Isolation and assessment of attachment bacteria and yeasts for the biological control of *Botrytis cinerea*.

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Abstract

The biological control of *Botrytis cinerea* Pers. infection by microbial agents applied to the host surface has been based on a wide range of mechanisms of which resource competition, antibiosis and induced host resistance have been considered the most important. A 1995 review of antagonistic mechanisms concluded that biocontrol agent (BCA) colonisation of the plant host was critical for successful biocontrol but that few isolates appear to achieve this. Recent research has shown a reduced epiphytic growth prior to penetration of *B. cinerea* when conidia are applied as dry spores. Such pre-penetration infection morphology would provide little opportunity for antibiosis, resource competition or induced host resistance. Contemporary *in vivo* plant tissue assays and *in vitro* agar plate-based-assays have perpetuated the traditional biocontrol model based on such mechanisms hence an alternative approach was required. BCA selection based on microbial adhesion to the pathogen itself appeared to offer such an approach.

An investigation of methods of *B. cinerea* conidial application showed that disease incidence was increased and development advanced from aerosol application of spores. Aerosol application was used as the standard technique for biocontrol experiments in the remainder of this study.

A total of 12 bacterial and eight yeast candidates were obtained from the attachment assay. *In vivo*, 15 reduced disease by more than 90% in at least one combination of incubation temperature (1°C ,7°C or 15°C) and BCA concentration (three-times to 60-times the *B. cinerea* population applied). When BCA application followed *B. cinerea* inoculation by up to 48 h, high biocontrol activity was observed. The five yeasts tested postharvest on kiwifruit conferred high biocontrol (>90%) when applied simultaneously or up to 48 h after *B. cinerea* inoculation. All eight bacterial and seven yeast BCA candidates also reduced disease incidence in stem wounds by more than 80% in glasshouse tomato plants.

In vitro investigations into antagonistic mechanisms suggested that antibiosis was unlikely to be important in all but two of these bacterial BCAs. Production of

endochitinase was common among the yeasts but there was no single presumptive mechanism for bacterial biocontrol. Variable levels of adhesion by BCA isolates were detected by light and electron microscopy and indicate that biocontrol may not be correlated quantitatively to the number of adhesion events. Adhesion of yeast and biocontrol activity were not affected by a monoclonal antibody to *B. cinerea.* However, bacterial adhesion and biocontrol activity were dramatically reduced indicating that the antibody blocked bacterial adhesion sites and that bacteria and yeast adhere to different sites on the pathogen.

A monoclonal antibody-based ELISA immunoassay was developed to measure vegetative biomass of *B. cinerea* in infected tomato stem tissue with or without BCAs. The key to the successful application of this ELISA assay was the extraction of the pathogen antigen from the plant tissue using 0.1M copper sulphate and salts solution. Significant reductions in pathogen growth were detected in host tissue co-inoculated with *B. cinerea* and BCA.

The attachment assay was an efficient isolation method that optimised use of laboratory resources and could be employed in future programmes as a presumptive test for biocontrol. With this determinative selection criterion, BCAs with desirable characteristics such as reduced importance of BCA application dose and timing were obtained. A comparison of these results with those in the literature led to the proposal for an alternative biocontrol model for *B. cinerea* that could supplement existing technologies.

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To Him who is the creator of all things that we seek to understand with our tools we call science and philosophy.

"He makes the grass grow for the cattle,
and plants for men to cultivate-
bringing forth food from the earth:
wine that gladdens the heart of man,
oil to make his face shine,
and bread that sustains his heart".
(Psalm 104: 14-15)

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General Introduction

The changing face of plant protection

1.1 Sustainable Agriculture

Klinkenberg (1995) wrote about changes in American farming based on techniques and management practices that are "sustainable", "economically viable", "environmentally sound" and "socially acceptable". Sustainable agriculture is a term that has come "into common usage as a broad ethical concept implying moral choices relating to the use and distribution of material goods within and between societies, both present and future" (Pearce *et al.*, (1988), cited by Hamblin, 1995). Among the various issues in sustainable agriculture, pesticide use and its perceived adverse effects on environment and human health has captured public attention, leading to a declining tolerance for crop production and protection using manufactured synthetic compounds. This perception is illustrated in a study comparing the frequency of events contributing to human deaths with a public survey of perceived killers (Berry, 1990). The public survey regarded nuclear power, motor vehicle accidents, hand guns and pesticides as killers but data presented by Upton (1982) (cited by Berry, 1990) showed that pesticides were ranked alongside food colouring and food preservatives. However this data does not take into account declines in human health or well-being nor are there adjustments made for social tolerances that vary with each event.

Views in the pesticide debate are formed from data in a background of moral choices and acceptance levels leading to lively and passionate debate between those who argue for the removal of pesticide use in agriculture and proponents of the status quo. Provocative publications expounding both views are evident in our libraries where, "Saving the planet with pesticides and plastics" by a proponent of the status quo (Avery, 1995) is a recent example. Yet it appears that some facts are objective enough for courts of law to make rulings. A Florida court of law in June 1996, awarded damages to parents of a child born blind, as it was proved that the condition was linked to the exposure of the pregnant mother to the fungicide, Benlate. Similar cases are

pending in Christchurch brought by parents of children born with no eyes or congenital blindness.

Regardless of the reason, social or scientific, the manner in which agriculture is practised is undergoing a transformation and is evident with many changes, for example, in pesticide use to reduce unnecessary exposure (MacIntyre, Allison & Penman, 1989) and the research and developmental costs incurred by companies involved in plant protection products. Estimates of US$80 million for pesticides compared with US$5 million for biocontrol-based product (Woodhead, O'Leary, O'Leary & Rabatin, 1990) are a reflection of more rigorous registration legislation that prejudice against synthetic pesticides.

1.2 Plant protection research

Research objectives within the scientific community involved in plant protection have similarly undergone a transformation, driven by funding priorities stipulated by public or private providers. This progression can be seen in the changing themes of the International Plant Protection Congress (IPPC), where the first in 1946, focused on the new synthetic compounds with pesticidal properties as answers to increasing food production. Later, "Plant protection for human welfare" (Brighton 1983), "Integrated Pest Management" (Rio de Janeiro 1991) and "Sustainable Crop Protection for the benefit of all" (The Hague 1995) all reflect a changing focus (Zadoks, 1995).

Within plant pathology, "Prelude to biological control" (Baker & Snyder, 1965) reflected doubt, uncertainty and scepticism which 20 years later Cook & Baker (1983) noted a new confidence "that biological control offers answers to many serious problems of modern agriculture and is an essential component in the development of a sustainable agriculture capable of continuing without interruption or diminution". This progress has largely been made in soil-borne diseases and exceeds that in foliar diseases (Fokkema, 1995). Thirty commercial products based on biocontrol have received registration in the world targeting viral (1), bacterial (12) and fungal (17) pathogens (Lumsden, Lewis & Fravel, 1995). In addition, commercial companies specialising in the advancement of agents and techniques from the laboratory to the user in the field have emerged globally (e.g. Ecogen) and within New Zealand (e.g. Crop Care Ltd. and

Agrimm Technologies). Their establishment reflects increasing optimism in biocontrol products as technologies are advanced and they are examples of the large multinational corporations or the smaller local/national biopesticide companies respectively that are becoming involved (Tormala, 1995).

Yet it appears that biocontrol progress has stalled. This is reflected in topics at the recent IPPC conference (The Hague, 1995) discussing political, commercial, legislative and biological limitations. Similarly the literature has reviewed issues such as biocontrol product registration and application (Woodhead *et al.*, 1990, Klingauf, 1995), the accidental or intentional release of genetically modified organisms (Pimentel, 1995; Whitten 1995) and the economics of biocontrol (Cullen & Whitten, 1995; Tormala, 1995). Biotic constraints have been more intensively examined and the reader is referred to reviews discussing general biocontrol (Boland, 1990; Lewis & Papavizas, 1991; Cook, 1993; Scott, 1995), biocontrol in the rhizosphere (Burpee, 1990; Deacon & Berry, 1993), biocontrol on the phylloplane (Blakeman & Fokkema, 1982; Andrews, 1990, 1992; Fokkema, 1993, 1995) biocontrol mechanisms (Fravel, 1988; Elad, 1996; Thomashow & Weller, 1996), screening techniques (Dhingra & Sinclair, 1985; Merriman & Russell, 1990) and post-harvest biocontrol (Wilson & Wisniewski, 1989). In contrast, project underfunding (Andrews, 1990), social and economic conditions in the market (Wilson & Wisniewski, 1989) were proposed as alternative reasons for the delays in biocontrol progress. Whipps (1994) recommended four general research priorities that have potential for future improvement; refinement of biocontrol agent screening methodologies, use of genetically modified organism (GMO) technology, application of antagonists as mixtures and investigation of fermentation methods for up-scaling agent production from research to commercial volumes.

1.3 *Botrytis cinerea* Pers. - A pathogen case study

Botrytis cinerea Pers. is distributed throughout the world, and it is a common pathogen of a wide variety of commercially important glasshouse, fruit, cut flower, ornamental and vegetable crops (Agrios, 1988). The pathogen causes preharvest and postharvest yield loss in roses (Redmond, Marois & MacDonald, 1987), red raspberries (McNicol, Williamson & Dolan 1985), strawberries (Jarvis, 1962, 1980; Sutton, 1995)

and stone fruits (Fourie & Holz, 1994). In New Zealand, the pathogen is responsible for NZ $6 million loss of kiwifruit during coolstorage and additional costs of NZ $ 4.5 million for labour and packaging to the New Zealand industry (Bautista-Banos, 1995). In glasshouse tomato crops, stem infections are a major problem in older plants (>11 months) with up to 40% of plants infected and is often the reason for the premature end to production. In the UK, production loss is estimated at £1.5 million (O'Neill, Shtienberg & Elad, 1997).

Cool, humid climates favour disease development, with optimal temperatures for growth and sporulation in the range, 18 - 23 °C however the pathogen remains active at lower temperatures (0 - 10°C) (Agrios, 1988). It is therefore a significant pathogen of products in prolonged coolstorage. The epidemiology of *B. cinerea* often has a significant phase on senescent host tissue particularly on floral or foliage parts from which spores later disperse onto the harvested product in a variety of ways. Powelson (1960) proposed that *B. cinerea*, isolated from up to 80% of necrotic floral parts of field strawberries, influenced the pre-harvest latent infection in unripe symptomless fruit. Similar conclusions were reached in later reports (Jarvis, 1962) and for red raspberries (McNicol *et al.*, 1985) and grapes (McCellan & Hewitt, 1973). In kiwifruit, Elmer *et al.* (1993) found that the major sources of *B. cinerea* inoculum were senescent flowers, foliage within the canopy and infections of green leaf tissue that contribute to contamination of the external fruit surface which was later redistributed to the picking scar during harvest. Flower crops are likewise infected from conidia landing on the host surface during preharvest where lesions develop in postharvest phases (Redmond *et al.*, 1987). In contrast, pear plum nectarine and apple, fruits appear to be invaded from mycelium on colonised residual floral parts (Fourie & Holz, 1994).

B. cinerea is a weak pathogen, and entry into host tissue is via natural openings or wounds (Rijkenberg, De Leeuw & Verhoeff, 1980), hence the description "an opportunist pathogen". Direct host penetration by conidia occurs in unwounded immature tomato fruit tissue where localised lesions form and seldom progress into rots (Rijkenberg *et al.*, 1980).

Fungicide resistance in *B. cinerea* populations is a significant management issue. Globally, strains of *B. cinerea* have been found that are tolerant to cyclic imides (captan), benzimidazole (benomyl), aromatic compounds (dichloran) (Agrios, 1988), dicarboximides (Locher, 1987) or to combinations of benzimidazole, dicarboximide and diethofencarb (Elad, Yunis & Katan, 1992). In New Zealand, resistant strains to both benzimidazole and dicarboximide fungicides were discovered in 1974-1976 (Hartill, 1986) and in 1981 (Beever & Brien, 1983). When resistance to these two fungicides has developed, it has become evident within two to five years of commercial introduction of the fungicide (Brent, 1987).

It is little wonder then that Whipps (1994) listed *B. cinerea* as a priority plant pathogen for future biocontrol research. The local and global impact of this pathogen through pre-harvest and post-harvest crop loss is considerable and the weaponry with which we can manage this disease has been eroded. Society no longer tolerates excessive use of synthetic fungicides thus biocontrol can potentially offer an alternative method for disease management.

1.4 Biocontrol of *B. cinerea.*

1.4.1 Introduction

Avenues for biocontrol of plant pathogens is reflected in the constituent parts of the disease triangle (the host, the pathogen and the environment) where each component has a role in determining the expression of disease. Basic abiotic parameters such as temperature, pH, water potential, radiation, surface charges, partial pressure of gases, ions and elements and carbon compounds (Cook & Baker, 1983) are indirectly involved (often mediated through some biotic system) in biocontrol and may be manipulated to the detriment of the pathogen.

It is essential that the aetiology, epidemiology and biotic tolerances of the pathogen, are thoroughly understood so that weakness in its life cycle or biology may be exploited and appropriate biocontrol systems applied.

The plant host is the third member of the complex interaction, and various methods have been utilised to enhance defence. Plant traits that exhibit disease resistance may be

selected through Mendelian genetic breeding, or inserted using modern molecular techniques. Alternatively, cross protection, induced resistance, or immunity are terms used to describe elevated defence status of the plant following inoculation of microbes or other treatment that stimulate appropriate physiological changes in the host. The plant also provides surfaces on which communities of microbes colonise, but have no known, direct influence on host physiology. Interactions between microbial populations may be modified through the manipulation of existing populations or the inundative release of introduced isolates of biocontrol agents (BCAs). The latter dominates the current approach to the biological control of *B. cinerea.*

1.4.2 The contemporary model of B. cinerea *biocontrol*

The complex interaction between moisture and nutrient conditions are considered to be primary determinants of *B. cinerea* growth during the epiphytic growth phase prior to host penetration (Andrews, 1992). Germination and growth of conidia is influenced by exogenous nutrient on the plant surface (Blakeman, 1975; Yoder & Whalen, 1975; Sharman & Heale, 1977, 1979; Harper & Strange, 1981) and further research has attempted to define this requirement using or mimicking gutation fluids (Warren, 1972; Frossard & Oertli, 1982), pollen grains (Chu Chou & Preece, 1968), removing this chemical resource by leaching (Sztejnberg & Blakeman, 1973) or by saprophytic competitive bacteria (Brodie & Blakeman 1976; Blakeman & Brodie, 1977).

Competition for phylloplane resources forms basis of the contemporary model for biocontrol of *B. cinerea,* provided that the respective niches of antagonist and pathogen overlap. Dubos (1992) and Elad (1996) argued that this mechanism is the most important and Andrews (1992) stated that colonisation and rapid population growth or activity to critical threshold levels to exclude any late-comers or weaker residents has become the central tenet in many other pathogen systems. However in recent research and reviews, there are questions that cast a shadow upon this biocontrol model, its relevance to the pathogen target and even the empirical screening assays used.

