway, 1984). For export mangoes both pre- and post-harvest control measures are carried out. Pre-harvest fungicides sprays are applied to protect the blossom and young fruit from immediate damage, and the maturing fruit from latent infections. The number of sprays can vary between six sprays per season as advised in the Philippines (Pordesimo, 1983), carried out mainly to protect the blossom and young fruit, and up to 25 sprays per season as applied in some orchards in Florida (Mitchell, in Thompson, 1987) whereby the maturing fruit also are protected. Fungicides which have been used are chlorothalonil, copper solutions, mancozeb, captafol, benomyl and prochloraz (McMillan, 1973, 1984; Pordesimo *et al.*, 1984; Thompson, 1987; Dodd *et al.*, 1989). Even with regular applications of fungicides before harvest, a post-harvest treatment is also necessary to be sure that adequate anthracnose control is achieved. In designing a rational control programme, it would be advantageous to be able to determine to what extent latent infections are present on the fruit, since in years where there is little rainfall, infection levels might be too low to warrant the use of a post-harvest treatment. Such a system was developed in Israel to determine the amount of latent infections caused by *Alternaria alternata*, present on mangoes (Prusky *et al.*, 1981, 1983). Fruit discs were plated out on agar and presence of *A. alternata* was recorded. *A. alternata* causes the major post-harvest disease on mangoes in Israel. When this methodology was adapted for use with *C. gloeosporioides* however, no satisfactory results were obtained to enable realistic estimates of infection to be determined (Jeffries *et al.*, 1990).

The most commonly used post-harvest treatment for anthracnose control is a hot water treatment (HWT) of 50 - 55°C for 5 to 10 min (Smoot & Segall, 1963; Quimio & Quimio, 1974) with the optional addition of benomyl at a rate of 500 - 1000ppm (Spalding & Reeder, 1972; Muirhead, 1976). Thompson (1987) has summarised research carried out on various post-harvest dips, with different dipping times, temperatures and fungicides. The HWT has been successfully carried out in many countries, although recently in Australia it has been advised to treat mangoes first in hot-benomyl, followed by a cold prochloraz dip (Bagshaw,

1989; Johnson *et al.*, 1990a). The hot-benomyl treatment alone was not sufficient in eradicating anthracnose. Care has to be taken with the HWT that the temperature of the water is not too high nor immersion is too long otherwise scalding of the fruit takes place (Bagshaw, 1989). Other adverse effects can be that soaking in water results in the lenticels becoming more prominent (Bagshaw, 1989) and some varieties, such as Carabao, loose their lustre after HWT (Quimio & Quimio, 1974). In general, ripening is accelerated by the HWT (Muirhead, 1976), but the reduction of post-harvest moulds developing compensates for this. A further problem, which arises from benomyl being used both pre- and post-harvest, especially where over 20 sprays are employed pre-harvest, is that the fungus becomes insensitive to the fungicide (Jeger & Plumbley, 1988). Resistant isolates have been found, first in Florida (Spalding, 1982), but have been reported from other areas as well (Jeger & Jeffries, 1988). Resistance against benomyl had been earlier reported for *C. musae* (Griffee, 1973).

Alternative post-harvest treatments are gamma-irradiation (Spalding & Reeder, 1986), but Johnson *et al.* (1990b) found that irradiation alone was not satisfactory in controlling anthracnose and fruit still needed to be treated with a fungicide. It was also shown that a dose of over 600 Gy resulted in unacceptable surface damage of the fruit (Spalding & Reeder, 1986; Johnson *et al.*, 1990b). Waxing of fruit is another alternative to reduce post-harvest moulds developing, and has been carried out on citrus fruit (Waks *et al.*, 1985), but waxing of mangoes can result in the development of off-flavours (Snowdon, 1990). Methods which have not been tried out commercially yet are washing mangoes with sodium hypochlorite (Pordesimo, 1984), controlled atmosphere storage with low oxygen and high carbon dioxide levels, which reduced anthracnose development in avocadoes (Spalding & Reeder, 1975) and low pressure storage (15mm Hg) which reduced anthracnose in papayas (Chau & Alvarez, 1983b).

A phenomenon that takes place with some *Colletotrichum* spp. and their hosts e.g. *C. lagenarium* on cucumbers and *C. lindemuthianum* on beans, is that induced resistance occurs. After initial contact with the pathogen, the hosts defence system responds more rapidly to subsequent infections, resulting in a reduction of disease severity and a delay in symptom development (see Dean & Kuć, 1987). This is, however, not a practical approach to controlling anthracnose disease in mangoes.

1.3 Biological control

1.3.1 Why biological control? - There are several reasons why the search for biological control agents has been very intense in the last years. The two most important ones are (i) the occurrence of fungicide resistant strains of pathogens and (ii) concern for the environmental implications of pesticide usage ('green' motives) and both of these will be discussed here.

As discussed before (1.2.3), benomyl has, in some areas, been extensively used for both pre- and post-harvest control, resulting in resistance occurring in populations of *C. gloeosporioides* (Spalding, 1982). Resistance to fungicides has especially been a problem with the more recently introduced, systemic fungicides. The systemic fungicides often only have one specific site of action as opposed to older protectant fungicides such as copper, which have multi-site activity (Dickinson & Lucas, 1982). Many genera have become resistant to systemic fungicides, including the fungicides belonging to the benzidamazoles (Delp, 1980). Strains resistant to benomyl do not appear to lose their 'fitness', as sometimes happens in strains resistant to other fungicides. Reversion of resistant strains back to sensitive strains also takes place naturally at a relatively high rate, reducing the resistance problem to some extent (Delp, 1980). Benomyl is a fungicide that interferes with microtubulin assembly, necessary for nuclear and cellular division, cell migration, organelle movement and the structure of the cell (Davidse, 1986). Insensitivity to the fungicide appears to be due to a decrease in binding affinity (Davidse, 1982). With the increased restrictions on the use of fungicides in developed countries, alternative fungicides are difficult to find. The widely used dithiocarbamate compounds maneb, mancozeb and zineb have been severely restricted in their use in the U.S.A. since it was found that after nearly forty years of use, they might be highly carcinogenic (Erlichman, 1989). They are, however, still widely used elsewhere. Since 1989 benomyl is no longer allowed for use as a post-harvest treatment in the U.S.A. (Sanchez, 1990). Post-harvest fungicides represent a relatively small market and chemical industries put little effort into the development of new products which can specifically be used post-harvest (Jeger & Jeffries, 1988). If new fungicides are developed, the risk of ineffectiveness developing is increased if the compound is used for both pre- and post-harvest application. One way of avoiding resistance occurring in pathogen populations is to use a rotation of fungicides (Eckert, 1982). In Florida however, only benomyl and copper are allowed for use on mangoes. Too many benomyl resistant strains are present in the orchards for this fungicide to be effective, and copper does not give satisfactory

control against anthracnose. In the 1990 season one of the major mango producers in Florida had to obtain an emergency licence, granted for only one year, to allow the use of isoprothiolane (K. Mitchell, pers. comm.). Alternative control methods are badly needed in this situation.

From a consumers point of view, pesticide-free products are highly desirable, as even those pesticides which are thought to be safe for use on food products, may in future be proven to have harmful effects, as was shown for the dithiocarbamates. The increased sale of organic produce, even in the bigger supermarkets, shows that there is a demand for pesticide-free crops. Until now, however, prices have been prohibitive for the public on a large scale. Changes have to come, and there is scope for the farming community as a whole, to reduce pesticide inputs where possible and to use integrated pest management programmes, not only to protect our health but also the environment.

In the developing countries the situation is slightly different. Whereas the developed world has been overproducing food, many developing countries struggle to produce enough food to satisfy their market. The green revolution has helped many countries to cope, but new cultivars of rice for instance, need a much greater input of fertilizers and pesticides than the traditionally grown cultivars (Khor, 1987). Poorer countries have also been used as an alternative market for pesticides not longer allowed for use in the richer countries, DDT is the best known example of this (Campbell, 1985; Crick, 1990). The use and application of pesticides is often not as strictly controlled in developing countries (Bello, 1982). The problems associated with this are that farmers do not obtain proper education or instructions of the risks involved. This leads to the application of pesticides in doses that are too high and safety precautions are ignored. In the Philippines it is quite normal to see a bare-handed labourer spraying mango trees with fungicides and insecticides with only a handkerchief in front of the mouth. Inadequate spraying equipment can lead to very large amounts applied per tree and wind-blown contamination may pollute the surroundings. The awareness of the risks involved in developing countries is, however, on the increase. A recent cover article in 'India Today' pointed out that India has the highest amounts of pesticide residues in food on a world scale, and the authors stressed that if no action was going to be taken soon, many people would suffer the effects of increased uptake of residues (Chengappa & Rajchatta, 1989). The government must be willing, however, to promote and put resources into the development of integrated pest management schemes, and to educate the farmers involved.

In reality it will not be possible to live in a world without pesticides, but an extended effort into the development of integrated pest management schemes should reduce the amounts of pesticides used.

Examples of research carried out at the Kinsleay Research Centre, Dublin (Ryan *et al.*, 1986) and the development and promotion of integrated control of *Phytophthora* rot of avocado (Coffey, 1987) show that it is possible in practice.

1.3.2 Pre-harvest biological control - Most biological control efforts have been put into the control of soil-borne diseases. The main reason for this is that effective fungicides have long been available for many foliar diseases, but for soil-borne diseases application of these is very difficult, and alternatives have been sought (Campbell, 1986). A discussion of these is outside the scope of this introduction and many books have been published on the subject (see Baker & Cook, 1974; Cook & Baker, 1983; Wood & Way, 1988; Campbell, 1989; Hornby, 1990). Interest in biological control of phylloplane fungi has a much shorter history, but recent interest in phylloplane microbiology has led to a series of symposia on this subject (see Preece & Dickinson, 1971; Dickinson & Preece, 1976; Blakeman, 1981; Fokkema & Van den Heuvel, 1986). There has been very little success in the development and commercialisation of biological control products for plant diseases, but this must be looked at in the context of the development of a novel fungicide which takes 10 - 15 years from the initiation of research to the legislation of a product. One commercially available product is Trichodermin, a *Trichoderma* sp. active against *Botrytis cinerea* on strawberries, developed in the Soviet Union and Hungary (in Elad, 1990). Early efforts in the search for biological control agents effective against several field diseases (cucumber anthracnose, early blight of tomatoes and northern leaf blight of corn) revealed problems which are still relevant today for the field application of potential antagonists. After a careful screening procedure carried out in the greenhouse (Leben, 1964; Leben & Daft, 1965), organism A180 (later identified as *Pseudomonas cepacia*, in Leben, 1985) was selected for extensive field trials. However, no disease control was observed in the field (Leben *et al.*, 1965) and the experiments were referred to later as the largest field failure observed in biological control (Leben, 1985). Reasons for the failure were that selection of potential antagonists was done from seedling plants which probably harbour microorganisms adapted to different environmental conditions than those found on mature plants (Leben, 1985) and only 1% of antagonists applied could be recovered after 24hrs of inoculation (Leben *et al.*, 1965). This work indicated two major problems, how to select a biological control agent and once selection has taken place, how can the organism be successfully established on the phylloplane?

There have been many examples of microorganisms showing biocontrol potential against a range of fungal phylloplane diseases. These include : *Chaetomium globosum* against apple scab, *Venturia inaequalis*

(Andrews *et al.*, 1983; Cullen & Andrews, 1984a; Cullen *et al.*, 1984; Boudreau & Andrews, 1987); a mixture of *Bacillus cereus*, *Bacillus mycoides* and *Bacillus* sp. against needle rust, *Melampsora medusa* (McBride, 1969); *Exophilia jeanselmei* against blight of roses, *Botrytis cinerea* (Redmond *et al.*, 1987); *Trichoderma viride*, *Alternaria alternata* and *Epicoccum purpurascens* against lettuce drop, *Sclerotinia sclerotiorum* (Mercier & Reeleder, 1987); *Ampelomyces quisqualis* against several genera of powdery mildews (Sztejnberg *et al.*, 1989); *Epicoccum purpurascens* against white mould, *Sclerotinia sclerotiorum* (Zhou & Reeleder, 1989); *Trichoderma viride* against *Botrytis cinerea* on grapes (in Papavizas & Lewis, 1988). The majority of the examples mentioned have not been developed beyond greenhouse testing for which conditions are favourable towards the establishment of the introduced antagonist. The control of apple scab with *Chaetomium globosum* was extensively tested before field trials were set up, but no satisfactory control was obtained because establishment was poor and the active compound, an antibiotic, was rapidly broken down on the leaf surface (Boudreau & Andrews, 1987). This shows once again that the phylloplane is a harsh environment and microorganisms have to be well adapted to survive the large fluctuations in temperature and relative humidity and, at times, high levels of U.V.radiation (Dickinson, 1986). There is also the need for an increased knowledge concerning natural phylloplane populations of microorganisms, (very little has been published for tropical crops) and how these populations respond to the introduction of another microorganism. Very useful information on this latter aspect has been provided by Kinkel *et al.* (1989a, b) showing that communities on leaves are formed from the sum of immigration, emigration and death, and to a lesser extent growth. This indicates that natural immigration does take place, thus introduced microorganisms should also be able to establish themselves on the leaf. There is, however, a natural limit to community size (Kinkel *et al.*, 1989b) which might limit the establishment of an introduced organism. The competitive ability of a potential antagonist needs to be assessed. Potential antagonists are often tested on greenhouse plants which have a much lower population levels of indigenous microorganisms than field grown plants (Blakeman & Brodie, 1977). The idea of introducing a single organism to effectively control disease is perhaps short-sighted (Spurr & Knudsen, 1985) and the introduction of mixed communities might offer a more viable proposal (Janisiewicz, 1988a).

One approach which can avoid the problems associated with the introduction of new microorganisms, is the manipulation of the environment with nutrients to encourage indigenous competition. Nutrients increase the number of saprophytic microorganisms present thus increasing the biological control potential of the

phylloplane community as a whole (Cullen & Andrews, 1984b; Jeger & Jeffries, 1988). An example of this is the application of a triple pesticide - foliar fertilizer mixture applied to mango trees in Malaysia (Lim & Khor, 1982). The spray changed the composition of the natural microflora present both qualitatively and quantitatively, due to the nutrients present in the mix and resulted in a reduction of damage caused by *C. gloeosporioides* and *Pestalotiopsis versicolor* present.

That biological control can work on the phylloplane is shown by the fact that the application of fungicides, in some instances, can result in an increase of disease, the so-called iatrogenic plant diseases (Griffiths, 1981). The best documented example of this occurs in coffee berry disease caused by *C. coffeanum* (as reviewed by Griffiths, 1981). Farmers in East Africa apply copper fungicides to coffee to increase yields up to 100% (the 'tonic effect' due to a decrease in leaf shed of sprayed plants). However, more coffee berry disease occurred on sprayed plants compared to unsprayed plants even though more inoculum was present in the bark of the latter. It seems most likely that the application of fungicides changed the natural balance of microorganisms which, if left undisturbed, was able to suppress outbreaks of coffee berry disease. At present research is under way to establish which components of the indigenous microflora are responsible for controlling coffee berry disease under natural conditions (Masabah & Waller, pers. comm.).

1.3.3 Post-harvest biological control - There are several advantages in applying a biological control agent after harvest compared with pre-harvest application. These are : 1 - once the fruit or vegetables have been harvested they become a high value commodity; 2 - the harvested crop represents a relatively small surface area to which to apply the biological control agent; 3 - the storage conditions can be regulated to suit the antagonist (Wilson & Pusey, 1985; Wilson, 1989; Wilson & Wisniewski, 1989; Jeffries & Jeger, 1990).

A list of potential post-harvest biological control agents is given in Table 1.1. Three of the examples, control of *Botrytis cinerea* on apples (Tronsmo & Raa, 1977; Tronsmo & Ystaas, 1980) and strawberries (Tronsmo & Dennis, 1977), control of *Monilinia laxa* on peaches (Melgarejo *et al.*, 1986) and control of *Penicillium funiculosum* on pineapple (Lim & Rohrbach, 1980) represent examples of pre-harvest field control measures taken to reduce post-harvest storage diseases. It is interesting to note that for all three examples use was made of filamentous fungi as biological control agents, while in all the examples in which biological control agents are applied post-harvest use was made of either bacteria or yeasts, which seem to be more suited to the post-harvest environment.

Table 1.1 Studies carried out on biological control of post-harvest pathogens of fruits. Studies are arranged according to antagonists used.

fruit	pathogen	antagonist	trials	authors
Bacteria				
citrus	*Alternaria citri* *Geotrichum candidum* *Penicillium digitatum*	*Bacillus subtilis*	laboratory	Vapinder Singh & Deverall, 1984
peach (+ nectarin, apricots and plums)	*Monilinia fructicola*	*Bacillus subtilis* B-3	packing house	Pusey & Wilson, 1984 Pusey *et al.*, 1986 Pusey *et al.*, 1988
cherry	*Monilinia fructicola* *Alternaria alternata*	*Bacillus subtilis* *Enterobacter aerogenes*	laboratory	Utkhede & Sholberg, 1986
peach	*Rhizopus stolonifer*	*Enterobacter cloacae*	laboratory	Wilson *et al.*, 1987 Wisniewski *et al.*, 1989
apple	*Penicillium expansum*	*Pseudomonas cepacia*	laboratory	Janisiewicz & Roitman, 1988
apple, pear	*Penicillium expansum* *Botrytis cinerea*	*Pseudomonas syringae* p.v. *lachrymans* *Acremonium breve*	laboratory	Janisiewicz 1987, 1988a
Yeasts				
citrus	*Penicillium digitatum* *Penicillium italicum* *Geotrichum candidum*	*Candida guilliermondii*	laboratory	Wilson & Chalutz, 1989 Droby *et al.*, 1989 Chalutz & Wilson, 1990
apple	*Botrytis cinerea*	*Candida guilliermondii*	laboratory	McLaughlin *et al.*, 1990
apple	*Botrytis cinerea*	*Candida guilliermondii*	laboratory	McLaughlin *et al.*, 1990
apple	*Botrytis cinerea*	*Cryptococcus laurentii*	laboratory	Roberts, 1990
Filamentous fungi				
peach	*Monilinia laxa*	*Penicillium frequentas*	field	Melgarejo *et al.*, 1986, 1989 DeCal *et al.*, 1988
pineapple	*Penicillium funiculosum*	*Penicillium funiculosum*	field	Lim & Rohrbach, 198
strawberry	*Botrytis cinerea*	*Trichoderma* sp.	field	Tronsmo & Dennis, 1977, 1978 Tronsmo & Raa, 1977 Tronsmo & Ystaas, 1980

Most research into post-harvest biological control is still in the experimental state since it is a relatively new technology. Compared to field applications it is relatively easy to simulate post-harvest conditions in the laboratory, and results will often indicate if large scale applications are feasible (Wilson & Wisniewski, 1989). To date only the application of *Bacillus subtilis* B-3 on peaches to control brown rot has been tested in a commercial packing house (Pusey *et al.*, 1988). Results were disappointing because disease levels were very low and residues of fungicides present in the commercial dipping tank obscured results. *B. subtilis* B-3 can be successfully incorporated into fruit wax and combined with the fungicide dichloran, which is necessary to control *Rhizopus stolonifer* against which B-3 is not active (Pusey *et al.*, 1986). The development of a post-harvest biological control agent active against *Rhizopus stolonifer* is now under way, and *Enterobacter cloacae* seems to be a good candidate (Wilson *et al.*, 1987b; Wisniewski *et al.*, 1989). Another important aspect is the potential of the biological control agent to remain active during cold storage (Wilson & Wisniewski, 1989), since most fruit will be kept at low temperatures for some period of time. Peaches treated with *B. subtilis* B-3 could be kept in cold storage (2 - 4°C) for up to three weeks and the bacterium still reduced infection when subsequently challenged by *M. fructicola* and kept at room temperature (Pusey *et al.*, 1986). *Candida guilliermondii* US-7 (identified previously as *Debaryomyces hansenii*) protected grapefruit against *Penicillium digitatum* for up to three weeks at 11°C (Chalutz & Wilson, 1990).

All the examples of post-harvest application of biological control agents are against wound pathogens. Wounding takes place during handling and infection occurs often through spores present in the dipping tanks (Spotts & Cervantes, 1986). A potential antagonist must be able to occupy these wounds and multiply within these wounds. That multiplication in wound sites does take place has been shown for *C. guilliermondii* US-7 which exhibited a 100-fold increase of numbers present in wounds on grapefruit (Droby *et al.*, 1989) and for *Cryptococcus laurentii* multiplication in wounds on apple occurred at 5°C (Roberts, 1990). Another feature that would be desirable would be that of wound healing (Janisiewicz, 1988b).

Latent infections are intrinsically different in that the propagule to be combatted is already present on the fruit, and no attempts in the control of latent infections through post-harvest biological control have been reported.

Once a biological control agent has been proven to be effective, technology present in the packing house is easily adapted for using biological control agents. In the U.S.A. *Candida guilliermondii* US-7 has been

patented and several other patents for post-harvest biological control agents are still pending (Sanchez, 1990).

1.3.4 Biological control of *C. gloeosporioides* - No real effort has been carried out into the development of a biological control system for *Colletotrichum*, and most observations have been made accidentally, or to observe a specific phenomenon. However, these observations provide us with useful information about interactions between *Colletotrichum* and other microorganisms. From the examples available (Table 1.2) several trends are visible. The most frequently made observation is that bacteria stimulate spore germination and appressorium formation (Lenné & Parbery, 1976; Blakeman & Parbery, 1977). This has been explained through nutrient competition taking place between *Colletotrichum* and the bacteria (Lenné & Parbery, 1976; Blakeman & Brodie, 1977), which results in adverse conditions for the fungus which responds by forming an appressorium, a survival structure (Emmett & Parbery, 1975). The other example given for this phenomenon is that iron blocks essential sites in the spore and needs to be removed prior to germination (Harper *et al.*, 1980; Graham & Harper, 1983). Removal takes place via iron chelating compounds present as anthranillic acid in host leachates (Swinburne, 1976; Harper & Swinburne, 1979) or siderophores produced by bacteria (McCracken & Swinburne, 1979, 1980; Slade *et al.*, 1986).

Other explanations for a decrease in mycelial growth observed *in vitro* and a reduction in disease development in greenhouse experiments are that antibiotics active against *Colletotrichum* are produced (Ettig, 1955, 1958; Leben, 1964; Leben & Daft, 1965), but no identification of these compounds has been made to date. *Streptomyces* spp. appear to work through this mode of action (Lockwood, 1959; Sharma & Gupta, 1982), although competition for nutrients also takes place (Hsu & Lockwood, 1969). It seems very likely that the *Streptomyces* spp. produce lytic enzymes as well, as do the unidentified bacteria (later referred to as *Bacillus* spp. by Blakeman & Parbery, 1977) described by Lenné & Parbery (1976). Williamson & Fokkema (1985) reported an interesting study of the interactions between two phylloplane yeasts, *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavescens*, and *C. graminicola*. Spore germination and appressorium formation of *C. graminicola* were not affected by the presence of the yeasts, but there was a reduction of 50% in the number of appressoria which formed infection pegs, resulting in a reduction of lesions developing on barley leaves. The proposed mode of action was that the yeasts competed with the appressoria for nutrients, and when insufficient nutrients were present, no infection peg

Table 1.2 Interactions between *Colletotrichum* spp. and microorganisms. The studies are arranged in chronological order.

species	antagonist	effect	proposed mode of action	authors
C. atramentarium	soil bacteria (*Bacillus subtilis*?)	*In vitro* inhibition of mycelial growth	antibiotics	Ettig, 1955, 1958
G. cingulata	*Streptomyces* spp.	inhibition of germination and lysing of hyphae	antibiotics, lytic enzymes	Lockwood, 1959
C. lagenarium	bacterium A180 *Pseudomonas cepacia*	reduction of anthracnose in green house trials, no field control	antibiotics	Leben, 1964 Leben & Daft, 1965 Leben *et al.*, 1965
G. cingulata	*Streptomyces* spp.	inhibition of mycelial growth and spore germination	antibiotics nutrient competition	Hsu & Lockwood, 1969
C. gloeosporioides	*Bacillus* sp.	stimulation of appressorium formation	not given	Lenné & Parbery, 1976
C. musae	leachates *Pseudomonas* UV3	stimulation of spore germination and appressorium formation	iron chelating compounds remove Fe from within the spore	Swinburne, 1976 Harper & Swinburne, 1979 McCracken & Swinburne, 1979, 1980 Harper *et al.*, 1980 Graham & Harper, 1983
C. acutatum	*Pseudomonas* UV3 & 14	stimulation of appressorium formation	competition for nutrients	Blakeman & Parbery, 1977
C. dematium f.sp. *spinaciae*	*Pseudomonas* 14	< germ tube length stimulation of appressorium formation	competition for amino acids	Blakeman & Brodie, 1977
C. gloeosporioides *C. falcatum*	*Streptomyces rochei*	reduction of anthracnose in green house	active filtrate	Sharma & Gupta, 1982
C. gloeosporioides	*Trichoderma viride* *Aspergillus flavus*	inhibition of mycelial growth	not given —	Khetmalas *et al.*, 1984 —
C. graminicola	*Sporobolomyces roseus* *Cryptococcus laurentii* var *flavescens*	reduced number of lesions	nutrient competition with appressorium	Williamson & Fokkema, 1985
C. acutatum	*Pseudomonas* UV3	stimulation of spore germination and appressorium formation	iron chelating siderophore —	Slade *et al.*, 1986 —
C. gloeosporioides	phylloplane bacteria from *Stylosanthes*	reduction of anthracnose	not given	Lenné, 1986
C. lindemuthianum	*Bacillus subtilis* *Enterobacter aerogens*	inhibition of mycelial growth	antifungal compound	Utkhede & Sholberg, 1986

was formed.

It is incidentally of interest that one *Colletotrichum* sp. has itself been used as a biological control agent. *C. gloeosporioides* f.sp. *aeschynomene* is used as a biological control agent ('Collego') to eradicate the weed northern jointvetch, which is a menace in rice and soybean fields in North America (see Charudattan, 1988). However, some doubts are raised about its use since *C. gloeosporioides* f.sp. *aeschynomene* is not as specific as first thought. The latency period of the fungus might cause problems to soybean (Cerkauskas, 1988) and the heterogeneity of the genus might make the population adapt and attack different hosts.

1.4 Objectives

The main aim of this research was to assess the potential for using a biological control agent against anthracnose on mangoes. To do this it was necessary to :

1 - Obtain a better understanding of the ecology and infection process of *C. gloeosporioides* on mango (Chapter 2).

2 - Isolate and test the natural microflora present on mangoes for their potential as biological agents of *C. gloeosporioides* (Chapter 3).

3 - Test potential antagonists in a post-harvest situation (Chapter 4).

4 - Test potential antagonists in a pre-harvest situation (Chapter 5).

5 - Determine the mode of action of the selected antagonist in order to improve the system (Chapter 6).

Part of the experiments described in Chapters 4 and 5 were carried out at the Post Harvest Training and Research Center (PHTRC), University of the Philippines, Los Baños, Philippines.

2 *C. gloeosporioides* isolates and spore germination studies

2.1 Objectives

The taxonomic position of *C. gloeosporioides* is not clear, and since von Arx (1957) reorganised the genus, several attempts have been made to clarify this. Both cultural and infection studies, along with isozyme patterns and biochemical tests have been used (Lenné, 1978; Cox, 1986; Fortune, 1987) but matters are still confused. Simmonds (1965) split *C. gloeosporioides* into two varieties, var. *minor* and var. *gloeosporioides* which are separated by the size of the ascospores. According to Simmonds (1965) var. *minor* is the causal agent of anthracnose in Australia. It has also been reported that another species, *C. acutatum* can cause anthracnose in mangoes (Fitzell, 1979). This fungus becomes dominant when *C. gloeosporioides* is eliminated by benomyl to which *C. acutatum* is not as sensitive as *C. gloeosporioides*. For this study isolates from different geographical areas were obtained, identified and their virulence was determined.

Much has been published on spore germination and infection processes in the genus *Colletotrichum*, but not specifically on the combination of *C. gloeosporioides* and mango (see Chapter 1). It was thus thought necessary, in order to devise an effective biological control system, to determine the events taking place during spore germination and infection, i.e. is an infection peg present during the latent stage?

The objectives of this chapter were :

1 - To place the *C. gloeosporioides* isolates within recognized taxonomic groups.

2 - To determine the events taking place during spore germination and infection by *C. gloeosporioides* on mango, to aid the development of a biological control system.

2.2 Materials and methods

All laboratory experiments described in this thesis were carried out at 25°C and with *C. gloeosporioides* isolate 24, unless mentioned otherwise. A list of media is given in Appendix 1.

Differences between treatments ($P<0.01$) were determined by analysis of variance using Genstat (Release 5; Payne *et al.*, 1987). On percentage data an angular transformation was performed before the data were subjected to analysis of variance.

2.2.1 *C. gloeosporioides* isolates - Isolates were obtained from lesions on diseased mangoes imported into the U.K. (a list of isolates is given in Appendix 2). Cultures were maintained on malt extract agar (MEA), and transferred approximately every three months. After 6 months cultures were transferred for a period of three months onto mango agar with the idea to retain their pathogenicity. Mango agar was prepared by depitting Kenyan pickling mangoes, liquidising the fruit flesh and peel, to which 15 g. of agar was added and the pH adjusted to 4 after which the whole mixture was autoclaved. For long term storage cultures were put onto MEA slopes and stored at 4°C under sterile paraffin oil. This method of storage turned out to be unsatisfactory and many cultures were lost. Other methods of long term storage were better, such as storage in liquid nitrogen or on silica at room temperature. For experiments, cultures were grown at 25°C in the dark, or, when spores were required, cultures were grown under a near-UV (365.5nm; 'black') light. For all growth experiments with *C. gloeosporioides* the initial inoculum consisted of an agar plug of mycelium, 0.5 cm diam., taken from the actively growing edge of a colony.

To determine any differences between isolates, growth on MEA (one plate only), sporulation and colony colour were recorded and compared.

2.2.2 Taxonomy of isolates - Two assays, which had highlighted differences between *C. gloeosporioides* var. *gloeosporioides* and var. *minor*, and *C. acutatum* in the study by Fortune (1987), were performed on all isolates. The first was to determine the number of nuclei in the spores and for this the Giemsa stain was used (see Johnston & Booth, 1983). Spores were air dried onto microscope slides and fixed in 3:1, ethanol : glacial acetic acid, for 10 min after which slides were rinsed once in 95% ethanol followed by one rinse in 70% ethanol and one rinse in dH_2O. Slides were immersed in 1M HCl at room temperature for 5 min, followed by 7 min in 1M HCl at 60°C. This was followed by 5 rinses in dH_2O and 5 rinses in phosphate buffer (pH 6.9; Dhingra & Sinclair, 1985). Slides were immersed in the Giemsa stain (1ml stain in 15ml of phosphate buffer; Giemsa improved R66, Gurr/BDH) for 2 hrs. after which slides were rinsed as before in phosphate buffer and dH_2O.

The second assay was radial growth on casein medium (see Appendix 1).

2.2.3 Virulence of isolates - Spore suspensions of *C. gloeosporioides* isolates were prepared by suspending spores from a 7 to 14 day old sporulating culture on MEA, in dH_2O with 0.01% Tween 80. Before the virulence trials were carried out, it was thought necessary to determine if spore concentration affected lesion

development. For this, three spore concentrations were tested, 10^4, $5x10^4$ and 10^5 spores/ml, which is equivalent to 100, 500 and 1000 spores/ 10μl drop. Daily observations revealed that both the time in which a lesion became visible and intensity of blackening was identical for the three concentrations tested. This was carried out with three isolates, 5, 20 and 24, and it was decided to continue work with a concentration of $5x10^4$ spores/ml.

All isolates were tested for virulence on three batches of mangoes, two lots of Kenyan pickling mangoes (which would probably not have received any fungicides) and one lot of Peruvian mangoes (var. Haden). Mangoes were prepared by surface sterilising in 1% chloros and allowing them to air dry. For each batch of mangoes there were 3 replicate mangoes, each receiving approx. twenty 10 μl drops containing $5x10^4$ spores/ml, and each representing a different *C. gloeosporioides* isolate. Mangoes were incubated in plastic boxes (27.5 x 15.5 x 18.0 cm) with a layer of wet tissue in the bottom, which resulted in a relative humidity (RH; measured by using a hygrometer) of >96% at 25°C (these will subsequently be referred to as moist chambers). Disease development was assessed at regular intervals and rated according to the amount and intensity of black colouration present in the lesion. The classes used are shown in Figure 2.1. Disease ratings were used to rank isolates for each separate batch of mangoes, after which rankings were combined to give each isolate a virulence rating. The virulence rating was divided into five groups : 1 - avirulent, 2 - low virulence, 3 - moderate virulence, 4 - moderately high virulence and 5 - high virulence.

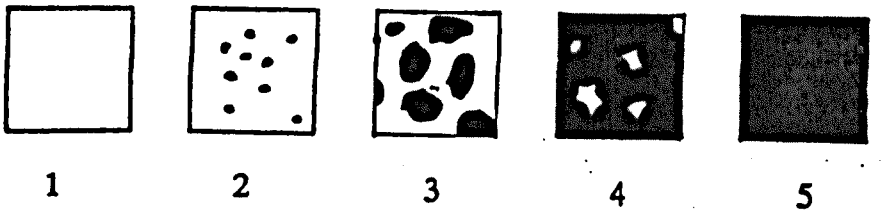


Figure 2.1 Disease rating: 1 - no black visible; 2 - less black than normal colour; 3 - same amount of black as normal colour; 4 - more black than normal colour; 5 - only black visible.

2.2.4 Host range of isolates - Plants were grown from seed and kept in a greenhouse at 20 - 30°C. For tomato, pepper and aubergine, leaves, flowers and developing fruit were sprayed with a spore suspension of *C. gloeosporioides* (see 2.2.3), 10^6 spores/ml, and the plants were incubated in plastic bags for a period of 24 hrs, after which the bag was removed. For papaya plants leaves were inoculated in the same way. Plants were regularly checked for anthracnose development and, for fruit and leaves, samples were taken after 48 hrs and incubated in moist chambers.

Detached ripe fruit of tomato, pepper, avocado and papaya were inoculated with 10 μl drops containing $5x10^4$ spores/ml (see 2.2.3) and incubated in moist chambers and checked for anthracnose development.

2.2.5 Spore germination and appressorium formation - Before spore germination experiments were carried out on glass slides, several other materials were tested to see if these could support spore germination and appressorium formation. These materials tested were filter paper (Whatman no.1, 2 and 6), membrane filters (cellulose nitrate 1HAWPO 1300, RA 1.2μ, SC 0.8μ, HA 0.45μ Millipore and WCN 0.2μ Whatman; cellulose acetate N47/45G 0.45μ Oxoid) and cellophane (32 SP, BCL) overlying tap water agar (TWA). Problems were experienced with all of these compared to glass slides. On filter paper growth was excessive and hyphae grew in between the fibres. Very few appressoria were formed on any of the membranes or on the cellophane. As it was easy to observe spores on glass slides and spores were stimulated into forming appressoria, slides were chosen for *in vitro* studies.

Another aspect that could have affected spore germination was whether or not it was necessary to wash spores. Washing removes the extra-cellular matrix which contains a self-inhibitor (Lingappa *et al.*, 1973; Lax *et al.*, 1985; Louis *et al.*, 1988). Washed spores were prepared by rinsing them three times in dH_2O, and spinning them down in between washes. Of 5 experiments with washed and unwashed spores, each consisting of 3 replicates of 100 spore counts, mean germination was 53% for washed spores and 59% for unwashed spores. That no difference was observed might be because the spore concentrations used were low ($5x10^4$ spores/ml) which resulted in a dilution of the self-inhibitor (Lax *et al.*, 1985; Louis *et al.*, 1988), and no reduction in spore germination was observed. Thus it was decided to continue work with unwashed spores.

Experiment a - Drops of 10μl containing $5x10^4$ spores/ml (see 2.2.3) were inoculated either on glass slides or on surface sterilised mangoes (see 2.2.3); both were incubated in moist chambers. Spore germination and appressorium formation on glass slides was first assessed at a range of different temperatures and 3x100

spores were counted for each treatment. The temperatures tested were 10, 13, 14, 15, 20, 25 and 30°C. Temperatures were tested in pairs and for each set of experiments (see Figure 2.2) a fresh spore suspension was prepared. Slides were assessed for spore germination (spores were recorded as having germinated as soon as a germ tube was visible) and appressorium formation (recorded as the percentage of germinated spores that had formed appressoria) under the light microscope (x100).

Experiment b - To evaluate the relationship between spore age and germination, cultures of *C. gloeosporioides* were grown under a black light and every 24 hrs newly sporulating mycelium was marked on the back of the Petri dish. The pattern of sporulation is easy to discern because under conditions of 12 hrs light/12 hrs dark most isolates of *C. gloeosporioides* form a diurnal pattern of sporulation. For spores ranging in age from 1 to 21 days, percentages of spore germination and appressorium formation were assessed. To evaluate differences in spore germination between isolates, two more isolates were treated in the same way. These were isolate 14, which according to the virulence rating was avirulent, and isolate 40, which had a moderate virulence rating (Appendix 2). In comparison, isolate 24 used for all the experiments had a high virulence rating. To reduce variation, 5x100 spores were counted.

Experiment c - To determine the timing of events taking place during germination of spores on glass slides, spores were incubated at 20, 25 and 30°C and sampled every other hour up to 12 hrs for 20 and 30°C, and hourly up to 12 hrs for 25°C, and again at 24 hrs for all temperatures (5x100 spores were counted).

Experiment d - To compare *in vitro* experiments with what takes place on the mango fruit, mangoes were inoculated and sampled at 12, 24, 36 and 48 hrs. Spore germination was simultaneously assessed on glass slides at six hourly intervals starting at 12 hrs (on glass slides 5x100 spores and on mangoes 3x100 spores were counted). Spore germination on mangoes was assessed by preparing samples for the scanning electron microscope (SEM) by critical point drying (CPD) the samples. This was done as follows : 1 cm^2 pieces of inoculated mango were cut from the fruit and put through an acetone series (25, 50, 75, 100, 100 and 100% acetone for a minimum of 15 min in each concentration). The samples were then critical point dried (Polaron E 3000) and sputter coated with a layer of gold (20 - 30 nm; Polaron E 5000) before being examined in a SEM (Philips 525 M or Hitachi S430 with a Hexland cryo attachment). To establish if this preparative process changed the appearance of the spores a few samples were cryo-fixed, coated with gold and examined directly. Temperatures always remained below -138°C.

In addition, some mangoes (var. Carabao) were inoculated while still on the tree at UPLB, Philippines, wrapped in plastic bags to allow germination to take place, and samples were taken at 24, 48 and 72 hrs and prepared for the SEM (CPD).

2.2.6 Effects of nutrients on spore germination and appressorium formation - Spores were germinated on glass slides as described (2.2.5) in the presence of peptone (soybean type III, Sigma) or D-glucose resulting in the following concentrations : 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% (w/v), and one additional treatment of peptone and glucose combined, both at 0.5% (5x100 spores were counted for each treatment).

2.2.7 Effect of pH on growth of *C. gloeosporioides* - Czapek Dox agar (CzDA) plates were prepared with the addition of a phosphate buffer (0.2M; Dhingra & Sinclair, 1985) and the pH was adjusted to 4, 5, 6, 7 and 8, which after autoclaving resulted in a pH of 3.9, 4.9, 6.0, 6.9 and 7.7. The same was done for TWA with the addition of 15g of sucrose/ litre medium (TWA-suc), which resulted in pH of 3.9, 5.0, 6.1, 6.9 and 7.8 after autoclaving. Plugs with mycelium were transferred onto these agars (5 plates/treatment) and radial growth of the fungus was assessed at 3, 5, 7, 9, 11, 13 and 15 days.

2.2.8 Effect of hot water treatment on spore germination - Tubes containing spore suspensions (see 2.2.3), $5x10^4$ spores/ml, were heated in water at 45, 50 and 55°C for 5, 10 and 15 min. After immersion, 10 μl drops were inoculated onto glass slides, incubated in a moist chamber for 24 hrs after which germination was assessed (see 2.2.5).

2.3 Results

2.3.1 *C. gloeosporioides* isolates - Growth, sporulation and colony colour are given in Appendix 2. All isolates grew between 9.0 and 13.6 mm/day on MEA, except isolate 26 with a rate of 5.5 mm/day and 46 with a rate of 7.4 mm/day. The mean radial growth rate of isolates coming from Brazilian mangoes var. Tommy Atkins was 11.9 mm/day compared to 10.9 mm/day for Venezuelan mangoes, 10.0 mm/day for isolates from Colombian mangoes, 10.4 mm/day for Florida mangoes, and 10.1 mm/day for Brazilian mangoes var. Ruby. Sporulation was moderate to excellent for most isolates, and only six isolates did not sporulate in culture. Sporulation seemed less prolific in isolates from Brazilian mangoes var. Ruby than in the other isolates. The majority of isolates were white or greyish white with the isolates from the Colombian mangoes being darkest in colour.

2.3.2 Taxonomy of isolates - All isolates contained one nucleus in the undivided spore indicating that they probably did not belong to *C. gloeosporioides* var. *minor*, which has a proportion of the spores containing two nuclei (Fortune, 1987). Growth on casein medium was moderate to good for all isolates. Fortune (1987) found that the *C. acutatum* isolates he tested grew very slowly on casein medium. None of the isolates tested here did so, although no isolate of *C. acutatum* was included for comparison, and hence growth rates are not presented. From these two assays it was assumed that all isolates were *C. gloeosporioides*, although too few tests were conducted to draw a firm conclusion.

2.3.3 Virulence of isolates - Virulence classes are given in Appendix 2 for all isolates. It was interesting to note that, for all three assessments, virulence was very similar and never varied more than two classes for each isolate. There were no isolates which did not cause any lesions at all, thus class 1 (avirulent), indicates that in two out of three trials no lesions were observed. For *in vitro* growth, sporulation and colour (Appendix 2), there did not appear to be a correlation between virulence and any of these characters, except that all four isolates with high virulence had excellent sporulation. The two isolates, 26 and 46, which had a low radial growth rate and did not sporulate well (see 2.3.1), were avirulent and of low virulence respectively.

2.3.4 Host range of isolates - The attempt to establish infection by *C. gloeosporioides* on whole plants was unsuccessful. Neither leaves, flowers or fruits had any visible lesions. Also detaching fruit and leaves did not result in any lesions. However lesions did appear on ripe avocadoes, papayas and tomatoes (albeit very slowly in the latter). It was not possible to obtain lesions on peppers by using spore suspensions, but penetration by the fungus did take place from agar plugs of *C. gloeosporioides* mycelium. The aim of these studies was to develop a model system in which it would have been possible to create latent infections on the plant, such as those which develop on fruit in the mango tree, and to use this model system to evaluate potential biological control agents. None of the combinations tested resulted in latent infections and therefore no model system was used.

2.3.5 Spore germination and appressorium formation -

Experiment a - Spore germination was low at 10°C (14%) but at 13°C germination was excellent at 95% (Figure 2.2). However germination was highly variable and at 25°C results ranged from 32 to 98%. The percentage of germinated spores forming appressoria was also variable and at 25°C results ranged between 25 and 86% (Figure 2.2). There was no obvious difference between experiments to account for this large variation except that in each set of experiments different batches of spores were used (see 2.2.5).