Williamson *et al.*, (1995), and Cole, Dewey & Hawes, (1996), observed germ tubes, from *B. cinerea* conidia applied as dry spores, directly penetrate rose petal and

bean leaf tissue respectively while epiphytic hyphal growth from conidia applied in liquid suspension continued to explore the plant surface. As Andrews (1992) regarded biotrophy and saprotrophy is part of a continuum, there is the possibility that the saprophytic nature of *B cinerea* growth has been overestimated. Since current laboratory technique uses spore suspensions in *in vivo* screening assays, the unnaturally large epiphytic phase could bias toward the selection of competitor-BCAs or antibiotic producers because the pathogen is more dependent on exogenous nutrient and has a greater surface area exposed to toxic metabolites. Any BCA selected against the wet-type *B. cinerea* would then be ineffective because this large epiphytic phase may be absent or greatly reduced following dry spore deposition in the field. In contrast screening for BCAs against biotrophic pathogens have tended to isolate mycoparasites (Kranz, 1981) because host penetration by biotrophs is independent of exogenous nutrient (Andrews, 1992). Therefore the absence of mycoparasites in previous selections *in vivo* against *B. cinerea* could be regarded as confirmation of the overexpression of the epiphytic growth phase assuming the expression of a particular antagonistic mechanism is the most appropriate for that pathogen.

Sufficient and aggressive colonisation of overlapping niches by a competitor-BCA is essential for biocontrol expression (Andrews, 1990, 1992), but Thomashow & Weller (1996) concluded that inadequate BCA colonisation of the infection court and inconsistent expression of antagonism activity were responsible for the poor commercial progress to the market place of BCAs against soil-borne pathogens. James, Suslow & Steinback (1985) examined rhizobacteria cell adhesion to radish roots as an initial trait for long term colonisation of the rhizoplane, but concluded that many features other than adhesion alone were involved. Andrews (1990, 1992) argued that if traits leading to colonisation could be identified, they would be a powerful selection criterion provided that such traits are correctly identified. If a trait for colonisation was identified, it is then assumed that these traits would be retained in the microbes throughout the screening process where isolates are maintained on artificial media (Boland, 1990). Wilson & Wisniewski (1989) and Andrews (1990) suggested that all media and accompanying conditions are selective. For example, the glycocalyx can be lost from bacteria cultured in some media (Andrews 1990) and Vesper (1987) was able to manipulate the presence or absence of

pilli on *Pseudomonas fluorescens* by altering the culture media. Such considerations have been given during fermentation processes, for example for *Trichoderma harzianum* (Harman, 1991) but not in the laboratory prior to bulk production for field trials or commercial release. Shifts in colonisation traits through repeated culturing on media could inadvertently result in poor colonisers being used. However the convenience that artificial media provides is difficult to overcome.

Results from previous biocontrol studies using competitor-BCAs suggest there is a close temporal and quantitative relationship between antagonist and *B. cinerea* populations. In many instances, plant host tissue is simultaneously co-inoculated with the pathogen and the BCA (Redmond *et al.*, 1987; Roberts 1990; Janisiewicz, 1992; McLaughlin, *et al.*, 1992; Janisiewicz, Peterson & Bors, 1994) while in other studies, the BCA is given prior access to the host by up to two days before pathogen inoculation (Malathrakis & Kritsotaki, 1992). In addition, the level of biocontrol expressed declines as the applied antagonist population is decreased or pathogen challenge is increased (Janisiewicz, 1988; Roberts 1990; Leifert, *et al.*, 1992a, Leifert, Sigee & Epton., 1992b; Janisiewicz *et al.*, 1994). It is questionable how relevant the timing of these applications are to the field environment because any user of a plant protection product based on such a BCA will have to apply the antagonist to the target site at an effective dose before *B. cinerea* arrival. Hammer & Marois (1989) recognised that postharvest BCAs would be required to antagonise pathogen populations already resident on rose petals in cut flower crops entering the postharvest phase. They delayed the application of a coryneform bacteria or a yeast (*Exophiala jeanselmei*) by up to 48 h and achieved protection of rose petals similar to fungicide treatment. The mode of antagonism was not proposed.

Another frequent mode of antagonism in *B. cinerea* biocontrol is antibiotic production, possibly a legacy of the period in research when *in vitro* plate tests identified and selected BCAs. The importance of antibiosis in plant protection has been debated (Fravel, 1988; Thomashow & Weller, 1996) with evidence from experiments using cell free extract from *in vitro* cultures, direct isolation of antibiotic from the plant host surface and the creation of mutant bacteria with modern genetic methods. Results from the later technique is regarded as more definitive where it was shown that nutrient was a critical determinant in soil-borne interactions (Thomashow & Weller, 1996). On the

phylloplane, quantities of nutrient resources are considerably lower compared with the rhizosphere (Andrews, 1992; Fokkema, 1995), therefore the acquisition of sufficient nutrient will be of greater importance. The issues of microbial colonisation in order to acquire these nutrients for antibiosis would appear to be similar to that of competition mediated biocontrol.

The risks of resistance developing in the target pathogen population to BCAs that act through the production of metabolites has been recognised (Droby, Chalutz, Wilson & Wisniewski, 1992; Elad, 1996; J Vanneste, personal communication) and was experimentally confirmed, when *Botryotinia fuckeliana* (de Bary) Whetzel was repeatedly exposed to *Bacillus subtillus,* CL27 and resistance to the metabolite from the BCA was detected in the target fungus (Li & Leifert, 1994).

Induced host resistance is a third biocontrol mechanism rapidly being adopted for *B. cinerea* biocontrol but is described this as one of the slower acting functions (Elad, 1996). Delays between treatment application and expression of resistance can be measured in days, during which the pathogen may still cause disease (T. Reglinski, personal communication). Again, microbial colonisation of the plant surface could conceivably be subject to the same considerations and limitations discussed earlier in this section.

1.4.3 Alternative, selectable cell characters for biocontrol

There have been many calls for alternative or improved ideas for the screening of biocontrol agents. Andrews (1990) stated that "Most of us are doing the same things repeatedly on different systems and are failing at it" implying the need for alternative ideas. In general biocontrol research, microbial adhesion to other microbes does not appear to have been exploited due to the absence of an efficient method of isolating microbes with such characteristics.

The biological significance of microbial attachment to surfaces in a variety of interactions is gaining credibility. This process is vital in initial phases of surface colonisation by a variety of fungi (Jones, 1994), bacteria (Marshall & Cruickshank, 1973; Marshall, 1980), yeasts (Douglas, 1987), the pathogenicity of plants (Nicholson & Epstein, 1992), animals (Douglas, 1987) and of plant pathogen biocontrol (Nelson *et al.*, 1986; Yang, Menge & Cooksey, 1994). In selecting microbes with a mode of antagonism other

than phylloplane niche competition, antibiosis or induced host resistance it may be possible to obtain biocontrol agents with quite different characteristics, for example, a much reduced reliance on the phylloplane to confer biocontrol. Given the hypothesis of the correlation between attachment and biological outcome, one would expect some form of activity, for example biocontrol, in microbes that attach to a pathogen surface.

1.5 Microbial adhesion as a criterion for biocontrol screening.

Early research classified microbial attachment mechanisms into two broad types (Marshall, 1980; Marshall & Bitton, 1980), a temporary (reversible) attachment phase which involves the electrostatic or hydrophobic positioning of the cell to a surface followed by a permanent, non-reversible attachment, characterised by the production of extra-cellular matrix materials. Formation of the first phase is instantaneous, holding the cell while the time dependent second phase progresses. The production of extra-cellular mucilage such as adhesion pads (Beckett, Tatnell & Taylor, 1990; Braun & Howard, 1994; Doss, Potter, Chastagner & Christian, 1993; Doss *et al.,* 1994), bacterial fimbrae and specialised structures such as holdfasts are involved in adhesion (Jones, 1994).

1.5.1 Microbial attachment to plants.

Nicholson & Epstein (1991), Manocha & Chen (1990), Beckett *et al.* (1990) and Braun & Howard (1994) stated that microbial adhesion of the pathogen propagule to the plant host was essential for pathogenesis where cell adhesion was inhibited by the addition of an agent such as Con A, IgG and D-mannose (Ding, Balsiger, Guggenbuhl & Hohl, 1994), detergents (Doss *et al.*, 1993), respiratory inhibitors (Young & Kauss, 1984), or the enzymic removal of specific surface molecules (Hinch & Clarke, 1980) or the creation of a mutant (Douglas Halperin & Nester, 1982). Subsequent pathogenesis functions were lost. The same conclusions were obtained in a number of non-pathogenic microbe - plant interactions, for example, *Rhizobium* sp. and pea root hair tips (Smit, Kijne & Lugtenberg, 1986; Dazzo *et al.*, 1984).

1.5.2 Microbial attachment to fungi (Mycolytic bacteria and yeasts)

Bacteria and yeasts capable of lysing and/or colonising fungi have been isolated from marine, aquatic, soil and phylloplane habitats. Early research did not focus on adhesion to fungi, but rather the suppression of *Fusarium* sp. (cited by Mitchell & Alexander, 1961), cereal rusts and *Puccinia* spp. by bacteria isolated from disease lesions and then observed within uredinospores using light microscopy (cited by Pon *et al.*, 1954). The incidence of *Puccinia recondita, P. graminis* f.sp. *tritici* and *P. coronata* infection on wheat and oats was reduced with the addition of broth cultures from *Bacillus pumilis* which lysed urediniospore germ tubes *in vitro* (Morgan, 1963). Mitchell & Alexander, (1961, 1963) and Mitchell & Hurwitz, (1965) detected fungal lysis by changes in absorbances of suspensions made from fungal cell wall preparations seeded with bacteria. Rotem, Clare & Carter (1976) used cell free extract or the addition of chloramphenicol to bacterial suspensions to determine the bacterial origin and the extracellular nature of the agent responsible for lysis. In addition to lysis of germ tubes, stimulation of appresorium formation was observed in the presence of bacteria designated I2 and I4 isolated from *Colletotricum gloeosporoides* (Lenne & Parberry, 1976).

In later studies, electron microscopy was used to visually verify fungal colonisation by other microbes. Despite observing perforations and bacterium-like structures on, within, or partially embedded in the wall of *Cochliobolus sativus* conidia and *Thielaviopsis basicola* chlamydospores, Old & Robertson (1969, 1970),Wong & Old (1974),Old & Patrick (1976) and Clough & Patrick (1976) were unable to isolate the causal agent(s). In a similar manner to Pon *et al.* (1954), Old & Robertson (1970) observed bacterial structures within conidia of the baited *C. sativus* and speculated that these were *Pseudomonas, Bacillus, Corynebacterium* and *Micrococcus* spp. Similar perforation morphologies led Old & Patrick (1976) to conclude that the same causal agent was responsible for the symptoms on *C. sativus, T. basicola* and on four other pigmented fungal species (Old & Wong 1975). The most likely candidates appeared to be amoebae of the Vampyrellidae family which are non culturable on media normally used for the maintenance of bacteria (Old 1977; Anderson & Patrick 1978). This would explain the lack of success of earlier attempts to isolate a causal agent.

Malajczuk, Nesbitt & Glenn (1977) observed bacteria colonising hyphae of *Phytophthora cinnamomi* and isolated *Bacillus* sp., *Streptomyces* sp., and *Pseudomonas* sp. These bacteria attached to the pathogen and exhibited inhibition zones in plate tests but rarely penetrated the hypha *in vitro*. Extracellular lytic metabolites were implicated as the mechanism responsible for the reduction in pathogen viability. Nesbitt, Malajczuk & Glenn (1981) observed bacteria attached to the hyphae of *P. cinnamomi* grown on agar then incubated in soil. None of the bacteria isolated attached to hyphae although lysis and antibiotic production was detected *in vitro*. The authors argued that attachment was not important for mycolysis. Other work using scanning electron microscopy (SEM) has shown adhesion to fungi by bacteria (Rovira & Campbell, 1975; Malajczuk, Pearce & Litchfield, 1984; Hadar, Harman, Taylor & Norton, 1983; Nelson *et al.*, 1986; Wisniewski, Wilson, & Hershberger, 1989; Toyota & Kimura, 1993; Yang *et al.*, 1994) or yeasts including *Pichia guilliermondii* (Wisniewski *et al.*, 1991) and *Debaromyces hansenii* (Wisniewski, Wilson, Chalutz & Hershberger, 1988). However these isolates have generally been chance selections using plant-based *in vivo* assays, where the technique bears little direct resemblance to the microbe mode of antagonism.

1.6 Proposed screening programme.

The hypothesis on which this work is based is that use of existing *in vivo* screening methods with inoculated host tissue is likely to perpetuate the selection of competitor or antibiosis-type BCAs. The reduced epiphytic growth observed from dry-inoculated *B. cinerea* conidia indicates that these antagonistic mechanisms may not be suitable because there is a limited biocontrol window and could conceivably be the reason for poor field performance. An alternative screening methodology is proposed based on the premise that attachment is crucial for subsequent biological activity and could be exploited in an assay designed to select from mixed populations, bacteria and yeasts capable of attaching to *B. cinerea*. This isolation procedure would function as a presumptive test for attachers to *B. cinerea* in an initial screening for BCAs.

Thesis Objectives.

The objectives of this thesis are to:

1) develop a procedure for the isolation of bacteria and yeasts with the ability to attach to *B. cinerea* hyphae and conidia,

2) evaluate such isolates for biocontrol activity against *B. cinerea* in the laboratory and in selected host crops in semi-commercial situations,

3) examine the importance of the microbial adhesion mechanism itself in the biocontrol event,

4) determine whether other activities of these isolates could potentially operate as antagonism mechanisms,

5) develop a method to measure pathogen biomass.

General Materials and Methods

2.1 *B. cinerea*

2.1.1 Cultures

B. cinerea, isolates BC 20, (HortResearch, Lincoln, New Zealand) Pezet (Swiss Federal Agricultural Station, Neon, Switzerland) and IPO 700 (IPO/DLO Wageningen, The Netherlands) were cultured on malt extract agar: 30 g/litre Maltexo (Maltexo NZ Ltd.), 15 g/litre bacteriological agar (Gibco-BRL Ltd.) and 5 g/litre peptone (Difco Ltd.) dissolved or suspended in reverse osmosis (RO) water. The medium was autoclaved at 121°C for 10 min (≤200 ml) or 15 min (>200 ml), cooled to approximately 50°C then poured into 90 mm diameter plastic petri plates.

Plates were inoculated with 5 mm agar plugs aseptically cut from stock *B. cinerea* cultures and incubated at 20°C under a 12 h Day/Night NUV light regime (Philips TLD 36W/08, located 40 cm above the cultures), for 8 days to induce sporulation.

2.1.2 Spore Suspensions

Approximately 15 ml sterile distilled water (SDW) was poured over a sporulating colony and gently agitated using a sterilised glass "hockey stick". The resulting suspension of conidia and mycelial debris was transferred to an autoclaved universal bottle where the suspension was shaken vigorously for 1 min to disperse clumps of conidia. The contents were transferred to a second universal bottle via a sterile cell strainer (Falcon 2350) with a pore diameter of 70 μm to remove mycelial debris. The suspension was again shaken immediately before a 10 μl aliquot was pipetted onto a haemocytometer slide. Spore concentrations were measured and adjusted with SDW as required.

2.2 Bacteria and Yeasts

2.2.1 Cultures

All bacterial isolates were cultured on nutrient agar (NA): 8 g/litre nutrient broth (Gibco-BRL) and 15 g/litre bacteriological agar (Gibco-BRL). Yeasts were cultured on nutrient yeast dextrose agar (NYDA): 8 g/litre nutrient broth (Gibco-

BRL), 5 g/litre yeast extract (Gibco-BRL), 10 g/litre D- glucose (Ajax Chemicals) and 15 g/litre bacteriological agar (Gibco-BRL). The pH of NYDA was adjusted from 7.0 to 4.5 using 6M HCl. Media were sterilised at 121°C for 10 min or 15 min depending on volume, cooled and *c.* 15 ml of molten media poured into each 90 mm diameter Petri plate. The final pH of NYDA was not changed by autoclaving.

Plates were inoculated by dipping a flamed sterile loop into suspensions of bacteria or yeasts and gently streaking onto the surface of the medium. Inoculated plates were incubated at 15°C in total darkness for 5 days which allowed maximal growth of the slower growing yeast isolates.

2.2.2 Cell Suspensions

After incubation, a jet of SDW (approximately 3 ml) from a pipette tip was used to dislodge cells from the colonies. Cell density in the suspension was increased by repeating the pipetting cycle using the same liquid until most of the cells were removed from the colonies. This method of suspending cells was preferable to the "hockey stick" as use of the later often resulted in clumps of cells that required dispersing. The "pipette" method minimised cell clumping with the repeated passage of suspension through the narrow pipette tip and the action of the jet removing a small number of cells at a time.

Suspensions were transferred to labelled, sterile 1.5 ml Ependorff tubes and centrifuged at 10,000 rpm for 5 min using a Biofuge A (Heraeus, Sepatech) (8,900 **g**). After the liquid was decanted off, 1.5 ml of SDW was added and the pellet was re-suspended with repeated pipetting.

Total cell counts were determined for each suspension using a Petroff-Hauser Counter (MNK-780-T, Weber Scientific International Ltd., England). Samples were vortexed immediately before a 10 µl aliquot was loaded onto the counting chamber. Since there were many samples to be counted, only one aliquot per sample was measured. Unstained bacteria could be observed using phase-contrast or dark-field microscopy where a minimum of 600 organisms were counted per sample. (Meynell & Meynell, 1970). The Petroff-Hauser chamber was used in preference to the Helber as the former included a heavier cover-slip which gave more accurate and precise volumes for counting.

Bacterial cell concentrations in experiments conducted at Oxford University (in Appendix Three), were determined by suspension absorbances using a spectrophotometer set at 585 nm. Concentrations of 1 x 10^8 to 1 x 10^9 cfu/ml were obtained with absorbances of 0.7 for isolates ox4, ox5 and absorbance of 0.5 for isolates ox6, ox7 and ox9. Yeast cell concentrations were determined using a haemocytometer.

2.3 Tomato Tissue

2.3.1 Growth of the plants

Tomato plants (*Lycopersicon esculentum* cv. 'MoneyMaker') were grown in bark-based medium; 500 g of 3-4 month osmocote, 50 g $FeSO_4$, 400 g dolomite and 400 g aglime per cubic metre of bark mix. Plastic trays containing 60 wells each of 50 ml volume were filled with this medium and sown with a single tomato seed per well. They were watered as required and glasshouse air temperature was maintained between 15°C - 28°C. Care was taken to ensure that the glasshouse air temperature did not exceed 30°C for prolonged periods (*c.* 12 h) as this rendered the plants more resistant to infection by *B. cinerea.*

Plants were grown for 6 wk by which time they were approximately 30 cm high and etiolated due to the high density of planting.

2.3.2 Tissue preparation

Stems of seedling plants were cut above cotyledon position and leaves removed. After surface sterilisation in a 0.04M sodium hypochlorite solution, (pH adjusted to 7.0 with 50% acetic acid) for 1 min they were rinsed in running tap water for 1 min to remove bleach residue.

Each stem was cut into 1 cm lengths using a hot sterile scalpel and pieces were pooled in a sterile beaker, which was tumbled to mix and randomise them before allocation for treatments.

2.4 Stem tissue holders (STH)

To facilitate efficient and convenient handling of tomato stem pieces, a system was developed to hold the stems vertically whilst the upper cut surfaces were spray inoculating (See Chapter Four). When stems were to be incubated for more than 8

days, a single plate was cut in half giving two plates with 48 (6 x 8) wells. The well bases were removed so that stems would rest directly on the moist sterile paper towels lining the plastic tray container thus maintaining turgidity.

A second "modular" STH type (MSTH) was developed for holding stems for single replicates or treatments during shorter incubations (< 8 days). This MSTH consisted of blocks of 12 wells (6 x 2) with the well bases remaining intact.

2.5 Enzyme linked immunosorbant assays (ELISA)

In Chapters Seven and Eight, ELISA techniques were used. In this section, the buffers, other standard solutions or suspensions and ELISA procedures are described.

2.5.1 Phosphate buffered saline (PBS)

For a 500 ml volume of 10-times concentrate add; NaCl 40.0 g, KCl 1.0 g, Na_2HPO_4 5.75 g and KH_2PO_4 1.0 g.

Bring to volume using distilled water.

Store at room temperature (R.T.).

For phosphate buffered saline plus Tween (PBST) add 2.5 ml of Tween 20 to the 500 ml concentrate stock.

Note: PBST will not store for prolonged periods at R.T.

2.5.2 Bicarbonate buffer (pH 9.6) (Bicarb)

A sodium carbonate - sodium bicarbonate buffer was prepared as described in McKenzie & Dawson (1969). Two separate 0.1M solutions of Na_2CO_3 and $NaHCO_3$ are dissolved in distilled water then appropriate volumes combined according to the desired pH (30ml and 70ml respectively for pH 9.6).

2.5.3 Preparation of B. cinerea antigen extract

Unless otherwise stated the following antigen stock extract was used.
Five millilitres of PBS was poured over a culture of *B. cinerea* grown on PDA for 8 days at 20°C. Isolates used were Pezet, IPO 700 or BC20 (see Section 2.1.1). The culture was agitated gently using a sterile glass hockey stick to suspend conidial and

mycelial material then centrifuged at 12,800 g for 3 min. The supernatant from different tubes was pooled before dividing into 1 ml aliquots for storage at -20°C .

2.5.4 Preparation of tomato plant extract used in immunological experiments

Fresh stems of six week old tomato seedlings (see Section 2.3.2) were harvested and immediately ground in 5-times weight:volume PBS using a mortar and pestle. Unless otherwise stated, PBS was used as the extractant buffer. The suspension was centrifuged at 12,800 g for 3 min and supernatant from different tubes was pooled before dividing into 1 ml aliquots for storage at -20°C.

2.5.5 Indirect-plate-trapped-antigen (PTA)-ELISA protocol using anti-mouse antibody conjugate (PTA-ELISA).

Coat surface of polystyrene wells (ICN Flo, Cat. No. 9502107, Labsystems Oy. Helsinki, Finland.) with 100 µl of the sample diluted in PBS. Incubate 16 h at 4°C.

Invert plate and flick out residue

Wash four times with PBST, flicking out residual solution each time.

Wash once with PBS.

Rinse briefly with distilled water. Dry immediately by gently banging the plate reverse side down on absorbent paper, finish drying by placing plate in sterile laminar air flow hood for 10 min at R.T.

Store sealed at 4°C until ready to use.

Add 100 µl undiluted hybridoma supernatant BC-KH4 to each well. Incubate at R.T. for 1 h.

Wash four times in PBST.

Add 100 µl Goat antimouse IgG +IgM horse radish peroxidase conjugate (Sigma, A 0412, St Louis, MO,USA) diluted 1 µl:1ml PBST and incubate for 1 h at R.T.

Wash four times in PBST.

Add 100 µl of substrate dissolved in buffer. Incubate at R.T. for 30 min for colour development.

Substrate and buffer are prepared as follows:

To 10 ml of the substrate buffer (5 ml 0.2M Na Acetate, 195 µl 0.2M Citric Acid, 5µl 30% H_2O_2 and 5 ml H_2O) add 100 µl of tetra- methylbenzidene (TMB) stock (100mg TMB (Sigma T2885, St Louis, MO USA) dissolved in 10ml DMSO) 100 µl per well), leave 30 min to develop blue colour.

Add 100 µl 3 M Sulphuric Acid to stop reaction, read yellow colour at 450 nm on ELISA reader (Dynatech MR 700).

2.5.6 Indirect-PTA-ELISA using the Biotin/ExtrAvidin detection procedure

Coat surface of polystyrene wells (ICN Flo, Cat. No. 9502107, Labsystems Oy. Helsinki, Finland.) with 100 µl of the sample diluted in PBS. Incubate 16 h at 4°C.

Invert plate and flick out residue

Wash four times with PBST, flicking out residual solution each time.

Wash once with PBS.

Rinse briefly with distilled water. Dry immediately by banging the plate reverse side down on absorbent paper, finish drying by placing the plate in sterile laminar air flow for 10 min at R.T.

Store sealed at 4°C until ready to use.

Add 100 µl of undiluted hybridoma supernatant BC-KH4 incubate at R.T. for 1 h.

Wash four times in PBST.

Add 100 µl goat antimouse whole molecule - Biotin conjugate (Sigma 6649, St Louis MO USA) diluted 1:1000 PBST (unless otherwise stated). Incubate for 1 h at R.T.

Wash four times in PBST.

Add 100 µl ExtrAvidin-Peroxidase (Sigma E2886, St Louis, MO USA) diluted 1:1000 PBST. Incubate for 1 h at R.T.

Wash four times in PBST.

Add 100 µl working substrate (10 ml substrate buffer + 100 µl TMB/DMSO substrate stock (See Section 8.3.3.5). Incubate for 30 min at R.T. to develop blue colour.

Add 100 µl 3 M Sulphuric Acid to stop reaction, read yellow colour at 450 nm on ELISA reader (Dynatech MR 5000).

2.6 Statistics

2.6.1 Introduction

Statistical methods are concerned with defining "real" treatment differences in data obtained from a sample. The selection of the method is dictated by the nature of the data (continuous versus discrete) and the conclusions that are desired. Analysis of variance (ANOVA), a mathematical procedure used to separate total variation into that caused by treatments or experimental variables from the remainder (the residual) was used in this work. There are four conditions that must be fulfilled before this procedure can be applied:

A. Independence.

Each data item in the set must be obtained independently to minimise any unknown linkages or gradients present in the environment that might be superimposed on the parameters (effects) being examined and therefore confound the conclusions obtained. Randomisation within a sample, allocation of treatments to a subject and overall experimental design are methods used to minimise this effect (S. Ganesh, personal communication).

B. Additivity

The assignment of variation to specific effects in ANOVA leads to the question of how these effects relate to one another. It is assumed that the model used for an ANOVA is additive according to the following equation (Mead & Curnow, 1983; S. Ganesh, personal communication).

$$Y_{ij} = \mu + \beta_j + \alpha_i + \varepsilon_{ij}$$

Where: Y=observation, μ= overall or common variance, β= variation from effect i, α= variation from effect j, and ε= random error (residual effect)

C. Homogeneity of variance

ANOVA is based on a constant level of variation within each treatment but departure from this assumption may occur when treatments alter the variability of the response. Data such as count data is particularly prone to departures from this assumption (Mead & Curnow, 1983; S. Ganesh, personal communication). Transforming the raw data can address this issue, using for example, logs, square-roots, reciprocals and arcsins.

D. Normality

The parameter of the population from which the sample data came is assumed to be normally distributed. In situations where this is not fulfilled, the use of transformation or non-parametric methods is recommended (Mead & Curnow, 1983; S. Ganesh, personal communication).

Departures from ANOVA assumptions were detected by programmes submitted to The SAS System (SAS) which included commands to plot residual and predicted variance values after fitting the statistical model to the raw and transformed data. Example SAS command files are detailed in Appendix One for simple experimental designs (Completely Randomised Block (Section A1.1)), complex designs (Nested (Section A1.2)) or combinations of designs (Section A1.3). Trends in the plotted points were used as a measure of the "goodness of model fit" to the data set. Where increasing or decreasing trends appeared in these plots, the data set from which the plot was obtained was appropriately transformed then re-analysed. However, there were some experimental designs, (for example, split plot or nested), where all variation in the data was included in the computer model in order to carry out the analysis. In such cases, a second model was formulated, "partially fitting" the model (Appendix A1.2) to the data which allowed the remaining residuals to be plotted and the need for transformation to be assessed.

2.6.2 General Data analysis

The F-statistic generated by ANOVA (the ratio of between to within sample variation) indicated whether there was at least one pair of treatments was significantly different. This was achieved by comparing the calculated F-statistic with threshold values available in tables. Where a factor was described in ANOVA as significant, the next step was to determine which treatments or levels within that factor were statistically different. Multiple comparison methods (MCP's) have been developed for this purpose and the selection of the correct comparison calculation was determined by the objective of the experiment. Fishers Protected Least Significant Difference (LSD) and Duncans Multiple Range Test (Duncans) control comparison-wise error rate, and Tukey's Honest Significant difference (Tukeys) analysis controls Experiment-wise error rate. Therefore, as a general rule, LSD was used in balanced experiments (those experiments where all treatments are replicated

the same number of times) and where the objective was to compare a single treatment effect with any one other (usually the control). If the experiment was unbalanced (those experiments where not all of the treatments were replicated the same number of times) then Duncan's analysis was used. In remaining experiments where a single treatment was compared with any of the others within the experiment, Tukeys analysis was used. One of the dangers in using MCP is to compare two specific pre-planned treatments, a function beyond the scope covered by MCP's. Ott (1993) described this practice as data dredging or data snooping and such direct comparisons are better addressed using customised comparisons (using "contrast" function in SAS).

2.6.3 Data presentation

Conventions for the presentation of data and statistical results vary. On occasions a data item is graphically presented and includes the treatment Standard Error of the Mean, or a measure for the Standard Error of the Difference. There has been a tendency to use these statistics as measures of the significance of treatment differences rather than a measure of variation. Parameters that describe a population (i.e. mean and standard deviation) are estimated from the sample. It can be shown mathematically and empirically that this estimate forms a distribution (t-distribution) which is similar to the population normal distribution. The computation of treatment Standard Error of the Mean (= $\sqrt{}$ treatment variance/n) does not include a "correction factor" such as $t_{0.05}$. In contrast, LSD (= $t_{0.05}$ x $\sqrt{2}$ x SEM) and confidence intervals do include such a correction term. Also note that with a large number of observations (>30) the t-distribution and the Normal distribution provide almost identical correction factors.