Experiment b - This experiment showed that there was no general trend for a relationship between spore age and percentage spore germination nor appressorium formation (Figure 2.3). The percentage of spores germinating seemed to decline with spore age for isolate 24. A preliminary trial with this isolate, not presented here, showed that spore germination was >70% at 21 days. All these experiments indicated that spore germination and appressorium formation are highly variable and are not related to spore age. Estrada (1990) found with several *C. gloeosporioides* isolates, that percentage of spore germination was higher for 7 day old spores than for 14 day old spores. Similarly Lenné (1978) found that 10 - 12 day old spores had highest rates of spore germination, but most appressoria were formed by spores aged between 12 and 16 days. In my subsequent experiments it was therefore decided to use spores between 7 and 14 days of age. For spore germination experiments, it is thus only possible to compare treatments within experiments set up on the same day with the same batch of spores. When comparing with other experiments, it is only possible to determine if similar trends are present, and absolute values for spore germination and appressorium formation should not be directly compared between experiments.

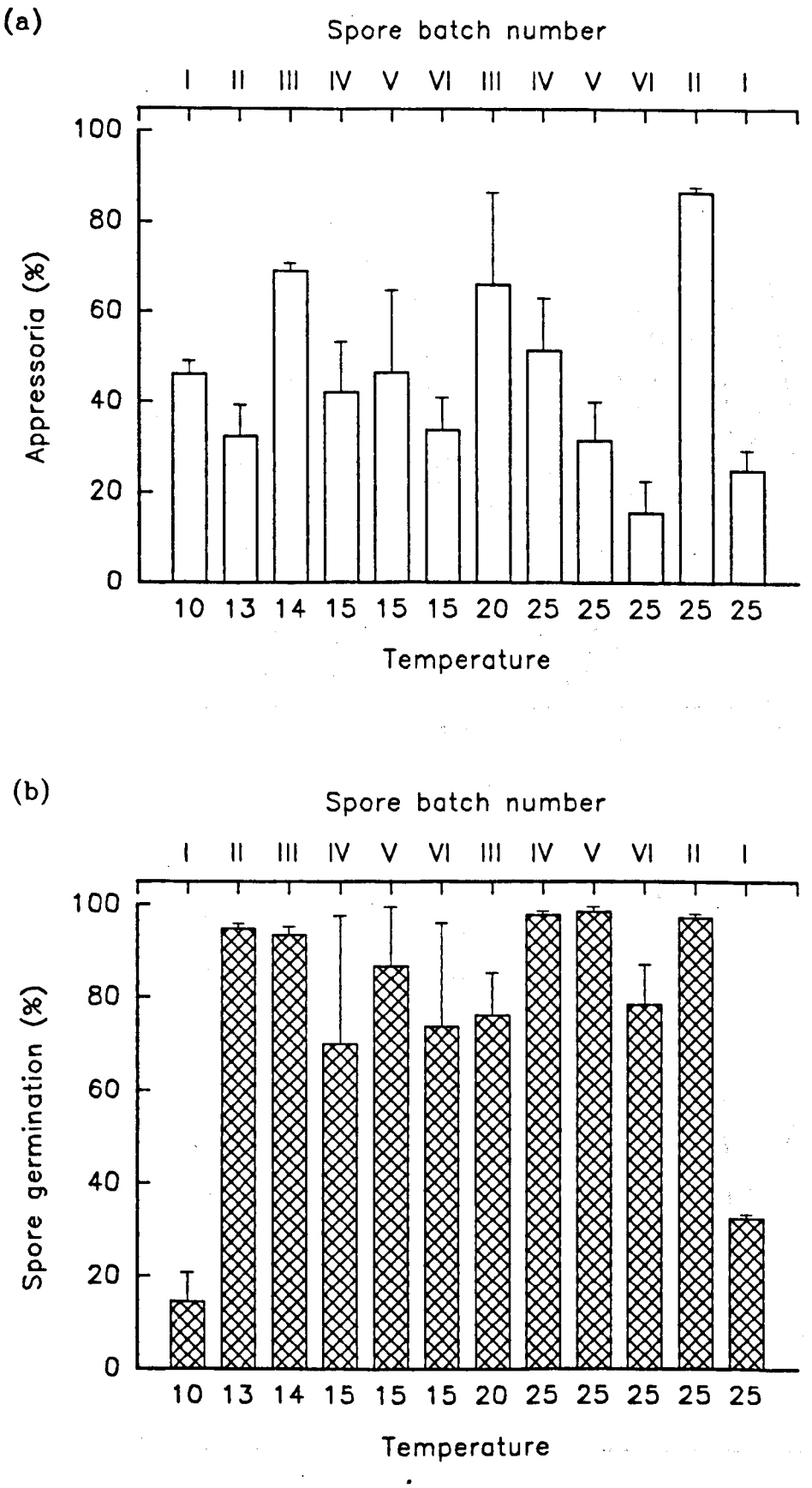


Figure 2.2 Percentage of (a) appressorium formation and (b) spore germination of *C. gloeosporioides* at different temperatures (roman numerals indicate experiment numbers i.e. the temperature treatments tested with the same batch of spores; vertical bars = S.E)

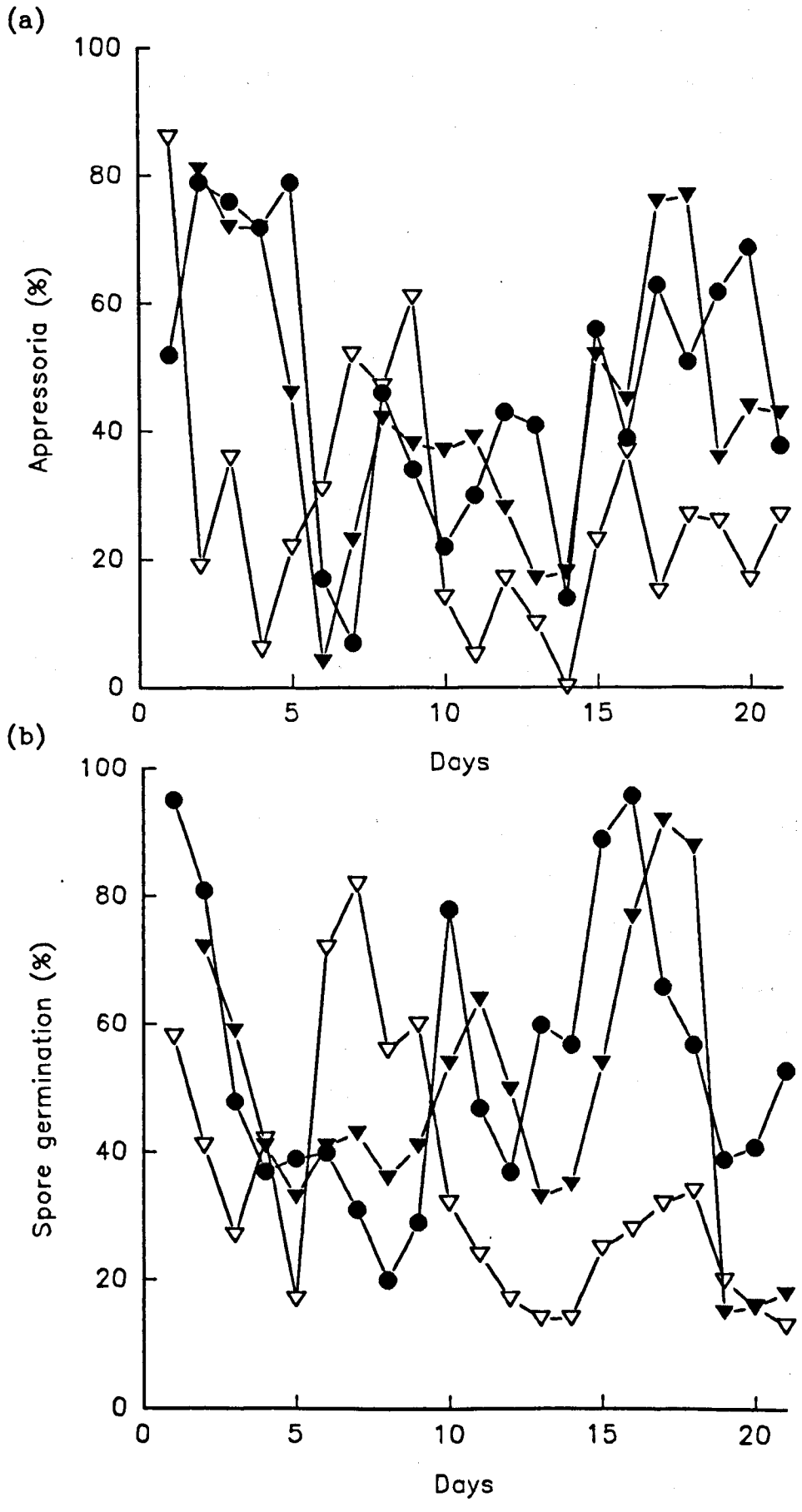


Figure 2.3 Percentage of (a) appressorium formation and (b) spore germination for 1 - 21 day old spores of isolates 14 (●), 24 (▽), and 40 (▼).

Experiment c - These observations showed that germ tube emergence started at 4 hrs at 25 and 30°C, but at 25°C a higher percentage of spores started to germinate in a shorter period of time than at 30°C (Figure 2.4). At 20°C spores did not start to germinate until 10 hrs and at 24 hrs only 44% of the spores had germinated compared to 56% at 25°C and 67% at 30°C. Interesting to note is that after 24 hrs at 30°C no appressoria had formed, while at 25°C 34% of germinated spores had formed appressoria and 7% at 20°C (Figure 2.4). From the earlier experiments it had been noted that the percentage of germinated spores forming appressoria could be as high as 66% at 20°C, and as high as 46% at 10°C (Figure 2.2). The timing and effects of temperature on events taking place during spore germination, might be very dependant on the isolate used though, because experiments with two isolates from the Philippines (Estrada, 1990) showed that hardly any spore germination (<10%) had taken place after 36 hrs at 15°C, yet spores germinated well at 35°C (57%) with the optimum temperature for these isolates being 30°C. Although no appressoria had formed after 18 hrs with these isolates, an average of 50% of germinated spores had formed appressoria after 36 hrs at 25 and 30°C, but at 20°C less than 10% of germinated spores had appressoria present. Both spore germination and appressorium formation were dependant on the relative humidity with the highest percentages of both at the highest RH (100%), but no spore germination nor appressorium formation had taken place at 90% RH (Estrada, 1990).

Experiment d - The germination pattern was the same on the mango surface as on the glass slides (Figure 2.5). However, after 48 hrs, the percentage of germinated spores forming appressoria was much higher on glass slides (44%) than on mangoes (6%). This experiment was repeated but no germination had taken place on the mangoes even after 48 hrs. This was probably due to high levels of fungicides in the peel, or a wax coating present that had not been anticipated.

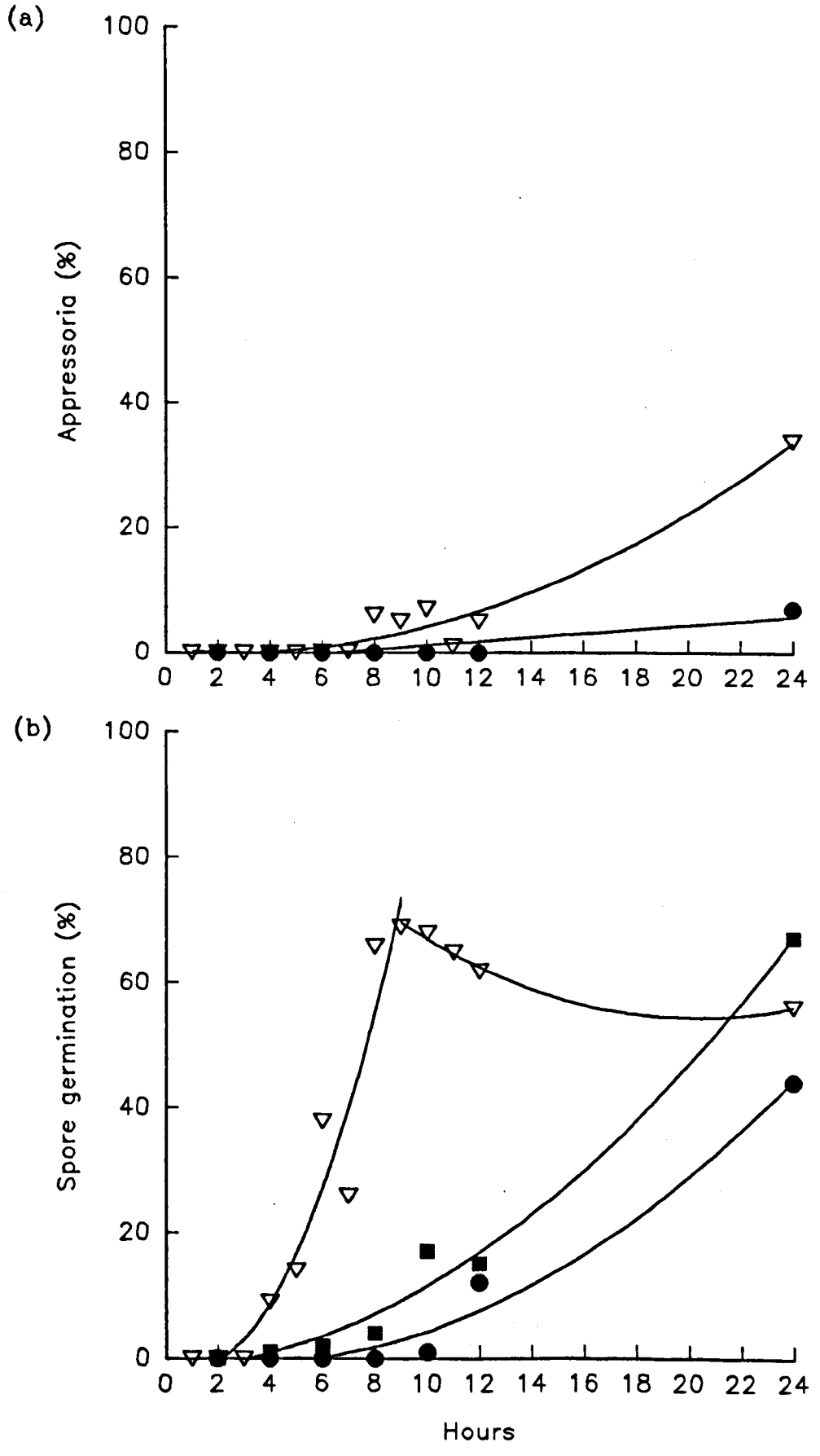


Figure 2.4 Percentage of (a) appressorium formation and (b) spore germination on glass slides at 20 (●), 25 (▽) and 30°C (■).

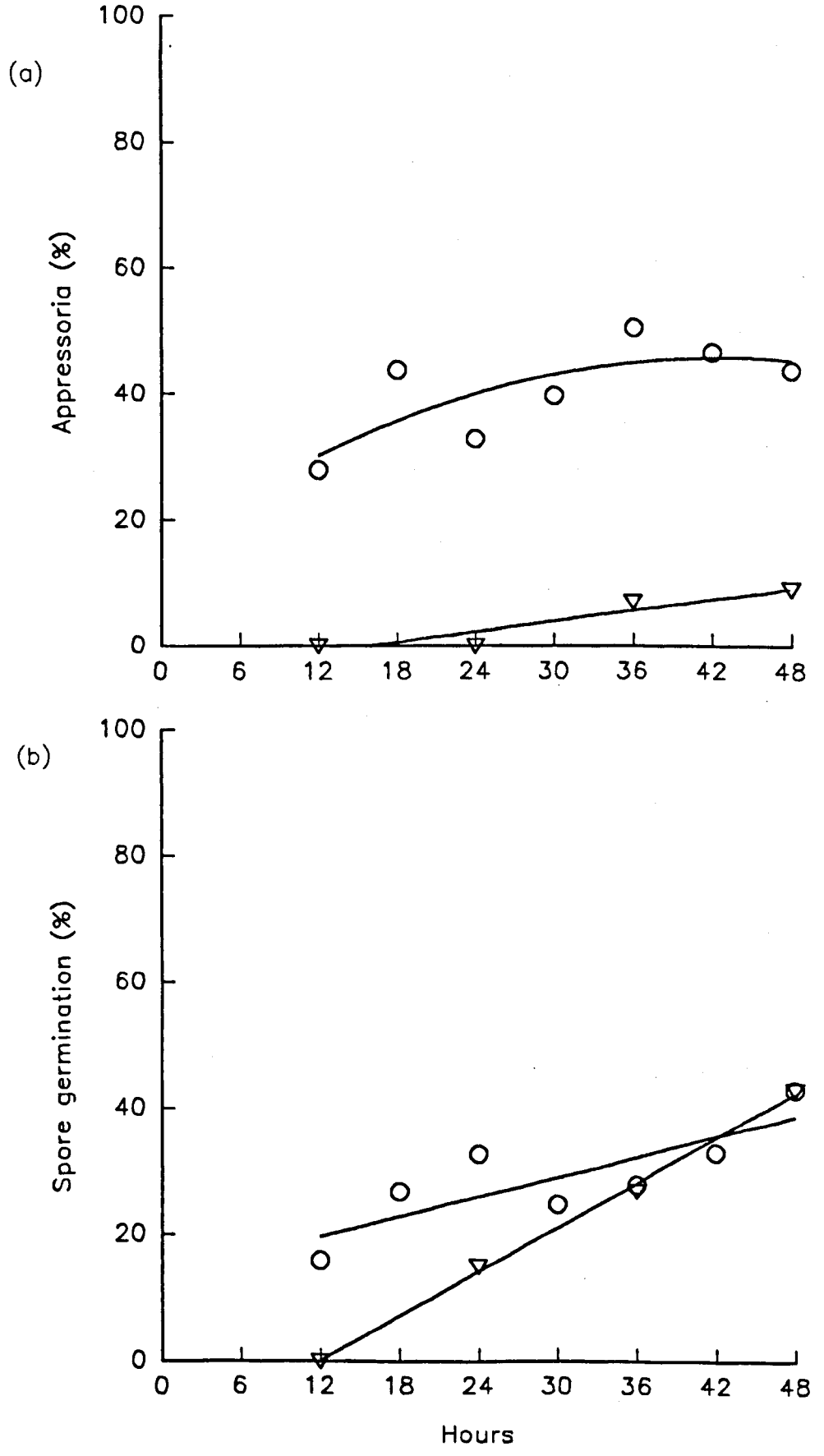


Figure 2.5 Percentage of (a) appressorium formation and (b) spore germination on glass (○) and on the mango surface (▽).

From all these experiments the following picture of events emerged : when submerged in water the spore swells slightly, the nucleus divides and a septum is formed, dividing the spore into two sections (Figure 2.6).

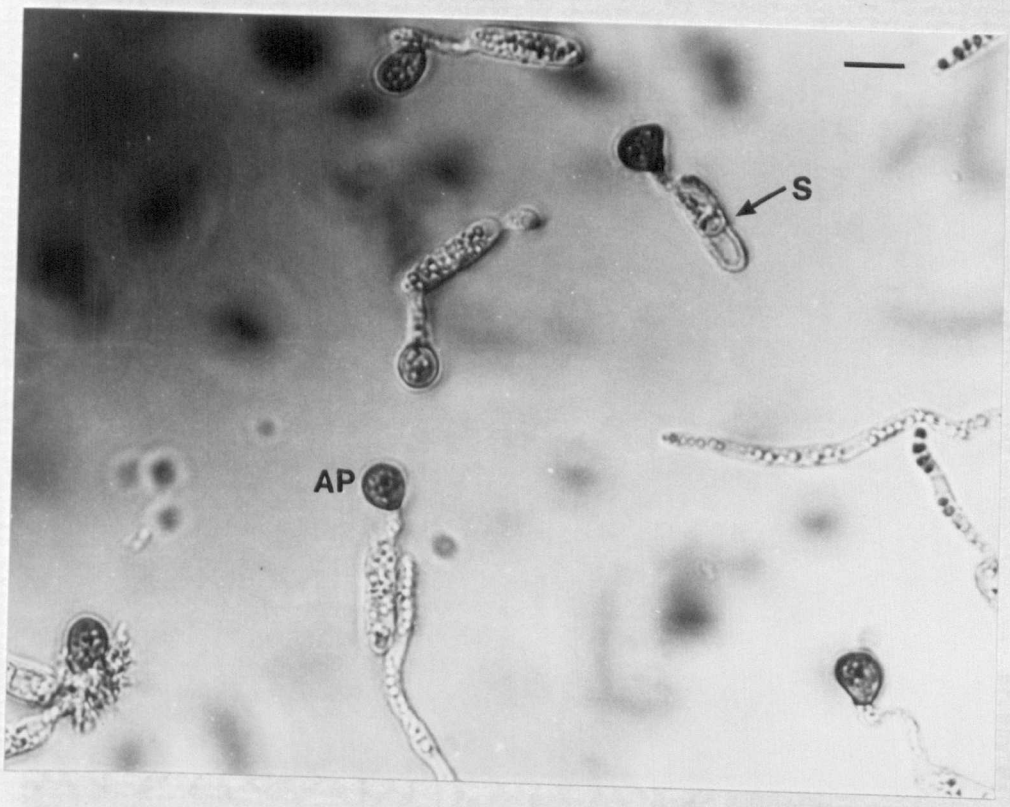
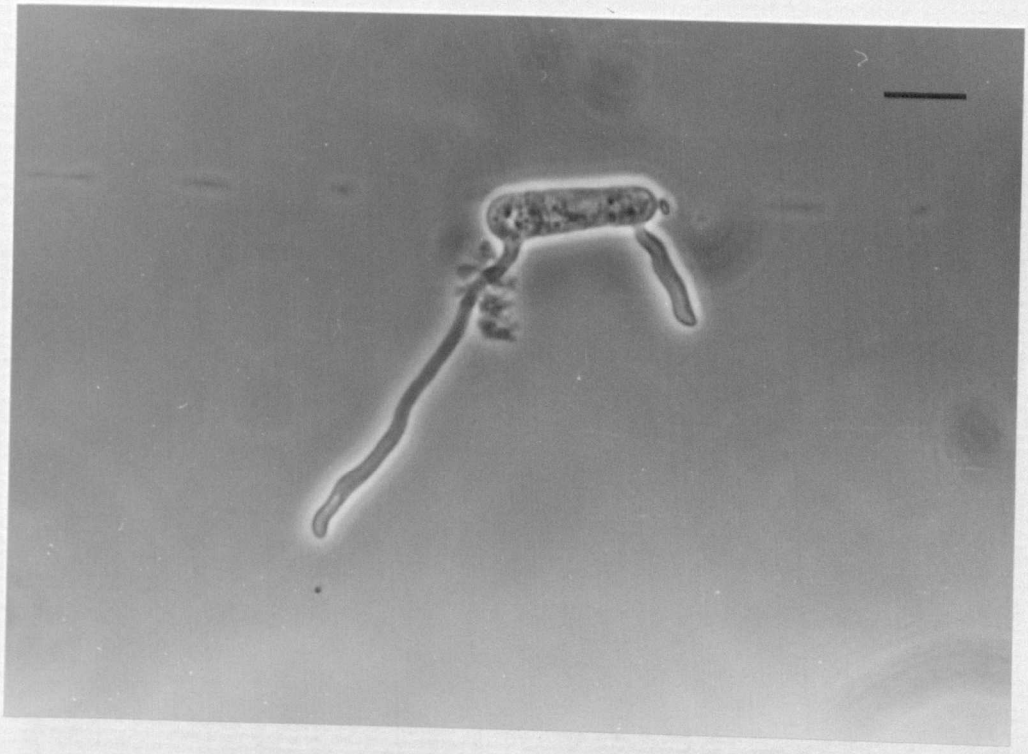


Figure 2.6 Germinated spores of *Colletotrichum gloeosporioides* on a glass slide with dark, melanized appressoria (AP). Note the septum (S) dividing the germinating spore in two (Phase contrast; bar= 10μm).

If conditions are optimum, a germ tube emerges from one side of the spore after 4 hrs, often followed by a second germ tube from the opposite side of the spore (Figure 2.7.a & 2.9.a). Further germ tubes can arise and, in some cases, a total of four germ tubes have been observed on a single spore. The germ tube starts to elongate and either an appressorium is formed on the end of the germ tube which can be variable in length, or the germ tube continues saprophytic growth and hyphae are formed. It is possible to find on a single spore that one germ tube has formed an appressorium, while the other germ tube continues to grow (Figure 2.7.b & 2.8.a). The first appressoria are formed after 6 hrs and appear as dark, melanized appressoria (Figure 2.6 & 2.8.b). On mangoes spore germination starts between 12 and 24 hrs, and the first appressoria are present between 24 and 36 hrs (Figure 2.9.b). However, the experiments with mangoes were

a)



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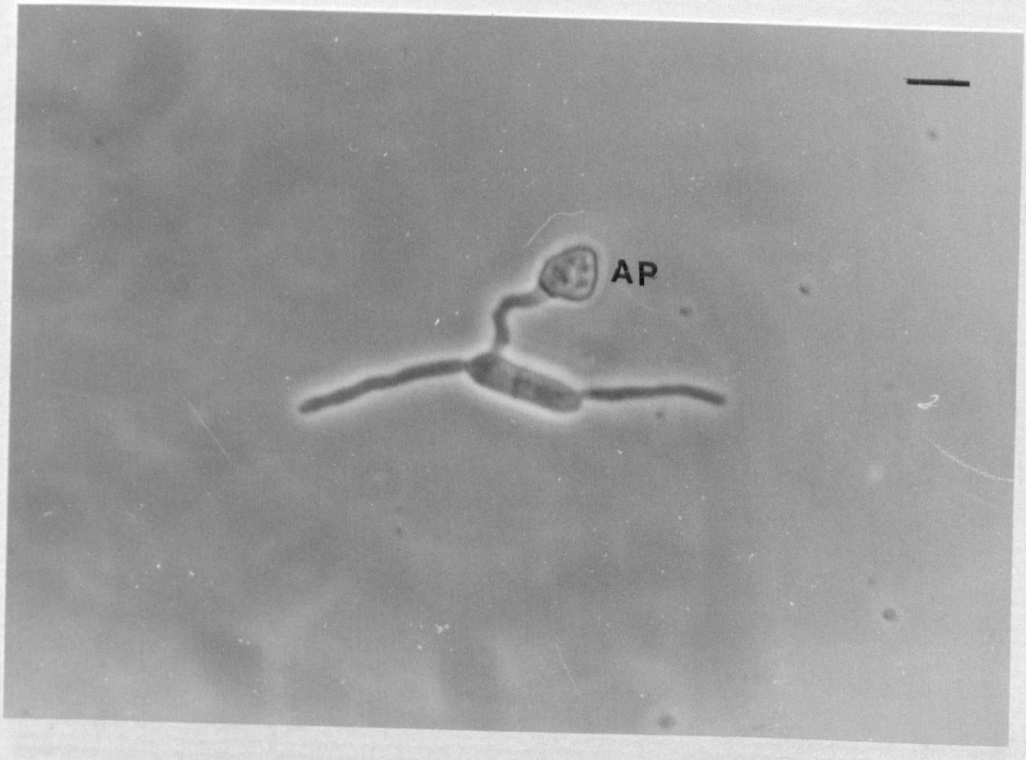
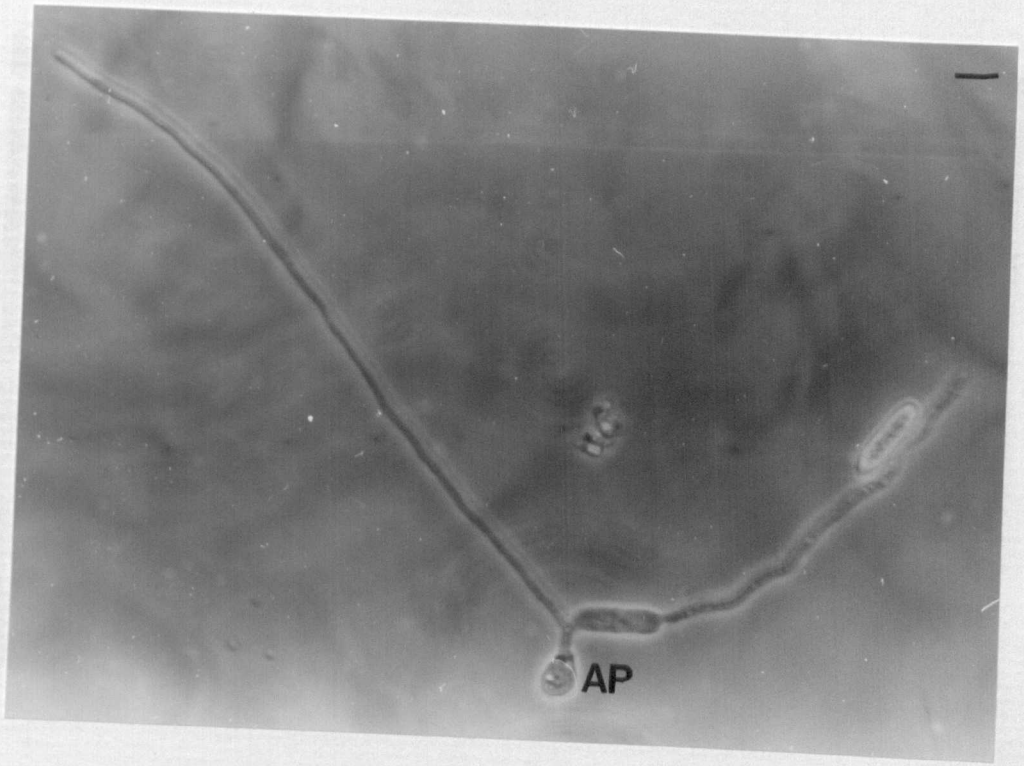


Figure 2.7 Germinated spores of *C. gloeosporioides* showing a) two germ tubes on opposite sides of the spore and b) three germ tubes of which one has formed an appressorium (AP; phase contrast; bar = 10μm)

a)



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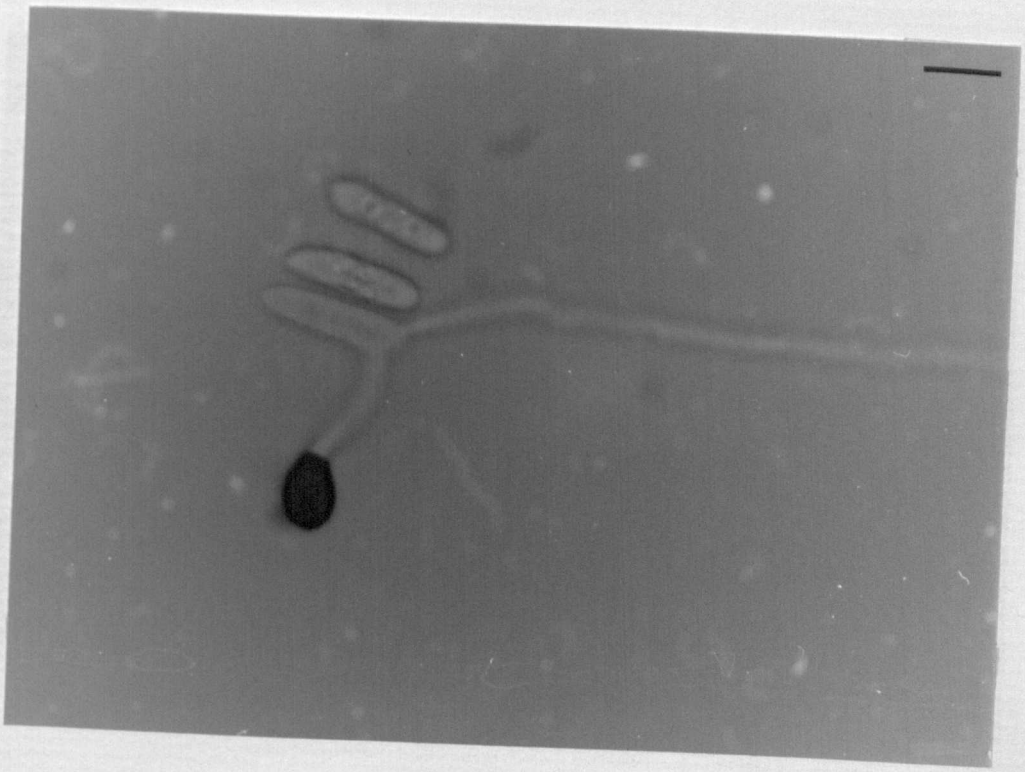
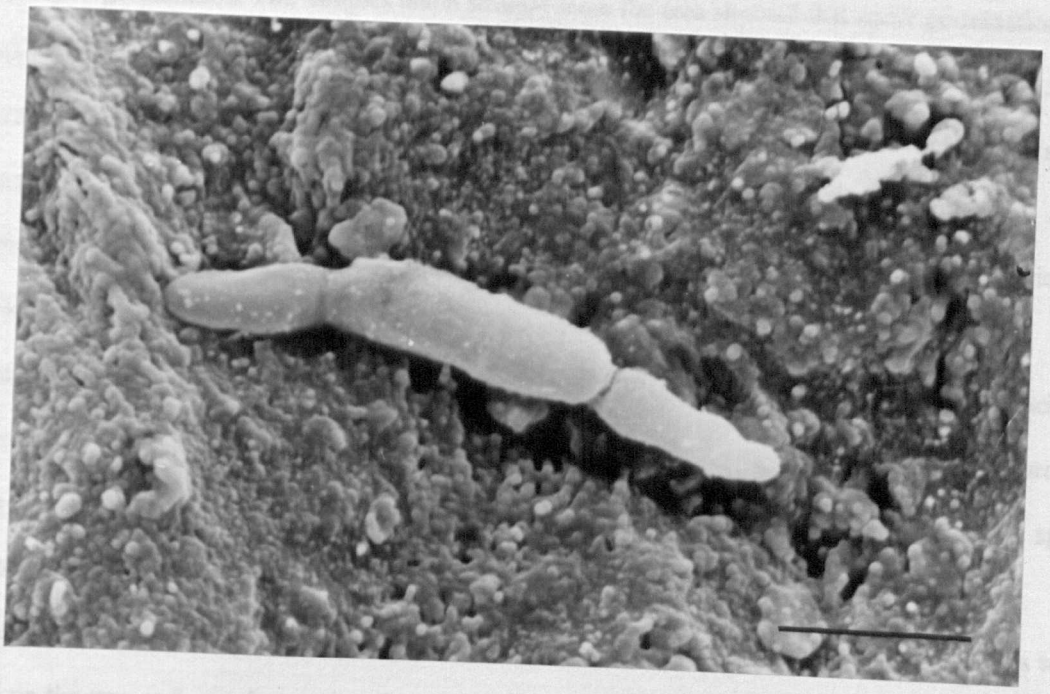


Figure 2.8 Germinated spores of *C. gloeosporioides* showing a) three germ tubes of which one has formed an appressorium (AP) while the other two have continued saprophytic growth (phase contrast) and b) a dark melanized appressorium (light microscopy; bar = 10μm)

a)



b)

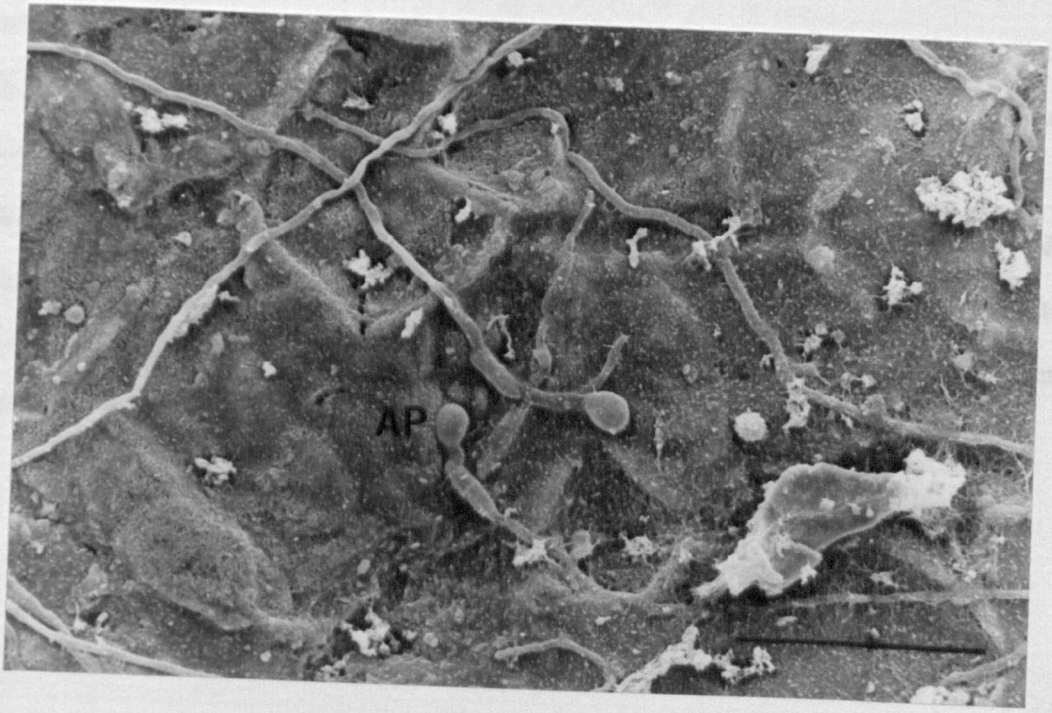


Figure 2.9 Germinated spores of *C. gloeosporioides* on mango a) 24 hrs after inoculation two germ tubes are visible (bar = 10μm) and b) 48 hrs after inoculation extensive saprophytic growth and appressoria (AP) are visible (bar = 50μm; S.E.M.)

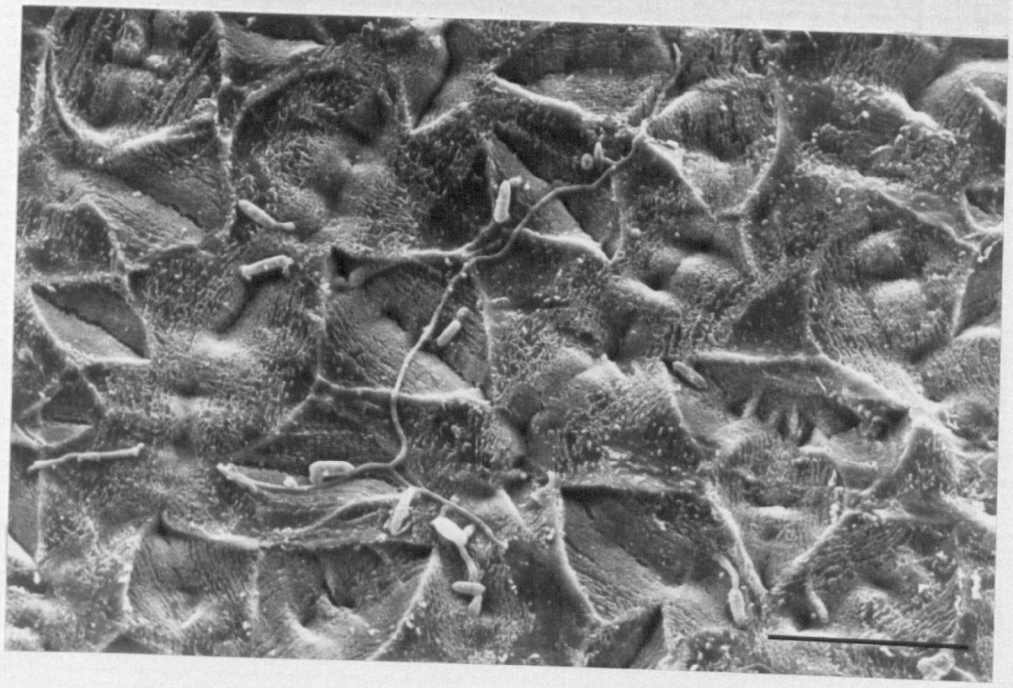
carried out with ripe, detached mangoes, which may have had fungicides on them, and this could have caused a delay in germination. The samples taken straight from the tree showed that spore germination and appressorium formation took place within 24 hrs on these immature, unsprayed mangoes (Figure 2.10 & 2.11.a). The timing of events correlated with that observed by Estrada (1990) who also assessed spore germination and appressorium formation on detached leaves, and detached fruit of different ages. As it appears that spores do not germinate until after 12 hrs on the fruit surface, this implies that a period of high humidity is required lasting a minimum of twelve hours.

The comparison between CPD prepared samples and cryo-fixed samples showed that a layer of mucilage was present on the latter which covered the spores (Figure 2.11.b). This layer has been reported before for *Colletotrichum graminicola* and its function seems to be that of an anti-desiccant, maintaining spore viability, and protecting the spore from phenols present in lesions (Nicholson *et al.*, 1989).

To determine if an infection peg had formed after the appressorium was visible, immature mangoes were inoculated on the tree and samples prepared for the transmission electron microscope. However it has not been possible to obtain good sections. This was due to the very thick cuticle the mango possesses (Figure 2.12.a). When ultra thin sectioning was attempted the resin would break away from the cuticle, particularly in the area that needed to be examined. Until further attention has been given to this it will remain uncertain if the latent infection of *C. gloeosporioides* in mango consists of the appressorium lying on the cuticle, or whether sub-cuticular hyphae are also part of the latent phase.