2.6.4 Statistical tests used in this thesis

Most of the data presented in this thesis work was analysed by ANOVA using The SAS System, (versions 6.10 in batch mode or the 6.12 PC SAS) using general linear modelling. Experimental design was chosen before carrying out the work so that a model appropriate to the conclusions desired and account for anticipated assumptions for ANOVA could be used. Generally the models used were the completely randomised block or completely randomised designs and are stated in

the materials and methods sections of the relevant experiments. Other, experimental models used included nested and split plot and repeated measures. Treatment effects were treated as fixed and modelled accordingly. In addition, some data sets in this thesis were incomplete and required "unbalanced" experimental data analysis.

Statistical analyses were carried out in a systematic manner in this thesis. After any data manipulation for treatment order or changing units, the model appropriate to the experiment design was applied to the data. The same model was also applied to transformed data using square root, $\log_{10}$, exponential functions and arcsin. In this study $\log_{10}$ or square root functions were sufficient to modify the data to comply with ANOVA assumptions. Second tier analyses of the data involved the use of MCP's, contrasts or regression.

There are several experiments presented in Chapter Eight (Development of an ELISA-based immunoassay) where ANOVA was not used to determine treatment differences. Instead, significant treatment differences were recognised by magnitudes of difference between ELISA absorbances. A measure of the spread in the data was provided by the 5% margin of error (MOE), calculated from 95% confidence interval calculations.

Development of the "Attachment Assay"

3.1 Introduction

3.1.1 The isolation of bacteria or yeasts colonising fungi

Baiting techniques have predominantly been used for obtaining mycolytic or attacher bacteria with mixed success. Mitchell & Alexander (1961, 1963) and Mitchell & Hurwitz (1965) used a modification of the method of Mueller & Durrell (1957) who developed a selective agar from macerated fungal cultures. Old & Patrick (1976) used a sandwich technique enclosing fungal propagules in filter membranes of various pore sizes to exclude all but bacteria sized organisms. These packets were then buried in the soil. Similar techniques were adopted by Homma (1984), Fradkin & Patrick (1985*a,b,c*) and Toyota & Kimura (1993). Clough & Patrick (1976) used nylon gauze in place of the filter membrane while Malajczuk, Nesbitt & Glenn (1977) and Nesbitt, Malajczuk & Glenn (1981) submerged agar disks pre-colonised with the target fungus in water extracts containing candidate microbes. Incubation times in which the pathogen bait remained in the various substrates ranged from 1 day (Toyota & Kimura, 1993) to 100 days (Wong & Old, 1974). Seemingly unrelated methods have also yielded fungal colonists where isolates of *Pseudomonas* sp., (Rovira & Campbell, 1975) and *Enterobacter cloacae* (Hadar, Harman, Taylor & Norton, 1983; Nelson *et al.*, 1986; Wilson, Franklin & Pusey, 1987) were identified using *in vitro or in vivo* screening methods for BCAs.

Attempts to retrieve the colonists from the fungal structure have relied on washing the baited fungus in SDW or saline (Fradkin & Patrick, 1985*a,c*), sonicating the colonised fungus (Toyota & Kimura, 1993) or immersion in nutrient solution followed by repeated subculturing to obtain pure isolates (Homma, 1984). However few of these were an efficient method for the extraction of fungal pathogen colonisers. None of the isolates selected by Nesbitt *et al.* (1981) and few isolates gathered by Toyota & Kimura (1993) colonised the respective fungal pathogen targets *in vitro.*

In these examples, the limited harvest of microbes may reflect limitations in the methods used. It is conceivable that the prolonged incubation times could have allowed contaminant microbes (those that do not colonise fungal bait) to adhere to the support materials used to contain or grow the bait. Colonisation of the agar on which the fungal

target was cultured by these microbes could be responsible for the absence of attacher bacteria isolated by Nesbitt *et al.* (1981) and despite Toyota & Kimura (1993) washing the colonised bait prior to isolating the mycolytic bacteria, the 24 h incubation in the manure may have been sufficient for firm adhesion of bacteria to the nylon gauze thus washing did not remove them. Initial processes responsible for contamination can be explained in literature relevant to microbial adhesion to inert surfaces.

3.1.2 Reversible microbial adhesion to inert macrosurfaces

Early research (Marshall, Stout & Mitchell, 1971; Marshall, 1980) recognised two distinct phases or classes; an instantaneous reversible phase and a time dependent irreversible phase (Marshall & Bitton, 1980) where the terms "reversible" and "irreversible" pertain to the ability of the researcher to reverse the adhesion process.

Instantaneous, reversible, non-specific attachment may involve a number of classes of interactions (ionic, dipolar, H-bonds and hydrophobic) to position the microbe at or near another surface. A more comprehensive list of chemical and non-chemical bonds for both attractive and repulsive forces was detailed by Daniels (1980). The importance of each of these classes vary with specific environment, bacterial species and the nature of the surface. In addition, Fletcher (1977, 1980) and Rutter & Vincent (1980) suggested that attachment may also be influenced by bacterial cell surface molecules and shape that change both with time and during the attachment process and cell motility that alter the force and frequency that cells impact on a surface.

Initial attachment processes of cells larger than bacteria, eg. yeast and fungal propagules have received less attention in the literature. Jones (1994) argued that initial processes like reversible attachment using passive mechanisms is unlikely to be as important for fungal propagules as their size excludes them from colloidal behaviour but other research has suggested otherwise. The initial adhesion process of *Uromyces vicia fabae* (Clement, Butt & Beckett, 1993; Clement, Porter, Butt & Beckett, 1994) and *B. cinerea* (Doss, Potter, Chastagner & Christian, 1993; Doss *et al.,* 1995) were shown to be mediated by hydrophobic interactions. Van Haecht, Bremaeker & Rouxhet (1984) and Douglas (1987) considered the passive mechanism model used for bacterial attachment was sufficient to explain yeast attachment.

The term "reversible attachment" immediately implies that conditions within physiological limits for minimising and maximising cell adherence have been

identified. The literature has shown that attachment is influenced by cell-related factors, surface factors and environment factors. Increasing cell number and time of exposure directly determines the number of collisions between cells and substrate which increase the probability of attachments forming (Fletcher, 1977; Gabriel Piette & Idziak, 1992). Bacterial species (McEldowney & Fletcher, 1986), cell age and the growth medium (Fletcher, 1977; McEldowney & Fletcher, 1986) appear to influence cell motility and the quantity and quality of surface molecules that affect reversible attachment. The hydrophobicity and surface charge of the surface itself vary widely and influence cell attachment (Fletcher & Loeb, 1979; Daniels, 1980). The environment in which the attachment interaction is taking place is another area studied where increasing temperature (Fletcher, 1977) increases cell motility and the frequency of collisions between cell and surface. Remaining parameters, pH (Nesbitt, Doyle & Taylor, 1982; McEldowney & Fletcher, 1986), presence of salts, buffers (Nesbitt *et al.,* 1982; Smith & Wollum, 1993), alcohols (Fletcher, 1983) or specific cations (Gabriel Piette & Idziak, 1992) do not exhibit predictable patterns.

There appears to be prima facie evidence that cell attachment leading to cell colonisation of the inert material used to contain the fungal baits is responsible for the high number of contaminant micro-organisms. There is no evidence in the literature on the isolation of mycolytic, attacher or coloniser bacteria that these processes were anticipated.

In the current study, attempts to bait attacher microbes by immersing *B. cinerea* colonised agar plugs into bacterial and yeast suspensions were unsuccessful. As a result, design emphasis shifted to a column wash system similar to a sand column assay used to detect attacher bacteria and remove unattached bacteria from sand particles (DeFlaun *et al.,* 1990).

3.1.3 The principle of the assay design

Candidate microbes were washed from the sample source (Fig. 3.1a) and were co-incubated with germinating *B. cinerea* spores (Fig. 3.1b). Unattached cells were removed by sieving the pathogen mycelium on a fine nylon mesh (Fig. 3.1c) then the mycelium with microbes attached was placed into liquid medium where reproduction by yeast and bacterial cells released progeny into the broth (Fig. 3.1d).

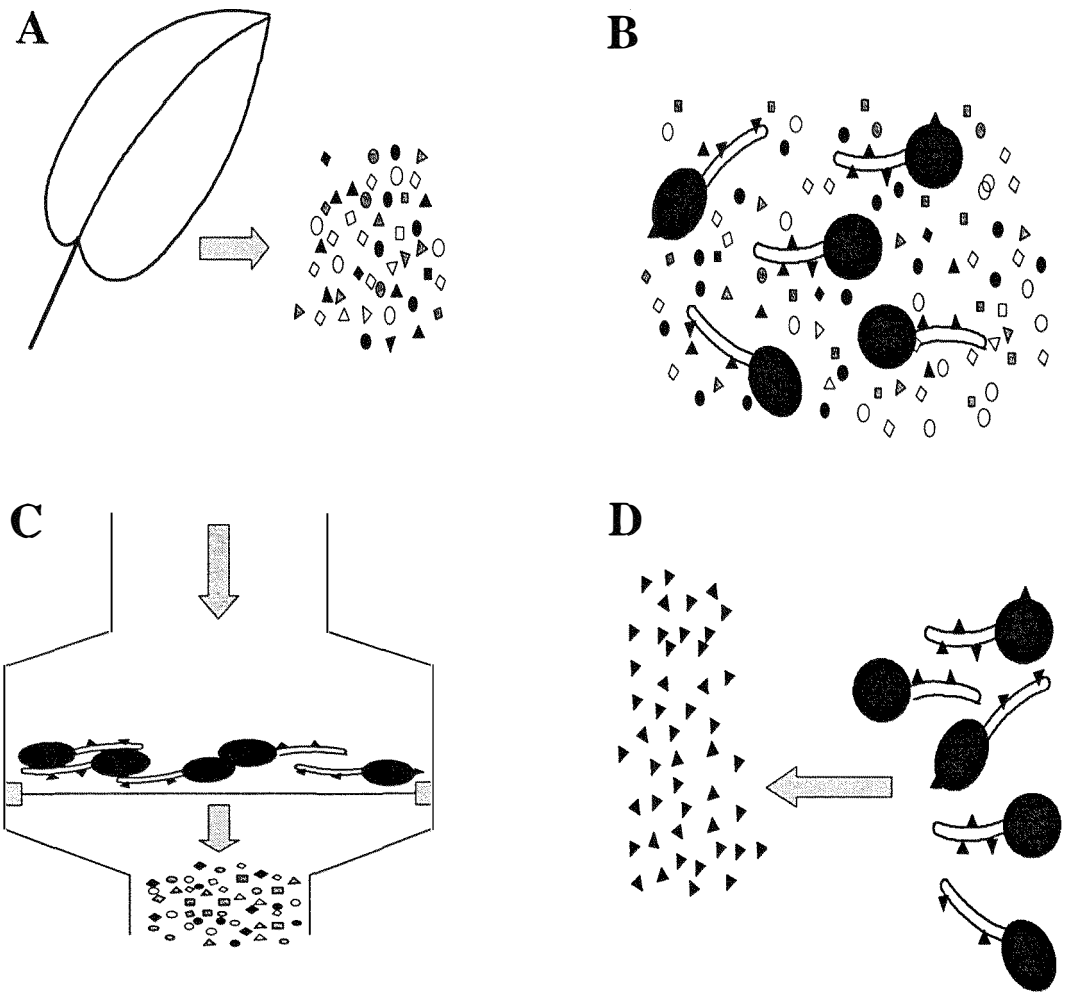


Fig. 3.1. Principle of the column based assay design. a) Leaves are washed, b) co-incubated with *B. cinerea*, c) unattached microbes are removed and d) attached microbes are isolated from *B. cinerea* .

3.2 Objectives

To efficiently separate bacteria and yeasts with the ability to attach to *B. cinerea* hyphae and conidia from those that do not. In the first phase of this work, the general assay design was developed and in the second phase, emphasis was placed on minimising contamination by live cells (colony forming units (cfu's)) on the inert support material used in the final assay design.

3.3 General note - Statistical Analysis

Data from experiments in this current study was transformed by $\log_{10}$ so that assumptions associated with ANOVA could be fulfilled. Hence apparatus contamination is expressed as "Wash efficiency" and calculated as:

$$Wash\ Efficiency(W.E.) = abs\ (\log 10 \frac{Number\ of\ colonies\ counted\ on\ each\ culture \times 8}{Total\ number\ of\ cells\ applied})$$

The response variable (W.E.) was fitted to statistical models in all experiments except one and two in Phase I of development. Secondary analyses were carried out as described in Chapter Two using either Tukeys MCP and contrasts or least-squares-means for balanced and unbalanced data respectively. Changes in microbe behaviour over time was analysed using a split plot experimental design whereas completely randomised block design was used for all of the remaining experiments. Each test isolate was analysed separately.

3.4 Phase I - Development of the attachment apparatus

3.4.1 Experiment One - Retention assessment

3.4.1.A Introduction

The first step in the assay development process was to create a measure for the efficacy of any wash procedure.

3.4.1.B Materials and Methods

A 13 mm disk cut from nylon mesh of pore size 20 µm (Swiss Silk Bolting Cloth Manufacturing Co. Ltd., Zurich) was immersed in a 1 ml suspension of *Debaromyces hansenii* cells (See Chapter Two (Section 2.2.2)), removed and placed into a 13 mm diameter filter holder (Sartorius 16514E). The mesh was washed by passing 50 ml SDW through the holder before being transferred into a sterile universal bottle reinforced with cellulose adhesive tape and containing 2 ml SDW and a 15 mm diameter glass marble. The bottle, marble and mesh were vortexed for six short bursts totalling 15 s. A 500 µl aliquot was pipetted directly onto NYDA (pH 5.4) and a second 500 µl aliquot was diluted 20-fold in SDW before plating onto agar media. Plates were incubated for 3 days at 15°C in total darkness before the colonies were counted.

This wash sequence was repeated three times for the same nylon mesh disk and three replicate disks were used.

3.4.1.C Results

The method of vortexing the SDW with a glass marble in a universal bottle was highly effective in removing cells from nylon mesh (Table 3.1).

Table 3.1. Mean number of *D. hansenii* cells washed from nylon mesh disks in 2 ml SDW, with a 15 mm diameter glass marble vortexed six times totalling 15 s.

	Wash #		
Wash number in 2 ml SDW	1	2	3
Mean number of *D. hansenii* cfu's washed from mesh	3966	7	4
LSD = 1314.9 (n=4, df=9, α=0.05)			

3.4.2 Experiment Two - The Prototype wash assay

3.4.2.A Materials and Methods

A prototype apparatus consisting of a vertical glass tube of 10 mm diameter and a polyethylene syringe barrel was connected to a 25 mm diameter filter holder (Sartorius SM 16517 E) via the luer lock. Within the filter holder a 25mm disk cut from a sheet of nylon mesh was positioned. The syringe tube/filter holder assembly (the mesh holder) was contaminated by pouring 5 ml of a *D. hansenii* cell suspension into it then immediately drained. The mesh holder was reconnected to the glass tube before 50 ml of SDW was poured through the apparatus. After washing, the nylon mesh disk was removed from the holder and replaced. This cycle of contamination, washing and replacing the nylon mesh disk was repeated six times and duplicated in a second identical apparatus with a reduced wash volume of 25 ml SDW. Mesh contamination after each cycle was assessed as described in Section 3.4.1.

3.4.2.B Results

Repeated use of the prototype apparatus resulted in a decline in the number of *D. hansenii* cfu's detected on the mesh after the first three contamination and wash cycles, then levels remained constant (Fig. 3.2). A similar pattern was observed in the duplicate apparatus where 25 ml SDW wash volume was used.

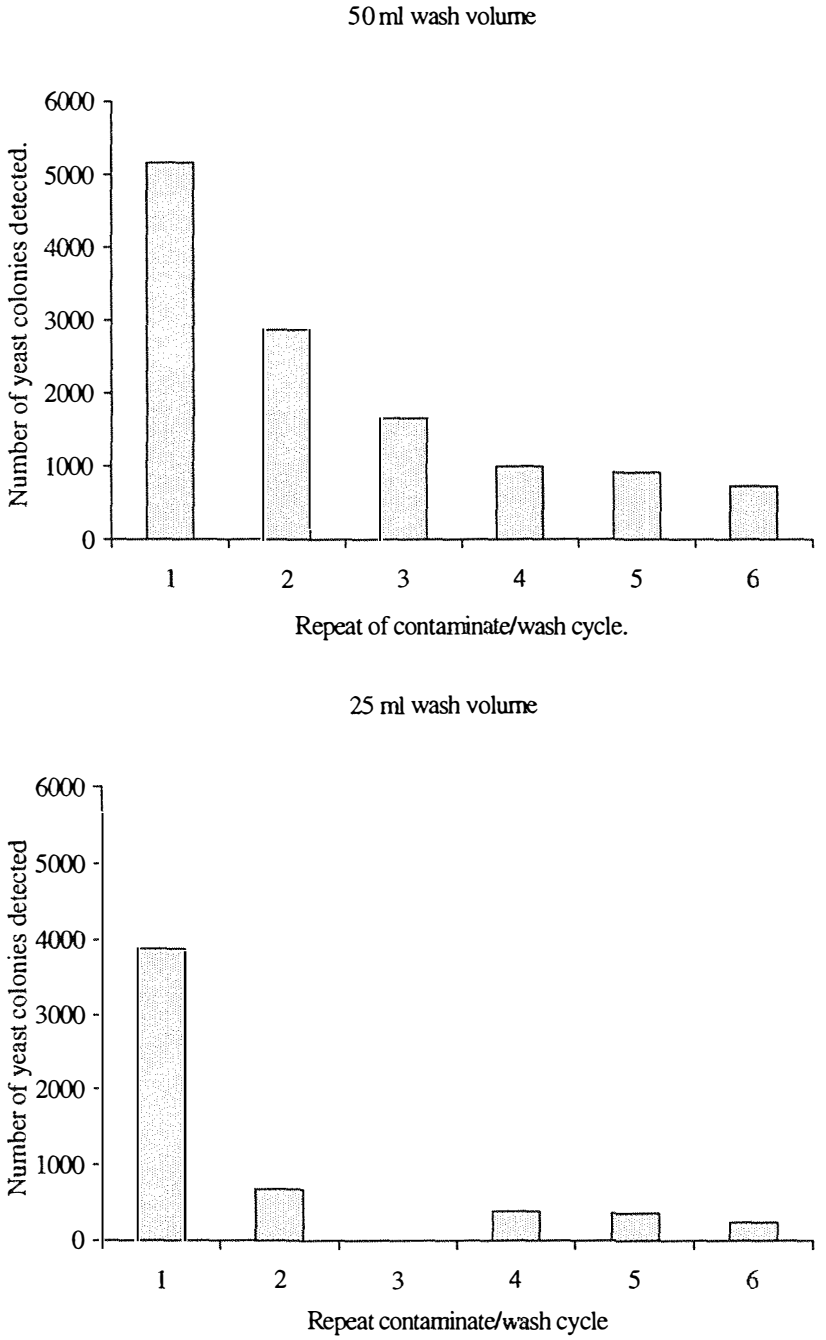


Fig. 3.2. *D. hansenii* contamination of the nylon mesh after cells were applied then the apparatus was washed with either 50 ml or 25 ml SDW. Repeat experiments were conducted on the same columns with new batches of nylon mesh.

3.4.3 Experiment Three - Modifications to the prototype assay

3.4.3.A Introduction

The prototype apparatus described in Section 3.4.2., was modified extensively during this phase of research. Observation of liquid flow patterns in the prototype identified the need to retain the 25 mm diameter filter holder and widen the entry into the holder to 10 mm. Experiments were conducted to examine the effect of additional changes to holder and protocol to further reduce cell contamination on the nylon mesh.

3.4.3.B Materials and Methods

Experiment A

The prototype mesh holder (A) and protocol were modified by the following treatments:

(a) pre-conditioning - the passage of three-volumes of the wash liquid through the entire apparatus before contamination

(b) removing the silicon 'O'-ring gasket and filter supports from inside the filter holder

(c) Adding a 5 ml wash step to the existing 50 ml. This new wash step was carried out after contamination by adding the wash liquid to the holder assembly, rotating four times then draining. The 50 ml wash volume was then applied as per the original protocol.

Experiment B

The original prototype (A) was compared to a second which incorporated all three treatment changes (B) in Experiment A. An alternative wash regime of two 25 ml washes using the second prototype was also compared. Treatment replication was increased from three to nine in this experiment to better resolve treatment effects.

Experiment C

In this experiment apparatus prototype B was compared to an identical apparatus but the second wash volume was increased from 50 ml to 70 ml while the first volume remained at 5 ml in both treatments.

Experiment D

In this experiment, three wash liquids were compared, SDW, 0.1% Tween 20 and di-sodium hydrogen orthophosphate/sodium dihydrogen orthophosphate buffer at pH 7.0 (prepared according to McKenzie & Dawson, 1969, p 489) using Prototype B procedure.

Experiment E

In this experiment, the wash procedure of 5 ml followed by 50 ml of 0.1% Tween 20 using prototype B was compared with the entire wash protocol repeated twice for a single sample. In order to repeat the wash procedure for the removal of contaminant microbes, it was necessary to anticipate the inclusion of *B. cinerea* mycelium in the system. In a full screening situation, the pathogen would be resting on the nylon mesh, which would be resuspended in SDW, then returned to the apparatus before recommencing washing. Therefore, between washes, the nylon mesh was removed from the apparatus, vortexed in 20 ml SDW in a universal bottle to simulate the resuspension of any *B. cinerea* mycelium on the mesh surface. The mesh was repositioned in the holder and the 20 ml SDW was poured through the syringe barrel/holder assembly to recontaminate the mesh with microbes removed.

3.4.3.C Results

Experiment A

Each of these treatments (a - c) significantly (P=0.001, 0.004 and 0.008 respectively) reduced the number of *D. hansenii* cfu's detected on the nylon mesh when compared with the un-modified prototype (Table 3.2).

Experiment B

In the second apparatus (Prototype B) combining all the improvements significantly increased ($P<0.0001$) Wash Efficiency (W.E.) 2.9 to 3.5 compared with Prototype A. The number of cfu's detected after using a wash regime of two 25 ml washes did not differ significantly ($P>0.05$) from the Prototype A procedure (Table 3.2).

Experiment C

There was no significant increase (P>0.05) in *D. hansenii* cfu removal from the nylon mesh by increasing the second wash volume from 50 ml to 70 ml using the prototype B apparatus (Table 3.2).

Experiment D

The addition of Tween 20 significantly increased (P=0.0004) W.E. from 3.9 to 4.2 from the SDW treatment but there was no significant difference (P>0.05) in using phosphate buffer (pH 7.0).

Experiment E

There was some evidence for a significant difference (P=0.03) between the two treatments (Table 3.2) in cfu detected on the mesh. A single replicate contained an outlier value that appeared responsible for the insignificant treatment effect thus washing the nylon mesh twice was included in the final protocol. Further increases in the number of washes for each mesh was not investigated as this procedure was already time consuming and would limit the number of samples processed with little potential gain in wash efficiency (W.E.)

Table 3.2. Summary of experiments (A to E) examining modifications to wash protocols and mesh holder for the removal of *D. hansenii* cfu's from nylon mesh. 1= Populations approximated, W.E.= Wash efficiency (see section 3.4.9) 2= Lsmeans (data was incomplete). Statistical analysis performed on $\log_{10}$ transformed data using customised comparisons (Contrasts) and Tukey multiple comparison test (α=0.05, df as indicated).

Mesh holder and protocol modifications	W.E.	Tukey multiple comparison test	Customised comparisons (ANOVA)	df	Mean number of cfu's on the mesh
Experiment (A) Prototype A - holder entry enlarged to 10 mm - holder diameter 25 mm - filter supports removed.	3.1	a	-	5	33,000[1]
(a) Pre-conditioning the holder and glass column	4.1	b	** (P=0.004)[2]	5	3288
(b) Silicon 'O'-ring gasket removed	4.4	b	** (P=0.001)[2]	5	1916
(c) Wash volume 5 ml added to usual 50 ml	3.9	b	** (P=0.008)[2]	5	5480
Experiment (B) Above improvements (a - c) combined =Prototype B					
(a) Prototype A	2.9	a	-	16	53,000[1]
(b) Prototype B	3.5	b	*** (P<0.0001)	16	14,000[1]
(c) Wash volumes changed to 25 ml + 25 ml	3.3	b	* (P=0.05)	16	23,000[1]
Experiment (C)					
(a) Prototype B	3.8	a	-	10	7569
(b) Wash volume changed to 70 ml	3.7	a	ns (p>0.05)	10	9417
Experiment (D) Comparison of wash liquids using Prototype B					
(a) SDW	3.9	a	-	10	5128
(b) Phosphate buffer (pH=7.0)	3.7	ab	* (P=0.02)	10	8317
(c) 0.1% Tween 20 solution	4.1	b	*** (P=0.0004)	10	3336
Experiment (E) Number of wash cycles					
(a) Once	4.4	a	-	9	1805
(b) Twice	4.7	a	* (P=0.03)	9	841

3.4.4 Summary

The final design of the mesh holder used in remaining experiments is illustrated in Fig. 3.3.

The "Mesh Holder" consisted of a polycarbonate filter holder (Sartorius SM 16517E) modified by removing all filter supports and the silicon gasket, cutting a 10 mm hole in the lid and inserting a 10 mm diameter polyethylene tube. Twenty five millimetre diameter disks of nylon were positioned as illustrated (Fig. 3.3).

The Mesh Holder was located on the end of a 10 mm diameter vertical glass tube that contained 50 ml of the wash liquid which flowed by gravity through the Holder (Fig. 3.4).

The basic wash procedure for removing unattached microbes from the nylon mesh started with "pre-conditioning " by pouring 50 ml of the wash liquid through the assembled apparatus, collecting the effluent and repeating the process three times. The Mesh Holder was removed from the glass tube and loaded with 5 ml of the microbe suspension to contaminate the nylon mesh then was immediately drained. Five millilitres of the wash liquid used in the pre conditioning was poured into the Holder, it was inverted four times, then drained. On re-assembling the apparatus, the remaining 45 ml was poured into the column then drained through the Holder. Residual wash liquid was flicked out of the Holder then the mesh was asceptically removed and placed in 10 ml of SDW in a Universal Bottle and vortexed in six short bursts over 15 sec. The same mesh was repositioned in the Holder and the SDW suspension from the universal bottle poured into the holder and immediately drained. Another 50 ml of the wash liquid was used to perform a second washing of the nylon mesh using the same 5 ml and 45 ml wash procedures.

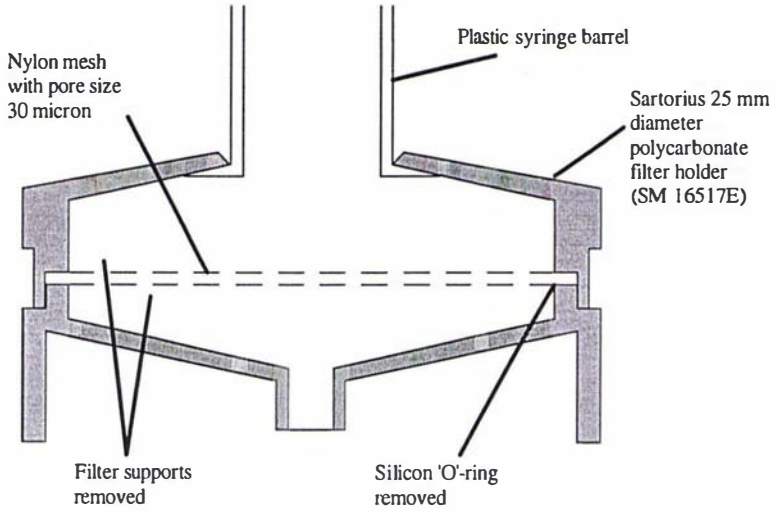


Fig. 3.3. Sectioned view of mesh holder, part of the wash apparatus.

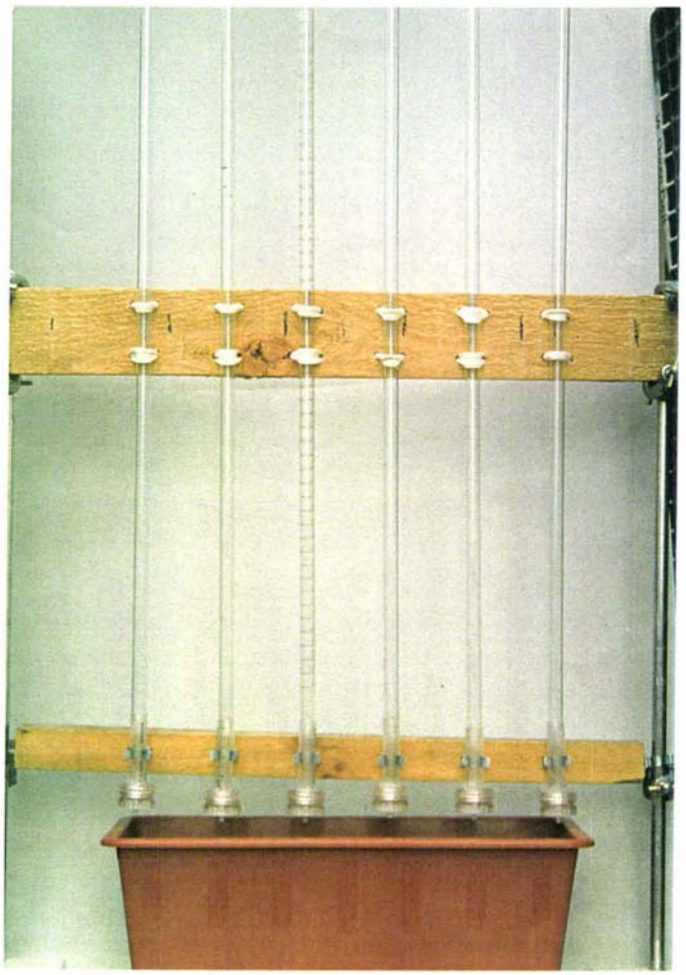


Fig. 3.4. Fully assembled wash apparatus with a frame to hold six fully assembled columns and drain to catch waste.