Two other observations on the behaviour of *C. gloeosporioides* spores were noted : (i) micro-conidiation takes place very easily, often coinciding with hyphal coiling (Figure 2.13) especially when the concentration of spores is very high (Lingappa & Lingappa, 1969) and (ii) hyphal anastomosis is frequent, even between germinating spores (Figure 2.12.b).

a)



b)

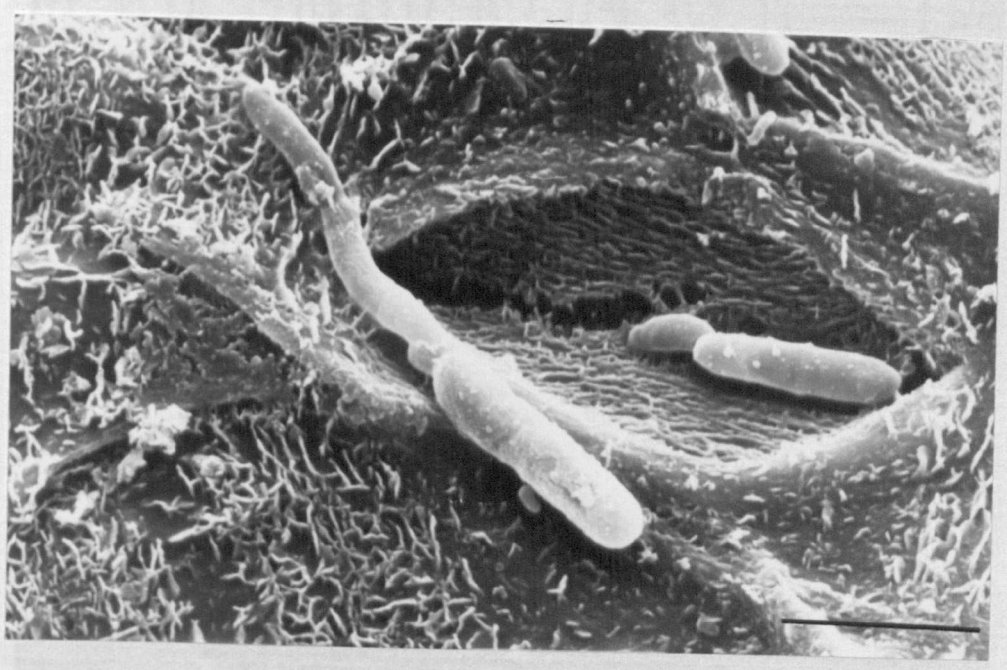
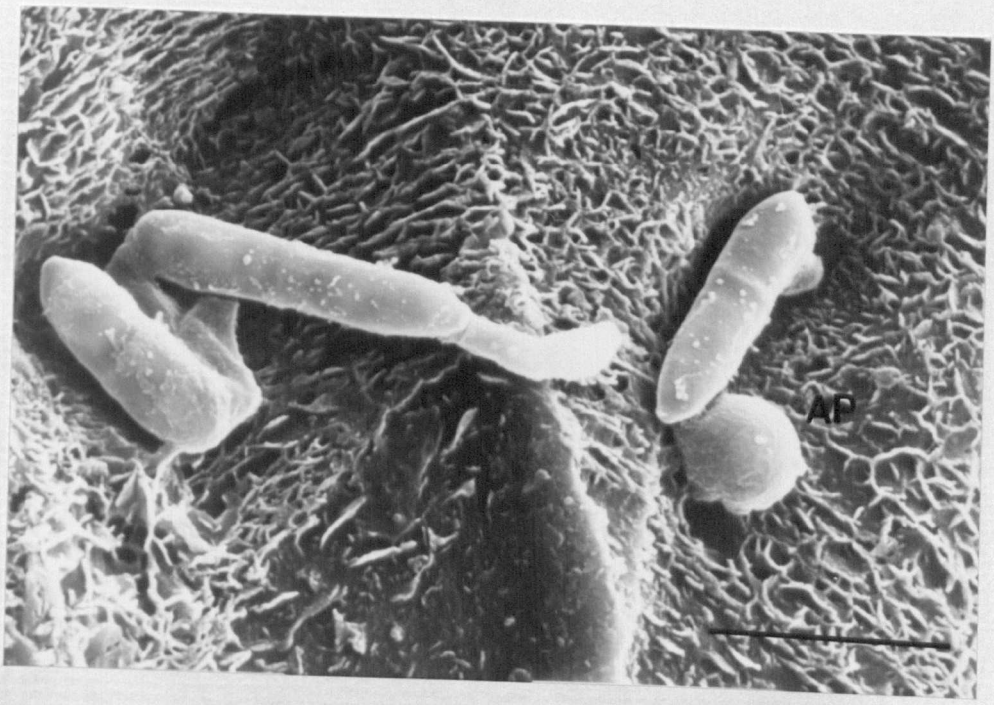


Figure 2.10 a) & b) Germinated spores on immature 'Carabao' mangoes, 24 hrs after inoculation on the tree (S.E.M.; A - bar = 50μm; B - bar = 10μm)

a)



b)

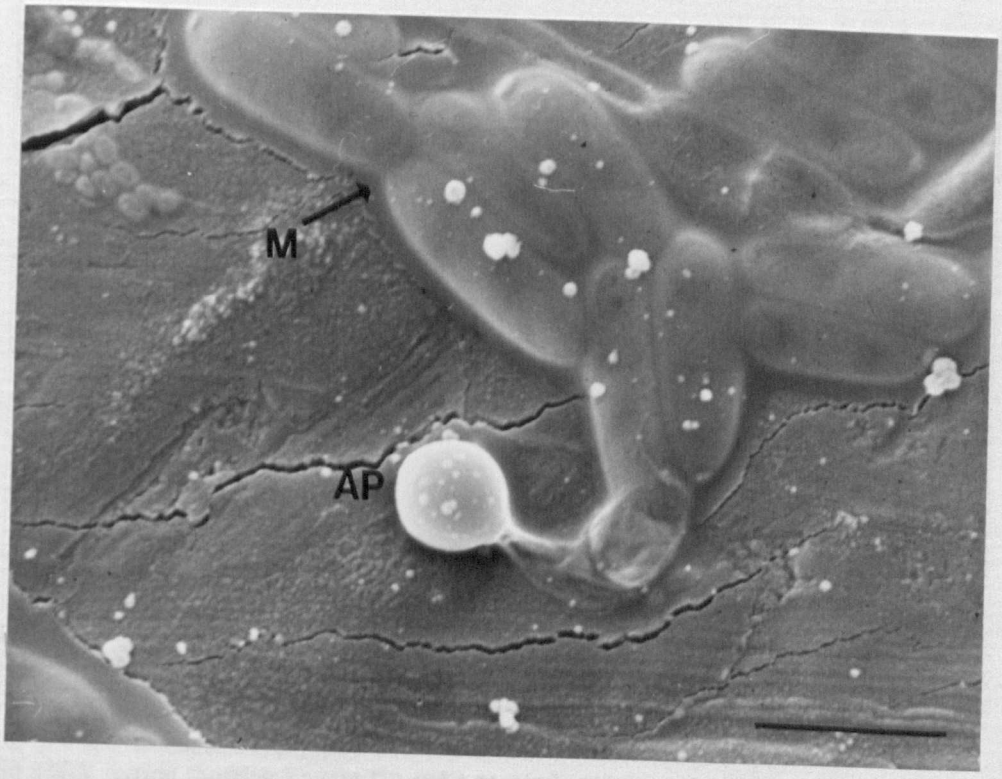
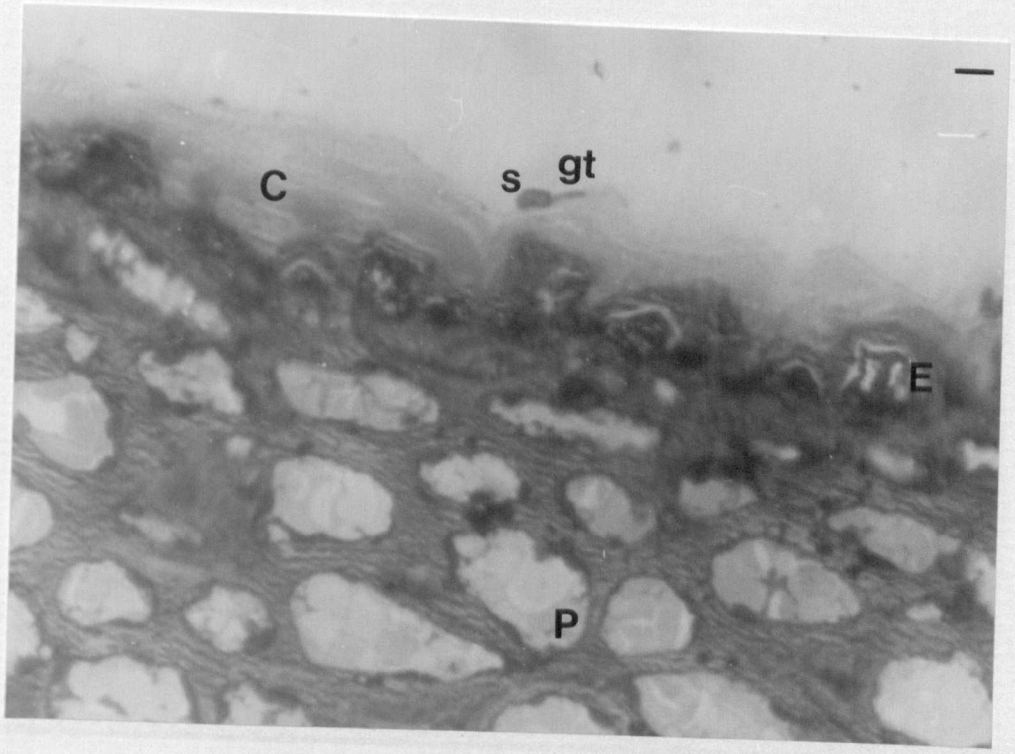


Figure 2.11 Germinated spores a) on immature 'Carabao' mango, 24 hrs after inoculation on the tree and b) on mango (cryofixed) showing the mucilage layer (M) present (AP = appressorium; S.E.M.; bar = 10μm)

a)



b)

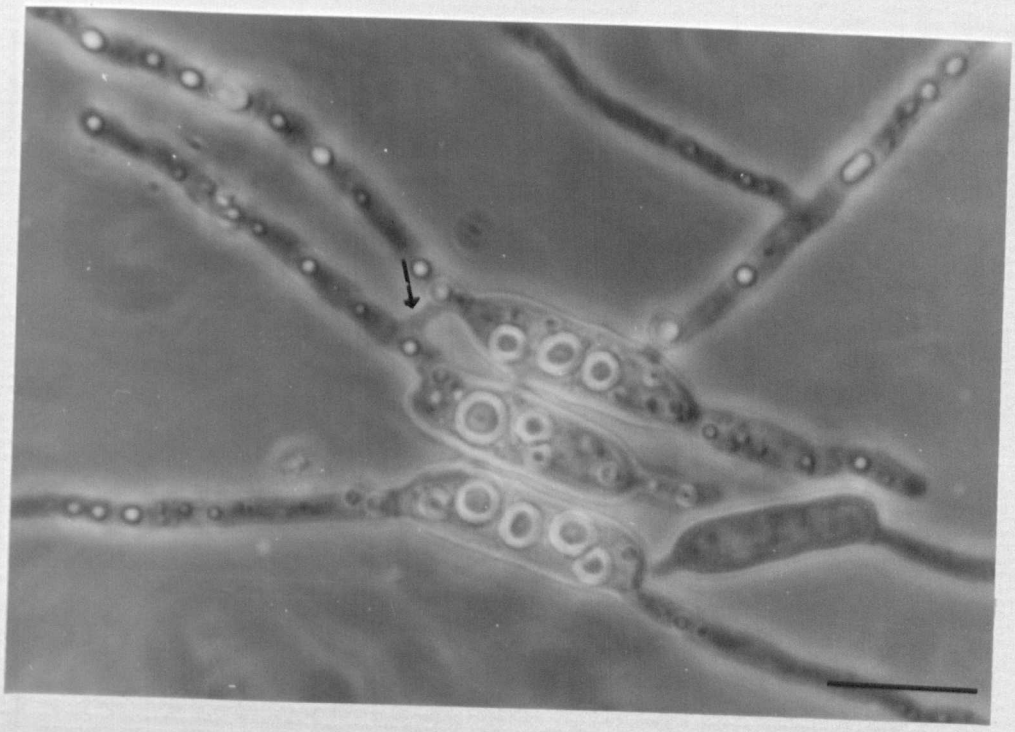
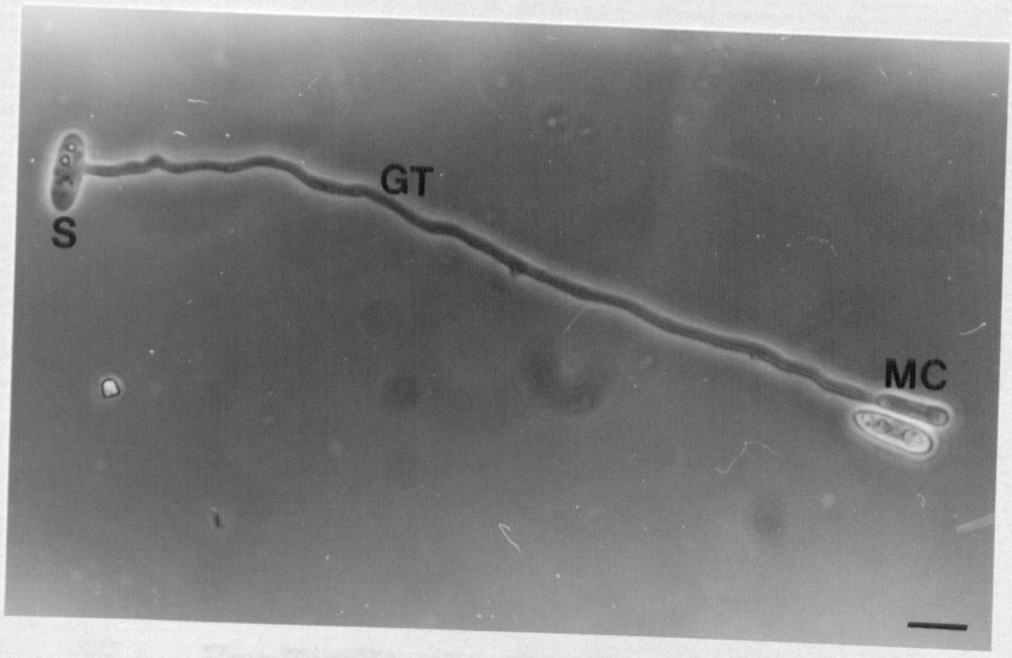


Figure 2.12 a) Thick section showing a spore (S) and a protruding germ tube (GT) on the surface of mango. Note (C) the thick cuticle. (E = epidermal cells; P = parenchyma cells; Light microscopy) and b) Germinated spores of *C. gloeosporioides* showing an anastomosis (→) taking place between adjacent hyphae (phase contrast; bar = 10μm).

a)



b)

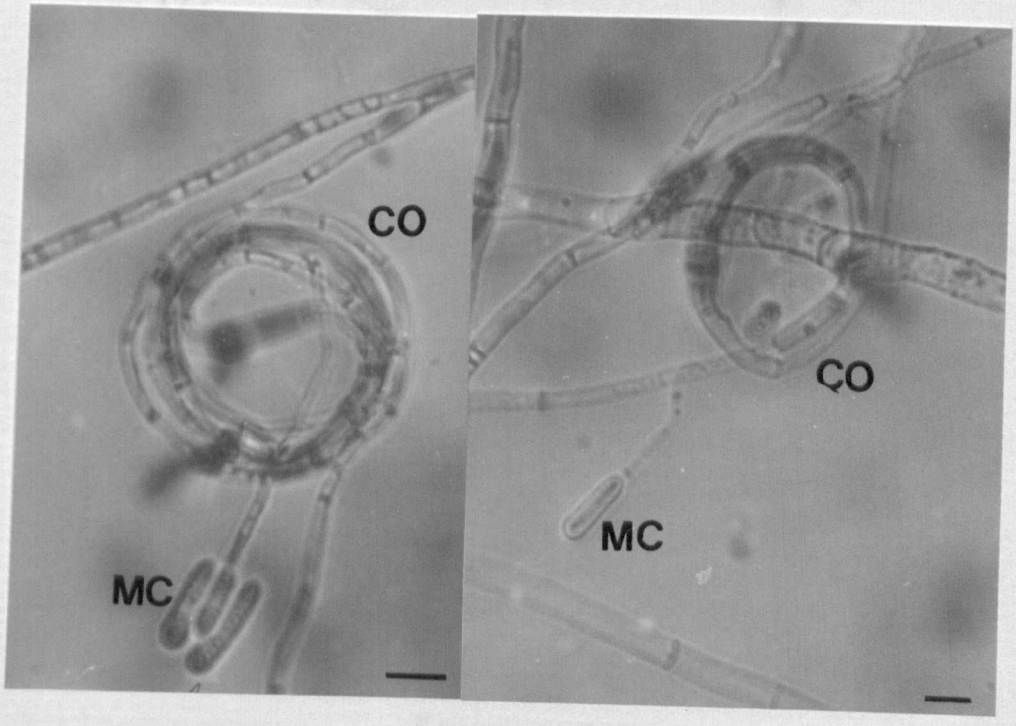


Figure 2.13 Microconidiation taking place on a) an extended germ tube (GT) and b) on saprophytic mycelium performing coiling (CO). (S = spore; MC = microconidium; phase contrast; bar = 10μm)

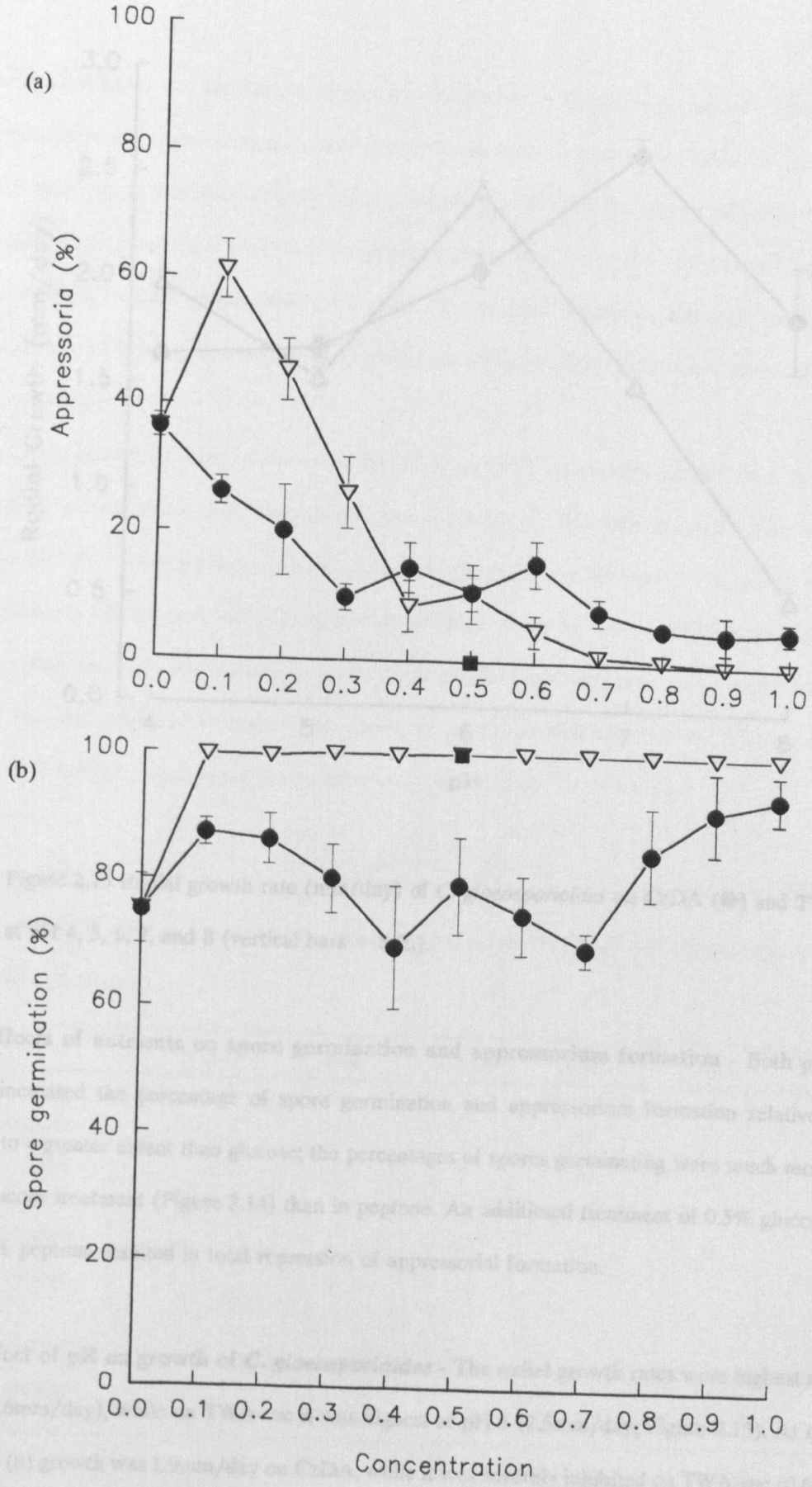


Figure 2.14 Percentage of a) appressorium formation and b) spore germination in the presence of increasing amounts of glucose (●) and peptone (▽). ■ indicates an additional treatment of 0.5% glucose and 0.5% peptone added simultaneously (vertical bars = S.E.).

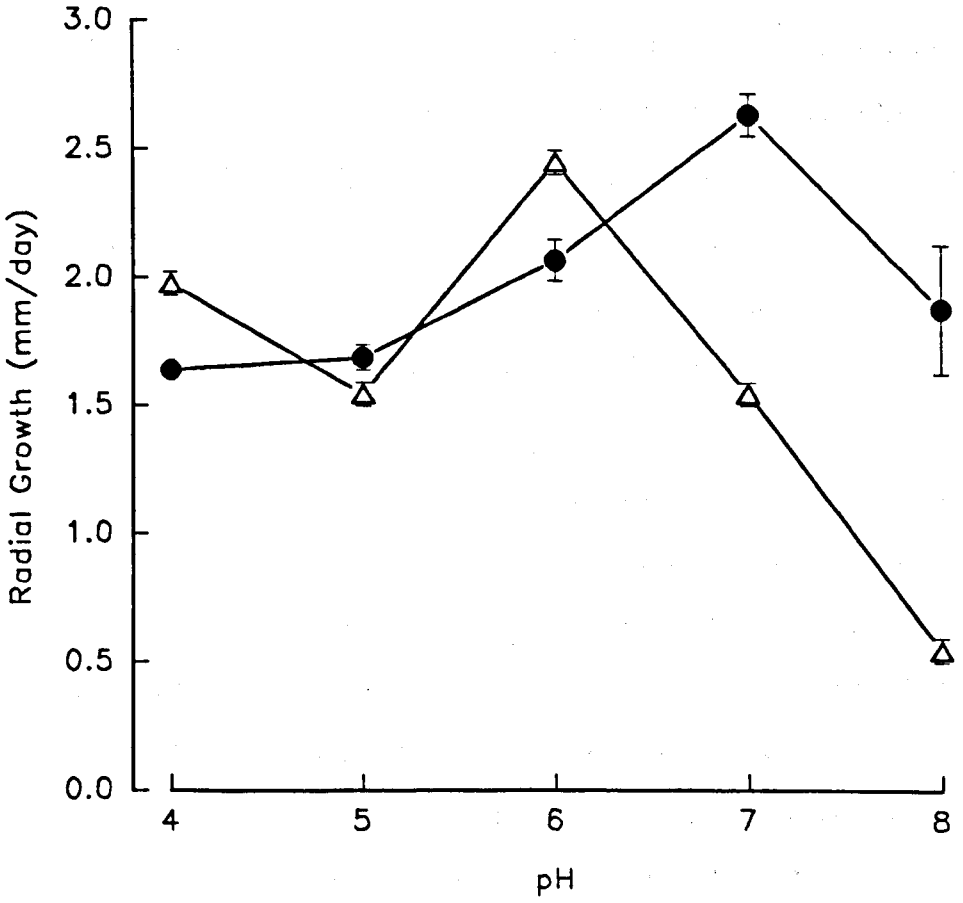


Figure 2.15 Radial growth rate (mm/day) of *C. gloeosporioides* on CzDA (●) and TWA-suc (▵) at pH 4, 5, 6, 7, and 8 (vertical bars = S.E.).

2.3.6 Effects of nutrients on spore germination and appressorium formation - Both peptone and glucose increased the percentage of spore germination and appressorium formation relative to water, peptone to a greater extent than glucose; the percentages of spores germinating were much more variable in the glucose treatment (Figure 2.14) than in peptone. An additional treatment of 0.5% glucose together with 0.5% peptone resulted in total repression of appressorial formation.

2.3.7 Effect of pH on growth of *C. gloeosporioides* - The radial growth rates were highest at pH 7 on CzDA (2.6mm/day), while on TWA-suc it was highest at pH 6 (2.5mm/day; Figure 2.15). At the highest pH tested (8) growth was 1.9mm/day on CzDA, while it was severely inhibited on TWA-suc (0.6mm/day), indicating that the lower nutrient status of TWA-suc agar made the fungus more susceptible to the adverse effects of the high pH. At the lowest pH (4) growth was hardly affected with rates of 1.6mm/day on CzDA and 2.0mm/day on TWA-suc agar.

2.3.8 Effect of hot water treatment on spore germination of *C. gloeosporioides* - After treatment at 55°C none of the spores germinated, even after only 5 min at this temperature (Table 2.1). After treatment at 50°C, germination was also severely reduced to 1% germination for spores subjected to 5 min heat treatment, and no germination took place by spores heated at 50°C for 10 min. Treatment of spores at 45°C, however, did not affect germination even after 15 min heat treatment, although the percentage of germinated spores forming appressoria was significantly reduced after 15 min heat treatment compared to the untreated spores.

It would have been interesting to determine the effect of HWT on appressorial viability, since this is the structure on the fruit that is most resistant to external conditions. The only attempt in this study to assess appressorial viability was carried out with neutral red (Ikediugwu & Webster, 1970) and it was observed that appressoria did not take up stain. Other possibilities would be to use a vital stain like fluorescein diacetate (Barak & Chet, 1986), but the appressorium may not be able to take up the stain through its thick wall. It was also attempted to trigger appressoria to germinate with ethylene and glucose, but this was unsuccessful, partly because appressoria germinate spontaneously on glass slides.

Table 2.1 Effect of simulated hot water treatment carried out at 45, 50 and 55°C on spore germination and appressorium formation.

	0 min	5 min	10 min	15 min	mean	S.E.
45°C						
% Germination	65	44	31	66	51	15.9
% Appressoria	16	16	6	2	10	3.2
50°C						-
% Germination		1	0	0	0	
%Appressoria		0	-	-	-	-
55°C						
% Germination		0	0	0	0	-
% Appressoria		-	-	-	-	-

2.4 Discussion

2.4.1 Taxonomy - From the limited tests carried out it seemed most likely that all isolates belonged to *C. gloeosporioides*. The distinction between var. *minor* and var. *gloeosporioides* is not very clear. The observation that var. *minor* contains two nuclei in a proportion of its spores was only made on one isolate (Fortune, 1987) and needs further verification. Simmonds (1965), who originally made the distinction between the two isolates, made the distinction between spore size on PDA. However, overlap between sizes is too large to make it a reliable character. More distinct are the sizes of the ascospores with a mean size for *Glomerella cingulata* of 18.3 x 5.4 μm, and for *G. cingulata* var. *minor* of 15.6 x 4.2 μm. In this study it was attempted to obtain ascospores by leaving cultures for a long time in the dark or under the black light, and by inoculating all possible combinations of isolates onto one plate. None of this, however, resulted in the formation of perithecia and hence the size of ascospores could not be determined. Cox & Irwin (1988) refined the differences between var. *gloeosporioides* and var. *minor* and made the distinction between the two isolates on their conidial width, with var. *gloeosporioides* having a conidial width between 3.0 and 4.2 μm and var. *minor* between 4.5 and 5.5 μm. Measurements on conidia have to be made on conidia produced in conidiomata since conidia produced on free hyphae are too variable in size. This measure could not be used on our isolates since by the time the above paper was published the majority of cultures were stored under paraffin oil and as discussed before (2.2.1) could not be recovered. I question, however, the importance of the distinction between var. *gloeosporioides* and var. *minor*, and since they are very hard to distinguish should they not both be referred to simply as *C. gloeosporioides*?

C. gloeosporioides is a heterogenous species, largely due to heterokaryosis (the frequent occurrence of anastomosis being an example of this) and parasexuality (Jeffries *et al.*, 1990). The host range is also enormous and even on a single host, different diseases caused by *C. gloeosporioides* have been observed, such as the occurrence of anthracnose and chocolate spot on papaya (Chau & Alvarez, 1983a). On *Stylosanthes* spp. in Australia two distinct disease types, A and B, were distinguished. Both types were caused by *C. gloeosporioides* but type A isolates had a wider host-range than type B (Vinijsanum *et al.*, 1987). Protein composition of both types were similar, but differences were observed in the double-stranded RNA's (Dale *et al.*, 1988).

The virulence trials carried out did result in a ranking, but variability between assessments was large and all it indicated was that on detached mangoes lesions are formed with different rates of spread. In conclusion I would say that this type of experimentation is unreliable. Eleven isolates of *C. gloeosporioides* from the Philippines showed distinct differences in their pathogenicity (Quimio and Quimio, 1974b), but pathogenicity was assessed in a similar way as in this study ranging from slight to severe infection. For other species of *Colletotrichum*, different races have been distinguished, which do or do not infect certain cultivars of the host plant. Examples are : *C. lindemuthianum* on bean (Tu, 1986; Alam & Rudolph, 1988; Drijfhout & Davis, 1989), *C. trifolii* on alfalfa (Churchill *et al.*, 1988) and *C. destructivum* on alfalfa (Boland & Brochu, 1989). However since no resistant cultivars of mango are known, the development of different races of *C. gloeosporioides* with respect to mango seems unlikely.

More work, apart from cultural characteristics is needed on the taxonomy of *C. gloeosporioides* and a quick method of identification would be advantageous. The importance of *C. acutatum* in anthracnose development deserves attention, since it was found that *C. acutatum* is less sensitive to fungicides as *C. gloeosporioides* (Fitzell, 1979). This means that it might also react in a different way than *C. gloeosporioides* to biological control agents. Malaysian isolates causing anthracnose on mangoes, were of a type in between *C. acutatum* and *C. gloeosporioides* (Fortune, 1987). It remains to be determined as to which type the Philippino isolates belong.

2.4.2 Spore germination and infection - Spore germination and appressorium formation is similar in all species of *Colletotrichum* (Lenné, 1978) and follows the pattern as described under 2.3.5. It appears that appressoria of *Colletotrichum* are situated on the leaf or fruit surface in a random fashion (Brown, 1975; Chau & Alvarez, 1983a) and appressorium formation on the fruit surface of mango also seems to follow this pattern. Appressorium formation in *C. graminicola* was dependant on the topography of the leaf rather than on host-exudates (Lapp & Skoropad, 1978).

Once the appressorium has been formed on the mango, the timing of events becomes uncertain, and this is the time when the latent period is initiated. The question still remains as to whether an infection peg penetrates the cuticle before the infection becomes latent, or if the appressorium lying on the cuticle is the latent state. This question is important for biological control because, if an infection peg does form, it is likely to be more difficult for an externally applied biological control agent to be able to control disease at

a subcuticular location.In this study we were not able to establish at which stage of the infection process the latent period is initiated due to the problems experienced with the ultrastructural work. This aspect requires more research to confirm the findings by Daquioag & Quimio (1979) that no subcuticular structures are formed before the latent period or maybe subcuticular structures are present as Simmonds (1963) suggested.

2.4.3 Effect of external factors on *C. gloeosporioides* - Spores of the *C. gloeosporioides* isolate (24) used in these experiments were able to germinate and form appressoria at temperatures as low as 10°C. However, for isolates from the Philippines, spore germination and appressorium formation was very poor, <10%, at 15°C (Estrada, 1990). Isolate 24 did not produce appressoria at 30°C, in contrast to the Philippino isolates which did produce appressoria at this temperature. This indicated that there are differences between isolates dependent on climatic conditioning. Lenné (1978), however, showed that for a large number of *Colletotrichum* spp. tested, under standard conditions there are no major differences between behavioural patterns. The only general distinction that could be made was that there was a shift in optimum temperature for spore germination and appressorium formation between temperate and sub-tropical isolates. For many isolates it has been observed that temperature range for spore germination is larger than for appressorium formation (see Parbery, 1981), as was found in this study. It has been suggested that at higher temperatures protein synthesis is disturbed (Tani *et al.*, 1977), which results in a shift of the metabolic pathway to form germ tubes rather than appressoria (Ishida & Akai, 1969).

Humidity is important for spore germination, and for an isolate of *C. gloeosporioides* from rubber (*Hevea brasiliensis*) it was determined that free surface water was necessary for germination (Wastie, 1972). For isolates from the Philippines it was observed that spore germination can take place at 30°C at a RH of 95%, where no free water was present (Estrada, 1990). A relationship between leaf wetness duration, temperature and disease incidence has been shown for *C. acutatum* on strawberry (Wilson *et al.*, 1990), *C. gloeosporioides* on mango (Fitzell *et al.*, 1984; Dodd *et al.*, 1991b) and *C. coccodes* on tomato (Dillard, 1989). Chakraborty *et al.* (1990) determined that on *Stylosanthes* in the field the most frequent leaf wetness periods lasted less than two hours, whereas the next most frequent periods lasted between 10 and 12 hours. At night leaf wetness periods lasted longer. Leaf wetness periods in tree canopies will follow a different pattern of duration, with longer periods experienced within the canopy than on the periphery of the tree.

It was nevertheless surprising to find that spore germination by *C. gloeosporioides* on mango fruit *in vitro* commenced between 12 and 24 hrs, and the percentage of germination continued to increase up to 36 hrs, as was also observed by Estrada (1990). Thus a long period of leaf wetness is required before a serious likelihood of infection is experienced. In comparison, infection of *Stylosanthes* by *C. gloeosporioides* was favoured by a period of 16 hrs or more of leaf wetness (Chakraborty *et al.*, 1990).

The optimum pH for growth of most isolates is around 6 (Lenné, 1978; Purkayashta & Sen Gupta, 1975; Dillard, 1988; Leu & Chang, 1988). Parbery (1981) stated that no species of *Colletotrichum* produced appressoria at a pH of 8 and above. In this study it was observed that sensitivity to a high pH decreased with increased nutrient status of the fungus. Effects of pH on spore germination and appressorium formation were not determined in this study while this might be more relevant than the effects on mycelial growth.

The effects of nutrients on germination confirm earlier work which also found that high levels of exogenous nutrients stimulate spore germination and decrease appressorium formation. This was first shown with orange extracts, which stimulated spore germination and appressorium formation of *C. lindemuthianum*, resulting in increased saprophytic growth and less disease development (Mercer *et al.*, 1970). One difference between their work and that described here, is that glucose alone had no effect on *C. lindemuthianum* but did in this study on *C. gloeosporioides*. Binyamini & Schiffmann-Nadel (1972b) found that increasing concentrations of sucrose, up to 5%, increased the percentage of spore germination in *C. gloeosporioides*. For a wide range of *Colletotrichum* spp., 1% peptone increased the percentage of spores germinating by an average of 10 - 15%, while the number of spores forming appressoria was reduced by 82% (Lenné, 1978). Glucose, sucrose and a mixture of glucose and peptone had the same effect and resulted in stimulation of mycelial growth. Glucose (0.1%) has also been shown to induce the appressoria of *C. gloeosporioides* to germinate (Dey, 1933). *C. piperatum* differed, in that increasing concentrations of sucrose, up to 0.1M, increased both the percentage of spore germination and appressorium formation; at sucrose concentrations above 0.1M both spore germination and appressorium formation were inhibited (Grover, 1971). Addition of amino acids did not increase either spore germination or appressorium formation, even in the presence of 0.1M sucrose.

For successful biological control it would be advantageous if appressorium formation could be reduced since appressoria are difficult structures to destroy whereas if saprophytic growth was stimulated, the resultant hyphae might be more easily attacked.

3 The isolation and selection of potential antagonists against *Colletotrichum gloeosporioides*

3.1 Objectives

The objectives of the experiments described in this chapter were to isolate and screen a large number of micro-organisms from environments in which *Colletotrichum* occurs, and to select organisms which have most potential as antagonists.

This was done by the following methods :

1 - Potential antagonists were isolated from mango leaf, blossom and fruit material and screened *in vitro* for their potential to inhibit mycelial growth and spore germination of *C. gloeosporioides.*

2 - Organisms that showed inhibition of both mycelial growth and spore germination were tested for their potential for inhibition of the development of post-harvest anthracnose lesions on mangoes

3 - Two antagonists, isolate 204, a *Bacillus* sp., and isolate 558, a *Pseudomonas* sp., were selected for further trials.

3.2 Materials & methods

3.2.1 Isolation - Bacteria, yeast and filamentous fungi were isolated from (i) Kenyan pickling mangoes and Peruvian mangoes cv. 'Haden' bought at Nine Elms Market, London, (ii) mango leaves collected in India, Sri Lanka, Tanzania, Thailand and Uganda, and (iii) mango blossom collected in the Philippines. Surfaces of fruit and leaves were swabbed with a sterile swab which was then shaken in 10 ml dH_2O. Mango blossom was directly vortexed in 10 ml of water. Dilution series were prepared and plated out on nutrient agar (NA; Oxoid) for isolation of bacteria and malt extract agar with antibiotics (MEA$^+$) for isolation of yeasts and filamentous fungi. From plates on which distinct colonies were visible, pure cultures of bacteria were isolated onto fresh plates of NA and yeasts were isolated onto plates of MEA. For isolation of fungi a square plug (0.5 cm^2) was cut from the edge of the colony and transferred to MEA. The fungal isolates were identified to genus level to obtain an indication of the resident population on mango fruit and leaves.

Additional filamentous fungi were obtained from culture collections and these are listed in Table 3.1.

Table 3.1 Origin and mean % inhibition performed in the test on MEA for the *Trichoderma* spp.

Isolate	Number	Source	% inhibition
Trichoderma sp.1	PJ1[1]		40
" " sp.2	EJT[2]	soil	36
" " sp.3	UKC[3] 17	compost	33
" " sp.4	This study	mango fruit	65
" " sp.5	This study	mango blossom	54
T. viride 1	IMI 298376		40
" " 2	UKC		25
T. harzianum 1	IMI 298372		58
" " 2	IMI 298373		62
T. koningii	UKC		30

[1] Obtained from P. Jeffries, University of Kent, Canterbury
[2] Obtained from E.J.Thompson, University of Kent, Canterbury
[3] Obtained from the culture collection at the University of Kent, Canterbury

3.2.2 Inhibition on MEA - Bacteria and yeasts, grown on NA and MEA respectively, were point inoculated at three equidistant points at 2.5cm round a mycelial plug (0.5 cm diameter) of *C. gloeosporioides* (Figure 3.1.a) and plugs of filamentous fungi were inoculated opposite a plug of *C. gloeosporioides* at a distance of 4cm (Figure 3.1.b) on MEA (plugs were always taken from the edge of an actively growing colony). Three replicate plates were inoculated for experiments with filamentous fungi.

After 5 days, the colony diameter of *C. gloeosporioides* was measured at R_2 (Figure 3.1.a&b) and percentage inhibition was calculated according to the following formula (Whipps, 1987) in which R_1 is the diameter of mycelial growth on a plate inoculated with *C. gloeosporioides* alone :

$$\%\text{inhibition} = \frac{R_1 - R_2}{R_1} \times 100 \qquad \text{(Whipps, 1987)}$$

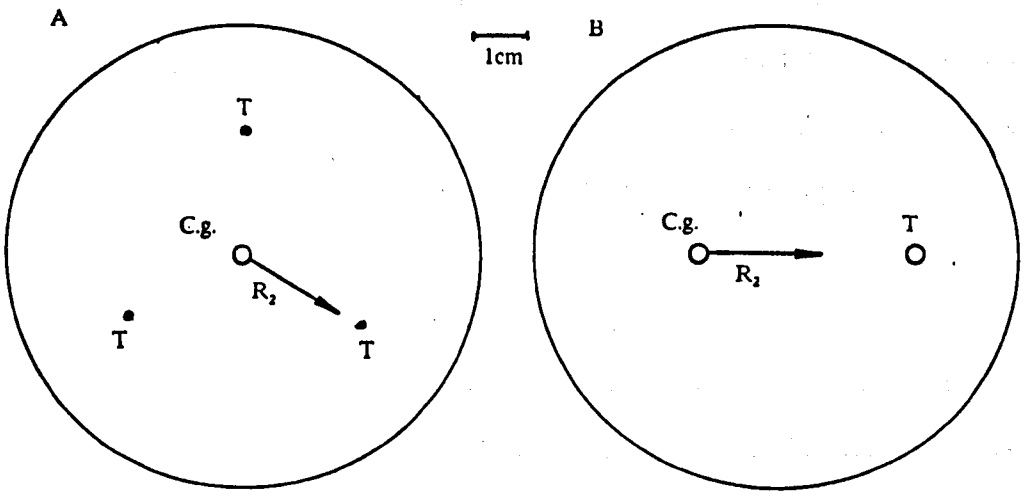


Figure 3.1 A) Three point inoculation of yeast and bacteria (T) with a plug of *Colletotrichum gloeosporioides* (C.g.) in the centre. B) Dual culture with a plug of the fungal isolate (T) to be tested on the right and a plug of *C. gloeosporioides* on the left (see text for explanation).

After another 3 weeks plates were assessed a second time and given a rating according to the coding listed below (adapted from Whipps, 1987) :

0 - Both organisms stopped growing where they met

1 - Antagonist overgrowing pathogen, pathogen has stopped growing

1- Antagonist inhibits pathogen, but does not overgrow the pathogen

1/2- Antagonist overgrowing pathogen, but pathogen is still growing

2/1- Pathogen overgrowing antagonist, but antagonist is still growing

2- Pathogen inhibits antagonist, but does not overgrow the antagonist

2 - Pathogen overgrowing antagonist, antagonist has stopped growing

3 - Mutual inhibition, zone ≤3mm

4 - Extreme inhibition, zone >3mm

3.2.3 Inhibition of spore germination - Bacteria and yeasts were grown in nutrient broth (NB; Oxoid CM67) and malt extract broth (MEB) respectively on an orbital shaker (160 rpm, 25°C) for 4 days. Cultures were diluted with dH_2O to approx. 10^7 cfu/ml and 50 μl was inoculated onto cellophane overlying TWA. To do this the cellophane was boiled twice in dH_2O, autoclaved and put onto the agar plates, covering the entire plate. Plates with cellophane where then exposed to U.V. light for 15 min to eradicate any contamination which could have occurred during handling. Approximately 100 spores of *C. gloeosporioides* were then added and mixed with the yeast or bacteria on the cellophane. Plates were examined daily for growth of *C. gloeosporioides*.

3.2.4 Antagonism on mango fruit - Before large numbers of antagonists were screened on fruit, an initial test was carried out to determine if it was relatively easy to infect the mangoes with *C. gloeosporioides* and which would be the best way to incubate the mangoes over several weeks. Fruits were first dipped in a suspension of *C. gloeosporioides* spores (see 2.2.3; 10^5 spores/ml), incubated in plastic bags for 15 hrs to stimulate spore germination, then transferred either into paper bags, paper bags with holes, plastic bags, or plastic bags with holes. Anthracnose lesions developed on all fruit, but slightly larger lesions were observed on the fruit incubated in plastic bags with holes, and the fruit incubated in plastic bags developed more secondary rots due to fungi such as *Aspergillus* and *Penicillium*. It was thus decided to incubate the mangoes in paper bags, after the antagonist dips, for the first experiment and in plastic bags, for the second experiment, to favour the antagonists.

Bacterial and yeast cultures were prepared as described in 3.2.3. and spore suspensions of *Trichoderma* were prepared by flooding sporulating plates of the fungus with sterile dH_2O, loosening the spores with a loop and diluting the spore suspension to give a final concentration of 10^5 spores/ml. All cultures were diluted with 0.5% peptone. Peptone was added to aid survival and establishment of the antagonists and to promote saprophytic growth of *C. gloeosporioides* (see 2.3.6).

Mangoes were surface sterilised in 1% bleach and left to dry, dipped in a spore suspension (see 2.2.3; 10^5 spores/ml) of *C. gloeosporioides*, and incubated in plastic bags for 15 hrs. After this incubation period plastic bags were removed and mangoes dipped in the antagonist suspensions, drip-dried and incubated in paper bags for the first experiment and plastic bags for the second experiment. For the two batches of mangoes used in the dipping experiments, disease development and breaking of colour, as an index for ripening (see Figure 3.2.a & b for disease and colour index) were assessed at 5, 10 & 19 days for the Haden mangoes,

and at 8, 13 & 18 days for the variety Tommy Atkins. Both batches of mangoes were imported from Brazil and did not ripen properly, presumably because they were harvested too early, but anthracnose development was nonetheless satisfactory for experimental purposes. The Tommy Atkins mangoes also developed a soft rot (bacterial or physiological) which hindered anthracnose assessment for the second and third assessments. Both batches of mangoes also developed stem end rot, but this did not hinder anthracnose assessment.