3.5 Phase II - Minimising apparatus contamination

3.5.1 Materials and Methods

3.5.1.A Micro-organisms and cultural conditions

Yeast isolates *Debaromyces hansenii* (isolate 211) and *Cryptococcus laurentii* (isolate 255), both attach to *B. cinerea* (C. Wilson, personal communication) and were obtained courtesy of Drs M. Wisniewski and C. Wilson ARS, USDA, Kearneysville, W.V., USA for use as standard yeasts. Bacterial isolates which attach to *B. cinerea* were not available at the time so an isolate of *Pseudomonas* sp. and of *Bacillus megaterium* were obtained from the culture collection of the Department of Plant Science, Massey University for use as standard bacteria for assay development. Yeasts and bacteria were cultured on NYDA and on NA respectively, at 15°C for 5 days with 12 h D/N. Each culture was suspended in SDW, centrifuged at 13,300 g for 5 min and the resulting pellet was resuspended in SDW.

Total cell counts of the suspensions were determined with a Pettroff Hauser counting chamber observing unstained cells with dark field microscopy. Suspensions were adjusted to 1 x 10^7 cells / ml and starved by storing the suspensions at 4°C overnight (16 h).

Since the purpose of this apparatus is to remove "field grown isolates" from the nylon mesh, mixtures of microbes obtained directly from the field were used in the final experiment described in this chapter rather than those from cultures. A mixture of microbes of unidentified species were obtained by gently washing four fully expanded leaves of Maple (*Acer* sp.) in 20 ml SDW and agitating vigorously for 2 min in a 150 mm x 100 mm plastic bag. After filtration through a 0.2 µm filter (Sartorius SM 113 07 025 N) the residue was resuspended in 10 ml SDW. Total cell counts were taken and the suspension was adjusted to 5x10^7 cells/ml. A 100 µl aliquot was pipetted onto three replicate plates of NA to estimate cfu in the suspensions. This value was used in the calculation of "Wash Efficiency" (Section 3.3).

3.5.1.B Changes in attachment behaviour of test cells with time

Four replicate suspensions of each test yeast and bacteria (*D. hansenii, C. laurentii*, *Pseudomonas* sp. and *B.megaterium*) (Chapter Two) and four mesh holders were prepared as described in Section 3.4.4. Changes in mesh contaminant behaviour were examined from suspensions in SDW stored static at 4°C and sampled at 0, 8, 16, and 24 h.

At each sample interval a 5 ml aliquot was pipetted from the first replicate of each test microbe suspension and placed into the first mesh holder. Microbial contamination on the mesh was assessed as described in Section 3.4.1. The same procedure was repeated for each of the replicate suspensions which were placed into the corresponding mesh holder. The experiment was carried out, and data analysed according to the split plot experimental design, with test microbe being the main plot and time as the split plot.

3.5.1.C Investigation into possible electrostatic behaviour

To estimate any electrostatic activity between cells of the test microbes and the nylon mesh, disks of nylon mesh were soaked in a 1.8 mM solution of $Fe(NO_3)_3$ for 16 h, rinsed in five changes of SDW then dried in sterile laminar air flow. Contaminant bacterial or yeast removal from both iron treated and iron untreated nylon meshes was carried out using the basic wash protocol (Section 3.4.4) and the nylon mesh assessed as described in Section 3.4.1. Two wash liquids were compared, SDW and sterile 0.1% Tween 20. Cells of the test microbe were starved by standing suspensions for 16 h at 4°C before pipetting a 5 ml aliquot into the mesh holder. There were four replicates of each treatment combination. The experiment and data analysis was carried out according to a completely randomised block design.

3.5.1.D Effect of wash solution pH and buffer

McIlvaines buffer (a Citric acid -Phosphate based buffer) was prepared at pH, 4.0, 5.0, 6.0, 7.0 and 7.6. according to the procedure of McKenzie & Dawson (1969). Tween 20 was added to give a final concentration of 0.1% (v/v). Wash procedure for each pH buffer is as described above with four replicate washings for each buffer. Suspensions of the four test microbes, (*D. hansenii*, *C. laurentii*, *Pseudomonas* sp. and *B. megaterium*), wash procedure for each pH buffer and assessment of mesh contamination were carried out with one procedural change

from that described in Section 3.4.1: di-sodium hydrogen orthophosphate/ sodium dihydrogen orthophosphate buffer pH 7.0 (McKenzie & Dawson, 1969) was used to suspend contaminants from the mesh to overcome the problem of residual buffer on the mesh affecting the pH of the suspension.

3.5.1.E Phylloplane washings with *B. cinerea* added

In this experiment, microbes obtained from phylloplane washings were used to simulate the designed purpose of the assay and apparatus. Variable number of *B. cinerea* germlings were added to examine the effect of impeded flows on WE.
A total of 3 x 10^7 conidia of *B. cinerea* were applied to the surface of washed cellophane that was overlaying 3% MEA then incubated for 12 h at 15°C. The germlings (30 to 40 µm long) were exposed to propylene oxide vapours for 30 min before venting in sterile laminar flow for an additional 30 min. Thirty millilitres of SDW was added to suspend the germlings then shaken vigorously to break up clumps of hyphae.

One millilitre aliquot of the phylloplane washing and 0, 1, 2, 3 ml of a suspension of *B. cinerea* germlings were pipetted into each Holder. Sterile Distilled Water was added to maintain the total volume in the Holder at 5 ml. This mixture was immediately drained and washed with McIlvaines buffer pH 6.0 plus 0.1% Tween 20 using the basic wash protocol already described (Section 3.4.4). Mesh contamination was assessed (Section 3.4.1) and aliquots spread on NA plates. This experiment and data analysis was carried out according to a completely randomised block design.

3.5.2 Results

3.5.2.A Changes in contaminant behaviour with time

Changes in the way bacteria and yeast cells interacted with the nylon mesh were examined as the cells were incubated in SDW suspension over a 24 h period. Significant differences in wash efficiency over time (P=0.03), with isolate (P=0.0001) and the interaction (P=0.02) were found (Fig. 3.5).

Mesh contamination by *B. megaterium* increased steadily between the first sample at time zero to the third sample (16 h) before declining. The number of *Pseudomonas* sp. cells detected increased in the second sample after 8 h then declined steadily in subsequent samples. Both yeasts showed very different behaviours. The number of *C. laurentii* cells detected on the nylon mesh increased throughout the duration of the experiment whereas that of *D hansenii* declined steadily. There were no significant differences between samples of either bacteria or of *C laurentii* (P>0.05) using customised comparisons in ANOVA but when *D hansenii* was suspended in SDW for more than 16 h there was moderate evidence in the data for a significant decline (P=0.05 (16 h) and P=0.04 (24 h)) in wash efficiency.

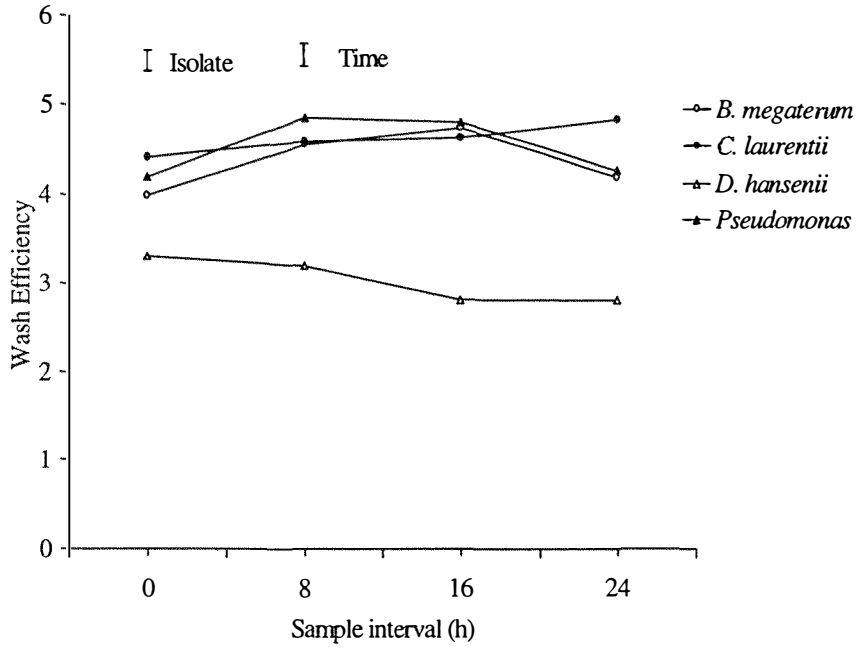


Fig. 3.5. Changes in contamination of the nylon mesh by *B. megaterium, C. laurentii, D. hansenii* and *Pseudomonas* sp. suspended in SDW. (Vertical bars represent LSD (α=0.05) for time (df=36) and isolate (df=9)

3.5.2.B Electrostatic behaviour

Treatment effects of the ferric ions were inconsistent among the four test isolates. (Table 3.3). A comparison of iron treated and untreated mesh, after washing with SDW, showed that significantly more cells (i.e. a lower W.E.) of *D. hansenii* ($P<0.05$) and *Pseudomonas* sp. ($P<0.01$) were recovered from treated than from untreated nylon mesh. More *B. megaterium* cells were also retained on treated mesh, but the difference was not statistically significant ($P=0.45$). There was no treatment effect on *C. laurentii*. The corresponding comparison using Tween 20 as the wash liquid, gave only one significant ($P<0.05$) difference: more *C. laurentii* cfu were recovered from iron treated than untreated mesh.

Effects of the wash liquid treatments (SDW and 0.1% Tween 20) were similarly variable. On untreated mesh, removal of *B. megaterium* cells was significantly improved ($P<0.01$) by using Tween 20 compared to SDW. There was a smaller but non significant ($P>0.05$) increase for *C. laurentii*. Wash efficiency (W.E.) did not significantly differ ($P>0.05$) for *D. hansenii* or *Pseudomonas* sp. ($P>0.5$) with wash liquid.

By washing the iron-treated nylon mesh with Tween 20, there were significant differences in cfu after Tween washing compared to SDW for *B. megaterium* ($P<0.005$) and *D. hansenii* ($P<0.05$) but not significant for *Pseudomonas* sp. ($P=0.08$). There was an insignificant increase in cfu with Tween 20 washing compared to SDW for *C. laurentii* ($P=0.09$).

Table 3.3. The wash efficiency of SDW or 0.1% Tween 20 for the removal of test microbes from nylon mesh pre-treated and not pre-treated with $Fe(NO_3)_3$. LSD values (df=6, n=4, α=0.05)

Isolate	Treatments				LSD
	SDW	SDWFe	Tw20	Tw20Fe	
Bacillus megaterium	3.62	3.53	4.02	4.17	0.28
Cryptococcus laurentii	4.37	4.39	4.54	3.97	0.58
Debaromyces hansenii	3.42	3.21	3.39	3.46	0.47
Pseudomonas sp.	5.03	3.89	4.60	4.28	1.14

3.5.2.C Effect of pH and Wash Buffer

All McIlvaine buffer / Tween 20 combinations significantly improved (P<0.0001) the removal of *D. hansenii* cells from the nylon mesh compared to Tween 20 in SDW. (Table 3.4.). Similarly, significantly more cells of *B. megaterum* were removed from the mesh by using buffer at pH 6.0 (P<0.01) or pH 7.0 (P<0.01) and for *Pseudomonas* sp. at pH 4.0, 7.0 and 7.6 (P<0.01). There were no significant differences between the pH buffers and SDW control for *C. laurentii.*

Table 3.4. The removal of bacteria and yeasts from nylon mesh using McIlvaines buffer at pH 4.0 to 7.6. LSD (df=10, n=4, α=0.05)

Isolate	Wash solution						LSD
	4.0	5.0	6.0	7.0	7.6	Tw	
Bacillus megaterium	3.60	4.04	4.11	4.17	3.97	3.81	0.24
Cryptococcus laurentii	3.48	3.86	4.06	3.86	3.73	3.99	0.90
Debaromyces hansenii	4.42	4.64	4.76	4.53	4.56	3.01	0.30
Pseudomonas sp.	3.42	3.31	3.14	3.49	3.77	2.42	0.90

3.5.2.D Removal of phylloplane washings

The data collected and analysed suggested no evidence for a significant difference between control (without *B. cinerea*) and treatments with increasing pathogen germling loads up to 3 x 10^6 germlings / mesh. Values for W.E. were between 1.2 and 2 (Fig. 3.6). When these values are back-transformed, contamination of the nylon mesh is approximately 1-2 cfu's per 100 viable cells added. From the phylloplane samples used in this experiment a maximum of 25 cfu's were detected as contaminants on the washed mesh with 3 x 10^6 germlings of *B. cinerea* added to its surface.

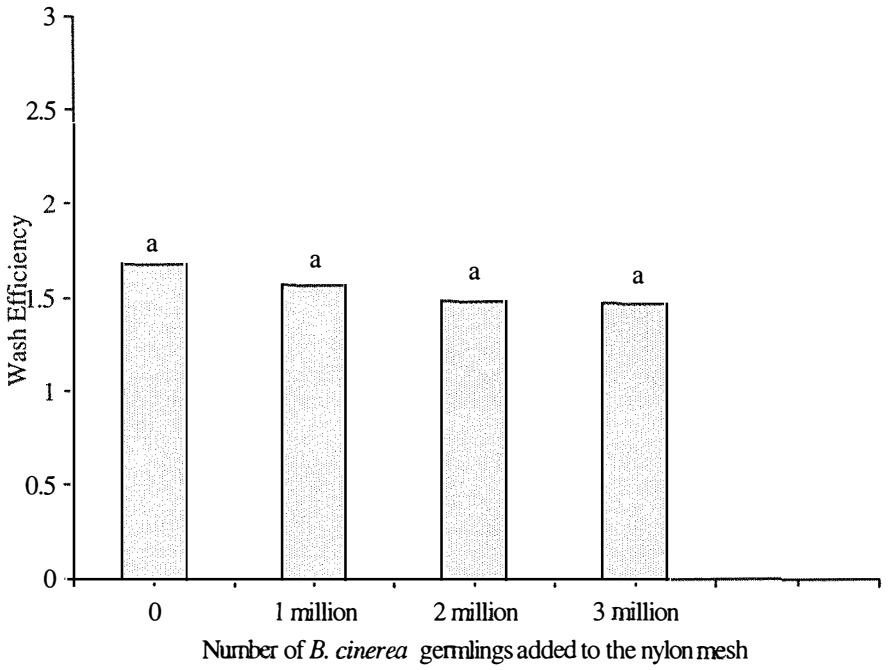


Fig. 3.6. Removal of cfu's (obtained from phylloplane washings of *Acer* sp.) from the nylon mesh with different numbers of *B. cinerea* germlings added to the surface. Different letters above each treatment represent significant differences (α=0.05) according to Duncans test (df=8).