3.2.5 Dip experiments, Philippines - Cultures were prepared as described in 3.2.2. but grown at ambient temperatures (23 - 30°C). Fruit were either dipped for 10 min in hot water at a temperature of 55°C (HWT), 500ppm benomyl (50°C), bacterial or yeast suspensions containing 0.5% peptone (ambient temperature, 27°C), or first dipped for 10 min in HWT followed by the bacterial and yeast dips at ambient temperatures. Assessments were carried out as described in Figure 3.2.

a)

b)

Figure 3.2 a) Colour index of Carabao mangoes. 1 - full green, 2 - breaking stage, tinge of yellow, 3 - more green than yellow, 4 - more yellow than green, 5 - tinge of green, and 6 - full yellow.

b) Disease index of Carabao mangoes. 1 - no spots, 2 - 1-3 spots, 3 - 4-6 spots, 4 - 7-12 spots, and 5 - >12 spots or 30% of the mango affected (Pordesimo, 1979).

3.3 Results

3.3.1 Isolation and inhibition on MEA - In total 247 bacteria, 210 yeast and 191 filamentous fungi were isolated and put through the first screening procedure. Numbers of isolates and place of origin are given in Table 3.2 and a list of the fungal genera is given in Table 3.3. Neither of these tables represent complete natural populations since the method of isolation does not allow all organisms to grow. The list of fungal genera includes many that are also pathogenic to mango; for example *Dothiorella* causes stem end rot on

Table 3.2 Source and type of organisms isolated from mango material and the success rate in the screening procedure.

Source	bacteria	yeast	fungi	to 2nd[1]	U.K.[2]	Phil.[3]	Final[4]
Kenya pickling mangoes	- 17 29	18 12 9	30 14 14	37	20	4	1
Peru mangoes	15	6	16	4	-	-	-
Sri Lanka leaves	70	62	63	24	5	1	-
Uganda leaves	25	14	23	10 (2)[5]	2	1	1
Tanzania leaves	25	40	22	13 (3)	8	1	-
Thailand leaves	11	15	5	6 (1)	2	-	-
India leaves	35	19	4	3	1	-	-
Philippines blossom	20	15	-	11 (8)	1	-	-
Other	10	-	-	10	5	-	-
Total	247	210	191	118	44	7	2

[1] - Number of bacteria and yeasts carried over to the second screening.
[2] - Number of bacteria which were tested *in vivo* on Brazilian mangoes in the U.K.
[3] - Number of bacteria and yeasts tested in the first trials in the Philippines.
[4] - Number of bacteria finally selected for further trials.
[5] - Number of isolates carried over showing <20% inhibition.

Table 3.3 Genera of filamentous fungi isolated from mango leaves, blossom and fruit and their mean inhibition zone as determined by the first screening procedure.

GENUS	NUMBER OF ISOLATES TESTED	MEAN INHIBITION (%)	MIN-MAX (%)
Alternaria	3	27	23-30
Aspergillus	12	30	4-41
Chaetomium	3	9	7-11
Choanephora	4	-	-
Cladosporium	31	10	0-23
Colletotrichum	4	30	26-33
Curvularia	2	33	31-35
Cylindrocarpon	1	27	-
Dothiorella/ Lasiodiplodia	13	64	42-77
Fusarium	30	28	4-46
Gliocladium	1	23	-
Mucor	1	4	-
Penicillium	46	21	-7-44
Pestalotiopsis	43	30	17-44
Phoma-like	6	25	12-36
Scopulariopsis	3	18	12-31
Trichoderma	2	60	54-65
Trichothecium	5	26	15-44
Verticillium ?	1	4	-

mangoes and *Penicillium* and *Aspergillus* are secondary post-harvest moulds. Because none of the fungal isolates, except for the *Trichoderma* spp., showed biocontrol potential (inhibition zones for the genera are given in Table 3.3), it was decided to concentrate on the yeasts, bacteria and the *Trichoderma* spp. All of the *Trichoderma* spp. including the two that were isolated from mango in this study, inhibited the growth of *C. gloeosporioides* by >25%, and 4 isolates by >50%, compared to that of the control, (Table 3.1). Of the bacteria and yeasts, 104 isolates showed >20% inhibition of *C. gloeosporioides* in dual culture compared to the growth of a colony of *C. gloeosporioides* in single culture. The extent of inhibition varied from a minimum of -7% (Isolate 712) up to a maximum of 73% (Isolate A; Appendix 3.1). Few isolates produced

a clearly visible inhibition zone with no growth between themselves and *C. gloeosporioides*. In total 118 organisms were selected for the second screening, the 104 organisms which showed ≥20% inhibition plus an additional 14 which showed inhibition ranking from -7% to 19%. Only 6 out of the 118 organisms carried over to the second screening showed an inhibition zone at the second assessment after a total of 26 days (see Appendix 3.1. for percentage inhibition and 2nd assessment for the 118 organisms carried over to the second screening).

3.3.2 Inhibition of spore germination - Of the 118 isolates tested, 33 inhibited spore germination of *C. gloeosporioides* when inoculated jointly onto the cellophane. In the presence of these 33 isolates no hyphae of *C. gloeosporioides* could be distinguished under the dissecting microscope (x50) and with eleven other isolates only very fine hyphae could be seen on the cellophane (see Appendix 3.1). All the other isolates allowed *C. gloeosporioides* spores to germinate and form mycelium which was easily observed with the naked eye. Thus in total 44 isolates inhibited both germination and growth after germination to some extent and these isolates were selected to be tested on fruit.

To examine what happened to the *C. gloeosporioides* spores in presence of the bacteria, dual inoculations of eleven isolates were made on glass slides. Ten μl of a $5x10^4$ spores/ml of *C. gloeosporioides* (see 2.2.3) and 10 μl of approximately 10^7 cfu/ml of bacteria in dH_2O, were inoculated simultaneously onto glass slides. Slides were incubated for 24hrs in a moist chamber and germination and appressorium formation were determined. Most isolates inhibited spore germination on glass slides, with the exception of isolate 208, no inhibition or stimulation; 210, showing 92% stimulation; 118, showing 93% stimulation; and 216, showing 214% stimulation of spore germination (Table 3.4). Isolate 558 was outstanding in that it inhibited spore germination completely, and consequently no appressoria were formed. The presence of all other isolates, except 222, resulted in an increase in appressorium formation by *C. gloeosporioides* compared with the control. In the case of co-inoculation with isolate 222, a slight decrease (8%) in frequency of appressorium formation was noted.

Table 3.4 Germination and appressorium formation of *C. gloeosporioides* spores on glass slides, in the presence of bacteria.

Isolate	% germination	% inhibition	% appressoria	% inhibition
control	29		26	
115	22	24	70	-169
118	56	-93	33	-27
202	12	59	54	-108
204	8	72	75	-188
208	30	0	60	-131
210	55	-92	70	-169
211	13	55	54	-108
215	15	48	31	-19
216	91	-214	96	-269
222	8	72	24	8
558	0	100	-	-
mean	27		54	
S.E.	3.6		3.6	

3.3.3 Fruit tests - It was decided to screen five *Trichoderma* isolates (*Trichoderma viride* 1 & 2 and *Trichoderma* spp. 2, 3 & 5) in addition to the 27 bacterial and 17 yeast isolates carried over from the spore germination test.

Disease indices (3 mangoes/treatment) for mangoes treated with each antagonist and for the two batches of mangoes are given in Appendix 3.2. Colour indices were recorded, but are not given here since there were no differences between treatments. Isolates were ranked according to disease index and rankings combined for all assessment days, and for the two batches of mangoes. This resulted in the ranking also given in Appendix 3.2. The following isolates were selected for further trials : bacterial isolates 104 (ranked 1), 202 (ranked 5), 204 (ranked 2) and 558 (ranked 27) and the yeast isolates 251 (ranked 11), 475 (ranked 8) and 651 (ranked 3). Isolate 558 was selected because of its excellent performance in inhibiting spore germination on glass slides, even though it was only ranked 27th in this fruit trial. None of the *Trichoderma* spp. were included because problems in applying the fungi to the fruit surface were experienced and hence it was decided to continue work only with the bacteria and yeasts.

3.3.4 Post-harvest dip experiments in the Philippines - Three batches of mangoes were used, all variety Carabao, from Iloilo, Pangasinan and Los Baños. The mangoes from Los Baños were harvested immature and dipped in the U.K. three days after harvesting, but otherwise treated the same as the mangoes from Iloilo and Pangasinan. The antagonist treatments were carried out with the bacterial isolates 104, 202, 204 and 558 and the yeast isolates 251, 475 and 651. Mangoes were assessed for disease and colour indices (see 3.2.5) at 5, 7 & 10 days for the mangoes from Iloilo (8 mangoes/treatment) and Pangasinan (7 mangoes/treatment), and at 7, 10 & 14 days for the mangoes from Los Baños (6 mangoes/treatment). Colour breaking was significantly faster in fruit from Iloilo and Pangasinan treated in hot water than in fruit treated in cold water (Tables 3.5.b & 3.6.b). There was, however, no significant effect of any treatment with antagonistic microorganisms on colour breaking of fruit from Iloilo, Pangasinan and Los Baños (Tables 3.5.b, 3.6.b & 3.7.b).

Anthracnose development was significantly less in hot water treated fruit than in cold dipped fruit, indicating that the hot water treatment alone was the most successful treatment (Tables 3.5.a, 3.6.a & 3.7.a). For all three batches of mangoes there was no significant reduction in disease of any of the antagonist treatments, compared to the control treatments, both for hot water treated fruit as well as cold water treated fruit. If a similar ranking is done as in 3.3.3 for %disease with cold antagonist dips, the following order of treatments is obtained, starting with the treatment which had less disease overall : 204, 104, 558, control, 202, 475, peptone, 651 and 251. However, it must be borne in mind that although we can rank the treatments, this was not significant and thereby only facilitates making a choice. The 'best' antagonist treatments were (of cold treated fruit) 204 (disease index 4.3), 475 (4.6), and 558 (4.4) for Iloilo mangoes (10 days; Table 3.5.a); 204 (3.7), 475 (4.1) and 651 (4.3) for Pangasinan mangoes (10 days; Table 3.6.a); and 104 (3.6), 204 (3.7) and 558 (3.4) for Los Baños mangoes (14 days;Table 3.7.a), compared to the controls (4.9 for Iloilo mangoes, 4.6 for Pangasinan mangoes and 4.2 for Los Baños mangoes). No antagonist treatment was as good for disease control as the HWT or the benomyl treatments which resulted in disease indices at 2.0, 3.4 and 1.2 for HWT, for Iloilo, Pangasinan and Los Baños mangoes resp., and 2.6 and 2.3 for hot benomyl treated fruit from Iloilo and Pangasinan resp.

From these results it was concluded that the treatments with antagonists had not resulted in any significant

Table 3.5 a) Disease and b) colour indices for mangoes from Iloilo (see text for explanation of treatments)

a) Disease

	Control	Peptone	104	202	204	251	475	558	651	mean	S.E.
5d.											
Cold	1.4	1.4	1.4	1.9	1.6	2.1	1.5	1.3	1.5	1.6	
Hot	1.0	1.0	1.0	1.1	1.1	1.3	1.1	1.1	1.1	1.1	.06
mean	1.2	1.2	1.2	1.5	1.4	1.7	1.3	1.2	1.3		
S.E.									.13		.19
7d.											
Cold	2.6	3.5	2.4	3.6	2.6	3.9	2.6	2.8	3.3	3.0	
Hot	1.4	1.5	2.0	1.1	1.4	1.4	1.4	1.3	1.5	1.4	.12
mean	2.0	2.5	2.2	2.4	2.0	2.6	2.0	2.0	2.4		
S.E.									.25		.36
10d.											
Cold	4.9	4.5	4.9	4.8	4.3	4.8	4.6	4.4	4.9	4.7	
Hot	2.0	1.9	2.3	2.1	2.8	2.6	1.8	2.1	2.8	2.3	.12
mean	3.4	3.2	3.6	3.4	3.5	3.7	3.2	3.3	3.8		
S.E.									.26		.37

b) Colour

	Control	Peptone	104	202	204	251	475	558	651	mean	S.E.
5d.											
Cold	2.0	3.1	2.9	2.8	3.0	3.6	2.9	2.9	2.9	2.9	
Hot	3.8	3.4	4.0	3.8	4.0	4.0	3.3	3.9	3.8	3.8	.09
mean	2.9	3.3	3.4	3.3	3.5	3.8	3.1	3.4	3.3		
S.E.									.20		.28
7d.											
Cold	3.1	3.4	3.1	3.3	3.4	3.8	3.4	3.4	3.3	3.3	
Hot	4.1	3.5	4.3	4.3	4.4	4.3	3.5	4.3	4.4	4.1	.12
mean	3.6	3.4	3.7	3.8	3.9	4.0	3.4	3.8	3.8	4.1	
S.E.									.25		.35
10d.											
Cold	3.8	4.0	4.1	3.9	4.4	4.5	4.1	4.3	3.9	4.1	
Hot	4.8	4.5	5.1	5.0	4.9	5.1	4.3	4.9	5.1	4.8	.10
mean	4.3	4.3	4.6	4.4	4.6	4.8	4.2	4.6	4.5		
S.E.									.22		.31

Table 3.6 a) Disease and b) colour indices for mangoes from Pangasinan (see text for explanation of treatments)

a) Disease

	Control	Peptone	104	202	204	251	475	558	651	mean	S.E.
5d.											
Cold	1.7	2.0	2.3	1.3	1.3	1.7	2.0	2.4	2.9	2.0	
Hot	1.1	1.0	1.0	1.1	1.1	1.2	1.3	1.3	1.1	1.1	.10
mean	1.4	1.5	1.6	1.2	1.2	1.4	1.6	1.9	2.0		
S.E.									.21		.29
7d.											
Cold	3.6	3.7	4.0	3.0	2.9	3.7	3.3	4.0	3.7	3.5	
Hot	1.4	2.1	1.7	1.6	1.7	1.8	1.9	1.7	1.6	1.7	.15
mean	2.5	2.9	2.9	2.3	2.3	2.8	2.6	2.9	2.6		
S.E.									.32		.46
10d.											
Cold	4.6	4.9	5.0	4.6	3.7	4.6	4.1	4.6	4.3	4.5	
Hot	3.4	3.7	3.0	3.0	2.7	3.3	2.6	3.4	3.4	3.2	.17
mean	4.0	4.3	4.0	3.8	3.2	3.9	3.4	4.0	3.9		
S.E.									.36		.52

b) Colour

	Control	Peptone	104	202	204	251	475	558	651	mean	S.E.
5d.											
Cold	3.9	4.0	3.9	4.1	3.0	3.1	3.6	4.1	4.3	3.8	
Hot	5.0	4.7	4.3	4.6	4.3	4.5	4.6	4.4	3.9	4.5	.13
mean	4.4	4.4	4.1	4.4	3.6	3.8	4.1	4.3	4.1		
S.E.									.27		.38
7d.											
Cold	4.7	4.4	4.4	5.0	3.7	4.4	4.3	5.0	4.6	4.5	
Hot	5.1	5.3	4.7	5.3	4.9	5.0	4.9	4.7	4.7	5.0	.09
mean	4.9	4.9	4.6	5.1	4.3	4.7	4.6	4.9	4.6		
S.E.									.19		.27
10d.											
Cold	5.0	5.1	4.7	5.1	4.6	5.0	5.1	5.1	5.3	5.0	
Hot	5.6	5.3	5.3	5.8	5.3	5.4	5.4	5.1	5.0	5.4	.08
mean	5.3	5.2	5.0	5.5	4.9	5.2	5.3	5.1	5.2		
S.E.									.17		.24

Table 3.7 a) Disease and b) colour indices for mangoes from Los Baños (see text for explanation of treatments)

	a) Disease index			b) Colour index		
Treatment	7 days	10 days	14 days	7 days	10 days	14 days
Control	1.7	2.8	4.2	3.3	3.8	4.7
Peptone	2.0	2.8	3.7	3.2	4.3	4.7
104	1.7	2.7	3.6	2.7	4.0	4.8
202	1.7	3.0	4.0	2.7	4.0	4.5
204	1.5	1.8	3.7	3.3	4.2	4.8
251	2.0	3.5	4.8	3.0	4.3	5.0
475	2.3	3.7	4.3	3.8	4.3	5.0
558	1.8	2.6	3.4	2.6	3.4	4.6
651	1.3	3.3	4.2	3.2	4.3	4.8
HWT	1.0	1.2	1.2	3.2	3.5	4.7
Mean	1.7	2.7	3.7	3.1	4.0	4.8
S.E.	.58	.75	.71	.53	.60	.36

control of anthracnose disease. However, one problem with these post-harvest treatments was that the normal practice of leaving mangoes to dry before packing them into boxes had been followed. This avoids post-harvest rots yet also creates an unfavourable climate for survival of the antagonists, since antagonists will either die or go into a resting state (*Bacillus* sp.) and they will not be able to actively antagonise *C. gloeosporioides*. Nevertheless, from the ranking and disease indices, it was decided to carry on future experiments with isolates 204 (a *Bacillus* sp.) and 558 (a *Pseudomonas* sp.) only.

3.3.5 Inhibition of stem end rot - From the mango dipping experiments described above it was clear that stem end rot is also a major post-harvest problem. It has been reported to be second in importance to anthracnose in terms of fruit loss (Cappellini & Lightner, 1988; Johnson *et al.*, 1990a). Stem end rot is caused by a complex of fungi, and Johnson *et al.* (1990a) have isolated 6 different species from lesions, with *Lasiodiplodia* and *Dothiorella* predominating. Cultural practices, such as leaving the pedicel intact, should reduce this disease to an acceptable level. However, both batches of mangoes from Brazil bought from fruit importers and intended for the commercial market suffered from this disease. It would be advantageous to

develop a biological control system that would reduce stem end rot in addition to anthracnose disease, either by using the same organism, or by using a mixture of organisms.

An initial test was carried out to determine the potential for antagonism against stem end rot by the isolates which were selected to determine their potential to control anthracnose *in vivo* (Appendix 3.1). These organisms were tested against two isolates of *Dothiorella / Lasiodiplodia*, one from Sri lanka and another isolate from a Kenyan pickling mango. The method used was a test of inhibition of growth on MEA (see 3.2.2). The stem end rot fungus is a fast growing fungus and only one bacterial isolate, 210, and one yeast isolate, 651, induced the formation of an inhibition zone between itself and the stem end rot fungus (Appendix 3.3). It is interesting to note that inhibition of isolate 1, from Sri Lanka, occurred more frequently than inhibition of isolate 2, from Kenya, indicating different responses by different isolates.

3.4 Discussion

The justification of an *in vitro* screen has long been a point of discussion among scientists looking for biological control agents (Campbell, 1986, 1989; Merriman & Russell, 1990). It has frequently been used because it is cheap, relatively fast and large numbers of organisms can be screened in Petri-dishes. Baker & Cook (1974) outlined why testing the inhibition of a pathogen by potential antagonists on agar plates has its disadvantages. They wrote principally in relation to soil pathogens but identical arguments can be used for phylloplane organisms. The main reasons for their criticism are: 1. Antibiotics may not be produced *in vivo*, but the antagonist does produce these compounds *in vitro* when nutrients are abundant; 2. Agar media may favour the antagonist rather than the pathogen and are selective for antibiosis; 3. There are no other organisms present that the antagonist has to compete with; 4. Environmental conditions in Petri-dish tests are different from the *in vivo* situation; 5. The pathogen is in a different state on the agar plate than it actually will be *in vivo*.

All these arguments are valid, especially when considering the production of secondary metabolites. Even if an antibiotic is produced *in vivo* it does not follow that it will actively play a role in antibiosis since external factors might deactivate the compound. The question of whether compounds are produced in large enough quantities to have any effect *in vivo* must also be considered. These problems are particularly apparent in soil where the soil can physically bind the antibiotic (Williams & Vickers, 1986), and further

microbiological breakdown of compounds could take place (Pramer & Starkey, 1951; Howell & Stipanovic, 1983). Few examples are known where it has been proven that antibiotics are both produced and are active *in vivo*, the best documented examples being the antibiotics pyrrolnitrin (Howell & Stipanovic, 1979) and phenazine-1-carboxylic acid (Thomashow *et al.*, 1990), both produced by fluorescent pseudomonads.

In order to avoid drawing conclusions from limited, *in vitro* screening, Whipps (1987) advised using a range of media, including media with a high and low nutrient status, to screen antagonists. This would have several advantages, but would reduce the number of organisms that could be screened and an initial selection will still have to be made. This study was begun by screening potential candidates on both MEA and TWA, but this yielded no great differences in activity and was abandoned due to the extra amount of work it generated. Antagonists were subsequently screened only on MEA. Besides nutrient status, other environmental conditions such as water potential (Campbell & Clor, 1985; Whipps & Magan, 1987) and pH (Dennis & Webster, 1971a; Ho & Ko, 1985) may influence the results obtained. A study carried out by Whipps & Magan (1987) showed that when both antagonist and pathogen were stressed by growing them under low water potential and/or low nutrient status, either the antagonist may not produce its antibiotic, or the pathogen may respond differently to antibiotics. That both the availability of water and nutrients are important on the leaf surface seems clear and both antagonists and pathogens will often live under conditions of stress (Campbell, 1985; Cullen & Andrews, 1984b).

Tronsmo & Raa (1977) carried out preliminary screens of organisms antagonistic to *Botrytis cinerea*, causal agent of dry eye rot in apple. Subsequently they found that their selected *Trichoderma* sp. was not able to control development of the pathogen at 4°C, while it could control this disease at temperatures of 9°C and above. They realised that their isolate was unable to grow below 9°C, while *B. cinerea* can still grow at temperatures as low as 3°C. The same was observed for a *Trichoderma* sp. used to control *B. cinerea* on strawberries, where temperatures in the field vary between 7 and 13°C (Tronsmo & Dennis, 1977). Once these researchers started to select organisms at the temperatures experienced in the field they observed effective control of *B. cinerea* with *Trichoderma* spp. The experiments described in this thesis have all been carried out at 25°C, which is the approximate average temperature in the Philippines. However, mangoes intended for the export market are transported at a temperature of 12°C, and it may therefore have been appropriate to carry out some experiments at both temperatures. Another consideration is the pathogen which is used for screening. Andrews *et al.* (1983) found that two isolates of *Venturia inaequalis*, a brown,

moderately virulent wild type strain, and a green, highly virulent mutant strain, varied in their response to a range of antagonists. But overall ranking of antagonists when tested against both strains, was similar for both the brown and green strain. In the experiments described here only isolate 24 was used for all *in vitro* screens, but whether this isolate responds in the same way as other isolates is considered in Chapter 6. The two isolates of the stem end rot fungus did show a differential response in the screen against a range of potential antagonists.

As the criticisms of using a limited *in vitro* screening system are many, it is worthwhile justifying why we did not consider screening directly on the fruit surface. The main reason for this was the cost. A study using fresh mangoes would have been possible in the Philippines in the height of the mango season, but the high price of mangoes in the U.K. prohibited carrying out trials on fruit in the U.K. The infection studies discussed in Chapter 2 were intended to yield a cheap model system in which *C. gloeosporioides* would make latent infections on another host, which would then have been used to screen large numbers of organisms *in vivo*. However no satisfactory model system was obtained. The use of initial screens on agar could be justified in that one screen was carried out under nutrient rich conditions and another under nutrient poor conditions simulating to some extent the leaf/fruit surface. We also looked at both the effect of antagonists on mycelial growth and on spore germination, taking two stages of the life cycle into consideration, to give a good indication of which candidates had potential. One observation that was made was that, of the eleven bacteria observed on glass slides, nine isolates did stimulate appressorium formation (Table 3.4), an observation also made by Parbery & Deverall (1977). This is a characteristic not desired for a biological control agent, since these structures will be the most difficult to attack. It would have been ideal to determine if appressoria were still viable after an encounter with an antagonist, but as discussed previously (2.3.8), a method to test appressorial viability was not available.

To overcome some of these arguments it was decided at this point to use a large number of isolates in an artificial *in vivo* screen in which appressoria would be present on the fruit surface, thus avoiding the problems of looking at appressoria in an artificial situation. These experiments were carried out in the U.K. and were designed such that they favoured growth of the antagonists. Common post-harvest practices in the Philippines do not favour microbial growth and survival on the fruit surface and may need to be adjusted in the light of experiments described here. Methods of application of a biological control agent to the fruit which are both acceptable for antagonist and host need to be devised to make biological control work, and this will be further discussed in Chapter 4.

Another factor that has to be taken into consideration is the initial selection of which organisms are going to be used in the screen. In this particular case organisms were taken from the fruit, leaf and blossom of mango, representing organisms present in both pre- and post-harvest environment. Cullen & Andrews (1984b) reason that organisms naturally present on leaves are r-strategist-type microbes with a high competitive potential and populations of these organisms tend to fluctuate drastically (Andrews & Rouse, 1982; Campbell, 1985). They also noted that the introduction of large numbers of exotic microbes might be necessary to make biological control work on the phylloplane. In this study it was not possible to sample anthracnose-free regions to investigate the possibility of finding antagonists there (Campbell, 1986), as the time-scale was too restricted. Lenné (1986) found in her work on *Stylosanthes capitata* that the resistance observed in this species against *C. gloeosporioides* in the rain forest was probably due to a population of antagonistic bacteria on the plant. It is not known if this is also true for other examples of the breaking of resistance when resistant lines are moved to other geographical areas. The example of the introduction of the so called resistant variety Julie into Dominica (Prior, 1987), where this variety was extremely susceptible to anthracnose, would be a prime example to study the resident microflora compared with that from an area in which this variety is resistant. So apart from environmental conditions which probably play a major role in the breaking of resistance it is not known if microorganisms are involved in resistance observed in mango. In this study bacteria and fungi were screened at random, though they were initially selected because they grew fairly rapidly on nutrient-rich media. The agar plate method discriminates against slow growing organisms, even though the plates were examined a second time after one week of incubation to see if any interesting organisms had appeared. This technique also does not allow all micro-organisms from the leaf surface to grow. For example no actinomycetes were obtained via this method. *Streptomyces* spp. had been used by Hsu & Lockwood (1969) as antagonists against *Glomerella cingulata*, and this is still a family that might offer good candidates, as long as they are not antibiotic producers (see Chapter 7). Because so little research had been done previously on the biological control of *C. gloeosporioides*, there was no indication as to which group of organisms would have potential for finding appropriate candidates, hence many isolates were tested in the first instance. The total number of organisms screened in this study, approx. 650, is nevertheless small compared to the number of organisms that are screened in industry to detect novel antibiotics (Williams & Vickers, 1986) and there is always the question as to where to set the limit of numbers.

Whatever organism is selected though it will be the investigator, who has to make a decision as to which organism to use for further trials. This choice will be based on the assumption that the biological system under investigation is understood. Even though this may look like an arbitrary decision to the outsider, with a good knowledge of the disease and of the environment in which the biological control is going to be applied, it should be possible to set criteria for a biological control agent on which to base a choice.

4 Post-harvest control with isolates 204 and 558

4.1 Objectives

To be able to determine the potential effectiveness of isolate 204 and 558, both isolates needed to be to tested under semi-commercial conditions. For this, a large scale post-harvest experiment was carried out at PHTRC, Philippines, using naturally infected mangoes. Apart from testing both organisms, this experiment would provide an opportunity to assess different methods of application. Several compounds were incorporated into the treatments to determine if they resulted in increased biological control activity. The compounds tested were (i) a commercial sticker, usually mixed with any fungicide treatment to enhance dispersal and adhesion to surfaces (Dixon, 1984); (ii) peptone, to promote saprophytic growth of *C. gloeosporioides* (see 2.4.3), and provide nutrients for the antagonists; (iii) incorporation into a hot water treatment; (iv) fruit wax; and (v) a sucrose polyester (Semperfresh), this a commercial fruit coating promoted to lengthen shelf life of fruit.

The overall objectives were :

1 - To assess if isolates 204 and 558 could be incorporated into existing commercial practices, it was necessary to assess the direct effect of a sticker, HWT, benomyl, sucrose polyester coating and fruit wax on the bacteria.

2 - To determine if both isolates were able to control anthracnose in a semi-commercial situation and to find the best method to apply the bacteria to the mango surface.

4.2 Materials & methods

4.2.1 Temperature survival of 204 & 558 - Suspensions of 204 and 558 were prepared by washing 3 day old cultures of both bacteria with dH_2O (see 3.2.3). Tubes with 10ml of bacterial suspensions ($8x10^3$ cfu/ml for 204 and 10^8 cfu/ml for 558) were incubated in water baths at 45°C (204), 50°C (204 & 558) and 55°C

(204). Samples were taken after 0, 15, 30, 60, 90 and 120 min. Dilution series were then prepared and samples spread onto NA plates. Colonies were counted after 48 hrs of incubation.

4.2.2 Effect of a sticker on 204 & 558 - Suspensions of 204 and 558 were prepared as in 4.2.1. and diluted in 0, .01, .05, .1, .5 and 1% sticker (Hyvis 5, polyisobutene, BP Chemicals Ltd.). Solutions were incubated at room temperature for 1 hr, dilution series prepared and plated out onto NA plates.

4.2.3 Effect of benomyl on 204 & 558 - The method used was similar to that described in 4.2.2, except that the sticker was replaced by five treatments of 0, 250, 500, 750 or 1000 ppm benomyl. The same concentrations of benomyl were also incorporated into NA, and both 204 and 558 streaked out onto these plates.

4.2.4 Effect of Semperfresh on 204 & 558 - The method used was similar to that described in 4.2.2, except that the sticker was replaced by 0, 0.5, 1, 1.5% sucrose polyester (Semperfresh, Semper Bio-Technology Ltd.).

4.2.5 Effect of a fruit wax on 204 & 558 - Fruit wax was prepared as follows :

Solution A -	Shellac	10g
	Triethanolamine	2g
	Water	100g
Solution B -	Sodium hydroxide	.6g
	Paraffin wax	16.4g
	Carnabau wax	5.5g
	Stearic acid	4.2g
	Water	100g

Solutions A and B were first dissolved separately at temperatures of 70°C and 93°C respectively, and then mixed together. When the solutions had cooled to a temperature of 50°C isolate 558 was incorporated into the mixture to obtain a final concentration of 10^7 cfu/ml. A sample was taken after 1 hr, dilution series prepared and plated out onto NA agar.

4.2.6 Post-harvest dips - a) Mangoes were harvested from three trees from the field site described in 5.2.2. These trees had not been used for any other experiment but had been sprayed with both fungicides and insecticides, as would be the normal practice, with the last fungicide spray approx. 6 weeks before harvest. For each treatment 5 mangoes from each tree were used, a total of 15 mangoes/treatment. Mangoes were dipped 24 hrs after harvest and stored at ambient temperature (23 - 30°C) in cardboard boxes.

b) Antagonist suspension were grown in MEB (204; see 6.3.3), or Czapek Dox liquid medium (CzDL; 558; see 6.3.3) for 4 days at ambient temperature on an orbital shaker. Bacterial suspensions were diluted for each treatment to a concentration of $5.5x10^6$ cfu/ml for isolate 204 and $6x10^7$ cfu/ml for isolate 558.

c) Treatments consisted of 204, 558 or no bacterial additions incorporated into (i) 0.1% sticker (Tenac, Shell); (ii) 0.5% peptone + 0.1% sticker; or (iii) a hot water treatment (50°C) + 0.1% sticker. Treated mangoes were either incubated dry or first incubated for a period of 48 hrs in plastic bags after which the plastic bags were removed and mangoes incubated dry for the remainder of the experiment.

Other treatments investigated were 204, 558 or no antagonist incorporated into a sucrose polyester (Semperfresh) and a fruit wax (Stafresh 320, FMC Corporation). Treatments following the normal practice were included as comparison: HWT (55°C), hot benomyl (50°C; 500ppm) and cold prochloraz (850ppm). See Table 4.1 for a summary of the treatments.

Table 4.1 Post-harvest treatments applied to 'Carabao' mangoes.

Treatment	isolate 204	isolate 558	no bacteria
0.1% sticker	✓	✓	✓
0.1% sticker + 0.5% peptone	✓	✓	✓
Hot water (50°C)	✓	✓	✓
Fruit wax	✓	✓	✓
Sucrose polyester	✓	✓	✓
Hot water (55°C)			✓
Hot benomyl (50°C)			✓
Prochloraz			✓

All mangoes were dipped for 10 minutes, except the mangoes for the fruit wax treatments, for which the manufacturers advice was followed and these fruit were dipped for only 2 minutes. Mangoes were assessed for disease and colour index (see 3.2.5) and for %disease at 7 and 10 days after dipping. %Disease was recorded as the area of the peel covered by anthracnose lesions.

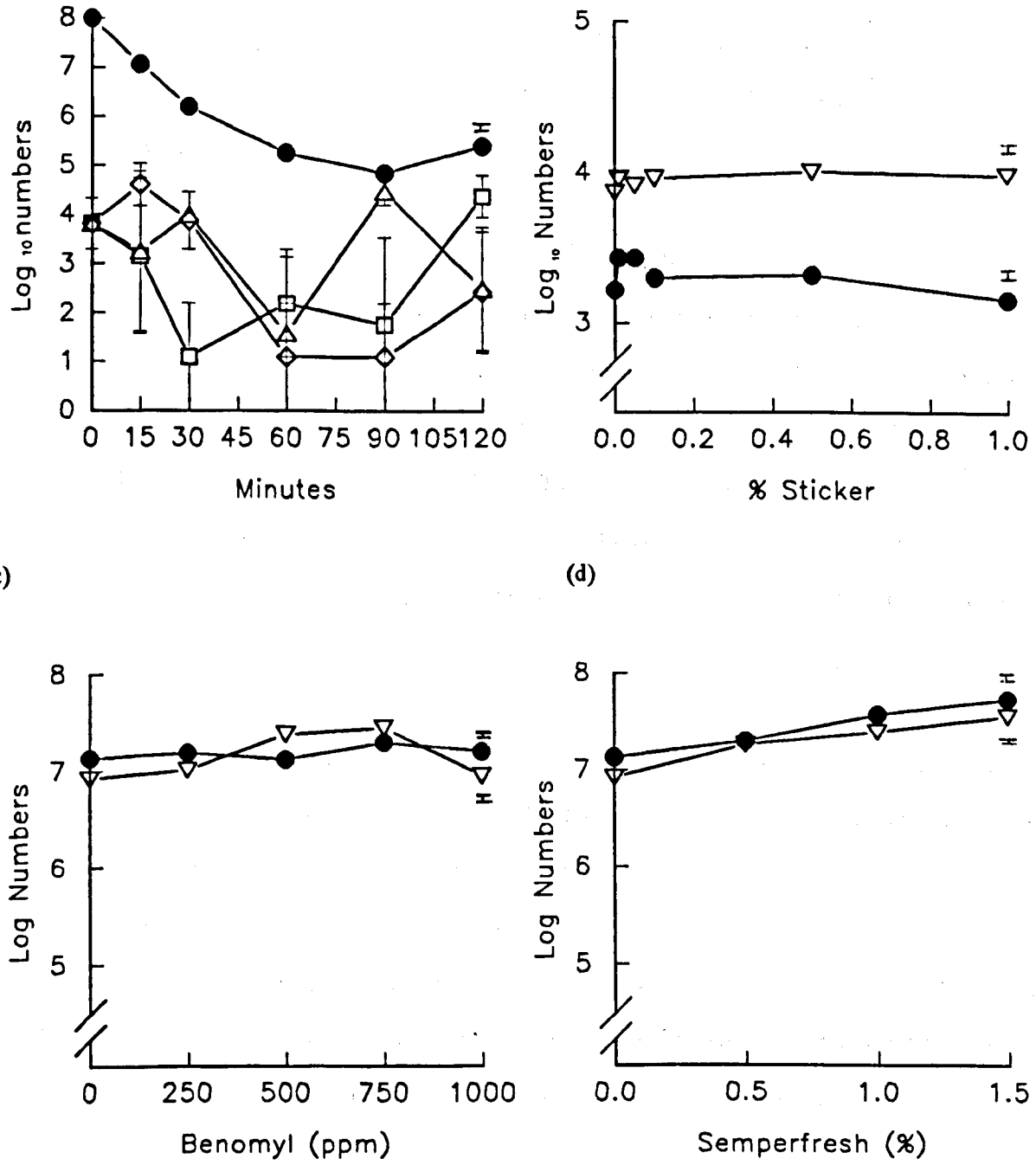
d) To assess the survival of both isolate 204 and 558 on the mango surface for the different treatments, one mango/treatment was sampled at 0, 5 & 10 days after dipping. Ten pieces of one cm^2 peel from each fruit were shaken in tubes containing 10ml dH_2O, a dilution series prepared and plated out on NA (isolate 204), and on KingB*agar (isolate 558). Colonies were counted after 48 hrs.

4.3 Results

4.3.1 Temperature survival of 204 & 558 - Isolate 204 survived a two hour exposure at all three temperatures. There was a slight fluctuation in numbers recovered, but after 2 hrs incubation counts were $4x10^3$ cfu/ml for those heated at 50 and 55°C and $3.9x10^4$ cfu/ml for those heated at 45°C. Numbers at the start of the experiment were $8x10^3$ cfu/ml at all three temperatures (Figure 4.1.a). For isolate 558 there was a slow but steady decline in numbers during incubation at 50°C from an initial inoculum level of 10^8 cfu/ml to $8.5x10^4$ cfu/ml after 2 hrs. Nevertheless numbers were still high ($1.2x10^7$ cfu/ml) after 15min at 50°C, which would allow a post-harvest dip lasting ten minutes to be carried out without significant loss of inoculum.

4.3.2 Effect of sticker on 204 & 558 - There was no detrimental effect of the sticker on either 204 or 558 at any concentration of sticker tested (see Figure 4.1.b). The sticker used in the post-harvest experiment in the Philippines was a different product (see 4.2.6) and at the time of this experiment it was not available for testing, nevertheless an effect cannot be ruled out.

4.3.3 Effect of benomyl on 204 & 558 - Growth of both 204 and 558 on NA with benomyl incorporated was not visually affected. Benomyl in solution also had no effect on numbers of bacteria recovered from the solution after 1 hr (Figure 4.1.c). Even the highest concentration of benomyl (1000ppm) yielded 10^7 cfu/ml for both bacteria, similar to the concentration of the control which received no benomyl.



e 4.1 Concentrations (log_{10}) after incubation in a) hot water (50°C, 558 = ●; 45°C, 204 = □; 50°C, 204 = ◇; 55°C, 204 = △); and of 204 (▽) and 558 (●) in b) increasing concentrations of sticker; c) increasing concentrations of benomyl; and d) increasing concentrations of semperfresh (vertical bars = S.E.).

4.3.4 Effect of Semperfresh on 204 & 558 - For both isolates a slight but not significant increase in numbers of bacteria was observed with increasing levels of Semperfresh (Figure 4.1.d) indicating that there was no harmful effect of the sucrose polyester on the two bacterial isolates.

4.3.5 Effect of fruit wax on 204 & 558 - The attempt to make fruit wax was not successful as pressurised equipment is necessary to obtain a liquid wax. The preparation made in the laboratory was not homogenous and an attempt to dip mangoes into this mixture was abandoned. Nevertheless the toxic effect of the mixture was assessed and, after 1 hr of incubation in the fruit wax mixture, the numbers of 558 were 10^5 cfu/ml compared to an initial concentration of 10^7 cfu/ml. This indicated a slight decrease in numbers recovered but overall there was no direct toxic effect of this paraffin based wax on 558. However the wax used in the Philippines was a commercial formula (see 4.2.6) which was water based and could not be tested for toxicity before the actual experiment.

4.3.6 Post-harvest dip experiments - There were no major differences in disease levels between the assessments carried out at 7 and 10 days (Tables 4.2 and 4.3) and since differences across experiments were more pronounced after 10 days these will be discussed first.

For %disease after 10 days, the main difference was that bagged mangoes had a significantly lower %disease than dry mangoes (Table 4.3). This can be explained by the distortion of ripening that occurred after the mangoes had been incubated in the plastic bags for 48 hrs. The colour index of bagged mangoes was 2.1 after 10 days (see Table 4.3) while the dry mangoes were already fully ripened with a colour index of 5.2. This difference in ripening can be seen in Figure 4.2 in which dry and bagged mangoes are shown after 10 days of incubation. Apart for a delay in ripening, this treatment also resulted in a much lower percentage of anthracnose development in bagged mangoes (2.8%), than in dry mangoes (11.1%; Table 4.3).

When disease development is compared between the antagonist treatments, isolate 558 reduced %disease significantly (4.8%) compared to both isolate 204 (7.3%) and the control treatment (8.9%; Table 4.3). For all separate treatments 558 had a lower percentage disease than both 204 or the control treatment.

There was no significant difference between %disease found for sticker treated mangoes (6.3%), peptone treated mangoes (6.7%) and hot water treated mangoes (7.9%; Table 4.3). Disease levels are relatively high for the hot water treated mangoes incubated dry, especially in the control treatment for which the percentage disease was 18.0% compared to the sticker control at 9.0% and the peptone control at 13.3%.

Table 4.2 a) %Disease and b) colour index for post-harvest treated mangoes at 7 days after harvest.

a) %Disease

		Control	204	558	mean	S.E.
Sticker,	Dry	1.0	2.0	2.3		
	Bag	1.8	0.3	0.0	1.2	
Peptone,	Dry	4.3	2.3	2.0		
	Bag	0.0	0.3	0.0	1.5	.32
Hot water,	Dry	5.0	3.5	2.0		
	Bag	0.0	0.0	0.0	1.8	
mean		2.0	1.4	1.1		
S.E.			.32			
Overall mean	Dry	3.5	2.6	2.1	2.7	
	Bag	0.7	0.1	0.0	0.3	.26

b) Colour index

		Control	204	558	mean	S.E.
Sticker,	Dry	3.6	3.9	3.5		
	Bag	1.5	1.4	1.1	2.5	
Peptone,	Dry	3.4	3.7	3.3		
	Bag	1.5	1.4	1.5	2.5	.09
Hot water,	Dry	4.1	4.4	2.9		
	Bag	1.9	2.2	1.5	2.8	
mean		2.7	2.8	2.3		
S.E.			.09			
Overall mean	Dry	3.7	4.0	3.2	3.6	
	Bag	1.6	1.6	1.4	1.6	.07

Table 4.3 a) %disease and b) colour index for post-harvest treated mangoes, 10 days after harvest.

a) %Disease

		Control	204	558	mean	S.E.
Sticker,	Dry	9.0	11.3	8.0		
	Bag	5.1	3.3	1.3	6.3	
Peptone,	Dry	13.3	10.3	7.3		
	Bag	4.7	2.4	2.0	6.7	1.36
Hot water,	Dry	18.0	14.0	8.7		
	Bag	3.3	2.2	1.3	7.9	
mean	Total	8.9	7.3	4.8		
S.E.			1.36			
Overall mean	Dry	13.4	11.9	8.0	11.1	
	Bag	4.4	2.6	1.5	2.8	1.11

b) Colour index

		Control	204	558	mean	S.E.
Sticker,	Dry	4.9	5.5	5.0		
	Bag	2.2	1.9	1.7	3.6	
Peptone,	Dry	4.8	5.3	4.9		
	Bag	1.5	1.9	1.7	3.3	.09
Hot water,	Dry	5.5	5.7	4.8		
	Bag	2.2	3.2	2.1	3.9	
mean		3.5	3.9	3.4		
S.E.			.09			
Overall mean	Dry	5.1	5.5	4.9	5.2	
	Bag	2.0	2.3	1.9	2.1	.07

Figure 4.2 Post-harvest treated mangoes after 10 days of ripening. On the left, mangoes which have been incubated for 48 hours in plastic bags and which still look green. On the right, mangoes which have been incubated dry and are fully ripened.

This hot water treatment was carried out at a slightly lower temperature (50°C) than a conventional hot water treatment (55°C) in order to give the bacteria a better chance of survival. As we have seen in section 4.3.1, at 50°C a drop in bacterial numbers is experienced for both organisms after prolonged exposure at this temperature (see Figure 4.1.a). In this post-harvest experiment, bacterial suspensions were kept for no longer than 30 minutes at 50°C in which dipping was carried out. Bacterial numbers were $6x10^4$ cfu/ml for 204 and $4x10^7$ cfu/ml for 558 at completion of the dip. This means that there was a decline in numbers of 204, but an increase in numbers of 558 as compared to the initial concentration of the suspension (initial concentration was $5.5x10^6$ cfu/ml for 204 and $6x10^6$ cfu/ml for 558).

The fact that the hot water treatment carried out at this lower temperature was not as effective as the conventional hot water treatment might be explained by the observation noted in section 2.3.8. The temperature for thermal death of spores of *C. gloeosporioides* lies between 45 and 50°C and although the effect of this temperature on appressoria is not known, the results indicate that the lower temperature at which this experiment was carried out (as low as 47°C) did not inhibit development of anthracnose lesions. The observation that the percentage of disease was even higher was because ripening was accelerated by

the hot water treatment; the colour index for hot water treated fruit was 3.9 while for sticker treated fruit this was 3.6, and for peptone treated fruit this was 3.3.

Fruit coatings - Incorporation of the antagonists into the sucrose polyester and fruit wax treatments did not significantly decrease anthracnose development relative to the control treatments (Table 4.4). %Disease for the sucrose polyester and the fruit wax were 6.0 and 5.7 respectively for 204 treated fruit, 8.3 and 9.0 respectively for 558 treated fruit and 9.0 and 5.7 respectively for control fruit. This indicated that neither of the fruit coatings supported the antagonists in disease control. There was also no effect of the fruit coatings on colour development (Table 4.4).

Table 4.4 a) %Disease and b) colour index assessed after 10 days, for mangoes either dipped in fruit wax or sucrose polyester.

a) %Disease

	Control	204	558	mean	S.E.
Fruitwax	6.3	5.7	9.0	7.0	1.16
Sucrose polyester	9.0	6.0	8.3	7.8	
mean	7.7	5.8	8.7	7.4	
S.E.		1.42			2.00

b) Colour index

	Control	204	558	mean	S.E.
Fruit wax	4.2	4.9	3.3	4.1	0.13
Sucrose polyester	4.9	4.8	4.6	4.8	
mean	4.5	4.8	4.0	4.4	
S.E.		0.17			0.23

Conventional treatments - For comparison conventional post-harvest treatments of HWT (55°C), benomyl and prochloraz were included. %Disease in HWT treated fruit was 5.7, in benomyl treated fruit 4.0 and in prochloraz treated fruit 5.3. In all cases, disease levels were less than in any of the antagonist treated fruit and indicated that the levels of control achieved using the biocontrol approach were not as good as conventional methods for controlling post-harvest anthracnose development. This observation leaves scope for improvement of the biological control method such that similar levels of control will be achieved. Colour indices were 5.8 for HWT fruit, 5.3 for benomyl treated fruit and 5.8 for prochloraz treated fruit.

Assessment at 7 days - %Disease and colour indices were also assessed at 7 days. Trends were similar to those of 10 days and results are summarised in Table 4.2. One exception was for mangoes to which 558 was applied with sticker and incubated dry. In this case %disease was higher than both the 204 and control treatments. For all other treatments fruit treated with 558 had a lower %disease than fruit treated with 204 or control fruit. Disease development in bagged fruit was hardly detectable after 7 days, and fruit were still green.

Survival of 204 and 558 on the mango surface - The initial inoculum concentration of the bacterial dips was $5.5x10^6$ cfu/ml for 204 and $6x10^7$ cfu/ml for 558. This resulted in bacterial numbers on the mango peel after dipping at $5x10^4$ cfu/cm^2 for 204 and 10^5 cfu/cm^2 for 558. For isolate 204, numbers of bacteria on the mango surface had decreased to between 10^4 and $3x10^4$ cfu/cm^2 after 5 days (Figure 4.3), only a slight decrease in numbers. However, where 204 had been applied with 0.5% peptone and incubated in plastic bags, an increase in bacterial numbers was observed to $0.8x10^5$ cfu/cm^2. This is remarkable in that, although these mangoes were first stored for 48 hrs in plastic bags, and bacterial numbers were expected to stay high, this period was followed by 3 days of dry incubation before samples were taken. It may be that the dry period induced isolate 204, a *Bacillus* sp., to form spores which survived the dry period, yet formed colonies when plated out onto agar. After 10 days numbers of the *Bacillus* were reduced to $<10^3$ cfu/cm^2 for all treatments, except for the mangoes which had peptone applied to them (10^4 cfu/cm^2) and for the mangoes which had been dipped in hot water and incubated in plastic bags ($9x10^3$ cfu/cm^2).

For isolate 558 there was no survival of the pseudomonads on the mango surface at day 5 (and consequently not at day 10). Because samples were not taken before day 5, the rate of decline in numbers is not known. To investigate whether the decline set in before the plastic bags were removed, a further experiment was

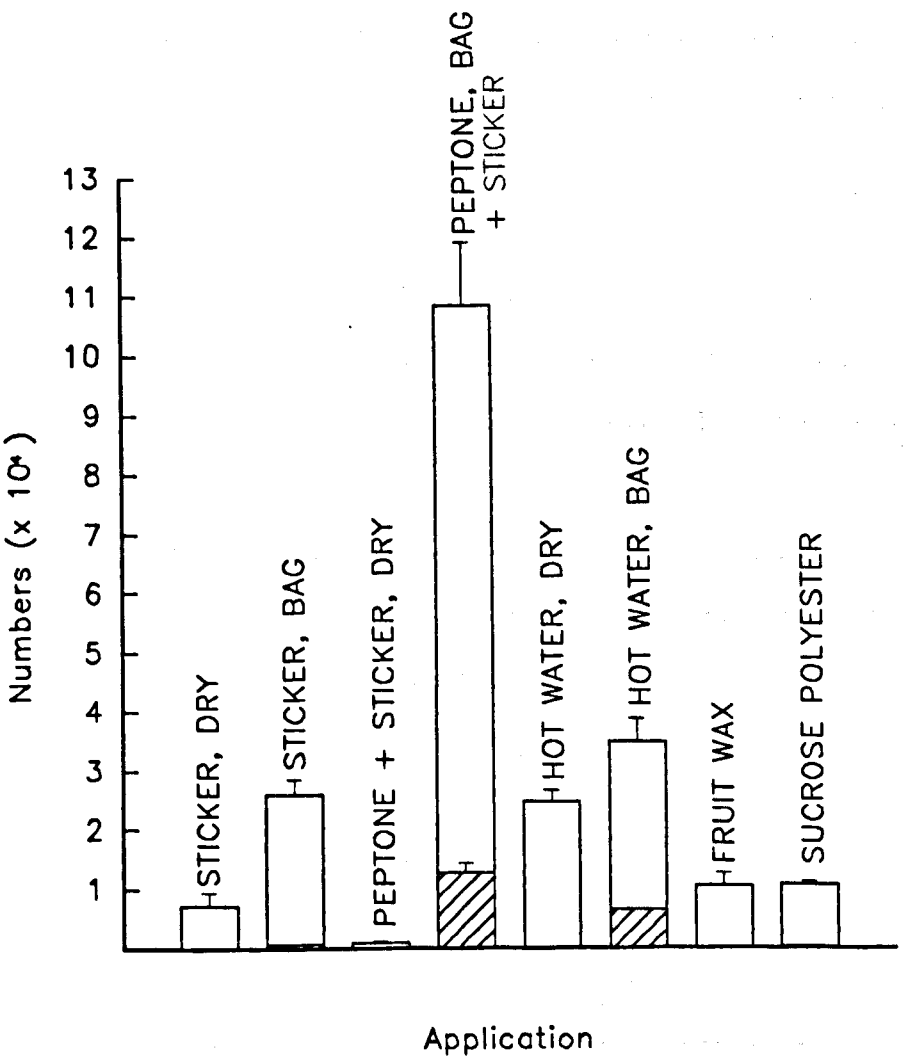


Figure 4.3 Numbers (cfu/cm^2 of fruit peel) of 204 on Carabao mangoes, 5 (open bars) and 10 (hatched bars) days after post-harvest treatment. The initial application was 10^5 cfu/cm^2 (vertical bars = S.E.).

set up with mangoes cv Carabao which were dipped in isolate 558 ($2x10^9$ cfu/ml) in 0.5% peptone + 0.1% sticker and incubated in plastic bags for 48 hrs. At time of dipping mangoes had received $5x10^6$ cfu/cm^2. After 24 hrs this was $4x10^6$ cfu/cm^2, and after 48 hrs this was $6x10^6$ cfu/cm^2. Thus up to 48 hrs the bacterial numbers were maintained at the initial concentration and the population decline must have begun after the plastic bags had been removed.

Effect of treatments on stem end rot - It was observed that in addition to anthracnose, stem end rot was also present in this batch of mangoes. To assess the effect of the post-harvest treatments applied on the development of stem end rot, the presence of this disease was recorded for all mangoes 14 days after harvest. These numbers are expressed as the percentage of mangoes/treatment which showed typical stem end rot symptoms. The overall mean percentage of mangoes affected by stem end rot was 45% for the control treatments, 43% for 204 treated fruit and 48% for 558 treated fruit (see Table 4.5). This indicated that there were no significant differences between the antagonist treatments and the control treatments on the development of stem end rot.

Table 4.5 Percentage of mangoes with stem end rot symptoms.

Application	Control	204	558	mean
Sticker, dry	71	80	60	70
Sticker, bag	54	40	36	43
Peptone, dry	60	67	54	60
Peptone, bag	27	47	57	44
Hot water, dry	13	40	57	37
Hot water, bag	85	36	23	48
Fruit wax	13	20	7	13
Sucrose polyester	36	53	53	47
HWT				27
Benomyl				7
Prochloraz				73
mean	45	48	43	43
S.E.	8.9	6.2	6.4	7.8

The only treatments that did appear to reduce stem end rot development were the fruit wax coating (13% stem end rot present), the HWT (27%) and the hot benomyl treatment (7%). Prochloraz is not effective against the stem end rot fungus. This was also observed by Johnson *et al.* (1990a) and confirmed by the high disease level (73%) observed in mangoes in this experiment. The fact that the fruit wax coating reduced the incidence of stem end rot could be due to the wax also coating the pedicel and thus depriving the stem end rot fungus of oxygen. Waks *et al.* (1985), however, found that all of four fruit waxes tested increased the occurrence of stem end rot in oranges to a greater or lesser extent.

4.4 Discussion

In this experiment it was attempted to establish if the bacteria selected, 204 & 558, were able to control anthracnose disease under semi-commercial conditions. To this end, mangoes were either incubated dry or in plastic bags. The effects of nutrients and fruit coatings on the effectiveness of bacteria was also assessed. Results showed that isolate 558 did significantly reduce disease incidence in naturally infected mangoes. Incubation in plastic bags was not successful since fruit quality was reduced. Of the other materials tested, neither the fruit wax and the sucrose polyester supported biological control activity. There was no advantage of using peptone as well as a sticker on final disease levels, although bacterial numbers were higher on peptone treated fruit.

The option of post-harvest application of biological control is appealing because, as already discussed (see Chapter 1), there are many problems attached to large scale applications of biological control agents in the field. The post-harvest environment allows the biological control agent to reach its target relatively easily, the commodity to be treated is of high value because it has been harvested already, and storage conditions such as temperature, are often controllable (Wilson & Pusey, 1985; Jeger & Jeffries, 1988). However, not all storage pathogens behave similarly and the treatment of wound pathogens is different from those that are already present on the fruit before harvest, such as the latent infections. All work to date has been carried out on wound pathogens with the major pathogens studied being *Penicillium expansum* (Janisiewicz, 1987), *P. digitatum* and *P. italicum* (Wilson & Chalutz, 1989), *Monilinia fructicola* (Pusey & Wilson, 1984), *Botrytis cinerea* (Roberts, 1990) and *Rhizopus stolonifer* (Wisniewski *et al.*, 1989). The advantages of

controlling wound pathogens compared with latent infections are that fruit are most likely to become damaged during harvest and post-harvest handling, so if an antagonist dip is applied directly after harvest, protection should be complete. A further advantage is that the wound site creates an ideal niche, not only for the pathogen, but also for the antagonist because humidity will be high and plenty of nutrients are available (Wilson & Pusey, 1985). As long as the antagonist can survive on the fruit surface, multiply and become active as soon as a wound arises, as is the case with *Candida guilliermondii* on citrus (Droby *et al.*, 1989), biological control should be successful. It has frequently been reported that biological control is more successful when the antagonist is applied before the pathogen (Janisiewicz, 1988a; Vapinder Singh & Deverall, 1984). In the case of wound pathogens, this means that the antagonist can occupy the wound site before the pathogen arrives. With latent infections a different situation arises. Infection takes place when the fruit are still on the tree, and after the appressorium has formed, an infection peg may penetrate the cuticle to form the latent infection (see 1.1.3). Spores will be dispersed on the fruit in a random fashion and for a biological control agent to be completely effective it has to be able to reach and control all these separate inocula, which all can give rise to lesions. If an infection peg is present within the cuticle, the situation becomes more complicated, as the biological control agent or its product has to be able to penetrate the cuticle directly to have access to the infection peg. It is interesting to note that isolate 558, which did not survive on the mango surface outside the plastic bag, did reduce disease levels compared to the control treatments even when the mangoes were not incubated in plastic bags.

The use of a fruit wax was successfully tested by Pusey *et al.* (1986) who found that two mineral oil and paraffin, and two water based fruit waxes were generally compatible with *Bacillus subtilis* isolate B3, although sometimes a negative effect was observed. The incorporation of B3 into a water based wax applied at a semi-commercial packing line reduced the severity of brown rot in peaches, caused by *Monilinia fructicola*, to comparable levels as those of fruit treated with benomyl incorporated into the wax (Pusey *et al.*, 1988). In our experiments, there was no successful control obtained with the incorporation of either 204 or 558 into the fruit wax used or into Semperfresh. The significant decreases in disease recorded for 558 incorporated into the other treatments, were not repeated using 558 and fruit wax or Semperfresh. This indicates that something in the wax and Semperfresh counteracts the activity of 558. Neither compound, however, was directly toxic to the bacteria used. The numbers of 204 on the fruit surface were as high in the fruit wax and Semperfresh treated fruit as they were for instance in the peptone treated fruit incubated dry (10^4 cfu/cm^2). Semperfresh may even stimulate bacterial growth, possibly due to the sucrose present

(see 4.3.4). This difference in success between Pusey's work and this study might be due to the fact that *Monilinia fructicola* is a wound pathogen. Bacteria have to be active in the wound space where oxygen and water shortage might not pose a problem, while for *Colletotrichum* infections bacteria have to be active all over the fruit surface. The layers of wax and sucrose polyester could result in dry, anaerobic conditions, while bacteria in wounds are protected from this. Another explanation could be that although bacteria can survive in either compound, conditions might become such that biological control activity cannot be expressed, e.g. inhibitory compounds are de-activated by the coatings. As we will see in Chapter 6, activity of 558 is coupled with an increase of pH, thus it is possible that both fruit wax and Semperfresh have a buffering capacity so that the increase in pH is counteracted and no biological control takes place.

Recent research at PHTRC has shown that any application to the mango surface that disturbs the respiration of the fruit, including plastic bags, fruit wax and Semperfresh should be avoided, because internal breakdown of the fruit flesh takes place and the fruit becomes inedible (see also Chaplin *et al.*, 1986; Miller *et al.*, 1986; Spalding & Reeder, 1986). The use of plastic bags with pin prick holes, or the use of gas permeable plastic is not, however, excluded. From a practical point of view, the cost of the treatment should not rise above the conventional hot benomyl treatment though.

It was a pity that the HWT with 204 and 558 was not carried out at a slightly higher temperature. This problem arose because dips were heated up indirectly and it took a long time for the solution to reach 50°C. Results from an undergraduate student project had shown that at 50°C cell counts for both 204 and 558 declined drastically within 15 min (Thomas, 1989). However the counts reported here were carried out on both bacteria after half an hour at 47°C revealed that bacterial numbers remained high. To confirm this I repeated the temperature curves, and also lengthened the time of exposure from 15 min to 2 hrs (the time a commercial packer will keep a fungicide dip in operation for). These experiments showed that 204 was not sensitive to the hot water treatments at any of the temperatures tested, although cell concentrations fluctuated enormously. Isolate 558 was only tested at 50°C and results showed that numbers decreased, from 10^8 to approx. 10^5 cfu/ml in two hours. The latter experiments indicated that both bacteria could have been applied at a higher temperature, in which case the HWT might have been more successful. It is interesting to note that early in the mango season, the manager of a commercial packing house (The Star, Manila) uses a hot benomyl dip carried out at 50°C and reduces this temperature to 46°C, to reduce cost, later in the season (April - May) when risk of anthracnose is less. The control of anthracnose that is observed at this temperature cannot be due to the hot water treatment as seen in the hot water dip experiments reported

here, but must be solely due to the effect of benomyl.

The effect of inoculum concentration on effectiveness of biological control for both isolates still has to be determined. Roberts (1990) showed a direct relationship between inoculum concentration and storage temperature of apples, on the effectiveness of *Cryptococcus laurentii* in controlling *B. cinerea*. Droby *et al.* (1989) found that control of *P. digitatum* was close to 100% at a concentration of *C. guilliermondii* of 10^9 cfu/ml while at a concentration of 10^6 cfu/ml control was only 40%. Janisiewicz (1987) determined the optimum concentration for a yeast isolate at 10^8 cfu/ml and for a bacterial isolate at $4x10^8$ cfu/ml. The effectiveness of treatment with *B. subtilis* improved when inoculum concentrations were increased from 10^6 to 10^7 cfu/ml (Pusey & Wilson, 1984). Many other researchers found a relationship between antagonist concentration and pathogen propagules present (Janisiewicz, 1988a; Wisniewski *et al.*, 1989). Most of the concentrations mentioned above were higher than used in this experiment ($5.5x10^6$ cfu/ml for 204 and $6x10^7$ cfu/ml for 558). These numbers were limited by the equipment available, and these were the maximum concentrations we could achieve at the field site in the Philippines.

A further possibility to enhance the biological control effect, is the incorporation of low concentrations of fungicide into the antagonist dips. Fungicide concentrations should be such that they weaken the pathogen and inhibit secondary invaders, and could be incorporated into the antagonist dips since both isolate 204 and 558 are resistant to benomyl. Droby (pers.comm) proposed the incorporation of low levels of fungicide, which in laboratory experiments enhanced the level of control of *P. digitatum* by *C. guilliermondii*. Pusey *et al.* (1986) incorporated 900ppm dichloran into a B3 dip to control *Rhizopus* sp., which causes *Rhizopus* rot which is second in importance to *Monilinia fructicola* in stone fruit. *M. fructicola* cannot be controlled by dichloran, while *Rhizopus* is not affected by B3, the combination of B3 and dichloran gave satisfactory control of both diseases.

Salt solutions such as $CaCl_2$, KCl and $CaCO_3$, enhanced the effectiveness of the yeast *C. guilliermondii* in controlling *B. cinerea* and *P. expansum* on apple (McLaughlin *et al.*, 1990). How this enhancement was achieved by the salts is as yet not known.

From the experiments described in this chapter we can conclude that under the circumstances provided, which were similar to commercial conditions, the level of control of anthracnose obtained using isolate 204 was not sufficient for potential exploitation. Further research on effects of bacterial concentrations and media used is required for this isolate. In contrast isolate 558 did exhibit a significant decrease of disease

compared to the control, except when incorporated into wax and Semperfresh. However, it must be noted that in this particular season it had been extremely dry, with levels of disease at approx. 10%, compared to disease levels in a 'normal' year of about 30% (R.D. Bugante, pers.comm.). Results might thus have been more striking in a normal year. Similar problems were experienced by Pusey *et al.* (1988) in their experiments on commercial packing lines, when they also depended on natural infection present.

Future work is proposed on the effect of incorporation of low concentrations of benomyl (about 50 - 100ppm) into the 558 treatment and further studies on how 558 exerts biological control if it does not survive on the dry fruit surface. Experiments have to be scaled up in size and preferably carried out on a commercial packing line. Pusey *et al.* (1988) found that, when going from laboratory experiments to a commercial packing line, satisfactory control was not maintained due to problems related to the packing line procedures.

Further research must also be carried out into the possibility of low temperature storage. Transport for export is carried out at a temperature of 12-13°C, whilst experiments described here were always carried out at a temperature of around 25°C.

5 Pre-harvest field experiment

5.1 Objectives

Latent infections of *C. gloeosporioides*, which give rise to lesions after harvest, are already established when the fruit are green and immature in the canopy (see Chapter 1). Apart from causing anthracnose on mature fruit, the fungus can also cause blossom blight and fruit drop. Both these symptoms will result in a reduced harvest, and in the case of blossom blight, fruit set can be reduced to zero. At present the routine employed for disease control in the Philippines is a scheduled regime consisting of 5 to 6 sprays of chlorothalonil, maneb, manzate or benomyl (Pordesimo, 1983). However standardised sprays have their disadvantages, such as that sprays might be applied when risk of infection is low. Abundant use of systemic fungicides, especially benomyl, can result in resistance being built up in the pathogen population (Spalding, 1982) and widespread use of fungicides may present ecological hazards. Pre-harvest control is a necessity to control both blossom blight and fruit drop, and to reduce inoculum sources near developing fruit. Pre-harvest approaches to biological control are rare (Chapter 1) and in order to test the feasibility of pre-harvest biological control of anthracnose in mango, isolate 558 was selected for testing in a small scale field trial carried out in a commercial orchard.

Isolate 558 was chosen because of its ability to reduce spore germination (see Chapter 3) which was seen as one of the major attributes necessary to reduce infection by *C. gloeosporioides* in the field. Because of practical limitations it was not possible to apply a full spray regime, so it was decided to spray at times when infections leading to blossom blight and fruit drop were most likely to occur. To allow 558 to grow and establish, nutrients were added to the spray. Research in Malaysia (Lim & Khor, 1982) had shown that nutrients applied in a fungicide (maneb plus zinc salt)/insecticide/foliar fertilizer mixture to mango leaves had a stimulating effect on the indigenous microflora present which resulted in a reduction in isolation of *C. gloeosporioides* from the blossoms and leaves.

The objectives of this experiment were :

1 - To assess if pre-harvest application of 558 could reduce post-harvest anthracnose development and increase fruit set (as a measure of presence of blossom blight and fruit drop).

2 - To determine if 558 could establish itself within the tree canopy and maintain reasonable population levels.

3 - To assess the effects of the different sprays applied on the microbial communities of the leaves and to see if this was related to anthracnose development.

5.2 Materials & methods

5.2.1 Pathogenicity test - Isolate 558 was tested on mango leaves (obtained from Kew botanical gardens) to ensure it was not the causal agent of bacterial blackspot (*Xanthomonas campestris* pv. *mangiferaeindicae*) or had any other negative effects on mango leaves. Detached leaves were put with their stems in agar containing benomyl, sprayed with 10^8 cfu/ml bacteria in CzDL until they were wet all over the surface and incubated in a moist chamber for two weeks. Examination after this period did not reveal any signs of lesions or other detrimental effects, thus it was presumed that 558 was not a potential pathogen.

5.2.2 Field site - The orchard in which the field experiment was carried out was located near Padre Garcia in the province Batangas, Luzon, Philippines (see Figure 5.1). All trees in the orchard were of the variety Carabao and had been planted approx. 20 years ago and never been pruned. The orchard was managed by a family who live on the compound and fruit is produced for the local market in Manila.

5.2.3 Treatment of trees - Trees were induced to flower with potassium nitrate (Bondad *et al.*, 1980) on the 30th of October 1989, and 24 trees should have been available to carry out the experiment. However, many trees were rendered unsuitable due to a typhoon in the middle of October. Several trees were

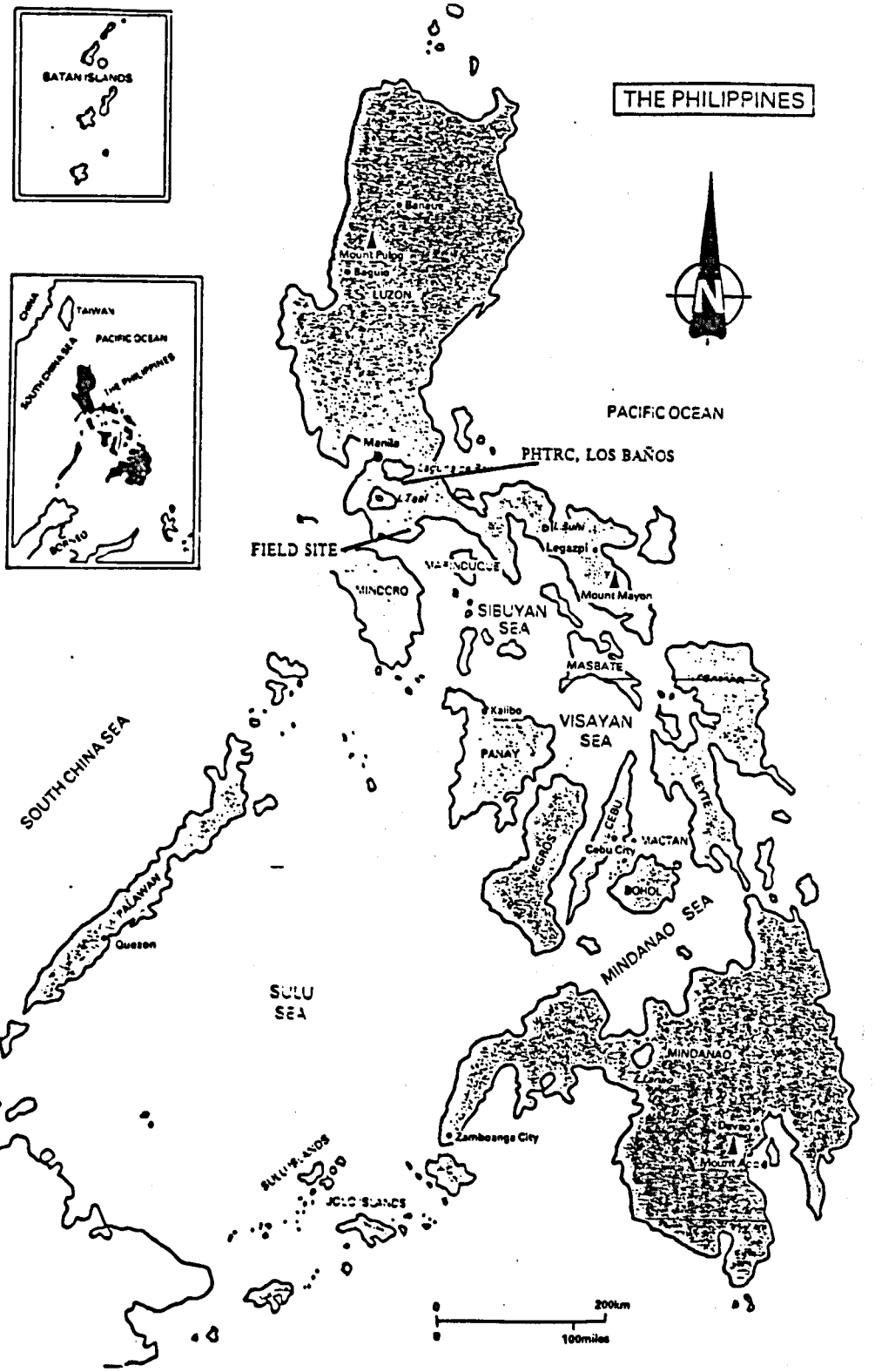


Figure 5.1 Map of the Philippines indicating the field site and PHTRC, Los Baños.

damaged, and responded to induction by producing new shoots ('flushing') rather than by producing flowers. Only four trees were determined suitable to carry out the experiment, and hence replications had to be carried out within single trees, rather than on separate trees.

5.2.4 Experimental treatments - Figure 5.2 outlines the events that take place from induction till harvest and the following treatments were applied :

Control	- no fungicide or bacterial spray applied	CON
nutrients	- one spray of nutrients applied at onset of flowering	NUT
1x558	- one spray of 558 applied on onset of flowering	558
2x558	- two sprays of 558 applied at onset of flowering and at fruit set	2x558
Benomyl	- one spray of benomyl applied at onset of flowering	BEN
Fungicide	- Six sprays of benomyl at set intervals (see Figure 5.2) as would be the normal farmers practice	6xBEN

5.2.5 Experimental lay-out - Because of the availability of four trees only, the experiment was carried out in a randomised block design in which each tree represented one block and contained six plots e.g. the six different treatments. Each plot was more or less one big branch in the tree. Care was taken to avoid the possibility of run-off of sprays and rain water from one treatment to the next. In each plot 25 panicles were labelled to be able to follow fruit set of these panicles. Figure 5.3 shows the lay-out of the plots within the trees.

5.2.6 Preparation of sprays - Bacterium 558 was grown up in CzDL on a shaking incubator for four days at room temperature (25-30°C). The final suspension was diluted (1:4) with CzDL, and the concentration of the spray was 9.4×10^7 cfu/ml. Each segment received 4 litre of this spray applied with a back pack sprayer. The nutrient spray consisted of CzDL only, at the same concentration as the spray containing bacteria. The fungicide sprays consisted of 250ppm benomyl.

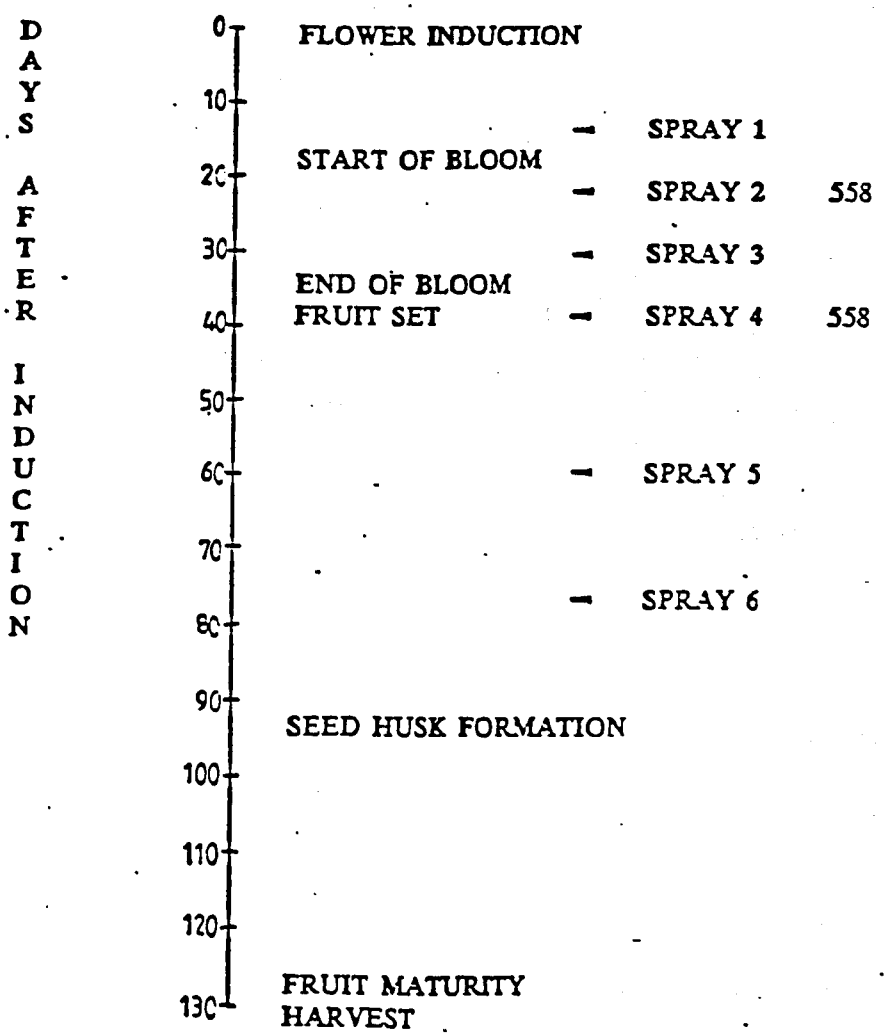


Figure 5.2 Table of events taking place during the development of mangoes. Indicated are the fungicide and antagonist applications.

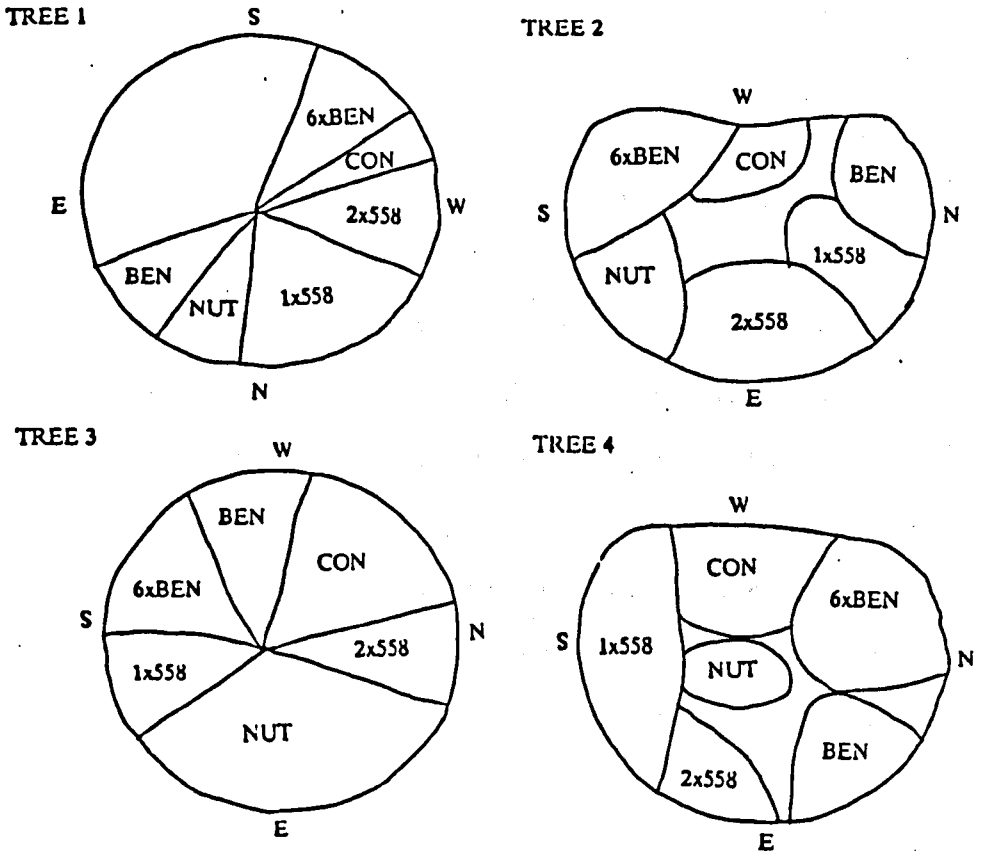


Figure 5.3 Experimental design of treatment plots within individual trees (see page 88 for explanation of treatments).

5.2.7 Measurements - Fruit were harvested according to commercial practice at 130 days after flower induction. Fruit set was recorded on the labelled panicles. Then 25 fruit/treatment/tree were picked and fresh weight of ten fruit was recorded. All 25 fruit/treatment/tree (total 600 fruit) were transported to PHTRC, Los Baños, incubated at room temperature (25-30 °C) and %disease (see 4.2.6) assessed at 10 days after harvest.

5.2.8 Population studies - To investigate how the treatments would affect the different microbial communities on the phylloplane and to assess how 558 would establish on the leaf surface, leaves were sampled at time of spray, 1 week after the first application of 558 and at time of harvest. Populations were assessed according to the leaf washing method (Dickinson, 1971).

Four leaves were sampled from each treatment/tree, and these were pooled for each treatment. These

sixteen leaves were cut into strips and 6 g of wet leaf material was shaken for 15 min in 100ml dH_2O with 20 g sand. This time was chosen because a preliminary trial in the U.K. showed that there did not appear to be a difference in total numbers after shaking at 15, 30, 60 and 120 min. Approximately 1 g of wet leaf material was equal to 40 cm^2 leaf = 80 cm^2 leaf surface. Simultaneous sampling of blossoms, as done by Lim & Khor (1982) could not be carried out due to the sparsity of flower spikes present.

Dilutions were prepared and plated out on either NA with cycloheximide (75 mg/litre) to assess bacterial populations, or KingB$^+$ to assess *P. fluorescens* / pseudomonad populations or MEA$^+$ to assess yeast and fungal populations. The introduced pseudomonad was recognised by its colony morphology with a low convex, entire edge and shiny appearance. The following groups of microorganisms were assessed : bacteria, pseudomonads, white yeasts, pink yeasts and filamentous fungi. For this latter group an attempt was made to identify them at least to genus level.

5.2.9 Meteorological data - There was no onsite recording, but data were obtained from the nearest weather station situated in Lipa City. Rainfall and temperature data are given in Table 5.1. It must be noted that the weather station was situated at an altitude approx. 200 meters higher than the orchard, which resulted in a slightly wetter climate than was experienced at the field site.

Table 5.1 Rainfall data and average temperature during rainfall period at Lipa City during the period of the field experiment

Date	Rainfall (mm)	Temperature (°C)
16-11-1989	0.8	22.1
22-11-1989	6.1	22.0
26-11-1989	1.1	22.4
16-12-1989	7.0	20.1
25-12-1989	3.6	
26-12-1989	3.6	21.2
9-1-1990	16.2	22.3
25-1-1990	10.2	22.1
26-1-1990	19.1	22.6

5.3 Results

5.3.1 Fruit measurements - Table 5.2 shows fruit set/panicle, fruit weight at harvest and disease assessment at 10 days after harvest. There was no significant difference between any of the treatments for any of the parameters. For fruit set the control treatment gave the highest number (1) of fruit/panicle, while the treatment receiving full fungicide application had the lowest number (0.5) of fruit/panicle. The other four treatments were between these values with 0.9 fruit/panicle for the nutrient treatment, 0.7 for one application of 558, 0.7 for two applications of 558 and 0.8 for one spray of benomyl (Table 5.2).
The lower fruit set for the full fungicide treatment might have been due to the higher number of sprays this treatment received and the damage to the blossom and young fruit this might have caused. However, all treatments received the same number of spray applications, since insecticide was applied to all treatments at all spray times. These results indicate that fruit set was not affected by fungicidal or nutrient treatment. It must be noted that fruit set was extremely low considering the number of flowers that each panicle contains. Fruit set was also highly variable with variations between no fruit/panicle to six fruit/panicle.

Table 5.2 Fruit set/panicle and fruit weight (n = 10) at harvest, and %disease at 10 days after harvest for all treatments.

	CON	NUT	1x558	2x558	1xBEN	6xBEN	mean	S.E.
Fruit set	1	0.9	0.7	0.7	0.8	0.5	0.8	0.16
Fruit weight (g)	221	236	230	229	229	242	231	6.1
%Disease	8.1	6.4	9.5	11.7	6.6	4.8	7.8	2.53

There was even less difference between fruit weight for the different treatments. Weight varies between 221 g/fruit for the control treatment and 242 g/fruit for the full fungicide treatment (Table 5.2). These results are not surprising because one expects fruit size to be dependant on water and nutrient availability rather than disease control measurements. Although it might have been expected that the application of nutrients on the foliage would have had some effect on fruit size, perhaps the levels of nutrients used were too low to achieve this.

There were no significant differences for disease levels between any of the treatments. The treatment in which 558 was applied twice had the highest %disease (11.7) followed by 1x558 (9.5%), control (8.1%), 1xBEN (6.6%), NUT (6.4%) and 6xBEN (4.8%; Table 5.2). This might be due to the fact that although 558 was not active on the blossom and fruit surface, by applying nutrients twice (as part of the bacterial spray), spore germination of *C. gloeosporioides* could have been higher in this treatment (2x558) and hence resulted in slightly higher disease levels. It was surprising that even the full fungicide treatment did not result in a significant reduction in anthracnose levels, but as mentioned before (see 4.4) in the season in which the field experiment was carried out there was very little rainfall (Table 5.1) and hence disease levels were very low. In a normal year one would expect the control treatment to have disease levels of around 30% (R.D. Bugante, pers.comm.). Another reason why low levels of anthracnose were observed is that control treatments were located in positions that would avoid run-off as much as possible as explained in the materials and methods. This meant that the control treatments would also receive less rain drip and splash containing anthracnose spores, resulting in lower levels of disease.

5.3.2 Survival of 558 - As already described none of the treatments reduced disease. This was partly due to the fact that 558 did not survive on the leaf surface. At time of spray leaves sprayed with 558 had received 1.6 $x10^5$ cfu/cm^2 leaf (Table 5.3). After one week, population levels of 558 on treated leaves had dropped to 108 cfu/cm^2 leaf, no different from the control (55 cfu pseudomonads/cm^2 leaf). At time of harvest no pseudomonads were observed in the control, nutrient or 558 treatments. For unknown reasons, at both one week after the first application of benomyl and at harvest, a population of pseudomonads had established on the leaves in both the benomyl treatments (Table 5.3).

Table 5.3 Presence of pseudomonads one week after spray and at harvest, for all treatments (cfu/cm^2).

	CON	NUT	1x558	2x558	BEN	6xBEN
at time of spray	56[1]	-	$1.65x10^5$	-	-	-
1 week after spray	55[1]	0	108[1]	-	475	-
at harvest	0	0	0	0	325	31[1]

[1] these numbers fall below the detectible levels and only indicate presence.

These pseudomonads were morphologically different from 558. This population could possibly have established because the fungicide treatment removed major competitors/inhibitors of these pseudomonads and created a niche in which they could establish themselves. A similar trend was found by Lim & Khor (1982), although not highlighted in their paper, in that the fungicide/insecticide/fertilizer sprayed leaves had a population of *Pseudomonas* sp. Q_2, not observed on unsprayed leaves.

The large fall in numbers of 558 only one week after application could partly be explained by the fact there was very heavy rainfall on the night after application. There is thus the possibility that many of the pseudomonads were washed off. Another important factor is that 558 had not been thoroughly tested on its ability to survive on the phylloplane. This was partly because we could not find a model system in which to test 558 in the greenhouse (see 2.3.4) and also because of the time scale in which this work had to be carried out.