3.6 Discussion

In most previous attempts to retrieve mycolytic bacteria from baited fungi relatively few attacher bacteria were isolated using these methods possibly because contaminant bacteria were colonists of the inert support material and were not removed by the wash steps (Toyota & Kimura, 1993). A fundamental change from earlier work was to reduce this exposure time of *B. cinerea* conidia and candidate attachers to the mesh to approximately 15 s by germinating the pathogen in phylloplane or soil extracts then sieving this suspension using nylon mesh. Reversible attachment has been described as instantaneous, but this appears to be a relative term used to distinguish the time taken until the irreversible attachment process begins. "Sedimentation periods" from 30 min (Morisaki, 1991), 2-5 h (Kjelleberg, Humphery & Marshall, 1983), 1.5 h (Nesbitt *et al.,* 1982), 2 h (Fletcher, 1983), 1 h (McEldowney & Fletcher, 1986) for bacteria and 24 h for yeasts (Van Haecht *et al.,* 1984) have been used in studies of irreversible adhesion of microbes to surfaces. Very brief exposure times such as those in this study, could have considerably reduced the contamination potential of the mesh but it was still unacceptably high (approximately 33,000 cfu's/mesh).

Initial experiments in the development of the assay achieved significant reductions in the number of *D. hansenii* cfu retained on the nylon mesh after washing. The removal of internal surfaces within the original filter holder eg. silicon 'O'-ring gaskets and filter supports reduced the potential for cell entrapment within sites created by these parts. Entrapment appears to be a major contributor to high cell retention in early experiments since a ten-fold reduction in cfu's was detected after removing these parts (Table 3.2). The practice of pre-conditioning the column and holder prior to contamination is not recorded in the literature and owes its origins to the unusual pattern of mesh contamination shown in Fig. 3.2. where contaminants declined with successive washes despite cycles of recontamination. This suggests that factors responsible for cell retention were removed by the repeated passage of liquid through the equipment (Table 3.2). One possible explanation is the generation of electrostatic charge on the dry polycarbonate and polyethylene plastic holder which the passage of a fluid may conduct away from the

mesh holder, effectively earthing the structure and preventing the charging of subsequent nylon mesh disks.

Changing from SDW to a 0.1% Tween 20 wash solution contributed to a further reduction in cell contamination of the mesh after washing (Table 3.2). The lower surface tension with the detergent may have enabled the liquid to permeate the contours of the holder more thoroughly to remove previously untouched cells. McEldowney & Fletcher (1986) and Smith & Wollum (1993) reported decreases in cell numbers on surfaces but when detergent was added to the liquid medium in which the attachment interaction took place. Adding Tween 20 in the present study is unlikely to have disrupted adhesion as the detergent was a constituent of the wash liquid and not of the liquid environment in which the microbe/nylon interaction was taking place.

Another assay feature was repeating the entire wash procedure for the same mesh sample. Although the treatment was significantly different from the single wash, the amount of contaminant removal was relatively low and was considered to be a threshold level in which further mechanical improvements (for example, increasing the number of washes) became impractical.

A consistent feature in all five of these experiments was the high variation in treatment means (Table 3.2). Replication was increased from three to nine in Experiment B, but had little effect in reducing this variability. A compromise of six replicates was later used as nine limited the number of treatments that could be carried out. Nesbitt *et al.* (1982), Fletcher (1983), Gabrielle-Piette & Idziak (1992) and Smith & Wollum (1993) all recorded similarly high treatment variations in adhesion experiments and this may be a consequence of heterogeneous cell populations which influenced cell adhesion as suggested by Morisaki (1991).

The complete removal of all microbes from the nylon mesh surface is an unrealistic expectation. Morisaki (1991) concluded that no single value for attachment force can be assigned to a cell population for a bacterial species which showed an exponential relationship between cells remaining and increased wash force, demonstrating that the removal of the last remnants of a bacterial population required ever increasing levels of force. However, cell attachment can be manipulated with physicochemical parameters including buffer selection (Smith & Wollum, 1993), pH (Nesbitt *et al.*, 1981; McEldowney & Fletcher, 1986), addition of surfactants (McEldowney & Fletcher,

1986) or specific cations (Nesbitt *et al.*, 1981; Gabriel Piette & Idziak, 1992) in the liquid in which the interaction is taking place. Cell starvation (Kjelleberg *et al.*, 1983; Van Haecht *et al.*, 1984) and treatment of inert surfaces with trivalent cations (Van Haecht *et al.*, 1984; Van Haecht, Bolipombo & Rouxhet, 1985; Mozes *et al.*, 1987) can also influence cell adhesion.

Kjelleberg *et al.* (1983) and Van Haecht *et al.* (1984) showed that selected bacteria and yeast cells starved in water suspension had enhanced adhesion behaviour. In the present study starving *B. megaterium, C. laurentii, D. hansenii* and *Pseudomonas* sp. in SDW did not consistently increase contamination of the mesh. There was a small increase for both bacteria after 8 or 16 h incubation which is consistent with the results of Kjelleberg *et al.* (1983). Declines in cfu detection after 16 h incubation may be the result of cell death in the suspension, although this was not measured. Van Haecht *et al.* (1984) found that increases in cell adhesion of *Saccharomyces cerevisae* during starvation conditions was due to changes in the cell wall and to the release of ionic material from the cell that contributed to the decline in repulsive forces. Such a process may have been responsible for the steady increase in mesh contamination by *C. laurentii* over the 24 h starvation period. However the steady decline in mesh contamination by *D. hansenii* has not been recorded in the literature and may have been due to a steady rate of cell death in the suspension population.

Immobilisation of cells by an inert surface can be enhanced with the pre-treatment of either surface with silica (Rouxhet *et al.*, 1981), Al (III) cations (Van Haecht *et al.*, 1985) and Fe (III) cations (Mozes *et al.*, 1987). These treatments appear to modify the repulsion forces present but should not be confused with the addition of cations to the liquid in which the interaction is actually taking place. In the present study, treating the mesh with Fe $(NO_3)_3$ increased the retention (measured as a decrease in W.E.) with moderate statistical evidence for increases in retention of three of the four test microbes examined. This is in agreement with the findings of Mozes *et al.* (1987) and indicates that the nylon mesh possessed the potential to interact with the cells in an electrostatic manner within the short exposure time. The observations of cell starvation and the effect of iron treatments has provided prima-facie evidence that electrostatic processes were operating within the mesh holder and on the nylon mesh itself during the contamination and wash procedures. A conventional approach to manipulate this interaction is to change the pH and buffer selection. Responses of cell adhesion to suspension pH vary

with pH value and buffer according to the specific conditions of the investigation. In this study, overall increases in the W.E. were observed where McIlvaines buffer was used as the wash liquid compared with SDW plus Tween 20. The effect of pH itself was less important with peak W.E.'s at pH 6.0 or 7.0 for three of the four test isolates. These pH values agreed with those of Morisaki (1991) who found that greater force was require to remove adhered cells at low pH and Mozes *et al.* (1987) who observed a decrease in immobilisation of *Moniliella pollinis* cells as pH increased from 4.0 to 7.0. However they differ from data of Nesbitt *et al.* (1982) who found bacterial attachment was minimal at pH values less then 5.0 and of Van Haecht *et al.* (1984) who observed maximum yeast adhesion between pH 4.0 and 9.0. Smith & Wollum (1993) observed marked buffer and pH effects on the number of *Bradyrhizobium* sp. cells attaching to *Glycine max* roots. These differences reflect the specific microbe and surface conditions of these interactions.

A very large decline in W.E. was observed where mixtures of "field" microbes replaced those cultured in the laboratory. W.E values of between 4.0 and 5.0 were routinely obtained using laboratory reared isolates but this was lowered dramatically to between 1.0 and 2.0 using phylloplane washings. In using such microbes, many unknown factors are introduced, for example species composition and energy/nutritional status of the cells in suspension. It could be argued that the dramatic decrease in W.E. was attributed to species not represented in the test microbes. Other factors influencing bacterial and yeast adhesion could be the quantitative and qualitative nature of materials in the cell wall, cell mobility, the efflux of ionic materials and physical distortion of the cell with respect to cell culture conditions, age and starvation (Fletcher, 1977; Van Haecht *et al.*, 1984) which have already been discussed. Cells from culture reared isolates were starved in anticipation of the very low nutrient status of the phylloplane but there appears to be other factors responsible for the different behaviour of these cells in SDW suspension.

Killed *B. cinerea* germlings were used to simulate increased impedance to liquid flow through the mesh holder despite Doss *et al.* (1993) showing that physiologically inactive cells retain their attachment behaviour. In this study *B. cinerea* germlings were used because substitutes such as nylon thread (Nesbitt *et al.*, 1978) were difficult to obtain with the correct diameter and length. However no attachers to hyphae of *B. cinerea* were found during microscopic examination after the short exposure period of

this experiment. As there was no significant increase in nylon mesh contamination with large numbers of *B. cinerea* germlings, 3 x 10^6 germlings/mesh was selected as the fungal bait loading.

The present study was an opportunity to apply information on cell attachment to inert surfaces, to the design of an apparatus and protocol for the selection of attacher microbes. Research primarily focused on the interaction between bacteria and yeast and the nylon mesh support used in the mesh holder, to minimise the cell contamination thought to be responsible for low yields in earlier work. The combined effects of mechanical and physico-chemical factors reduced mesh contamination by phylloplane microbe species to approximately 25 cfu's from 17,000 phylloplane cfu's added in the presence of 3 x 10^6 *B. cinerea* germlings.

However it remains unknown whether this reduction in contaminant microbe levels is sufficient to allow those attached to *B. cinerea* to dominate in populations isolated during BCA screening.

Use of the Potter Tower for inoculating tomato stem pieces with *B. cinerea* conidia, yeast or bacterial cells

4.1 Introduction

There is evidence that the method of inoculation of *B. cinerea* influences subsequent pathology. Generally, conidial suspensions in SDW have been used although dry inoculum were harvested and discharged into a cloud over the surface of the gynoecium (McNicol, Williamson & Dolan, 1985; Williamson, McNicol & Dolan, 1987) or fruits (McNicol, Williamson & Dolan, 1990) of red raspberry; strawberry flowers (Bristow, McNicol & Williamson, 1986) and rose petals (Williamson *et al.*, 1995). Fourie & Holz (1994), Spotts & Holz (1996) and Salinas *et al.* (1989) used settling towers to inoculate dry *B. cinerea* conidia onto floral parts of plum and nectarine and onto gerbera flowers. Williamson & Duncan (1989) punctured and inoculated raspberry fruitlets with dry conidia on a sterile needle.

There are few reports of direct comparisons of inoculation methods with *B. cinerea* and the conclusions are inconsistent. Bristow *et al.* (1986) compared unwashed dry application of spores with micro-droplet suspension techniques and found no significant difference in average hyphal length but Pie & Brouwer (1993) observed fewer lesions from dusted dry conidia compared with sprayed or dipped suspensions. Similarly, O'Neill, Shteinberg & Elad (1997) found more rapid disease development in tomato stem pieces when *B. cinerea* spores were applied in suspension compared with dry inoculation, but there was no difference in the number of diseased stems. Williamson *et al.* (1995) and Cole, Dewey & Hawes (1996) used SEM to compare infection of host tissue from dry *B. cinerea* conidia with wet application. The conidia and germ tubes from dry spores directly penetrated the host tissue and appeared elevated from the host surface due to forces exerted on the conidium either by the penetration process or as an artifact of SEM preparation. By comparison, hyphal growth from wet inoculum was considerably more extensive on the host surface in both reports.

Dry inoculation techniques suffer one limitation, the inability to control quantity and destination of a spore cloud to ensure precise and accurate inoculum loads on the target surface which is particularly important in plant protection bioassays (Reifschneider & Boiteux, 1988). Artificial inoculation of Erysiphaceae has stimulated development of

several dry methods using still air towers (Reifschneider & Boiteux 1988) but it appears that many researchers still favour suspensions to maintain a high degree of accuracy and precision.

The Potter Tower was originally developed as a method for the precise and accurate delivery of insecticide aerosol in entomological studies (Potter, 1952). In this study, a procedure was developed to use the tower to give a precise and accurate delivery of microbial propagules for use in bioassays for biocontrol.

4.2 Objectives.

To:

1) Determine the deposition patterns of *B. cinerea* propagules and yeast or bacterial cells using the tower

2) Compare disease development in host tissue inoculated with *B. cinerea* conidia in pipetted suspensions or in an aerosol

3) Identify factors influencing the deposition process within the Potter Tower.

4.3 Section One: Deposition patterns of *B. cinerea* and bacterial or yeast cells

4.3.1 Experiment One: Calibration of the Potter Tower

See Appendix Two

4.3.2 Experiment Two: Dispersal of B. cinerea conidia.

4.3.2.1 Objective

To examine the relationship between the concentration of *B. cinerea* conidia in the liquid reservoir and the density of conidial dispersion on the spray table.

4.3.2.2 Materials and Methods

B. cinerea conidia from isolate BC 20 were suspended in SDW and suspension concentrations adjusted to, $5x10^6$, $7.5x10^6$, $1x10^7$, $2.5x10^7$ and $5x10^7$ spores/ml. (See Chapter Two, Section 2.1 for preparation details).

One ml of each suspension was loaded into the liquid reservoir of the Potter Tower and conidia were applied at 100 mm Hg to nine numbered microscope cover slips (22mm x 22mm) covered in Potato dextrose agar (PDA) arranged in a 3 x 3 grid ("The arena") on the spray table. The nozzle was rinsed by atomising 3 ml of SDW between applications to remove residual conidia. Each treatment was applied to fresh uninoculated agar surfaces according to a completely randomised block design, replicated four times.

The inoculated agar arenas were stained with 0.05% cotton blue in lactophenol using a second, spare Potter Tower nozzle to spray the conidia. The arena was placed into sterile petri dishes and stored at 4°C until assessed. The orientation and numbering of each slide was carefully maintained to ensure the entire inoculated surface could be accurately reconstructed.

To aid in the density assessment, a microscope slide (25 mm x 60 mm) was customised by marking a grid of 2 mm x 2 mm squares within the coverslip dimensions (22 mm x 22 mm). Two glass guides were positioned beside this marked zone to ensure precise location of the agar coated coverslips during counting. These

2 mm squares ("quadrates") were numbered so that specific quadrate sites could be selected for counting using a random number generator.