5.3.3 Population counts - For all the communities, for both the nutrient and bacterial treatments, one week after the first spray was applied, an increase in numbers was observed compared to the control (Figure 5.4). This was to be expected since both treatments contained the same amount of nutrients, making the conditions of the leaf surface more favourable to microbial multiplication. This increase was significant for the communities of bacteria, white yeasts and filamentous fungi. For communities of pink yeasts there was a similar, but not significant, increase but these yeasts appeared in very low numbers on the petri dishes, making variation very high. Three months later, at time of harvest, most of these stimulatory effects had disappeared. There was, however, a significant increase in numbers of fungi for the 1x558 treatment, with numbers for the control at 5.8×10^3 cfu/cm^2 and for the 1x558 treated leaves at 1.3×10^5/cm^2. There is no obvious explanation for this effect since 558 did not survive and could not have caused a direct effect. The other explanation could be that there was some long lasting effect of the nutrients. The nutrient treatment alone, however, received the same level of nutrients and numbers of filamentous fungi of 3.1×10^4/cm^2 were recorded, which were not significantly different from the control. If these fluctuations were not due to nutrients, it may be that they are natural fluctuations of microbial populations on the leaf surface. Alternatively, they may reflect inadequacies of the methodology. Population counts by leaf washings have often been criticised in that they overestimate heavily sporulating fungi and underestimate other

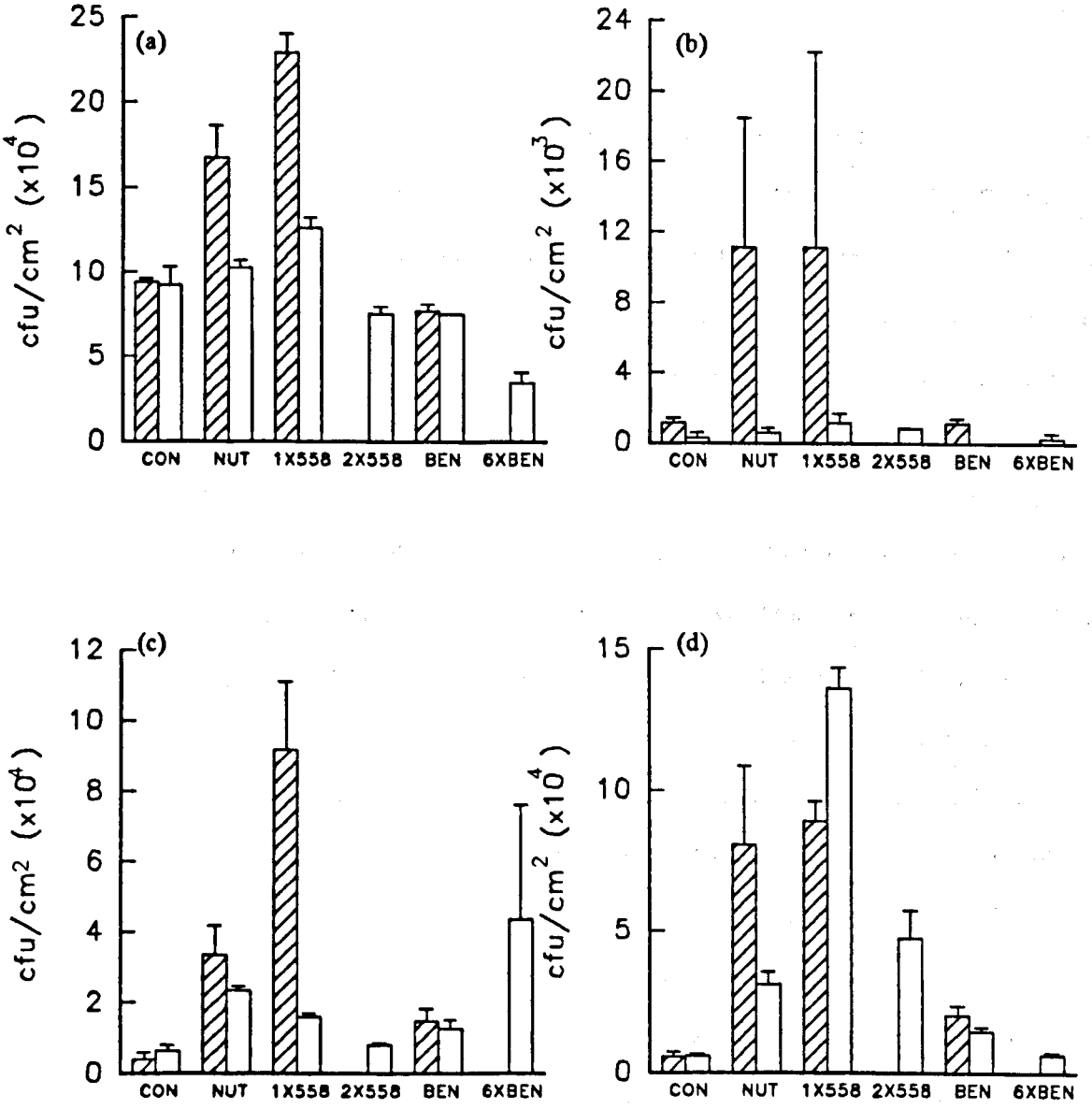


Figure 5.4 Concentrations (cfu/cm^2 of leaf) of a) bacteria; b) pink yeasts; c) white yeasts; and d) filamentous fungi, one week after pre-harvest applications (hatched bars) and at harvest (open bars)(vertical bars = S.E.).

microbes which do not grow well on the isolation medium or might not get a change to grow due to competition on the Petri dish (Dickinson, 1971; Last & Warren, 1972; Davenport, 1976). There could also be a direct toxic effect e.g. from the agro-chemicals present on the leaves and consequently in leaf washings, resulting in erroneous estimations of population size (Dickinson, 1971), though Andrews & Kenerley (1978) did not find proof for this in their experiments. Another factor that might have affected counts is the position of leaves in the tree canopy, which can affect the microbial population, and even individual leaves show a variability in immigration (Kinkel *et al.*, 1989a). Since only a limited number of trees were available, samples could only be taken from the different segments and these did not represent all possible positions in the tree. As can be seen in Figure 5.3, the control treatments would have had leaf samples taken from the upper, more exposed parts of the tree, which might have resulted in lower numbers of total counts. Surprisingly, the benomyl treatment did not seem to have any effect on the numbers of yeasts and filamentous fungi isolated. The numbers recorded at harvest for this treatment were $6x10^3$ cfu filamentous fungi/cm^2, which was not significantly different from the control. One week after a single spray of benomyl there appeared to be a slight increase in the number of white yeasts and filamentous fungi (Figure 5.4). This is interesting considering that this orchard almost certainly did not receive benomyl before, because of the high cost of this fungicide. This means that the natural microflora present on these trees could not have built up benomyl resistance and it is unlikely that the effect of benomyl would last less than one week. These results are contradictory to research by other authors who found that fungicide applications, such as zineb (Dickinson, 1973), captan (Hislop & Cox, 1969), mancozeb, dichlone (Pandey, 1988) and metiram (Andrews & Kenerley, 1978) had reduced the total numbers of fungi after multiple sprays had been applied (see also Hislop, 1976; Andrews, 1981). The results do, however, agree with those of Pandey (1988) who found a decrease in total numbers of fungi after two sprays with benomyl, but found that numbers started to increase by the third spray and by the fifth spray numbers were slightly higher than those found on unsprayed guava leaves. The same author also found that sensitivity to benomyl differs between species, as was also observed by Andrews & Kenerley (1978), who found that unsprayed leaves supported a 50% higher species diversity than metiram-treated apple leaves. Differences in this latter study were attributed to a higher resistance of some species to the fungicide. Hislop & Cox (1969) found that the effect of captan

on species diversity was only temporarily and species composition on apple leaves sprayed with captan in the previous season was similar to those on unsprayed leaves. The fungal genera found in this study are given for each separate treatment in Table 5.4; there does not appear to be a different taxonomic composition depending on the treatment. The genera are similar to those found by Lim & Khor (1982; Table 5.4).

It must be noted that a significant decrease in post-harvest anthracnose development was also not observed in those trees receiving the full fungicide treatment. This is probably a result of the very dry weather experienced, which resulted in low levels of disease being present, rather than due to the ineffectiveness of the fungicide.

Table 5.4 List of genera found on MEA from leaf washings of all treatments, compared to the study of Lim & Khor (1982). - indicates absence and X presence of genus, N this treatment could not have been sampled one week after spraying.

	CON	NUT	1x5	2x5	BEN	6xB	L&K[2]
Aspergillus	X -[1]	X -	X -	N X	X X	N X	X
Cladosporium	X X	X X	X X	N X	X X	N X	X
Colletotrichum	X -	- -	- -	N -	- -	N -	X
Curvularia	- -	- -	- -	N -	- X	N -	X
Fusarium	- -	- X	- X	N X	X X	N X	X
Gliocladium	- -	- -	- -	N -	- X	N -	-
Lasiodiplodia	- X	- X	X X	N -	X X	N X	-
Neurospora	X -	- -	- -	N -	- -	N -	-
Penicillium	- -	- X	- X	N X	X X	N X	X
Pestalotiopsis	X X	X X	X X	N X	X X	N -	X
Va[4]	X -	- -	X -	N -	X -	N -	
Vb	- -	X -	- -	N -	- -	N -	
VIIb	- -	- -	X -	N -	- -	N -	
XVIII	- -	X -	- -	N -	X -	N -	
XX	X -	- -	- -	N -	- -	N -	
green fluffy	- -	- X	- -	N -	- -	N -	
spreading white	- -	- -	- X	N X	- X	N -	
small white	- X	- X	- X	N -	- X	N X	
Acremonium							X
Gonabotryum							X
Scolecobasidium							X
Total no. of genera	9	10	9	6[3]	12	6[3]	10

[1] presence at 1 week after spray, followed by at harvest.
[2] presence in study of Lim & Khor (1982).
[3] Both 2x558 and 6xBEN have less genera, this could be explained by the fact that at harvest species diversity was less than at one week after spraying.
[4] Genus not identified.

5.4 Discussion

That no control of anthracnose was observed at all was disappointing but influenced by the dry weather. Tronsmo & Ystaas (1980) carried out trials during two field seasons with *Trichoderma harzianum* to control *Botrytis cinerea* on apple. In both seasons disease incidence was low, with less than 1% infected apples in the first season and 10% in the second season. However the second year resulted in a small but significant decrease of infected apples in the *T. harzianum* treated trees. This emphasises that firm conclusions cannot be made about the efficacy of a biological control agent on the basis of one trial only.

The fact that there were hardly any pseudomonads on the leaf surface on all three sampling dates should have discouraged further experimentation if known beforehand. From previous reports in the literature, pseudomonads represent about 50% of the phyllosphere population on mango leaves (Lim & Khor, 1982). It has also been shown from studies carried out in temperate climates that pseudomonads are commonly isolated from the phyllosphere (Brodie & Blakeman, 1976; Spurr, 1981; Gross *et al.*, 1983; Outryve *et al.*, 1989), but according to Fokkema & Schippers (1986) never comprise more than 5-10% of the total bacterial population present. There are some examples of biological control on the phylloplane using a *Pseudomonas fluorescens*. An isolate from a grass, *Lolium perenne*, reduced lesion development caused by *Drechslera dictyoides* (Austin *et al.*, 1978). Ruinen (1961) mentioned the presence of pseudomonads in tropical rain forests. This last report might give an indication why there is a discrepancy between the number of pseudomonads found in this study and the study by Lim & Khor (1982). Large numbers of phyllosphere microbes were found on leaves in tropical rain forests with layers of up to 20 μm thick (Ruinen, 1961), obviously supported by the high humidity experienced in this ecosystem. Climatic conditions of the study of Lim & Khor (1982) are not known, but it is possible that humidity in their orchard was higher than experienced in our experiment. A leaf wetness sensor at the orchard in Batangas recorded 8 periods of over 24hrs or more leaf wetness (Dodd *et al.*, 1991a) but even short dry periods may have been enough to cause the death of all the pseudomonads (Spurr & Knudsen, 1985). Differences in microbial populations found between different sites are not always easily explained. A study into the occurrence of ice nucleation bacteria, *Pseudomonas syringae*, in the Pacific North-West of the United States, showed that 30% of fruit tree orchards in the Yakima Valley contained these bacteria compared to 75% of orchards in the Hood

River Valley (Gross *et al.*, 1983). Since there were no clear environmental differences between the areas no explanation for this difference could be given. Thus for mango orchards, it may be necessary to obtain a general impression of the microbial populations found in many different areas, before the selection of a biological control agent that will be applicable to all these areas can be made. I do not think it will be any good just to develop a biological control system for one particular area because that will never be economically feasible (Upadhyay & Rai, 1988).

Isolate 558 was isolated from a mango leaf, although it is questionable whether the isolate was actively growing since it might have arrived on the leaf in dust. Many bacteria do arrive on leaf surfaces from the outside environment and are not residents (Leben, 1961).

One encouraging factor in the potential of 558 for biological control is that this isolate produces plenty of exo-polysaccharides in CzDL, as was noted during experiments to obtain cell free cultures (6.2.4). The exo-polysaccharides were also visible on electron micrographs of 558 inoculated in the laboratory onto the mango surface (Figure 6.8). These exo-polysaccharides could play an important role in the adhesion of bacteria to surfaces, and also protect them from desiccation and U.V.radiation (Dickinson, 1986). However it is not certain that 558 does produce these exo-polysaccharides on the leaf surface and further studies into this matter are required.

A biological control agent should really be tested in a small scale *in vivo* trial (Andrews, 1985). This was not possible, however, with mango trees, and an alternative model system using *C. gloeosporioides* was not available. A combination of *Phaseolus vulgaris* and *C. lindemuthianum* might serve as a model system to carry out future *in vivo* testing, but might behave differently than *C. gloeosporioides* on mango. *C. lindemuthianum* was, however, inhibited by 558 in the cellophane overlay screens for antagonism (see 6.3.11). The effect of 558 on spore germination of *C. lindemuthianum* was not determined.

Leben & Daft (1964) tested a whole range of different organisms on plants in a moist chamber and in the greenhouse which resulted in the selection of isolate A180 obtained from a cucumber seedling (Leben, 1964). This isolate reduced cucumber anthracnose (*C. lagenarium*), early blight of tomato (*Alternaria solani*), and northern leaf blight of corn (*Setosphaeria turcica*) in greenhouse tests (Leben & Daft, 1965). To further test this isolate against apple scab (*Venturia inaequalis*), cucumber anthracnose and early blight of tomatoes, extensive field trials were carried out. Disease developed, however, on all A180 treated plants

and when tests were carried out to determine the survival of the bacteria 24 hrs after spraying, it was found that only 1% of the inoculum had remained viable. They also determined that more bacteria survived when applied in nutrients, soybean meal broth, than when applied in water (Leben *et al.*, 1965). Another study in which the authors had been very careful in selecting their antagonist was that of Andrews and co-workers for control of *V. inaequalis*, causing apple scab, with *Chaetomium globosum*. A total of 3 *in vitro* and 3 *in vivo* experiments, including tests on mycelial growth, spore germination and germ tube length, lesion size, disease symptoms and sporulation *in vivo*, resulted in 8 potential candidates of which *C. globosum* performed best overall (Andrews *et al.*, 1983). When this antagonist was tested in the field, only 25% control of apple scab was observed (Cullen *et al.*, 1984a). This was explained by the low survival of spores on the leaf surface and the instability of chemotin, the antibiotic produced by *C. globosum* thought to be responsible for inhibiting *V. inaequalis* (Cullen & Andrews, 1984a). Chemotin leaks passively from the spores, but once on the leaf surface the physical environment becomes unfavourable to this antibiotic and it is no longer active (Boudreau & Andrews, 1987).

From the above examples it is obvious that the establishment of an antagonist must be a major consideration. Microbial immigration studies revealed that it was possible to introduce *Aureobasidium pullulans* to surface sterilised apple leaves, yet *C. globosum* failed to establish itself even though no competition was present (Kinkel *et al.*, 1989b). In another study the same investigators failed to establish *A. pullulans* on apple leaves in an attempt to control *V. inaequalis* (Cullen *et al.*, 1984a). It was thought that in this latter experiment *A. pullulans* was inhibited through desiccation. These observations indicate that it is difficult to predict the circumstances under which an antagonist is going to survive, but they are obviously influenced by the resident population, the physical and chemical state of the leaf surface, micro-climate and 'fitness' of the antagonist (Blakeman, 1973; see also Dickinson, 1976). The success rate of biological control approaches will be higher using greenhouse crops since environmental conditions are more stable (Campbell, 1986). For example, Redmond *et al.* (1987) obtained 63% control of *Botrytis cinerea* with the yeast *Exophiala jeanselmei* on greenhouse grown roses, while the iprodione treated roses reduced infection of *B. cinerea* by 74%. The way an antagonist is applied is also important and, in general, the simultaneous application of nutrients (Leben, 1964; McBride, 1969) and sticker-spreader compounds increase the rate of success (Spurr, 1981; Cullen & Andrews, 1984b). The use of forecasting models has also been promoted

(Knudsen & Spurr, 1988) and have been tested in a limited number of case studies. However, these models need a large amount of experimentation to determine all the possible factors which could influence the prediction and they are only at an early stage of development. Knudsen & Spurr (1985) used a computer analysis system to assess the quantitative relationship between antagonist and pathogen, in this case *Pseudomonas maltophilia* and *Bacillus mycoides* as antagonists of *Alternaria alternata*, causing tobacco leaf spot. Dik (pers.comm.) developed a simulation model to evaluate the role of yeasts in the integrated control of necrotrophic pathogens such as found on wheat. These pathogens were found to be less sensitive to the fungicides applied due to the high nutrient status (pollen and honeydew) on the leaves in absence of their major competitors, the yeasts, which were drastically reduced in numbers by the fungicide application.

In the experiments described in this chapter it was obvious that 558 did not survive on the phylloplane, and this could have been due to several reasons. Firstly, although the spraying was carried out on a cloudy day, it was applied in the morning and the humidity might have been too low for 558 to survive. Most microorganisms require >95% RH to survive (Blakeman, 1985), while in sunlight the RH on the leaf surface is between 40 and 65% (Diem, 1971). There was a torrential rain storm on the evening following spraying which could have washed off most of the bacteria if they were not adhering firmly. The technique for quantifying numbers on the leaf surface indicates that washing for 15min can remove many bacteria. Another explanation could be that the force of the spray, even though it was applied by a backpack sprayer, could in combination with the nutrients or the pH of the medium become toxic to the bacteria. The effect of the different insecticides used in this study on 558 was not determined, but in general insecticides do not appear to have a direct toxic effect on bacteria (Hislop, 1976). Successful establishment could be enhanced by applying the bacteria in the evening when humidity is higher, and will remain high till the next morning. This would be possible in an experimental trial, but might pose problems to the farmer. Another possibility is the incorporation of a biological control spray into a strategic spraying programme as developed by Fitzell *et al.* (1984) for mangoes in north-east Australia. This system has been tested out by Dodd *et al.* (1991a, b) on the Carabao mango in the Philippines. The idea behind the strategic spray programme is that sprays are only applied when risk of infection is highest. For mango anthracnose this is when there is a prolonged period of leaf wetness (Fitzell & Peak, 1984) or high relative humidity (Dodd *et al.*, 1991b). Both these criteria would be conducive for a successful microbial spray because initial survival of the microorganisms

would be enhanced. In addition there is more chance that the spray might be successful since bacteria and spores of *C. gloeosporioides* would simultaneously be present in an active state on the fruit and blossom surface.

In theory, the best strategy would be to establish an antagonistic population within the resident tree microflora. This does not imply, however, that the antagonist will then colonize rapidly growing parts, such as the flower panicles and developing fruit, which are at high risk from anthracnose infection. It might be more feasible to use yeasts as biological control agents rather than bacteria. Yeasts offer certain advantages, for example, they appear to be less susceptible to population fluctuations caused by external factors, such as desiccation (Bashi & Fokkema, 1977; Fokkema, 1981), although migration to new areas is slow or non-existent (Fokkema *et al.*, 1979). On temperate crops there is a succession of microbial populations on new leaves. The initial colonizers are bacteria, living on low levels of nutrients (Blakeman, 1972), followed by yeasts which have higher nutrient requirements, and then filamentous fungi (Dickinson, 1976; Blakeman, 1985, 1988). Yeasts arrive on the leaf surface via airborne and rain splashed particles (Fokkema *et al.*, 1979), bacteria are more mobile and can colonize virgin leaf areas (Leben, 1961). There is, however, a problem in extrapolating these observations to the tropical ecosystem. Most population studies have been carried out in the temperate climates on deciduous trees, most tropical trees are, on the other hand, evergreen and hence will have a different succession and leaf population. The environmental fluctuations caused by the seasons in temperate climates is also much less in tropical areas.

In the experiments described here, bacteria were the dominant population in terms of numbers, present. This confirms the results of Lim & Khor (1982) who also found that bacteria were dominant on mango leaves, but yeasts were dominant on blossoms. Another consideration in assessing the potential of yeasts is that *Aureobasidium pullulans*, a yeast which looked a promising candidate in the initial screen (isolate 251; Chapter 3), can be part of the sooty mould microflora. This microbial overgrowth on leaves often develops after an attack by pink wax scale, on the honeydew deposited on leaves and fruit by this insect, and could reduce the market price of fruit. There are many examples in which phylloplane yeasts have been able to reduce fungal diseases in experimental studies (see Blakeman & Fokkema, 1982), including the inhibition of *C. graminicola* on maize by *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavescens* (Williamson & Fokkema, 1985).

The use of nutrient sprays to enhance competition by antagonists present among the resident microflora is an area where much more work needs to be done. In some of the larger orchards in the Philippines it is standard practice to apply a foliar fertilizer during blossom and fruit set. It would be interesting to determine how different compositions of foliar fertilizers stimulate different components of the phylloplane microflora, and to determine which combination would best enhance an introduced biological control agent. In one study it was found that bacteria are poor competitors for sugars but are scavengers of amino acids, while yeast are much more able to use sugars (Blakeman & Brodie, 1977). It might also be possible to alter the endogenous population with the addition of nutrients and hence stimulate biological control. The effect of urea sprayed on apple trees just before leaf shed to control the overwintering of *V. inaequalis* in the leaf litter, is due to the positive effect urea has on the microflora, stimulating saprophytic fungi and gram-negative bacteria (Burchill & Cook, 1971). Another example of nutrient addition enhancing disease control is the reduction of disease caused by *Pseudomonas syringae*. Addition of amino acids to leaves of *Phaseolus vulgaris* resulted in a reduced population of fluorescent pseudomonads and through competition, less disease was observed as a consequence (Morris & Rouse, 1985).

In summary, these experiments have given us an increased insight into the problems attached to biological control in the field even though results were negative in relation to developing a biological control strategy. With increased impetus it should be possible to develop a pre-harvest biological control system against *C. gloeosporioides*, providing it will be economical. At present, though, it does appear that the post-harvest approach to disease control will be more feasible.

6 Identification and mode of action of isolates 204 and 558

6.1.Objectives

To successfully introduce an antagonist, a microorganism that adversely affects another organism growing in association with it (Baker & Cook, 1974), we need to understand the mechanism(s) through which the target pathogen is inhibited. With knowledge of the mechanisms involved it should be possible to optimise biological control and make it a more viable proposal (Blakeman & Fokkema, 1982). The possible modes of action of antagonists (see also Baker & Cook, 1974) are:

1) Antibiosis - this could be achieved through the production of antibiotics, volatile compounds, lytic enzymes or a modification of the environment e.g. change in pH. Any of these can result in a condition which is directly lethal to the pathogen, or which inhibits the pathogen so that it is not able to express pathogenicity. Very few studies have proven that antibiosis actually takes place *in vivo*, most studies imply that antibiotics are involved. One of the best examples demonstrating *in vivo* production of antibiotics is the production of phenazine by *Pseudomonas fluorescens* where the antibiotic was recovered from the rhizosphere (Thomashow *et al.*, 1990).

2) Competition - both nutrients and suitable space are in short supply on the leaf surface (Cullen & Andrews, 1984b) and microorganisms need to be able to compete for either to be able to survive. For example nutrient competition for essential amino acids, sugars or iron, could be important. In relation to competition for iron, whether the antagonist produces siderophores (iron-chelating compounds).

3) Parasitism - Direct parasitism, in which one organism lives off another organism, has in biological control only been reported for one fungal species parasitising another fungal species (see Ayers & Adams, 1981). Examples of this on the phylloplane are *Trichoderma* spp. (see Tronsmo, 1986) and *Ampelomyces quisqualis* (Sztejnberg *et al.*, 1989).

Thus to be able to improve biological control activity of 204 and 558 it was necessary to :

1 - Identify both isolates, 204 and 558, and make an evaluation of the feasibility to use them as biological control agents.

2 - Determine the mode of action and nutrient requirements of both organisms, to be able to decide on how to improve their action.

6.2 Materials & methods

Not all experiments were carried out with both isolates and for each experiment the isolates used are indicated between brackets.

6.2.1 Identification (204 & 558) - Identification of isolates was performed using analytical profile index (API) identification kits, API 50 CHB for isolate 204 and API 20 E for isolate 558.

6.2.2 Production of volatile compounds (204 & 558) - Bacteria were streaked onto NA plates, covering the entire plate, and incubated for 48 hrs. A plug of *C. gloeosporioides* was inoculated onto MEA and the two Petri dishes were taped together, with the plate containing *C. gloeosporioides* inverted (Dennis & Webster, 1971b). The control was provided by taping an uninoculated plate of NA over a plate inoculated with *C. gloeosporioides*. Growth of *C. gloeosporioides* was measured at 3, 5, 7, 9, 11, 13 and 15 days. The experiment was conducted twice with 3 and 5 replicates/treatment respectively.

6.2.3 Cellophane overlay assay (204 & 558) - Plates of TWA, MEA and Czapek Dox agar (CzDA), were covered with a circle of cellophane (see 3.2.3 for preparation of cellophane covered plates). Bacterial suspensions in dH_2O were inoculated onto the cellophane and dispersed with a spreader to form an even covering. Plates were incubated for 5 or 10 days, after which the cellophane was removed and a plug of *C. gloeosporioides* inoculated onto the centre of the plate (Heatley, 1947; Gibbs, 1967). Treatments were replicated 3 times and growth of *C. gloeosporioides* was measured at 3, 5, 7, 9, 11, 13 and 15 days.

6.2.4 Cell-free cultures (204 & 558) - In order to determine whether inhibitory compounds were secreted into the growth medium, cell free extracts were made. Bacteria were grown in NB (204), and CzDL (558), over a period of 2 or 4 days. Cultures were then centrifuged at 12,000xg for 60 min and filter sterilised through a 0.2 μm filter (Cellulose nitrate, Millipore). These filtrates were incorporated into TWA, one part being incorporated directly, whilst the other part was first autoclaved (15min, 20 psi). A plug of *C. gloeosporioides* was inoculated into the centre of the plate and growth of *C. gloeosporioides* was measured at 3, 5, 7, 9, 11, 13, and 15 days. Controls were TWA and NB (for isolate 204) or CzDA (for isolate 558). Treatments were replicated 5 times.

6.2.5 Antibiotic production (204 & 558) - Several methods of extraction were employed.

Method 1. Bacteria were grown on cellophane overlying agar as described in 6.2.3. After 5 days the cellophane was removed and the agar was cut into small pieces. Each agar plate was covered with 10 ml of solvent in a universal vial and agitated for a period of 1 hr. Solvents were pipetted off and dried down over nitrogen gas to 1/20 of their original volume.

Method 2. Bacteria were grown in MEB or CzDL and, after 5 days of growth, cell free cultures were prepared (6.2.4). Equal parts of broth were washed with solvents after which the solvent fractions were dried down as in method 1.

Method 3. (558) This method was used by Howell & Stipanovic (1979) to detect pyrrolnitrin. Isolate 558 was grown for 5 days on KingB agar, and 10 plates were extracted with 200ml of an 80% aqueous acetone solution. After extraction the extract was filtered through cheese cloth, centrifuged (10,000 r.p.m.) and reduced on a rotary evaporator (<50°C). One gram of NaCl was added to the aqueous fraction, and this fraction was washed twice with diethyl ether. The ether fractions were combined and dried under vacuum to give the final concentrated extract.

Method 4. (558) This method was used by Thomashow & Weller (1988) to detect phenazine. Cultures of 558 were grown in both CzDL and minimal medium (MM) medium. CzDL was either incubated stationary, or on a shaker at 25°C. MM cultures were either incubated stationary at 25 and 30°C, or on a shaker at 25°C. Cell free suspension were prepared (6.2.4). These and whole cultures were extracted twice with equal volumes of benzene. Pooled extracts were scanned with the spectrophotometer (Philips, PU 8720 UV/VIS scanning spectrophotometer) at 349.5 nm. The presence of phenazine was also tested by growing cultures on potato dextrose agar (PDA, Difco), on which phenazine producing colonies form a brown pigment. A

dark zone should also surround these colonies when they are examined under long wave U.V. (365 nm).

To assay the fractions obtained by the methods described above, the following bio-assays were employed: 1. 50μl of solvent or filtrate was pipetted onto bio-assay filters (Antibiotic Assay discs, Whatman) which were then placed onto a MEA plate on which also a plug of *C. gloeosporioides* was inoculated at a distance of 4cm. 2. 100μl solvent or filtrate was inoculated into wells of 0.5cm diameter cut into MEA plates. A plug of *C. gloeosporioides* was then inoculated onto the plate at a distance of 4cm. 3. 100 μl samples of solvent or filtrate were spotted (using a Desaga autospotter) onto a Thin Layer Chromatography (TLC) plate (Kieselgel 60, Merck) after which this plate was developed in one of the following solvent systems : a. 8:2 methanol : chloroform; b. 1:1 Diethyl ether : cyclohexane; c. 5:5 methanol : chloroform (Braithwaite & Smith, 1985). After development, plates were thoroughly dried to remove any hazardous vapours from the solvents which could inhibit fungal growth. Plates were bioassayed by either spraying a spore suspension of *C. gloeosporioides* spores in MEB directly onto the plate or by pouring a thin layer (0.5 cm) of a spore suspension of *C. gloeosporioides* in MEA over the plate.

Experiment a - In this experiment method 1 was used for extraction, with the following solvents : methanol (204 & 558), 80% acetone (558), chloroform (204 & 558), cyclohexane (204 & 558), ethyl acetate (204), diethyl ether (204 & 558) and dimethyl sulphoxide (DMSO; 204 & 558). Extracts were tested for their inhibitory activity in wells, on filter papers and spotted onto TLC plates and developed in solvent systems a and b. TLC plates were overlayed with a spore suspension of *C. gloeosporioides* in MEA.

Experiment b - To investigate 204 further, a cell free suspension of 204 grown for 48 hrs in NB was prepared and extracted according to method 2 with chloroform, butanol, ethyl acetate and diethyl ether. TLC plates were developed in solvent system 1 and bioassayed with a spore suspension.

Experiment c - To check 558 another time, CzDL and -Fe/CzDL (see 6.2.7) in which 558 had been growing for 5 days, were extracted according to method 2 with chloroform, diethyl ether, ethyl acetate, cyclohexane and dichloromethane. Extracts and pure filtrates were spotted directly onto TLC plates, developed in solvent system 1 and bioassayed with a spore suspension.

Experiment d - Method 3 was carried out with 558, and additionally, extracts were spotted onto a TLC plate which was developed in solvent system 1 and bioassayed with a spore suspension.

Experiment e - Method 4 was carried out with 558, and, additionally, extracts were spotted onto TLC plates, developed in system 1, and bioassayed with a spore suspension.

6.2.6 Effect of carbon and nitrogen ratios on effectivity (558) - A range of modified CzDA was prepared by altering the amount of carbon and nitrogen present in the medium. The following agars were prepared : 100% sucrose with either 100, 80, 60, 40 and 20% $NaNO_3$ and 100% $NaNO_3$ with either 80, 60, 40 and 20% sucrose. Other elements were supplied in the normal quantities found in CzDA (see Appendix 1) except for one treatment which contained 100% sucrose and 100% $NaNO_3$ but no other nutrients. The cellophane overlay method (6.2.3) was performed on these agars, and treatments were replicated five times.

6.2.7 pH (558) - The pH was measured of CzDA on which 558 had grown (using the cellophane overlay method; 6.2.3; 3 replicate plates/day) during a period of 7 days, and during a period of 10 days of CzDL in which 558 was growing. The growth of 558 in liquid culture was measured as the optical density (O.D.) which was calibrated at 253 nm and this calibration curve is given in Figure 6.1 (3 replicate readings/point). To determine if 558 would still show antagonism on buffered medium, CzDA was prepared with the inclusion of a phosphate buffer (0.2M; Dhingra & Sinclair, 1985).

6.2.8 Siderophore production (558) - Iron free glassware was prepared by soaking glassware in 0.5M HCl overnight, rinsing it thoroughly in dH_2O and drying it in an oven. Iron free water was prepared by passing dH_2O through a column of chelating resin (sodium form, 50 to 100 mesh, Sigma) after the pH of the column was adjusted to 6.3 by rinsing four times with 0.5M sodium acetate (McCracken & Swinburne, 1979). Iron free dH_2O and analytical grade chemicals were then used to prepare CzDL, omitting the ferrous sulphate. Isolate 558 was grown in normal CzDL and iron free CzDL (-Fe/CzDL). After 5 days of growth, cell free filtrates were prepared (6.2.4) and filtrates were examined for siderophore production by adding drops of a $FeCl_3$ solution (1g/litre) into the filtrate. The formation of a brown pigment is an indication that iron chelating compounds are present (Philson & Llinás, 1982).

A spore germination test (see 2.2.5) was set up to assess the effect of the siderophore produced by 558 and a commercially available iron chelator, ethylene diamine tetra acetic acid (EDTA), on spore germination. For this experiment spore suspensions were prepared (see 2.2.3) from a culture of *C. gloeosporioides* which had grown on iron free CzDA (-Fe/CzDA).

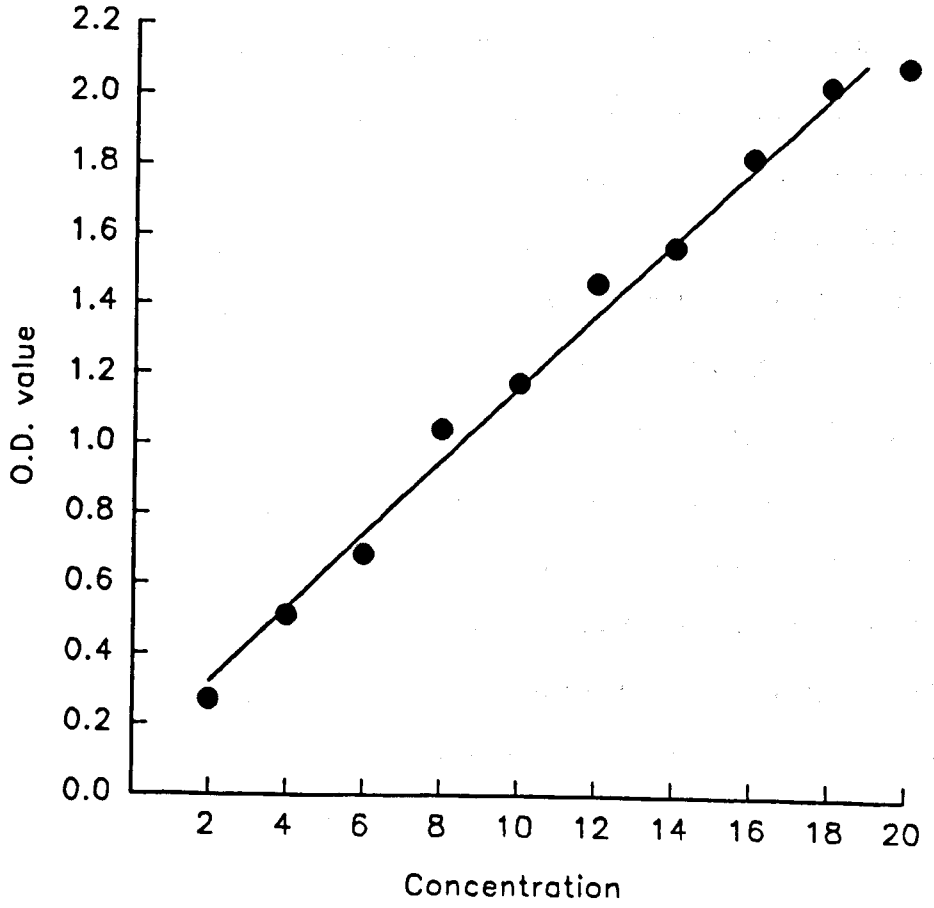


Figure 6.1 Calibration curve of optical density (O.D.) of 558 grown in CzDL in relation to the cell concentration (cfu/ml x 10^7; $y = 9.340 x + 0.0926$; $r^2 = 98.8\%$)

6.2.9 Microscopical observations of interactions (204 & 558) - Mangoes were surface sterilised and dual inoculations of 10 μl bacterial suspension in 0.5% peptone (10^7 cfu/ml) and 10 μl spore suspension of *C. gloeosporioides* ($5x10^4$ spores/ml; see 2.2.3) were made. Mangoes were incubated in moist chambers (2.2.3). Samples were taken after 24 and 48 hrs, prepared for the SEM (CPD, see 2.2.5) and examined.

6.2.10 Temperature range (558) - CzDA plates with 558 on cellophane (see 6.2.3) were incubated at 10, 15, 20, 30 and 37°C for 5 days. After this time the cellophane was removed and a plug of *C. gloeosporioides* inoculated onto the centre of the plate. At this stage all plates were transferred to 25°C and growth measured at 3, 5, 7, 9, 11, 13 and 15 days (5 plates/ treatment).

6.2.11 Taxonomic spectrum of inhibition (558) - To determine if 558 could also inhibit *in vitro* growth of additional *C. gloeosporioides* isolates, other *Colletotrichum* species and other pathogens the cellophane overlay assay (6.2.3) was carried out using the following fungi : *C. gloeosporioides* mango isolates 19, 24, 25, 29 and 40 (see Appendix 2.1 for place of origin), SLI4 and PI4 (obtained from J.C. Dodd, University of Kent, isolates from Sri Lanka and Philippines respectively), IMI 321880 (obtained from C. Prior, Commonwealth Institute for Biological Control, isolate from Dominica), *C. gloeosporioides* papaya isolate (obtained from R.A. Plumbley, Natural Resources Institute), *C. capsici* (obtained from B. Ritchie, International Mycological Institute, Kew), *C.lindemuthianum*, *C. lagenarium*, *C. musae*, *C. gloeosporioides* (isolate from avocado), *C. coffeanum*, *C. acutatum* and *C. atramentarium* (the last seven isolates were obtained from R.J. O'Connell, Long Ashton Research Station). Other pathogens used for the cellophane overlay assay were : *Penicillium digitatum*, *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora citricola*, *Fusarium solani* and *Lasiodiplodia* (Sri Lankan isolate, see 3.3.5). Growth was measured at 3, 5, 7, 9, 11, 13 and 15 days, except for *R. solani*, *Py. ultimum*, *Ph. citricola* and *Lasiodiplodia* for which growth was measured daily (5 plates/treatment).

6.3 Results

6.3.1 Identification - By using the API strips it was determined that isolate 204 was *Bacillus cereus*, and isolate 558 was *Pseudomonas fluorescens*. To confirm these findings both cultures were send to NCIMB (Aberdeen) who obtained identical identifications.

6.3.2 Volatile compounds - The average radial growth rate was similar for all three treatments (Table 6.1). This indicated that no inhibitory volatile compounds were produced by either 204 or 558.

Table 6.1 Radial growth (in mm/day) for *C. gloeosporioides* grown inverted over a plate with 204, 558 or no bacterium (Con) present.

	Con	204	558	S.E.
Exp. I	3.4	3.1	3.0	
Exp. II	3.4	3.4	3.3	0.17

6.3.3 Cellophane overlay assay - On TWA there was no difference in radial growth rate of *C. gloeosporioides* on plates on which 204 or 558 had grown previously, compared to the control plates. Radial growth of *C. gloeosporioides* on the plates which had 558 grown on it before, was 0.3 mm/day less than the control (Table 6.2). On MEA plates on which 204 had been grown, *C. gloeosporioides* had the lowest radial growth rate with 2.8 mm/day, followed by plates on which 558 had grown, with 3.4 mm/day and the control plates with 4.4 mm/day. The largest difference was, however, observed on CzDA. On CzDA plates on which 558 had grown, *C. gloeosporioides* had a radial growth rate of 0.6 mm/day compared to the control with 4.2 mm/day, a decrease of 86%. On plates on which 204 had been grown the radial growth rate of *C. gloeosporioides* more or less equalled the control with 4.0 mm/day (Table 6.2).

C. gloeosporioides was left to grow on the CzDA plates on which 558 had grown before, for up to 3 weeks and it was observed that up to 2 weeks growth was very slow, with an abnormal growth pattern which appeared as 'feathery' growth. After two weeks the fungus started growing again from the original agar plug, with a normal morphology, but a sparse mycelium.

Table 6.2 Radial growth rate (mm/day) of *C. gloeosporioides* on agar on which previously 204, 558 or no bacterium had grown.

	Con	204	558
TWA	2.4	2.3	2.1
MEA	4.4	2.8	3.4
CzDA	4.2	4.0	0.6
mean	3.7	3.0	2.0
S.E.	0.29	0.22	0.37

This biphasic growth pattern was also observed for *C. lindemuthianum* by Bailey *et al.* (1976) in relation to fungal growth on agar in which phytoalexins had been incorporated. These researchers showed that this growth pattern was due to breakdown of the phytoalexin, phaseolin, by *C. lindemuthianum*. This growth pattern highlights some of the problems of using colony diameter as a measurement. When the growth pattern is feathery it is difficult to measure the diameter, and growth from the plug was measured randomly. To avoid these problems a measure of total biomass is necessary. Initially total chitin was determined as a measurement of fungal growth. It was possible to process whole plates, but the method was messy and labour intensive. It was subsequently decided that colony diameter gave sufficient information for the purpose of the assessment. Another method, if equipment was available, would be the use of an advanced optical density reader.

6.3.4 Cell free cultures - The results obtained from these experiments cannot be explained. Firstly, comparing the radial growth rates of *C. gloeosporioides* grown in the presence of culture filtrates of 558 with those of the control growth rates (3.0 mm/day for TWA and 4.3 mm/day for CzDA; Table 6.3). The mean growth rate for *C. gloeosporioides* on media containing 558-filtrate (48 hrs) was 3.2 mm/day; on media containing autoclaved 558-filtrate (48 hrs) 3.2 mm/day; on media containing 558-filtrate (96 hrs) 3.1 mm/day; and on media containing autoclaved 558-filtrate (96 hrs) 3.0 mm/day. But as can be seen in Table 6.3 variation between the four identical experiments carried out was enormous and no conclusions could be made from these experiments. Only one similar experiment was carried out with isolate 204 which

showed the following daily growth rates of *C. gloeosporioides* : on media containing 204-filtrate (48 hrs) 0.8 mm/day; on media containing autoclaved 204-filtrate (48 hrs) 1.1 mm/day compared to a normal growth rate of *C. gloeosporioides* on NA of 3.3 mm/day. This indicates that an inhibitory compound is formed by 204 growing in liquid culture, but these experiments were abandoned in favour of those involving the extraction of possible inhibitory compounds.

Table 6.3 Radial growth rate of *C. gloeosporioides* (mm/day) on TWA with 558 filtrate incorporated (Fil = pure filtrate, A.Fil = autoclaved filtrate) and on control plates of TWA and CzDA.

	Control		48hrs growth		96hrs growth	
	TWA	CzDA	Fil	A.Fil	Fil	A.Fil
Exp. I	3.0	4.2	4.7	2.0	0.6	3.6
Exp. II	2.7	4.5	3.0	3.6	3.4	4.3
Exp. III	3.0	3.9	2.0	4.0	4.0	1.6
Exp. IV	3.2	4.6	-	-	4.4	2.6
mean	3.0	4.3	3.2	3.2	3.1	3.0
S.E.	0.06	0.07	0.26	0.23	0.34	0.27

6.3.5 Antibiotic production - *Experiment a* - None of these bioassays yielded any inhibition of growth of *C. gloeosporioides* by the extracts of 204 and 558. The only observation made was that the hyphae round the well containing the chloroform extract from 558, formed a dark pigmentation (presumably melanin). A similar observation was made for the well containing the ethyl acetate extract from 558, which also increased sporulation of the fungus round the well. Changes in morphology were not observed for similar extracts applied to the filter papers, but concentrations of these were 50% of those in the wells. The extracts from 204 did not cause any changes in morphology. On the TLC plates the methanol extract from 204 showed a small, clear spot at the baseline in both solvent systems, and for the cyclohexane extract from 558 there was a spot where increased sporulation was visible at a rf-value of 0.07 in solvent system 1. To examine this latter spot further, a more polar solvent system was used, system 3, and compared to system 1. Another plate was developed in two dimensions, first in solvent system 1, followed by solvent system 3. However the agar overlay method did not reveal any spots this time.

Experiment b - There was a clear spot near the solvent front for the butanol extract, but none of the other

extracts yielded any clear spots. This experiment was repeated with the butanol extract and a butanol control, and both bioassay systems were employed. The TLC plate sprayed with spores showed spots in all the lanes which contained butanol, but the agar overlay method did not show up these spots. This indicated that the agar overlay bioassay was not sensitive enough to indicate glutaraldehydes, toxic to *C. gloeosporioides*, and present as contaminants in the butanol. No further use was made of the agar overlay bioassay. In this experiment the crude filtrates were also tested on TLC plates, but also this time no spots were observed.

Experiment c - The filtrates nor the extracts from 558 showed any spots after development in solvent system 1 and after bioassaying by spraying a spore suspension over the plate.

Experiment d - The assays carried out according to the method of Howell & Stipanovic (1979; method 3) with extracts from 558 did not inhibit growth of *C. gloeosporioides*, indicating that neither pyrrolnitrin or other inhibitory compounds were present in these extracts.

Experiment e - The benzene extracts (method 4) did not increase the O.D. at 349.5 nm over the control (benzene alone), indicating that no phenazine was produced. This was confirmed by the fact that on PDA, colonies did not produce a brown pigment nor was a clear zone visible when examined under long wave U.V. There were also no spots were observed on the TLC plates.

In summary no detectable inhibitory compounds against *C. gloeosporioides* were produced by either 204 or 558 in agar or in liquid culture. Isolate 558 also did also not produce pyrrolnitrin or phenazine.

6.3.6 Effect of carbon and nitrogen ratios on effectivity - Isolate 558 had formed a dense growth on the cellophane overlying all the different agars, except on that over the agar which contained no other elements except sucrose and $NaNO_3$. Differences in the colour of the film of pseudomonads formed on the cellophane were observed (Figure 6.2), which was to a lesser extent reflected in the colour of the underlying agar into which these compounds had exuded. On complete CzDA 558 produced a typical bright yellow pigment which became less intense in agar containing 80 and 60% sucrose, and the colour of 558 became white cream on agar containing 40% and white on agar containing 20% sucrose. Colour of 558 was dull yellow on agar containing 80% $NaNO_3$, cream on agar containing 60%, white cream on agar containing 40% and white on agar containing 20% $NaNO_3$ (see Figure 6.2). This colour was correlated to the amount of growth of *C. gloeosporioides* observed on these agars.

Figure 6.2 Growth of 558 overlying CzDA containing different concentrations (%) of sucrose (S) and $NaNO_3$ (N). Note the different colours.

Radial growth rate of *C. gloeosporioides* was lowest on the agars containing most nutrients, with increasing radial growth rates with decreasing amounts of carbon and nitrogen (Figure 6.3). Radial growth rate of *C. gloeosporioides* was 0.4 mm/day for complete CzDA on which 558 had grown. In the presence of 100% sucrose radial growth rate of *C. gloeosporioides* on plates on which 558 had grown was 0.6 mm/day for 80%$NaNO_3$, 1.3 mm/day for 60% $NaNO_3$, 3.2 mm/day for 40% $NaNO_3$, and 3.1 mm/day for 20% $NaNO_3$. In the presence of 100% $NaNO_3$ radial growth rate of *C. gloeosporioides* on plates on which 558 had grown was 0.4 mm/day for 80% sucrose, 0.5 mm/day for 60% sucrose, 1.3 mm/day for 40% sucrose and 2.8 mm/day for 20% sucrose (Figure 6.3). This indicates that the inhibition of radial growth rates of *C. gloeosporioides* on plates on which 558 had grown is dependant on the amount of available sucrose and $NaNO_3$ to 558. The percentage inhibition of *C. gloeosporioides* observed was more dependant on the amount of $NaNO_3$ available than the amount of sucrose available to 558. Growth rate of *C. gloeosporioides* was identical for all control plates containing the different amounts of sucrose and $NaNO_3$ (Figure 6.3).

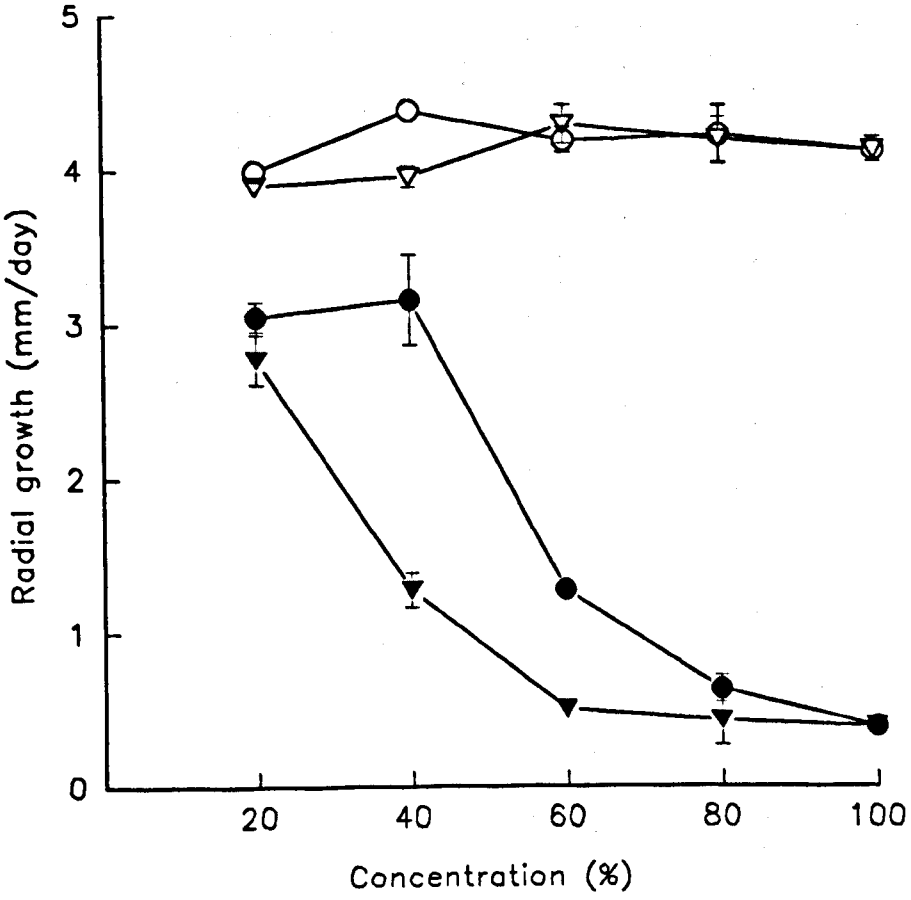


Figure 6.3 Radial growth rate (mm/day) of *C. gloeosporioides* on agar containing increasing concentrations (%) of sucrose (▽,▼) and $NaNO_3$ (○,●) on which 558 had previously grown (▼,●) and on control plates (▽,○; vertical bars = S.E.).

To test if any of the minor components of CzDA had an influence on the inhibitory action observed, a range of agars was prepared containing 0 or 50% of each element combined with the others in their standard amounts. Thus 7 agars (50% Mg was absent) were tested in addition to full strength CzDA. Growth of 558 was similar to that of the control (full strength CzDA) on all these agars except on the agar which did not contain Mg. Growth of *C. gloeosporioides* on control plates which not had 558 growing on them occurred at rates between 4.7 (0% KCl) and 5 mm/day (complete CzDA; Figure 6.4), thus there was no direct effect of these agars on the growth of *C. gloeosporioides*. The growth rate of *C. gloeosporioides* on the agar plates on which 558 had previously grown varied between 0.5 and 0.9 mm/day (Figure 6.4). Each agar thus still inhibited growth of *C. gloeosporioides*, indicating that the separate minor components present in CzDA are of no importance towards the inhibitory action observed.

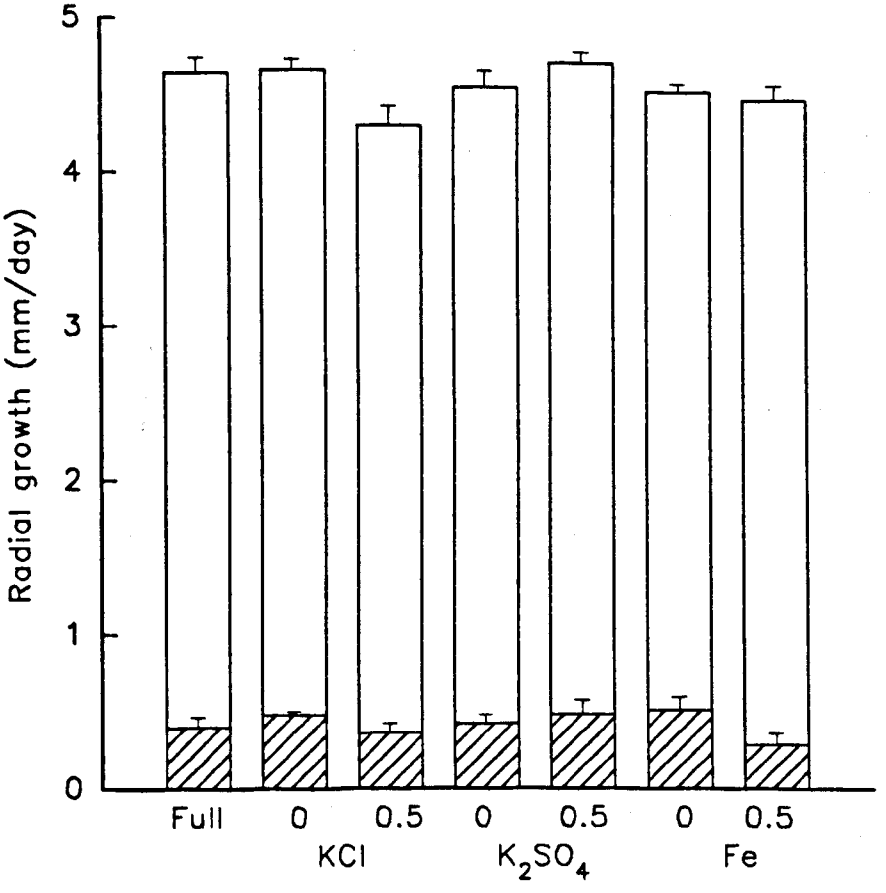


Figure 6.4 Radial growth rate (mm/day) of *C. gloeosporioides* on CzDA containing full, 0, or 50% of the normal concentration of KCl, K_2SO_4 or Fe (Control plates, open bars; plates which had 558 grown on them previously, hatched bars; vertical bars = S.E.).

6.3.7 pH - In liquid culture the pH initially decreased from 6.2 to 4.9 by 3 days after inoculation. The pH then increased rapidly until at 6 days after inoculation and stabilised at pH 8.8 - 9. The decrease coincided with the log phase of growth and the increase in pH correlated with the stationary phase of growth of 558 (Figure 6.5).

The measurements in solid media did not show this decrease in pH (Table 6.4), but the pH measurements were rather variable and this was reflected in the results that were obtained in many of the cellophane overlay experiments. This variability in pH was very clear in an experiment set up to determine if buffered CzDA would still support the inhibitory action observed by 558. Buffered plates had a pH of 6.5 (from 6.8 at the start of the experiment) after 5 days of growth of 558. However, the 4 unbuffered plates differed greatly in their final pH, with pH's of 4.3, 5.7, 8.6 and 8.7 being recorded. The two plates of higher pH showed inhibition, and *C. gloeosporioides* grew with a radial growth rate of 1 mm/day, compared to the control plates on which *C. gloeosporioides* grew with a radial growth rate of 4.4 mm/day.

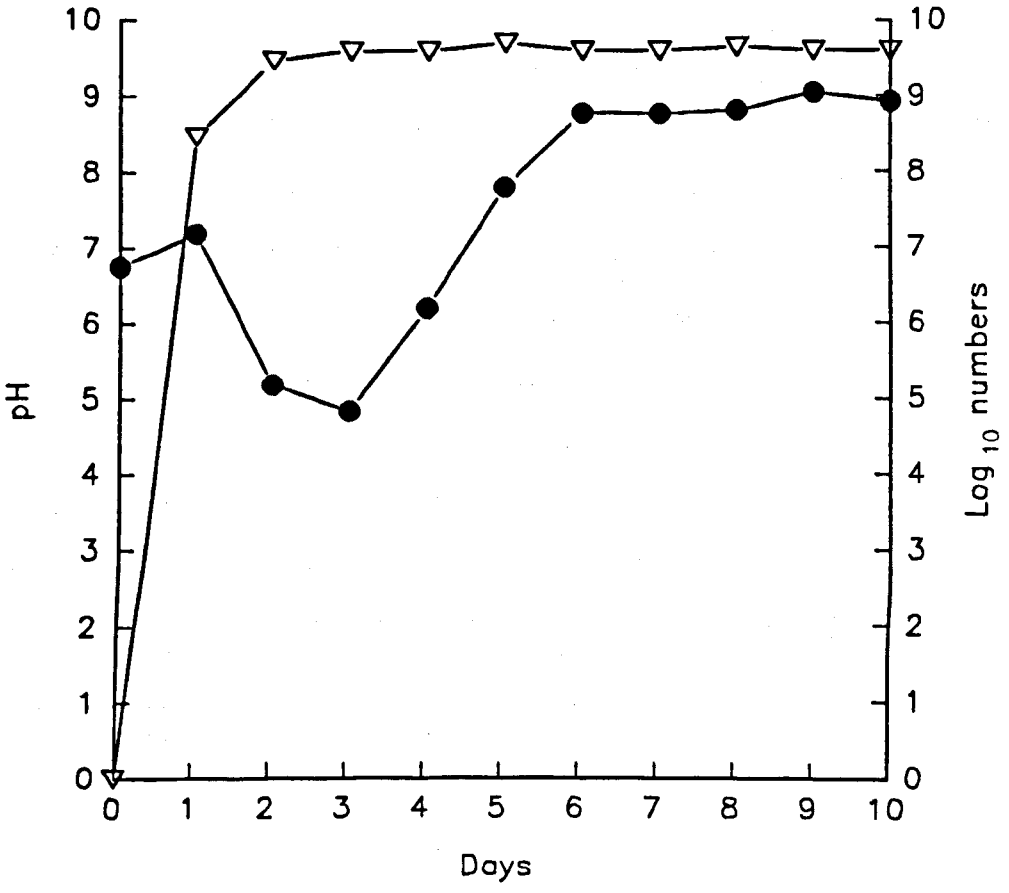


Figure 6.5 Concentration of 558 (log_{10}; ▽) and pH of the medium (●) against time.

Table 6.4 Radial growth rate (mm/day) of *C. gloeosporioides* on unbuffered CzDA on which 558 had previously grown over a period of 0 to 7 days. Indicated are the pH of the agar after growth of 558 and the % inhibition of growth of *C. gloeosporioides* compared to that on plates on which 558 had not previously grown.

Days	0	1	2	3	4	5	6	7	S.E.
pH	6.2	7.4	7.5	8.8	8.9	9.0	8.9	9.0	0.17
Growth (mm/day)	5.2	-[1]	1.4	1.9	0.7	0.6	0.6	0.6	0.33
% Inhibition	-	-	73	63	87	88	88	88	

[1] No measurements of growth due to contamination of the plates

On the plates with the lower final pH *C. gloeosporioides* had similar radial growth rates to those on the control plates, at 5.1 mm/day for pH 4.3 and 4.9 mm/day for pH 5.7. On the buffered plates on which 558 had grown, *C. gloeosporioides* had a slightly higher radial growth rate (3.2 mm/day) than on the buffered control plates (2.7 mm/day; Table 6.5). This indicated that pH affects growth of *C. gloeosporioides*, certainly in nutrient limiting conditions (see 2.3.7) where a high pH (>8) can severely limit growth. Further experimentation is required however to confirm the pH effect.

Table 6.5 Radial growth rates (mm/day; ± S.E.) of *C. gloeosporioides* on normal and buffered CzDA and pH, with or without previous growth of 558, and %inhibition.

	Normal agar	pH	Buffered agar	pH
Control	4.4 (± 0.10)	6.8	2.7 (± 0.09)	6.8
+558	3.0 (± 0.67)	6.8 (±0.95)	3.2 (± 0.21)	6.6 (±0.04)
%Inhibition	32		-19	

6.3.8 Siderophore production - -Fe/CzDL in which 558 had grown appeared bright yellow-green and fluoresced when examined under short and long wave U.V. light. The addition of $FeCl_3$ produced a brown pigment in the cell free extract indicating that a siderophore was produced by 558 in this medium. This brown pigment did not appear in the normal CzDL in which 558 had grown, nor in the original -Fe/CzDL, after addition of $FeCl_3$.

Table 6.6 Percentage spore germination and appressorium formation of spores of *C. gloeosporioides* in the presence of EDTA, Fe and glucose (Glu).

Treatment	Con	EDTA	Fe	EDTA + Fe	EDTA + Glu	EDTA + Fe +Glu	mean	S.E.
% Germination	35	34	50	44	51	49	44	8.3
% Appressoria	45	21	27	39	13	18	27	8.0

The commercial siderophore, EDTA, did not affect germination of *C. gloeosporioides* spores. Percentage germination of spores in presence of EDTA was similar (34%) to that of the control, -Fe/dH_2O (35%). The addition of Fe increased the percentage of spore germination slightly to 50%. None of the other combinations increased spore germination (Table 6.6). All treatments reduced appressorium formation from a control value of 45% of germinated spores forming appressoria, with the lowest treatment, EDTA + glucose with a value of 13% of germinated spores forming appressoria (Table 6.6). This low percentage germinated spores forming appressoria, 13%, was due to the glucose present in the medium (see 2.3.6). The spore germination experiment that was set up with the cell free extracts of 558 grown in -Fe/CzDL to determine the effect of these extracts on spore germination and appressorium formation, resulted in excessive growth of *C. gloeosporioides* on the glass slide. This could have been due to nutrients still present in the filtrate or other, stimulatory, compounds present. The controls for this experiment were spores in dH_2O or CzDL and spores in the presence of a living culture of 558 in either dH_2O or CzDL. These treatments showed that although the cell free extracts did not inhibit spore germination, both 558 in dH_2O and in CzDL did inhibit significantly reduce spore germination compared to the controls. In dH_2O, 558 did stimulate appressorium formation, but due to the presence of nutrients, 558 in CzDL did not stimulate appressorium formation and no appressoria were observed (Figure 6.6).

6.3.9 Microscopical observations - From the scanning electron microscopy there did not seem to be a direct parasitic or lytic action of either 204 or 558 on the hyphae of *C. gloeosporioides*. Both isolates formed exopolysaccharides (Figures 6.7 & 6.8) which presumably adhered the cells to the surface (see 5.4). In samples prepared by CPD these polysaccharides just appeared fibrilar due to desiccation by the method of preparation. The concentration of 558 appeared highest round the hyphal tips and the appressoria (Figure 6.8), maybe because exudates were present there. It was observed that bacteria of 558 were not homogeneously dispersed over the surface of the mango and near the edge of the drop, spores were found with no bacteria present. This suggests that 558 was not motile while on the fruit surface. Isolate 204 appeared to be randomly dispersed, with the same frequency near as away from spores (Figure 6.7). Observations made by SEM always have to be interpreted cautiously since mode of preparation might result in artifacts. From the observation made here there are no clear indication to the mode of action of either isolate.

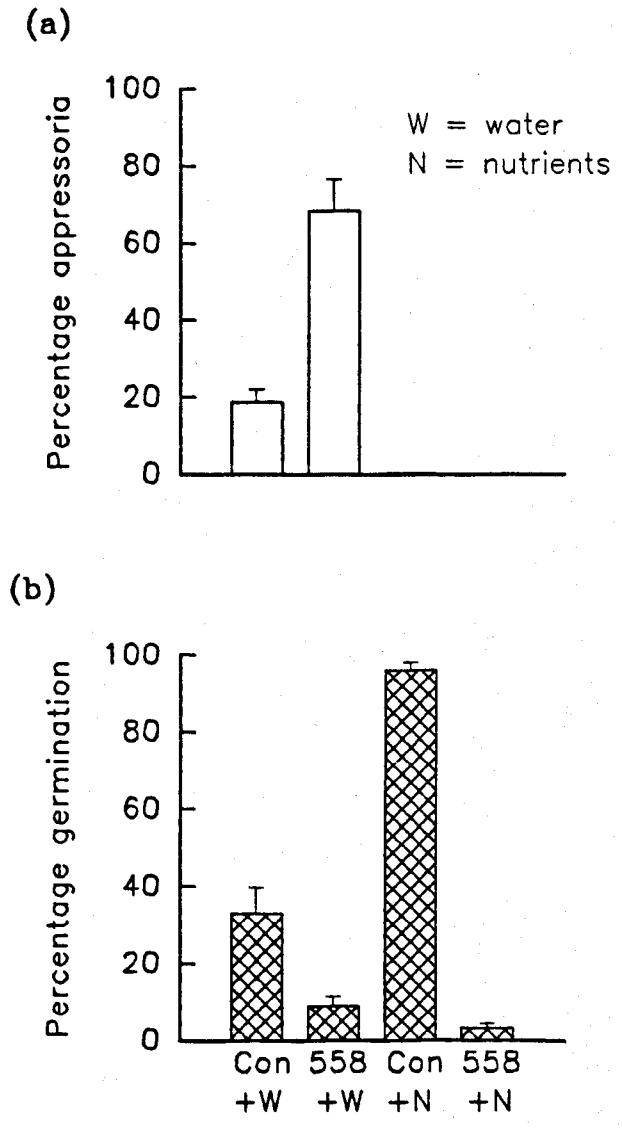
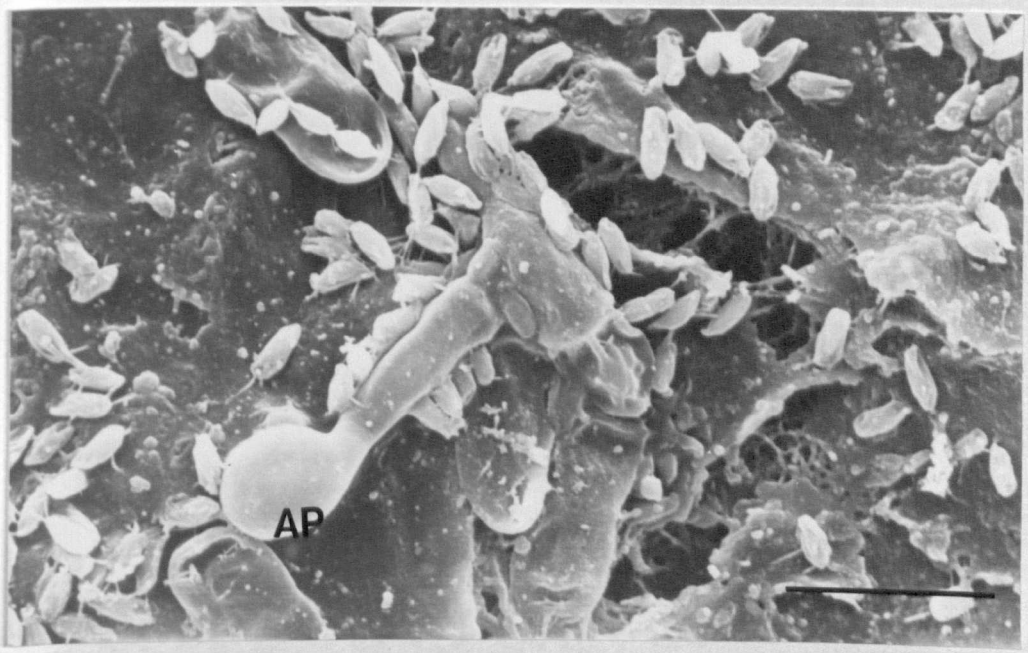


Figure 6.6 Percentage of a) appressorium formation and b) spore germination of spores of *C. gloeosporioides* in water or CzDL (nutrients) with or without 558 present (vertical bars= S.E.).

(a)



(b)

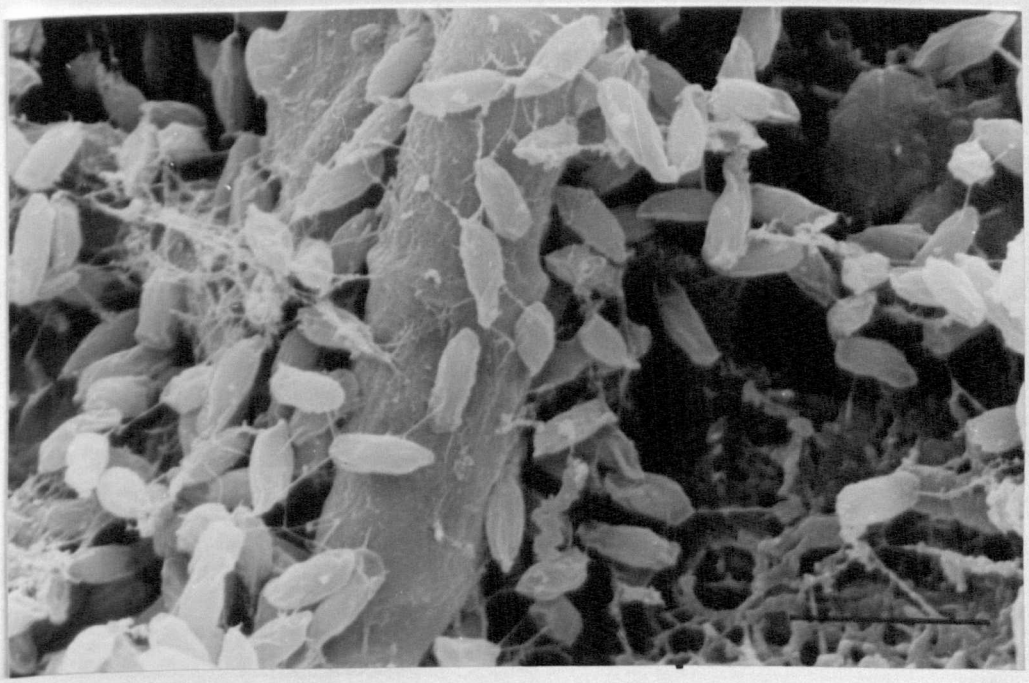
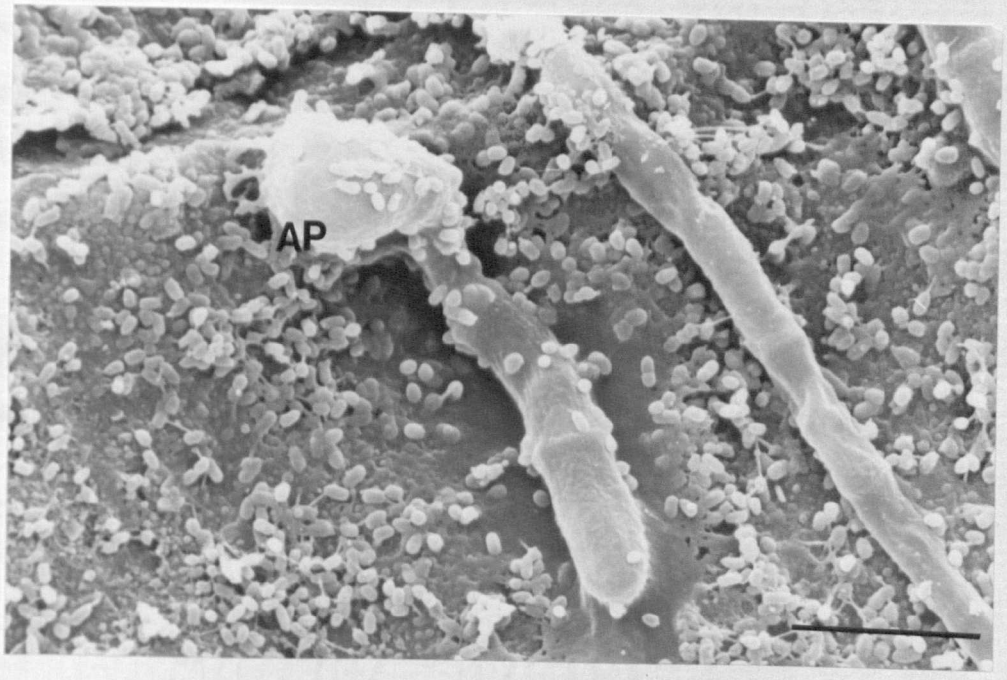


Figure 6.7 Dual inoculation of isolate 204 and *C. gloeosporioides* on mango.

(AP = appressorium; a) bar = 10μm; b) note the desiccated polysaccharide strands; bar = 5μm; SEM)

(a)



(b)

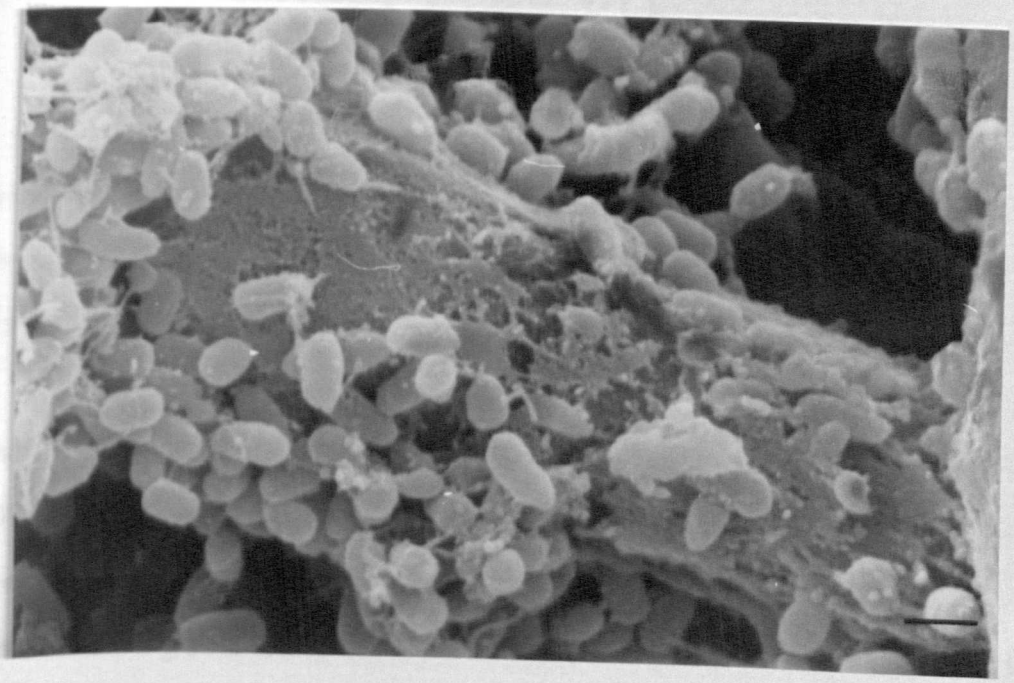


Figure 6.8 Dual inoculation of isolate 558 and *C. gloeosporioides* on mango.

(AP = appressorium; a) bar = 10μm; b) note the desiccated polysaccharide strands; bar = 1μm; SEM)

6.3.10 Temperature range - Isolate 558 did not grow at 37°C, but grew well at 10°C. The film of bacteria was not as shiny at 10°C as it was at the other temperatures, perhaps because less polysaccharides were produces at this low temperature. Growth of *C. gloeosporioides* (at 25°C) was inhibited to the greatest extent on plates where 558 has been allowed to grow at 15, 20 and 25°C. Radial growth rates of *C. gloeosporioides* on plates on which 558 had grown at these temperatures were 0.3, 0.4 and 0.5 mm/day respectively, compared to the radial growth rate of the control at 5.2 mm/day. Growth of *C. gloeosporioides* was less inhibited on plates on which 558 had grown at 10 and 30°C, with radial growth rates of 2.8 and 1.9 mm/day respectively (Table 6.7).

Table 6.7 Radial growth rates (mm/day) and % inhibition of *C. gloeosporioides* after 558 had been allowed to grow at a range of temperatures.

Temperature (°C)	10	15	20	25	30	Con
Growth (mm/day)	2.3	0.3	0.4	0.5	1.7	4.7
S.E.	0.20	0.11	0.12	0.09	0.22	0.09
%Inhibition	51	94	91	89	64	-

That isolate 558 still inhibited growth of *C. gloeosporioides* on plates on which this isolate had grown at 15°C, showed that the action of inhibition observed in this test, was still effective at this temperature. The advised storage temperature for mangoes is 12-13°C (see Chapter 1) and it has yet to be determined if 558 is still inhibitory to *C. gloeosporioides* at this slightly lower temperature.

6.3.11 Taxonomic spectrum of inhibition - The growth of all isolates of *Colletotrichum* tested was inhibited by ≥ 50% in the cellophane overlay test (Table 6.8), except for isolate 25. This isolate had a very slow growth rate even on normal CzDA (1.1 mm/day) compared to most other isolates. *C. lindemuthianum* and *C. lagenarium* also had a growth rate of less than 2 mm/day on control plates of CzDA, but their growth was inhibited by 558 by 68% and 71% respectively.

Considering the growth of the other pathogens, that of *R. solani* and *Lasiodiplodia* was inhibited by over 90%, followed by *Py. ultimum* (65%), *Ph. citricola* (64%) and *P. digitatum* (59%). Growth of *F. solani* was inhibited the least (37%; Table 6.8)

Table 6.8 Radial growth rates (mm/day) of *Colletotrichum* isolates and other pathogens on normal (Con) and on CzDA on which 558 had grown previously using the cellophane overlay method, and %inhibition.

Isolate	Con (± S.E)	+558 (± S.E)	%inhibition
19	3.6 (± 0.09)	0.4 (± 0.06)	89
24	4.9 (± 0.03)	0.4 (± 0.03)	92
25	1.1 (-)	0.8 (± 0.03)	27
29	4.8 (± 0.12)	0 (-)	100
40	4.1 (-)	0.5 (± 0.08)	88
SLI4	4.4 (-)	0.4 (± 0.03)	91
PI4	4.4 (± 0.14)	0.5 (± 0.04)	89
IMI 321880	4.4 (± 0.05)	0.2 (± 0.03)	95
C. capsici	4.1 (± 0.05)	0.6 (± 0.11)	85
C. lindemuthianum	1.9 (± 0.05)	0.8 (± 0.04)	50
C. lagenarium	2.1 (± 0.05)	0.2 (± 0.03)	90
C. musae	2.8 (± 0.05)	0.8 (-)	71
C. gloeosporioides[1]	5.4 (± 0.07)	0.8 (± 0.10)	85
C. coffeanum	5.1 (± 0.06)	0.8 (± 0.04)	84
C. acutatum	1.9 (± 0.03)	0.6 (-)	68
C. atramentarium	3.1 (± 0.05)	0.8 (± 0.05)	74
C. gloeosporioides[2]	3.1 (± 0.05)	0.8 (± 0.18)	74
P. digitatum	5.6 (± 0.04)	2.3 (± 0.19)	59
R. solani	12.3 (± 0.09)	1.0 (± 0.14)	92
Py. ultimum	22.5 (± 0.22)	7.8 (± 0.61)	65
Ph. citricola	19.5 (± 0.46)	7.1 (± 0.86)	64
F. solani	4.1 (± 0.05)	2.6 (± 0.15)	37
Lasiodiplodia	14.3 (± 0.26)	1.3 (± 0.15)	91

[1] Isolate from avocado

[2] Isolate from papaya

These results must be interpreted carefully because all what is probably being measured is the tolerance of the respective isolate to grow at a relatively high pH (> 7.5) and what we observe might not be related to the antagonistic properties of 558 to these pathogens *in vivo*. A more effective test to confirm some of these results would be to test the effect of 558 on disease development of *C. lindemuthianum* on beans or of *C. lagenarium* on cucumbers in the green house.

6.4 Discussion

6.4.1 Isolate 204 - The fact that this isolate is a *Bacillus cereus* will make it unlikely that legislation would ever be allowed to use it as a fruit dip. Some strains of *B. cereus* are reported to be mild human pathogens that can cause food poisoning (Collins & Lyne, 1984), although this does not necessarily mean that 204 will do so. The use of *B. cereus* as a biological control agent has already been proposed, as in the study of Handelsman *et al.* (1990), where the organism was applied as a root dip or soil drench. In their study, a *B. cereus* isolate with great biological control potential was used against zoospores of *Phytophthora megasperma* f.sp. *medicaginis* causing damping-off of alfalfa seedlings. The proposed mode of action for this isolate (UW85) was that it increased the pH of the medium it was growing in by removing calcium and excreting ammonia into the solution. This resulted in a condition in which zoospores of *Phytophthora* lysed (Gilbert *et al.*, 1990). However there were also two active compounds present in cell free filtrate of UW85 (Handelsman *et al.*, 1988) and the lysis hypothesis was dismissed as a laboratory artefact (G.S. Gilbert, pers.comm.). It has been reported before that *B. cereus* produces anti-fungal compounds, including mycocerein (Wakayama *et al.*, 1984) and tunicamycin (Kamogashira *et al.*, 1988).

Doherty & Preece (1978) isolated *B. cereus* from over 60% of uredospore samples of *Puccinia allii* collected all over Great Britain. *B. cereus* completely inhibited uredospore germination but inhibition of spore germination was not found when cell free extracts were tested in wells, and the mode of action was not further determined. The same was true for an isolate of *B. cereus* subsp. *mycoides* which under controlled environmental conditions reduced tobacco brown-spot disease (*Alternaria alternata*). Cell free extracts did not affect the fungus and mode of action was not further determined (Fravel & Spurr, 1977). A mixture of *B. cereus*, *B. mycoides* and a *Bacillus* sp. reduced disease caused by needle rust, *Melampsora medusae*, while the cell free extracts did not (McBride, 1969). Mode of action by these bacteria proposed, but not proven,

were antibiotic production, competition for nutrients or space, or lysis of the basidiospores or germ tubes. Two other species in this genus which show biological control potential are *B. pumilus* which lyses uredospores of *Puccinia recondita* (Morgan, 1963), and *B. subtilis*, a species on which the vast majority of research has been carried out (summarised by Pusey, 1989). *B. subtilis* isolate B3 has been used as a post-harvest biological control agent of *Monilinia fructicola*, the causal agent of brown rot in stone fruit. This isolate produces iturin antibiotics, cyclic peptides with a wide anti-fungal spectrum (McKeen *et al.*, 1986; Gueldner *et al.*, 1988). Utkhede & Sholberg (1986) tested 21 isolates of *B. subtilis* on cherries against *Monilinia fructicola* and *Alternaria alternata*. All isolates gave different levels of control and were ranked differently against the two diseases. The mode of action was not determined, but it would have been interesting to see if all these isolates produced the same antifungal compounds and if disease control was related to the type and amount of antibiotic produced. Swinburne and coworkers used *B. subtilis* to protect leave scars on apple from fungal colonists, mainly *Nectria galligena* (Swinburne, 1973; Swinburne *et al.*, 1975). A mixture of antibiotics, including bacillomycin, produced by the *B. subtilis* was thought to be responsible for the control observed. Bacillomycin was the first antifungal compound described for *B. subtilis* (Babad *et al.*, 1954), followed by the description of mycobacillin (Majumdar & Bosse, 1958; see Katz & Demain (1977) for an overview of antibiotics produced by *Bacillus* spp.). It is interesting to note that antibiotic production by *Bacillus* spp. often coincides with sporulation in these bacteria (Swinburne *et al.*, 1975; Katz & Demain, 1977). The fact that *Bacillus* spp. produce spores would in some ways make them good candidates for biological control agents because they should be able to survive adverse conditions when no biological control activity is required e.g. after a harvest. However, for the type of biological control that is required in the post-harvest situation an organism needs to have the potential to be either active over a period of up to 4 weeks or the mode of action expressed must be fungicidal rather than fungistatic.

The reasons for not continuing work with isolate 204 are twofold : (i) because it is a *Bacillus cereus*, and could be mildly pathogenic, and (ii) because in the large post harvest trial carried out in the Philippines it did not show biological control activity. Thus little emphasis has been put into conducting experiments with 204 for these reasons. For future work it is still worthwhile considering whether there are antibiotics produced by this isolate of *Bacillus* or by other isolates, that have the potential to control *C. gloeosporioides* and hence could be used as an antibiotic treatment. In the filtrate experiments (6.3.4) both the filtrate and

the autoclaved filtrate of 204 grown in NB (48 hrs) inhibited growth of *C. gloeosporioides* after incorporation into agar. Even though no inhibitory compounds were detected with the solvent extractions, these experiment were not conclusive.

6.4.2 Isolate 558 - Much research has been done on fluorescent pseudomonads, especially with reference to the control of soil-borne diseases. This is partly because a larger proportion of the soil bacteria are fluorescent pseudomonads compared to those colonising the phylloplane (Fokkema & Schippers, 1986), and the fact that they are the dominant type of bacteria in soil. The classical example of biological control with pseudomonads is their role in the decline effect observed in relation to take-all disease (*Gaeumannomyces graminis* var *tritici*) in wheat (Cook & Rovira, 1984). They also have effects as plant growth-promoting rhizobacteria (Schippers *et al.*, 1987). A detailed account of biological control in soil (see Hornby, 1990) is outside the scope of this discussion.

Two general explanations have been put forward to explain how pseudomonads may affect phytopathogenic fungi. These are (i) the production of siderophores (6.4.2.a) and (ii) the production of antibiotics (6.4.2.b). Some indication of competition for amino acids has also been observed (Blakeman & Brodie, 1977) and this will be discussed under the section on spore germination (6.4.3).

6.4.2.a Antibiotic production by pseudomonads - The two main antibiotics reported in the literature that play a role in biological control are pyrrolnitrin (Howell & Stipanovic, 1979) and phenazine compounds (Thomashow & Weller, 1988). Many pseudomonads produce several antibiotics at the same time. An example is *Pseudomonas* Pf-5, which controlled disease caused by both *Rhizoctonia solani* and *Pythium ultimum* on cotton seedlings (Howell & Stipanovic, 1980). The first antibiotic which was detected to be produced by this isolate was pyrrolnitrin (Howell & Stipanovic, 1979). *In vitro* testing, however, revealed that this antibiotic reduced growth of *R. solani*, but not growth of *P. ultimum*. Further examination yielded a second antibiotic produced by Pf-5, pyoluteorin, which was inhibitory to *P. ultimum* (Howell & Stipanovic, 1980). Pyrrolnitrin is contained in the cell until it lyses, while pyoluteorin is secreted extracellularly when it is produced. Homma *et al.* (1989) found that *P. cepacia* RB 425 produced two pseudanes as well as pyrrolnitrin. Of the three antibiotics pyrrolnitrin was most effective against a range of phytopathogenic fungi. It is interesting to note that in this study pyrrolnitrin reduced *in vitro* growth of *P. ultimum* by 75% at the

highest concentration, 10 μg/ml, while Howell & Stipanovic (1980) had found no inhibition of *P. ultimum* with the same compound. Roitman *et al.* (1990) found that strain LT4-12-W of *P. cepacia* (known to reduce development of *Penicillium expansum*, a storage rot in apples; Janisiewicz & Roitman, 1987) also produced pyrrolnitrin and, in addition, three related chlorinated phenyl pyrrole antibiotics. All four compounds showed antifungal activity against *P. expansum*, *Botrytis cinerea* and a *Mucor* sp. *in vitro* (Roitman *et al.*, 1990). Another major group of antibiotics, the phenazine antibiotics (Kanner *et al.*, 1978), are thought to be involved in suppression of plant disease. Phenazine-1-carboxylic acid suppresses development of *Gaeumannomyces graminis* var. *tritici*, causal agent of take-all in wheat, and of *P. ultimum* (Gurrusiddaiah *et al.*, 1986) and 1-hydroxyphenazine, a degradation product of pyocyanine, reduced disease caused by *Septoria tritici* and *Puccinia recondita* on wheat seedlings (Levy *et al.*, 1989). Phenazine-1-carboxylic acid is produced by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84. Several mutant strains obtained through transposon mutagenesis, deficient in phenazine production were not able to reduce take-all in wheat to the same extent as the parent strain 2-79 (Thomashow & Weller, 1988). No antibiotic could be recovered from roots inoculated with either mutants of 2-79 or 30-84, while roots inoculated with either parent strain yielded up to 43 ng phenazine/g root (Thomashow *et al.*, 1990). This clearly demonstrates that both these strains produce antibiotics *in vivo*, and that the antibiotic is directly involved in disease control. Flaishman *et al.* (1990) reported a similar study with *P. aeruginosa* Lec-1 (Levy *et al.*, 1988) in which they produced mutants through transposon mutagenesis, lacking in production of pyoverdine, a siderophore, or pyocyanine, a phenazine antibiotic. Like the parent strain, the mutant lacking pyoverdine production still suppressed growth of *S. tritici* in agar, and also pycnidium formation on wheat leaves, while the mutants lacking pyocyanine had partly lost this ability (Flaishman *et al.*, 1990). This indicated that pyoverdine did not contribute to suppression of *S. tritici*, but pyocyanine did so. The other part of the suppression was attributed, although not proven, to HCN production. Involvement of cyanide is also indicated in the suppression of root rot of tobacco (*Thielaviopsis basicola*) by another isolate of *P. fluorescens* (Voisard *et al.*, 1989).

In the results presented here for isolate 558, evidence indicates that isolate 558 did not produce an antibiotic with activity against *C. gloeosporioides* under the conditions tested. This could mean that a) no antibiotic was produced by this isolate, b) the bio-assay was not sensitive enough or c) nutritional conditions were not favourable for antibiotic production. It seems unlikely, however, that 558 produces antibiotics responsible

for the inhibition of *C. gloeosporioides*, but until fully confirmed this hypothesis cannot be discarded.

6.4.2.b Siderophore production by pseudomonads - The results showed that isolate 558 produced a siderophore. Members of the *P. fluorescens/P. putida* group are known to produce siderophores of the pyoverdine type under iron deficient conditions (Demange *et al.*, 1986). These are seen as the yellow-green, fluorescent pigment produced in iron deficient media such as KingB, and have a high affinity for iron. Kloepper *et al.* (1980) proposed the competition principle in that plant growth-promoting rhizobacteria (PGPR) competed with deleterious micro-organisms for available iron, obtaining it at the expense of the latter by chelation with siderophores produced by the PGPR. Leong and co-workers (see Leong, 1986) found that both *Pseudomonas* B10 and pseudobactin, the siderophore produced by this isolate, enhanced plant growth of potatoes, and after examination it was shown that application of both B10 and pseudobactin had significantly reduced the fungal population of the roots compared to the control. Non-fluorescent mutants of B10 did not show this effect. A similar approach was carried out with *P. putida* WCS358 (Geels & Schippers, 1983) for which mutants lacking pseudobactin production were obtained by transposon mutagenesis (Marugg *et al.*, 1985). These siderophore lacking mutants did not increase root development of potato, even though they did establish themselves in the rhizosphere, while the parent strain did enhance root development (Bakker *et al.*, 1987). They also showed that although the mutant did not produce siderophores it was still able to utilize the siderophore produced by the parent strain (Schippers *et al.*, 1987). They also showed that not all isolates were able to use siderophores from other strains, while some isolates seem to be able to use all the siderophores they encountered. This phenomenon has great implications for the competitive ability of *Pseudomonas* strains.

Apart from playing a role in PGPR systems, siderophores are also considered to be involved in the biological control of plant diseases. For soils suppressive to both take-all of wheat and wilt of flax (*Fusarium oxysporum f.sp. lini*), the suppressiveness could be eliminated by the addition of a 50 μM solution of Fe-EDTA (in Leong, 1986). The same workers could induce conducive soils to become suppressive to *Fusarium* wilt and take-all disease by inoculating the soil with *P. putida* B10 or its siderophore pseudobactin, and addition of Fe-EDTA again reverted this suppression. Leong (1986) strongly advocates the importance of siderophores produced by fluorescent pseudomonads in the disease suppressiveness of certain soils. It has been questioned, however, if the role of siderophores is important to disease suppression.

The example mentioned above, in which the siderophore lacking mutant of *P. aeruginosa* Lec-1 (Flaishman *et al.*, 1990) still suppressed disease to the same extent as the parent strain, showed that for this isolate the siderophore does not take part in disease control. Campbell *et al.* (1986) selected antagonists *in vivo* against *Gaeumannomyces graminis* var *tritici* and found that, of all microorganisms showing biological control potential, 20% were fluorescent pseudomonads, but no evidence for the involvement of siderophores was obtained. In another study, an iron-antagonised, antifungal compound was found to be produced by *Pseudomonas* NZ130, and appeared to be common to many other *Pseudomonas* isolates (Gill & Warren, 1988). Because iron diminished the effectivity of this antibiotic, the reduction in disease suppression observed with increasing amounts of available iron, could easily be confused by explaining that the mode of action was through competition for iron by siderophores rather than antibiotic activity. From the research carried out so far on this antibiotic, it was not clear if iron acted directly on the antibiotic or that under iron-limiting conditions the fungus was weakened and more susceptible to the antibiotic (Gill & Warren, 1988). Many other questions remain (Buyer *et al.*, 1990), for instance, can the pathogen itself produce siderophores competing for the available iron? or can the pathogen use the siderophore produced by the pseudomonad?; it has also not been shown yet that siderophores are produced *in situ*. Although monoclonal antibodies have been developed against pseudobactin B10 (Buyer *et al.*, 1990) *in situ* experiments have not been reported yet using this monoclonal antibody. Another question is the iron availability on the phylloplane, is iron in such short supply that siderophore production is induced? For the iron content of leachates of banana fruit it was determined that this was low enough to allow induction of siderophore production (McCracken & Swinburne, 1979). However, the pH of the environment (acid conditions) might be such that iron is freely available (Campbell *et al.*, 1986). One problem which is quite specific to *Colletotrichum* is that it has been shown that spore germination is stimulated by siderophores (Swinburne, 1986), but this will be further discussed in 6.4.3.

As far as isolate 558 is concerned the conclusion is that it produces a siderophore but the significance of this siderophore in disease control will not be known until further studies have been carried out. The effect of the purified siderophore on anthracnose development should be examined, and the construction of a siderophore lacking mutant could be attempted to determine if such a mutant can still inhibit disease development.

6.4.3 Spore germination - In the limited experiment carried out here to determine the effect of a commercially available siderophore, EDTA, on spore germination of *C. gloeosporioides*, there was no significant effect of either EDTA or Fe on spore germination (Table 6.8). EDTA reduced appressorium formation slightly, as was also observed in the treatments containing glucose. It was necessary to add glucose because first attempts to study the *effect of the cell free filtrate of -Fe/CzDL* on spore germination resulted in excessive mycelial growth on the glass slide. This occurred even though the pH of the filtrate was 8.8 and it was not possible to count either spore germination or appressorium formation. The same was true for the cell free filtrate of normal CzDL which had a pH of 8.1. It was thought that the observed effect could have been due to the sugars still present in the medium, and in the absence of the purified siderophore it was not possible to determine its direct effect on spore germination by *C. gloeosporioides*. The addition of EDTA + glucose and EDTA + Fe + glucose to spore suspensions resulted in a significant stimulation of spore germination and a reduction in appressorium formation, but did not result in the excessive growth as observed with the cell free filtrates. This indicated that there was another substance present in the filtrate that may have stimulated spore germination. Whatever the explanation, the results are inconclusive and further studies are needed to determine the effect of the 558 siderophore on spore germination by *C. gloeosporioides*. It was interesting to note that an experiment carried out with whole cells of 558, both cells of 558 in dH_2O and in CzDL inhibited spore germination while the cell free extracts did not!

Swinburne (1976) found that both anthranillic acid and banana leachates stimulated spore germination of *C. musae* resulting in shorter germ tubes and an increased number of appressoria. *C. musae* is capable of converting anthranillic acid into 2,3-dihydroxybenzoic acid which chelates iron (Harper & Swinburne, 1979). Under iron limiting conditions, cultures of *Pseudomonas* sp. UV3 stimulated spore germination of *C. acutatum* (Blakeman & Parbery, 1977) and *C. musae* (McCracken & Swinburne, 1979). McCracken & Swinburne (1979) also showed that cell free extracts of UV3, and the siderophore purified from it, stimulated spore germination and appressorium formation by *C. musae*. The explanation of this phenomenon was that it was not due to increased uptake of iron by *C. musae*, since ferrous and not ferric iron was involved (fungi take up iron in the ferric form, McCracken & Swinburne, 1979). Neither did anthranillic acid influence the rate of iron uptake by spores which again indicated that iron uptake was not responsible for the observations (Graham & Harper, 1983). Harper *et al.* (1980) concluded that conidial iron first needs to be chelated and removed from binding sites within the spores to allow the spores to germinate, although

chelated siderophores were not excreted from the spores and must have been transported within the spore (Graham & Harper, 1983). The ability of spores to germinate was related to their conidial iron content and percentage germination was highest for the spores with the lowest iron content. Spore germination was independent of conidial iron in the presence of anthranillic acid, indicating again the inhibitory effect of iron present in the spores on spore germination (Harper *et al.*, 1980). It is a pity these researchers did not determine the iron content of spores harvested from banana fruit to compare *in vivo* concentrations with their experimental results.

For *C. acutatum* the relationship between siderophores produced by UV3 and increased germination was determined (Slade *et al.*, 1986), although when the initial observation of increased spore germination in the presence of UV3 was made it was thought that UV3, which is highly competitive for amino acids, deprived spores of these essential nitrogen sources, resulting in an increased appressorium formation. Two isolates which did not compete well for amino acids had a lower stimulatory effect on appressorium formation (Blakeman & Parbery, 1977). To confirm this spores of *C. acutatum* were leached to remove nutrients and this also resulted in increased appressorium formation, while spore germination was not affected. It was observed that for other fungi, e.g. *Botrytis cinerea*, competition for amino acids between UV3 and the fungus resulted in a reduction of spore germination (Blakeman & Brodie, 1977).

6.4.4 What is the mode of action of 558? - From the results presented in this chapter it can said that the production of volatile compounds by 558 and direct lysing of hyphae does not take place. It also seems very unlikely that antibiotics are produced. Two observations that were confirmed were that (i) isolate 558 produces a siderophore and (ii) the pH of the medium *in vitro* is sharply increased after about 5 days. We will not know if either of these factors play a role in disease control unless these phenomena can be shown to take place on the fruit surface. Newhook (1951) observed that several isolates of *B. subtilis* and *Pseudomonas* sp. raised the pH both of NA *in vitro* and of lettuce leaves *in vivo* as high as 8.2, resulting in a decrease in growth of *B. cinerea*. However he concluded that control was due to antibiotic production by the bacteria independent of pH, although the rise in pH must have contributed to disease control. To date it has not been possible to measure the pH on the fruit surface, but it would be interesting to see if the rise in pH also takes place *in vivo*. The experiments on media with a buffered pH (6.3.11), where normal growth was observed, show indirectly that no inhibitory compound (e.g. an antibiotic), is secreted into the

agar by 558, and further that inhibition does not occur through nutrient competition because growth on buffered 558-CzDA, which would have had nutrients depleted by growth of 558, and buffered CzDA is similar. That nutrient competition does not play a role in this assay was also confirmed by the addition of nutrients to 558-CzDA, after which growth of *C. gloeosporioides* was still inhibited. However caution has to be taken in drawing conclusions from any assay test, because the observations from the cellophane overlay test are effects observed on mycelial growth and results might be quite different if spore germination or appressorium formation is considered.

As discussed earlier, mutants lacking in siderophore production need to be obtained, and determination made as to whether these are still capable of controlling disease to prove the importance of the siderophore. Caution would have to be taken that in creating a mutant, the fitness of the organism does not change. For further studies of pH effects, it should be possible to measure the pH of the fruit surface and relate this to disease control, it might be that the pH of the dip containing 558 is high, and the dipping medium itself controls disease rather than a direct effect of 558. This could be confirmed by dipping mangoes in solutions of different pH's and determining if high pH solutions on their own can control disease. A similar approach was taken by Magan & co-workers, who are attempting to control *C. musae* on bananas with acid treatments and some encouraging results have been obtained (Magan *et al.*, 1990, abstr. AAB/BPPS meeting, April 1990). *C. gloeosporioides* grew better in a near neutral pH medium (2.3.7), and grew only at a pH > 3 (Gavino, 1990). The approach of low and high pH treatments also be considered for controlling anthracnose on mangoes, as long as this does not reduce fruit quality.

The effect of bacteria on spore germination and appressorium formation by *C. gloeosporioides*, is unlikely to be determined by one factor alone and from the literature it seems convincing that both iron (and consequently siderophores), and nutrients are involved in interactions between bacteria and spores of *Colletotrichum*. It is not yet clear how isolate 558, which produces a siderophore and reduces spore germination in the presence of nutrients, fits in to this picture. Results indicate that a further factor besides siderophores and nutrients, is responsible for the observed effects. These may be through an increase in pH, but then spore germination should have been low in the cell free extracts with pH's of 8.1 and 8.8, or maybe 558 produces HCN, as has been shown for other pseudomonads (Voisard *et al.*, 1989; Schippers *et al.*, 1987); alternatively an unknown factor may be involved.

It has also been suggested that application of bacteria can evoke a host response resulting in resistance against infection by the pathogen (Austin *et al.*, 1978). In avocado it has been found that as long as levels

of dienes stay high, infection by *C. gloeosporioides* does not take place (Karni *et al.*, 1989). These high levels of diene can be artificially maintained by CO_2 treatment (Prusky *et al.*, abstr. AAB/BPPS meeting, April 1990). Maybe could bacteria do the same?

7 General discussion and conclusions

7.1 Pre- or post-harvest biological control?

From the experiments presented in this thesis the choice between pre- or post-harvest approaches to biological control is straightforward, since there are many more problems attached to pre-harvest control. For field treatments, many questions remain : (i) chances of survival of an introduced organism are small; (ii) when the biological control agent is introduced successfully, does it retain its activity on the leaf surface?; (iii) is the disease control by the introduced organism as effective as conventional, chemical methods of control?; and (iv) is the field application going to be cost-effective? There are, however, some advantages to seeking a pre-harvest approach in the case of anthracnose disease. For example pre-harvest agents deal with the spore, rather than with an already established appressorium as is the case with post-harvest treatments. A second reason for not abandoning attempts to develop a pre-harvest biological control system, is that, in the U.S.A at least, there are problems in finding suitable effective chemicals which are still permitted for agricultural use (see 1.3.1). Alternatives are urgently required.

For some types of anthracnose, such as those on beans, cucumbers and rubber, there is no choice between pre- or post-harvest control, since the disease causes the greatest problems in the field. With the aid of a model system to test candidates *in vivo*, such as *C. lindemuthianum* on beans it should be possible to develop a pre-harvest biological control system. Once a biological control system has been developed for one species of *Colletotrichum* this might be readily extrapolated for use on other host/*Colletotrichum* combinations. Infection patterns for most *Colletotrichum* spp. are similar and from the experiments carried out in this research project, especially those on the taxonomic spectrum of antagonism of 558 (6.3.11), it seems likely that if a biological control system is developed it will be applicable to anthracnose developing on a variety of hosts.

For mango, however, post-harvest biological control is the most attractive of the two options, even though the problem of quiescent infections must be dealt with. Within the limited time scale of the research carried out here, it was possible to show that post-harvest application of bacteria can successfully reduce anthracnose disease on mangoes. Apart from the problems that have to be considered in dealing with a quiescent infection, it is likely that successful post-harvest biological control could be more easily obtained

than pre-harvest biological control. There are several advantages to post-harvest biological control: (i) the harvested commodity is of high value; (ii) environmental conditions can be controlled; and (iii) the harvested product has a small volume (Wilson & Pusey, 1985; Jeffries & Jeger, 1990; see also 1.3.3). An additional reason why there may be a greater rate of success in applying post-harvest biological control in the Philippines is that there are only a few packing houses which deal with mangoes for export. There are, however, many farmers who produce mangoes, since mango production is still very much a 'back-yard' operation and commercial orchards are still very small. It will be much easier to transfer technology to a few packing houses where the technique will almost certainly be applied correctly, than to transfer technology to all the individual farmers.

In conclusion, whilst the development of a post-harvest biological control agent for anthracnose on mangoes is the main priority, efforts into finding a system applicable to the pre-harvest situation should be continued.

7.2 The ideal organism

Before a description of the ideal organism for post-harvest biological control of anthracnose on mango can be made, all factors involved have to be considered, from the mango to the consumer. To begin with the mango itself, we must evaluate what takes place between harvest and post-harvest treatment. In the Philippines mangoes intended for the export market should be transported to the packing house within 24hrs, where they receive a post-harvest treatment. This is not always possible and it can sometimes take up to three days before mangoes receive the post-harvest treatment. This delay in post-harvest treatment could mean that the disease has developed from a quiescent into an active infection. Mangoes are, in general, harvested at the mature-green stage (Pantastico *et al.*, 1984). The timing of when the quiescent infection of *C. gloeosporioides* on the mango becomes active and continues to infect the fruit is not known and further ultrastructural work is required to determine the train of events. Dodd *et al.* (1991a) found that a post-harvest treatment with benomyl was successful if applied on the same day or one day after harvest, but did not control anthracnose disease to a satisfactory level if applied three days after harvest. This indicates that further penetration of the fruit by the fungus takes place shortly after harvest.

After post-harvest treatment, mangoes are stored at 12-13°C for a period of up to 4 weeks (Salunkhe & Desai, 1984). Once they reach their destination a further period of up to two weeks at ambient temperatures

will follow from display in the shop to consumption. This period from harvest until consumption could be as long as 6 weeks, and during this time mangoes have to remain free of disease. Thus a potential biological control agent must be able to control disease for this length of time. This probably means that, in some instances, the biological control agent must be able to inhibit growth of subcuticular hyphae. The biological control agent must either remain active over the whole period required, or the mode of action has to be fungicidal.

Thus what must the potential biological control agent do? For post-harvest biological control it is not necessary to reduce spore germination, germ tube elongation or appressorium formation, since these processes will have taken place before harvest. But a biological control agent will have to be able to destroy or inhibit either the appressorium, the infection peg or subcuticular hyphae. These are not easy tasks. As discussed before (1.1.4), the appressorium is fairly resistant to external forces (due to the melanization), including antibiotics and lytic enzymes produced by other microorganisms (Bell & Wheeler, 1986). The likelihood that a biological control agent would be able to destroy the appressorium is small. A better option might be a reduction in the number of infection pegs formed from the appressorium or inhibition of their growth. There is one example of this in the literature, in which two phylloplane yeasts, *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavescens*, caused a reduction in the number of infection pegs formed from appressoria of *C. graminicola* (Williamson & Fokkema, 1985). These authors considered that the observed phenomenon was due to nutrient competition taking place between the yeasts and the appressoria. This, however, seems unlikely. The appressorium appears to be a self-contained structure which penetrates through a combination of mechanical force and the production of cutinases (see 1.1.4). At the early stages of penetration, it is unlikely that external nutrients are required for this process, although this is not proven. Later during the progression of the infection, nutrients are necessary, which are released from the host tissue through cellulases (Suzuki *et al.*, 1983). It seems likely that the yeasts disrupted the infection process in some other way. The yeasts may have acted directly on the appressorium by the action of a lytic enzyme, not destroying the appressorium but damaging it so that turgor was lost and hence mechanical penetration could not take place [as was the case in appressoria of melanin deficient mutants of *Magnaporthe grisea* (Howard & Ferrari, 1989)]. Alternatively, penetration may have taken place, but before the infection peg was clearly visible it could have been inhibited by antibiotics or lytic enzymes produced by the yeasts, or through nutrient competition. Either possibility might result in an unsuccessful infection.

Another way that penetration may be prevented is by the effect of external factors, such as nutrients, on

the appressorium. Perhaps substances such as peptone, glucose, or ethylene, for example, stimulate the appressorium to form an infection peg, while the underlying host tissue is not conducive to infection. The formation of infection peg can either result in penetration which is subsequently arrested, as happens with the infection pegs formed by hyaline appressoria of *C. musae* on banana (Muirhead & Deverall, 1981), or the infection peg goes sideways, resulting in saprophytic growth on the surface, instead of downwards into the fruit. This would result in hyphae on the fruit surface, which are more susceptible to inhibition by a potential biological control agent. Sideways growth of infection pegs was observed in *C. gloeosporioides* on glass slides when 0.1% glucose was added to appressoria (Dey, 1933), and in appressoria lacking melanin (Wolkow *et al.*, 1983; Kubo *et al.*, 1982a, b, 1987).

A further possibility is that bacteria might stimulate resistance mechanisms in the host tissue, through formation of compounds such as antifungal resorcinols and dienes that inhibit development of *A. alternata* in mango and of *C. gloeosporioides* in avocado respectively as described by Prusky and co-workers (Droby *et al.*, 1986, 1987; Cojocaru *et al.*, 1986; Karni *et al.*, 1989). If bacteria could induce the formation of these compounds they could thus inhibit infection by *C. gloeosporioides*.

A final stage in the infection process at which the biological control agent might have to work is against subcuticular hyphae. The potential biological control agent would need to produce an antibiotic compound, which could penetrate the cuticle. The production of antibiotics, however, is not the most desirable property of a biological control agent to be applied to fruit. The reason for this is that the antibiotic is a 'foreign' chemical that can be toxic to, or cause allergic reactions in human beings, and quantities produced on the fruit surface will be unknown. It will be more difficult to obtain legislation for use of a biological control agent which produces antibiotics than for a non-producer (Jeffries & Jeger, 1990). Another disadvantage is that if the antibiotic producer is also used pre-harvest, resistance against the antibiotic could develop in the *Colletotrichum* population, similar to the situation which has occurred for systemic fungicides.

So what is the ideal organism? It would need the following properties: (i) it should be able to survive on the fruit surface for prolonged periods; (ii) it preferably should not produce antibiotics, but act through competition for nutrients, lytic enzymes, or a modification of the environment such that this becomes unfavourable to growth of *C. gloeosporioides*; (iii) it should control stem end rot as well as anthracnose disease, or a second control measure would still be required to reduce incidence of this disease; (iv) it must be safe for human consumption; (v) it must be easy to produce and apply; and (vi) it has to be better and

cheaper than conventional treatments, unless legislation no longer allows these conventional treatments to be used.

A final consideration must be public acceptability. Even if a successful and safe biological control system is developed, which passes all legislative requirements, the consumer still has to accept fruit treated with a microorganism. 'Bacteria' have a bad public image, especially since the recent problems of *Salmonella* and *Listeria* contamination of food. It would need careful marketing before fruit dipped in bacteria are accepted by the general public for consumption. A related aspect, which has often been mentioned in relation to biological control, is that fruit will never be as clean, e.g. no lesions present at all, as chemically treated fruit (Campbell, 1986). If the general public wants pesticide-free food, the public insistence on blemish-free fruit has to be changed.

7.3 Is biological control of quiescent infections feasible?

In the work presented here, it was shown possible to obtain a significant reduction in the incidence of anthracnose disease in mangoes that had been treated post-harvest with 558, compared to mangoes which had received no post-harvest treatment. The mechanism by which this was achieved is not yet clear. It was shown, however, that 558 produces a siderophore, and its growth causes a local increase in pH. Competition for other nutrients, beside iron, might also take place. Interactions between *Colletotrichum* spp. and iron chelating compounds, including siderophores, have all been made in relation to spore germination and appressorium formation. The effect they have on infection peg formation and penetration has not been reported as yet. But the production of siderophores is not a property desired for pre-harvest biological control. Swinburne & co-workers (see Table 1.2 for references) showed that siderophores increase spore germination and appressorium formation. It is interesting to note that in the spore germination experiments carried out here (3.3.2) 558 reduced spore germination of *C. gloeosporioides*, even though it produces a siderophore. In the post-harvest situation spore germination has already taken place and the interactions of the siderophore with the appressorium and infection peg might be of a different nature than that which is observed for spore germination.

The explanation that inhibition of infection could take place through an increase in pH might be preferable. It still has to be proven, however, that an increase of pH takes place *in situ* on the fruit surface and reduces

successful lesion formation from quiescent infections. If this is the case, it might be that a microbial component is not required for control, and alkaline dips might be sufficient to achieve equivalent control, and would present a cheaper option.

7.4 Future research

As already emphasised in this discussion, one of the main priorities should be to understand the chronology of events taking place during the infection process and the quiescent stage. With respect to 558, the main priorities are to determine the mode of action of 558, so that rational steps might be taken to improve the biological control system; to carry out more extensive post-harvest trials with this isolate, to determine if the observed control of anthracnose observed is consistent; and to find the best way of applying 558. However, 558 is not the only organism that should be considered for biological control of anthracnose. Other groups of microorganisms should be assessed in view of our increased knowledge of what is required of a biological control agent to control anthracnose. The yeasts are one of the major groups that deserves more attention (Williamson & Fokkema, 1985).

There are problems in conducting this type of study in the U.K. with tropical fruit, partly because authentic material is not easily available. In the early stages, to isolate potential antagonists I was dependant on people abroad collecting material, and either carrying it home in their luggage, or sending it by post. Apart from the delay in the processing of material, there would be advantages in being able to collect material oneself. In this study material was collected from many different countries, but isolates were then tested in the Philippines. It may have been better to consider isolates only from the Philippines, because they would be adapted to the prevailing climatic conditions. It might also create difficulties in legislation in introducing non-indigenous isolates into the Philippines.

Test material in the U.K. was also not ideal, as mangoes bought in the U.K. had an unknown history of fungicidal treatment. Mangoes needed to be artificially inoculated with *C. gloeosporioides* to secure anthracnose development, but this always left the question as to whether true quiescent infections had established. It would have been much more satisfactory to work with mangoes which had been naturally infected, and to be able to carry out many more post-harvest trials. Now that the basic framework of the project has been completed, future research should be carried out in a mango producing country.

7.5 Conclusions

Isolate 558 has been proved to reduce anthracnose in mangoes when applied post-harvest. The method of application of the bacteria, however, is not yet entirely satisfactory and deserves more research. The mechanism by which 558 controls anthracnose is not fully understood, but a siderophore is produced and an increase in pH, observed in artificial media, may be involved. Pre-harvest control of anthracnose was not successful with 558 because this isolate could not be established on the phylloplane. Biological control of anthracnose has potential, but more research is necessary to develop it, and before a commercial product is on the market, or before a biological control component is included in production practices.

To conclude with Poincaré, I have the feeling that I have accumulated an enormous heap of facts, which do not yet make a house, but hopefully have made the foundation for one.

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Appendix 1

Media

All quantities are given in g/litre dH_2O.

Casein medium	KH_2PO_4	1
	KCl	0.5
	$MgSO_4.7H_2O$	0.2
	$CaCl.2H_2O$	0.1
	Skimmed milk (Difco)	0.3
	Glucose	10
	Agar	15
Czapek Dox liquid medium (CzDL)	$NaNO_3$	2
	KCl	0.5
	Magnesium glycerophosphate	0.5
	$FeSO_4.7H_2O$	0.01
	K_2SO_4	0.35
	Sucrose	30
Czapek Dox agar (CzDA)	as CzDL with the addition of	
	agar	15
KingB (King *et al.*, 1954)	$MgSO_4.7H_2O$	1.5
	K_2HPO_4 anhydrous	1.5
	Glycerol	10
	Proteose peptone (Oxoid)	20
	Agar	15
KingB$^+$ (selective for *Pseudomonas fluorescens*)	as KingB with the addition of	
	Ampicillin	50mg
	Chloramphenicol	12.5mg
Malt extract agar (MEA)	Malt extract (Oxoid)	30
	Mycological peptone (Oxoid)	5
	Agar	15
Malt extract agar$^+$ (MEA$^+$)	as MEA with the addition of	
	Streptomycin	50mg
	Penicillin	30mg
Malt extract broth (MEB)	Malt extract	17
	Mycological peptone	3
Minimal medium (MM) (Kanner *et al.*, 1978)	Na_2HPO_4	4
	KH_2PO_4	1.5
	NH_4Cl	1
	$MgSO_4.7H_2O$	0.2
	$FeNH_4$	5mg
	Glucose	10
Tap water agar (TWA)	Agar	15
	with dH_2O replaced by tap water	

Appendix 2

C. gloeosporioides isolates obtained from lesions on mangoes bought in the U.K.

No.	Cou[1]	Var[2]	Growth[3]	Sporulation[4]	Colour[5]	Virulence[6]
2	S	A	10.1	++	W	M
4	V	?	10.1	++	W	MH
5	V	?	10.4	++	W/G	M
7	V	?	12.3	+++	W	MH
8	C	H	10.1	+	B	ML
9	C	H	9.8	++	W/G	ML
11	C	H	10.9	+++	W/G	ML
12	C	H	9.3	+	G	M
14	C	H	9.6	-	B	L
15	C	H	9.3	-	B	M
16	C	H	10.9	+	G	MH
18	C	H	10.1	-	G	M
19	F	?	10.4	++	W/G	M
20	F	?	11.2	+++	W	MH
21	F	?	10.1	++	W	M
22	F	?	11.2	+++	W/G	H
23	F	?	9.6	++	W/G	MH
24	F	?	11.2	+++	W/G	H
25	F	?	12.5	+	W	M
26	F	?	5.5	+	W	L
27	F	?	12.3	+	W	M
28	F	?	9.8	-	W	M
29	F	?	11.7	+++	W	M
30	F	?	10.4	++	W	L
31	F	?	10.1	++	W	M
32	F	?	9.8	++	W/G	M
33	B	TA	12.3	+	W/G	ML
35	B	TA	11.5	++	W/G	MH
36	B	TA	13.6	++	W	ML
37	B	TA	10.7	+++	W	M
38	B	TA	11.2	+++	W/G	ML
39	B	TA	12.8	-	G	ML
40	B	TA	11.2	++	W	M
41	B	TA	11.2	+++	W	H
42	B	TA	12.5	+++	W/G	H
43	B	R	10.1	±	W	MH
44	B	R	11.5	++	W	M
45	B	R	10.4	++	W	MH
46	B	R	7.4	-	B	ML
47	B	R	10.9	+	W	M
48	B	R	9.6	±	W	MH
49	B	R	9.8	+	W	MH
50	B	R	10.9	+	W/G	MH
51	B	R	9.6	±	B	M
52	B	R	9.6	++	W	M
53	B	R	9.0	±	W	ML
54	B	R	10.9	++	W	M
55	B	R	11.5	+++	W	M
56	B	R	11.5	++	W	M
57	B	R	11.2	++	W	MH
58	B	R	9.8	+	W	MH

59	B	R	9.0	+	W	M
60	B	R	8.5	-	B	MH
61	B	R	10.1	++	W/G	M

[1] Country of origin: B = Brasil, V = Venezuela, F = Florida, C = Colombia, S = Senegal
[2] Variety: R = Ruby, TA = Tommy Atkins, H = Haden, A = Amelie, ? = Unknown
[3] Radial growth rate on MEA (mm/day)
[4] On MEA under a black light; - - no sporulation, ± - a few spores present, + - moderate sporulation, + + - good sporulation, + + + - excellent sporulation
[5] Colour of surface mycelium; W - white, W/G - greyish white, G - grey, B - black
[6] L - avirulent, ML - low virulence, M - moderate virulence, MH - moderately high virulence, H - high virulence

Appendix 3.1

Results for the two *in vitro* tests, inhibition of mycelial growth on MEA, and spore germination on cellophane, for isolates which produced over 20% inhibition in the first test.

ISOLATE	organism[1]	origin[2]	inhibition[3]	2nd ass.[4]	germ[5]	to test on fruit[6]
8	Y	K1	26	2/1	-	Y
20	Y	K1	26	2/1	±	Y
38	Y	K1	33	3	M	-
39	Y	K1	26	2-	M	-
47	Y	K1	22	2-	M	-
101	B	K2	24	2	-	Y
102	B	K2	24	2	-	Y
104	B	K2	24	2	-	Y
105	B	K2	21	2	-	Y
106	B	K2	30	2	-	Y
109	B	K2	30	2	-	Y
113	B	K2	27	2	-	Y
114	B	K2	30	2	±	Y
116	B	K2	36	2-	-	Y
118	B	K2	30	2	-	Y
121	Y	K2	33	2	M	-
122	Y	K2	27	2	M	-
123	Y	K2	30	2	-	Y
127	Y	K2	36	2	M	-
128	Y	K2	33	2-	F	-
129	Y	K2	27	2	M	-
130	Y	K2	24	2	±	Y
141	Y	K2	35	0	M	-
202	B	K3	30	2	-	Y
204	B	K3	37	ND	-	Y
208	B	K3	22	2	-	Y
210	B	K3	52	1-	-	Y
211	B	K3	67	ND	M	-
216	B	K3	56	ND	C	-
222	B	K3	22	0	M	-
228	Y	K3	22	0	M	-
246	Au	K3	31	0	M	-

248	B	K3	34	2	M	-
250	B	K3	28	0	M	-
251	Au	K3	31	0+	-	Y
253	Y	K3	28	0	-	Y
273	Y	P	28	0+	M	-
276	Au	P	31	0+	M	-
277	Au	P	30	3	M	-
279	Au	P	31	0+	M	-
284	B	K3	33	0+	M	-
326	B	SL1	20	2	-	Y
337	B	SL2	33	ND	C	-
353	Y	SL1	60	1	M	-
354	Y	SL1	32	0	F	-
355	Y	SL1	36	0	-	Y
359	Y	SL2	50	ND	F	-
376	Y	SL1	41	0	M	-
377	Y	SL1	33	0	F	-
380	Y	SL1	52	0	M	-
381	Y	SL3	56	0	F	-
383	Y	SL3	53	1	-	Y
385	Y	SL3	33	0	C	-
388	Y	SL2	44	0	M	-
415	Y	SL6	41	4	-	Y
417	Au	SL6	41	1-	M	-
464	Au	SL5	26	1-	M	-
465	Au	SL5	26	1-	F	-
470	Y	SL5	26	0	M	-
475	Y	SL6	22	0	±	Y
479	Au	SL6	30	1-	M	-
488	B	SL6	33	2	F	-
489	B	SL6	36	0	M	-
492	B	SL6	33	3	M	-
493	B	SL6	21	2	C	-
507	B	TM4	15	0	-	Y
517	B	TM5	9	2	M	-
523	B	TM2	42	0	-	Y

530	B	TM3	46	0	-	Y
534	B	UK1	36	0	M	-
536	B	UK2	21	2	-	Y
539	B	UK2	36	0	M	-
541	B	UK3	9	2	M	-
543	B	UK3	27	2	C	-
546	B	UK3	40	1/2	M	-
547	Y	UK1	21	0	C	-
552	Au	UK2	36	0	M	-
557	Au	UK3	21	0	M	-
558	B	US	19	1/2	-	Y
566	Y	TM1	24	2	±	Y
572	Y	TM2	42	0	-	Y
585	Y	TM4	39	0	M	-
586	Y	TM4	42	0	M	-
588	Y	TM4	33	2	±	Y
594	B	TM5	3	2	M	-
598	Au	TL	42	0	M	-
650	Au	TM2	27	0	-	Y
651	B	TM3	39	1	-	Y
707	Y	B2	23	0	M	-
712	B	IH1	-7	2	-	Y
725	B	IH3	23	2	M	-
742	B	IH3	58	4	C	-
776	Au	B2	31	0	M	-
777	Au	B2	27	0	±	Y
778	Au	B2	46	0	M	-
779	Au	B2	31	0	-	Y
780	Au	B2	31	0	M	-
801	Y	Ph	28	ND	M	-
804	Y	Ph	8	ND	±	Y
808	Y	Ph	28	ND	M	-
812	Y	Ph	58	ND	M	-
814	Y	Ph	-4	ND	M	-
817	Y	Ph	0	ND	M	-
821	Y	Ph	-4	ND	M	-

822	Y	Ph	0	ND	M	-
827	Y	Ph	4	ND	M	-
831	Y	Ph	8	ND	M	-
832	Y	Ph	15	ND	M	-
Ps	B	UKC	49	0	-	Y
JCD5	B	SL	50	0	-	Y
A	B	Cont	73	1	C	-
B	B	Cont	70	1	C	-
C	B	Cont	57	0	±	Y
D	B	Cont	57	1	M	-
I1	B	Cont	54	ND	M	-
C38	B	Cont	50	ND	M	-
C517	B	Cont	58	4	±	Y
C19	B	Cont	23	ND	±	Y

1 - B= bacterium, Y= yeast, Au= *Aureobasidium*
2 - K= Kenyan pickling mango
P= Peruvian mango
SL= leaves from Sri Lanka
TM= leaves from Tanzania, Mombo
TL= leaves from Tanzania, Lushoto
UK= leaves from Uganda, Kampala
UL= leaves from Uganda, Luwero triangle
IH= leaves from India, Hyderabad
B= leaves from Thailand, Bangkok
Ph= blossom from the Philippines
UKC= from University of Kent
Cont= Contaminant
3 - %inhibition on MEA
4 - 2nd assessment on MEA (see text for coding)
5 - -= no spores had germinated
±= tiny fragments of hyphae
F= fine mycelial growth
M= well devloped mycelium
6 - isolates followed by a Y were tested on fruit

Appendix 3.2

Disease indices for isolates tested on Brazilian mangoes.

Isolate	5d.	10d.	19d.	8d.	13d.	18d.	mean[1]	ranking
8	2.5	4.0	4.5	1.0	1.0	1.0	2.3	11
20	2.3	4.0	4.3	4.0	5.0	5.0	4.1	35
101	1.3	2.7	3.7	1.0	1.3	1.7	2.0	4
102	3.0	4.0	5.0	2.0	4.5	4.5	3.8	32
104[2]	1.0	1.3	2.7	1.0	1.0	3.0	1.7	1
105	2.0	3.0	3.5	1.0	2.0	3.0	2.4	7
106	3.3	5.0	5.0	3.0	5.0	5.0	4.4	40
109	2.7	3.7	5.0	1.0	2.3	3.5	3.0	20
113	3.3	5.0	5.0	3.3	5.0	5.0	4.4	41
114	1.0	3.3	5.0	1.3	2.0	2.5	2.5	16
116	2.0	3.3	5.0	1.0	1.0	1.7	2.3	17
118	1.7	3.0	4.3	-	-	-	-	-
120	1.7	3.7	5.0	1.0	1.0	1.3	2.3	13
130	2.3	3.7	4.7	2.3	4.3	5.0	3.7	28
142	3.0	5.0	5.0	1.0	1.7	1.7	2.9	21
202	1.0	2.0	5.0	1.0	1.7	2.5	2.2	5
204	1.0	1.0	4.7	1.0	1.3	1.3	1.7	2
208	1.0	3.0	5.0	-	-	-	-	-
210	2.3	3.7	5.0	1.0	1.7	2.3	2.7	18
246	1.3	2.7	5.0	3.0	5.0	5.0	7.3	26
251	1.7	2.0	4.0	2.0	3.7	4.7	3.0	11
253	2.0	3.7	4.7	3.3	5.0	5.0	4.0	34
355	1.7	3.3	5.0	3.0	5.0	5.0	3.8	33
383	1.7	3.0	5.0	3.3	4.7	5.0	3.8	31
415	1.0	3.0	3.3	2.0	3.7	4.3	2.9	6
475	1.7	3.7	4.7	1.0	1.3	2.0	2.4	8
507	1.3	3.3	4.3	1.3	3.3	3.7	2.9	10
523	1.3	4.0	5.0	2.3	4.0	4.7	3.6	22
530	2.0	4.3	5.0	2.3	4.3	4.3	3.7	29
536	2.7	4.7	5.0	1.3	2.0	2.5	3.0	23
558	1.7	2.3	5.0	2.7	5.0	5.0	3.6	27

566	3.0	3.5	5.0	2.7	5.0	5.0	4.0	36
572	1.0	3.3	4.7	3.3	5.0	5.0	3.7	25
650	2.0	4.7	5.0	3.0	5.0	5.0	4.1	38
651	1.0	2.7	4.7	1.0	1.0	1.0	1.9	3
712	2.0	3.0	5.0	1.0	1.0	2.0	2.3	15
777	2.7	4.7	5.0	3.0	4.7	5.0	4.2	38
779	1.7	3.3	5.0	2.7	4.7	5.0	3.7	30
Ps	1.7	1.7	3.3	2.3	4.3	4.3	2.9	9
C517	2.7	4.3	5.0	2.3	5.0	5.0	4.1	37
JCD5	1.7	3.0	4.3	2.3	4.3	5.0	3.4	19
C	1.7	2.0	5.0	2.7	2.7	5.0	3.2	13
T.vir1	1.7	2.0	3.3	3.0	4.3	5.0	3.2	-
T.vir2	1.0	1.3	2.7	3.0	5.0	5.0	3.0	-
Tr.sp2	1.0	1.0	1.7	3.0	5.0	5.0	2.8	-
Tr.sp3	1.3	1.7	2.0	3.3	4.7	5.0	3.0	-
Tr.sp5	1.5	3.0	2.3	3.0	5.0	5.0	3.3	-
control	1.7	3.2	5.0	2.8	4.4	4.6	3.6	24

[1] - mean disease ratings of the two batches of mangoes pooling all the ratings together.
[2] - underlined isolates were selected for trials in the Philippines.

Appendix 3.3

Inhibition of two isolates of the stem end rot fungus, isolate 1, from Sri Lanka, and isolate 2, from Kenya, by a selection of antagonists.

	Isolate 1		Isolate 2	
Antagonist	%inhibition	2nd assess.	%inhibition	2nd assess.
8	42	2	0	2
20	47	2	44	2
101	47	2	0	2
102	44	2	0	2
104	42	2	42	2
105	0	2	0	2
106	44	2/1	49	2
109	47	2	0	2/1
113	40	2/1	51	2/1
114	47	2	0	2
116	44	2/1	0	2
118	-	-	47	2
130	0	2	63	3
202	0	2	0	2
204	53	0	42	0
208	0	2	0	2
210	60	3	53	3
246	0	2	43	2
251	56	-	42	0
253	0	2	0	2
355	0	2	0	2
383	47	0	42	2
415	0	0	0	0
475	47	0	0	2
507	42	2	0	2
523	47	2	49	2
530	0	2	0	2
536	0	2	0	2
558	63	1	44	0
572	42	1	44	2
650	0	2	0	0

651	77	4	56	3
712	0	2	0	2
777	47	0	42	2
779	53	0	51	1
Ps	44	0	53	2
JCD5	47	2	44	-
C517	53	0	0	2
Tr.2	66	1	64	1
Tr.3	62	0	62	0
Tr.4	70	1	60	1
Tr.5	62	1	62	1
T.vir.1	60	1	49	1
T.vir.2	53	0	47	-
T.kon.1	70	0	51	-
T.kon.2	53	0	51	0
T.har.372	58	1	57	1
T.har.373	66	1	66	1