Each quadrate site was further subdivided into 64 squares in an 8 x 8 grid using a numbered eyepiece graticule ("The graticules") at 40x magnification (10x eyepiece and 4x objective lenses) inserted into a light microscope (Olympus BH-2). Each quadrate was subsampled by randomly selecting graticules and all the conidia within that area were counted. For treatments $5x10^6$ spores/ml and $7.5x10^6$ spores/ml 32 graticules were selected; treatments $1x10^7$ spores/ml and $2.5x10^7$ spores/ml 24 graticules were selected and treatment $5x10^7$ spores/ml a diagonal transect of 8 graticules was selected. In each subsample, the data was multiplied by the appropriate area ratio to give a spore population per quadrate.

A repeat inoculation was carried out and the agar arenas were incubated at 15°C overnight to allow the spores to germinate. The samples were then sprayed with 0.05% cotton blue in lactophenol before being examined for germination using light microscopy.

Data analysis was carried out according to a nested experimental design. In addition, spatial analysis was also carried out using Lloyds index of patchiness and Iwao's patchiness regression in order to quantify the pattern of spore deposition relative to theoretical indices of 1= random, <1 = uniform and >1 = aggregate spatial distributions.

$$\text{Lloyds index of patchiness} = \frac{\bar{x} + (s^2 / \bar{x} - 1)}{\bar{x}}$$

$$\text{Iwao's patchiness regression} = \bar{x} + (s^2 / \bar{x} - 1) \text{ versus } \bar{x}$$

Where: s^2 is sample variance

$\bar{x}$ is sample mean

4.3.2.3 Results and discussion

There were significant differences in spore density as spore concentration in the reservoir increased ($P<0.0001$) (Fig. 4.1) and between the nine coverslip zones

(P<0.0001) but no significant differences between replicate applications (P>0.05). The coefficient of determination (R^2) given in Fig. 4.1 of 98% shows the majority of variation in the data is explained by the linear regression model.

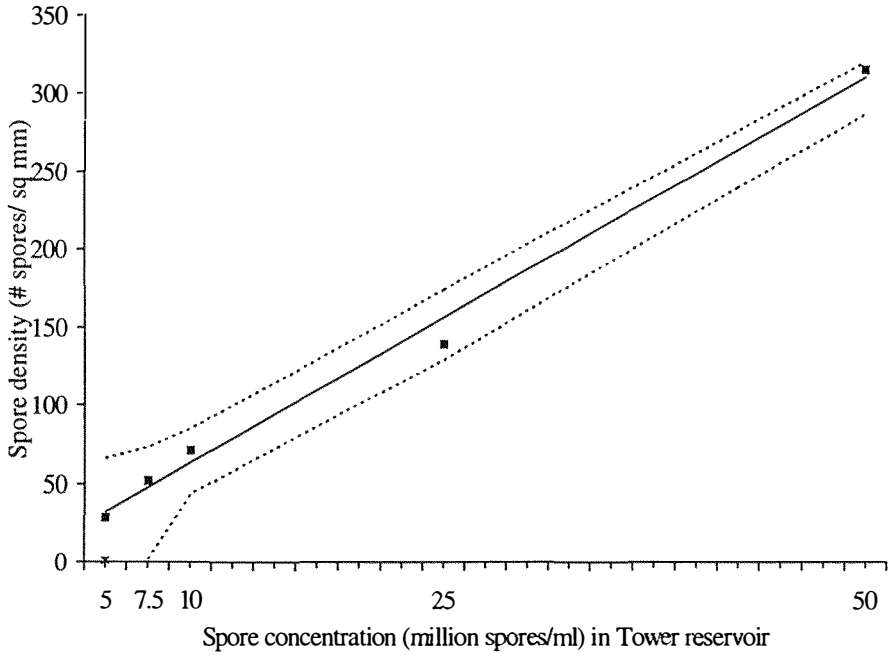


Fig. 4.1. Conidial density of *B. cinerea* applied to PDA using the Potter Tower at 100 mm Hg nozzle pressure. Dashed lines represent upper and lower 95% predictor bounds for the regression relationship. Parameter values: $y=6.1 \times 10^{-6}x + 1.743$; $r^2=0.98$; $P<0.0001$.

Values for the statistic, Lloyds index of patchiness (LIP) ranged from 0.99 to 1.1 and were regressed against treatment mean densities to form Iwao's patchiness regression (IPR) (Fig. 4.2.). The regression equation obtained from this relationship was significantly (P<0.05) different from a theoretical slope of 1.0 which is the spatial pattern for a perfectly random population (indicated as a dashed line in Fig. 4.2).

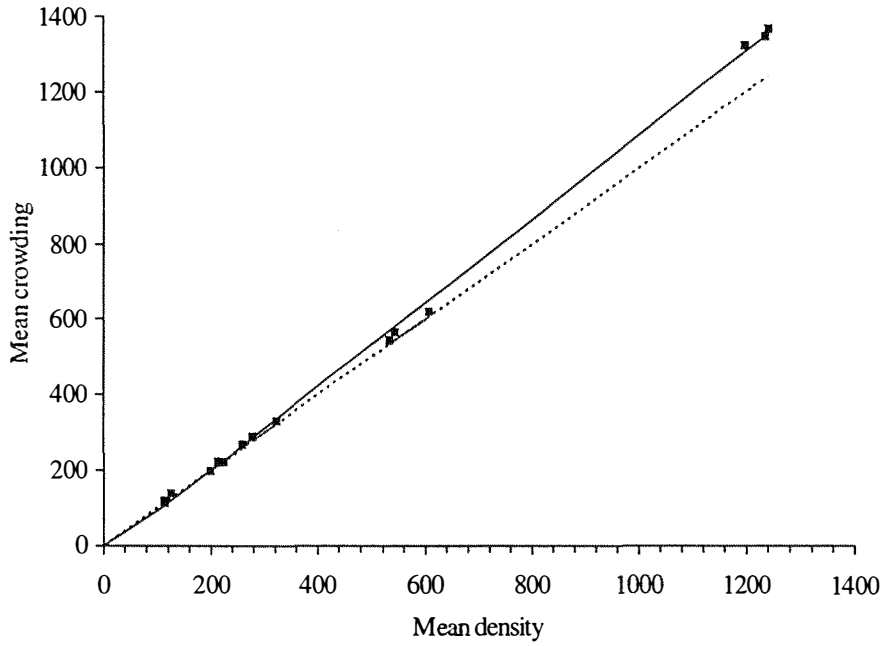


Fig. 4.2. Iwao's patchiness regression of conidia applied to spray table by Potter Tower at 100 mm Hg nozzle pressure compared with a random population distribution (dashed line, slope =1.0). Parameter values: y=1.10x - 21.5; r^2=0.99; P<0.0001.

In the second part of this experiment, the viability tests showed that all of the *B. cinerea* spores examined had germinated.

This method of inoculating *B. cinerea* conidia onto the spray table was inefficient. At the highest spore density ($5x10^7$ spores/mm^2) only 2% of spores pipetted into the reservoir landed in the agar arena which is a value less than the 9% for SDW. The carrier alone would be expected to be a good predictor of microbe deposition and possible causes of this difference are not obvious from the data. It is possible that a small reduction in the surface tension of the *B. cinerea* suspension caused by release of metabolite from the spores was sufficient to alter the spray pattern of the water itself. The contact angle (a measure of liquid surface tension) was lowered when conidia of *Pyrenopeziza brassicae* were added to SDW on cabbage leaves and it was proposed that this was due to soluble fungal exudates (Davis & Evans, 1990). The impact of a change in surface tension on spray deposition patterns are not known but physical properties of the liquid affect spray droplet formation during atomisation (Stevens, 1993).

A number of spatial statistics have been developed for the calculation of population dispersal including Poisson and Negative Binomial. These provide qualitative evidence of randomness provided there is a general agreement between the data and the models. However there are situations where none or more than one

of these distributions fit the data (Elmer, 1990). Dispersion indices such as Variance to Mean ratios (VTM), LIP and IPR give quantitative values that show the degree of model fit to designated values <1, 1 and >1 (spatial pattern, uniform, random or aggregated respectively). The later two indices, LIP and IPR, have an added advantage of being independent of population density (Elmer, 1990) and were used in this experiment. Both spatial statistics indicate the deposition pattern was not random but tended to be aggregated, particularly at the highest spore density. It is possible, given such a high spore concentration some agglutination took place in suspension forming clumps of spores that did not completely disperse after atomisation.

The cause of a significant difference between the nine coverslips used that made up the arenas is not immediately obvious in this experiment but may be due to increased accuracy of spore counts used in this experiment versus the method of weight determination used in Appendix Two that revealed a bias in the nozzle direction.

4.3.3 Experiment Three: Deposition of Yeast and Bacteria cells.

4.3.3.1 Objective

To examine the relationship between suspension concentration of *Saccharomyces cerevisae* and *Pseudomonas* sp. and the density with which these cells were dispersed onto the spray table.

4.3.3.2 Materials and methods

Isolates of *Saccharomyces cerevisae* and *Pseudomonas* sp. obtained from the culture collection at the Plant Science Department, Massey University were grown on NYDA and NA respectively and cell suspensions were made as described in Chapter Two (Section 2.2). Concentrations were adjusted to 2.5 x 10^6 , 2.5 x 10^7 , 2,5 x 10^8 , 2.5 x 10^9 and 2.5 x 10^{10} cells/ml with SDW.

Three sterile, 13mm diameter glass coverslips were positioned in three marked locations on the spray table and 1 ml of each suspension was pipetted into the liquid reservoir and applied to the spray table at 100 mm Hg nozzle pressure. There were three replicate applications for each microbe.

Treated coverslips were immediately removed and placed into a universal bottle containing 4 ml of SDW and a 15 mm diameter sterile glass marble. Yeast or bacterial cells were washed from the coverslip by vortexing the bottles in six short bursts totalling 15 sec. Three determinations of each replicate were made by applying a 100 µl aliquot and a ten-fold dilution of this aliquot onto NYDA or NA. Culture plates were incubated at 15°C, in total darkness for 48 h before counting colonies.

The experiment and data analysis was carried out according to a completely randomised block design.

4.3.3.3 Results and discussion

As suspensions with higher concentrations of bacteria or yeast were applied, the $\log_{10}$ of cfu density on the spray table significantly increased ($P<0.0001$). For both bacteria and yeast, $\log_{10}$ cfu density and $\log_{10}$ concentration were modelled using linear regression and coefficients of determination were 99% respectively. (Figs 4.3 and 4.4).

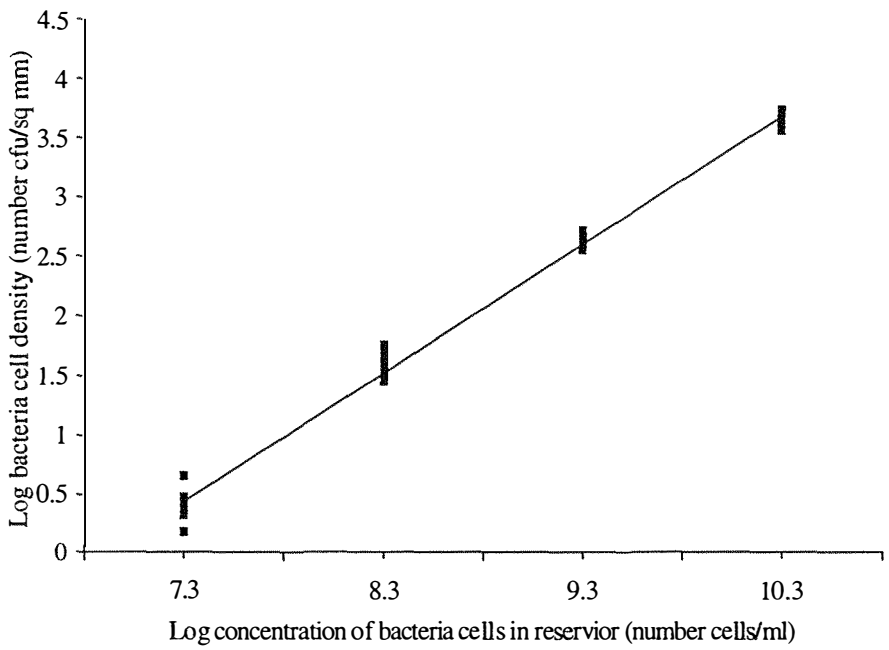


Fig. 4.3. Log_{10} viable bacteria density (cfu/mm^2) following the application of a range of concentrations of cell suspensions in SDW using the Potter Tower at a nozzle pressure of 100 mm Hg. (Replicates =3, determinations =3). Parameter values: $y=1.07x - 7.43$; $r^2=0.99$; $p<0.0001$.

Back-transformed, yeast and bacterial cfu densities of 7200 cfu/mm^2 and 3900 cfu/mm^2 respectively were obtained when $2x10^{10}$ cells/ml (log value 10.3) was pipetted into the liquid reservoir. A measure of cfu in the original suspensions were not determined.

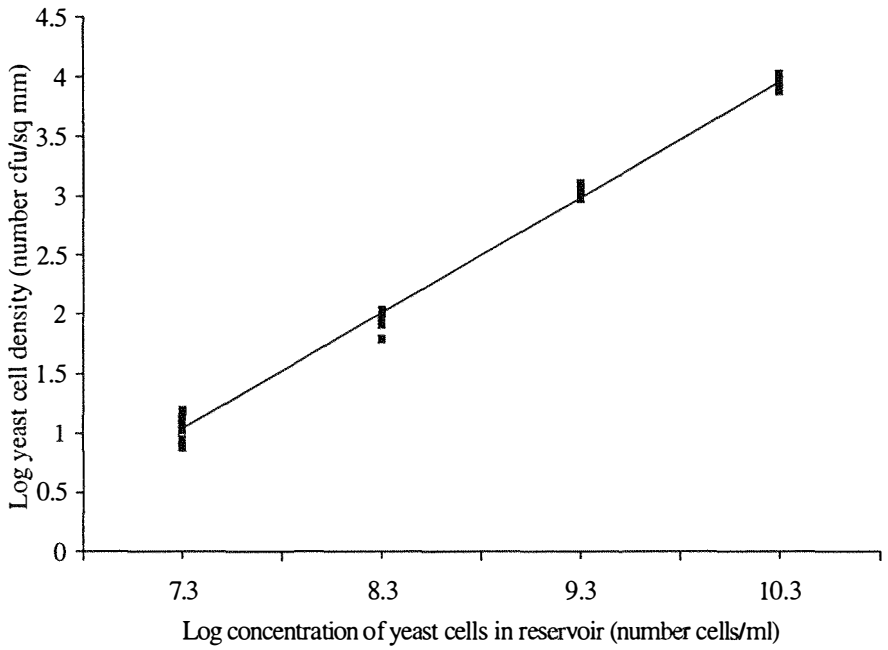


Fig. 4.4. Log_{10} viable yeast density (cfu/mm^2) on the spray table following the application of a range of cell concentrations in SDW using the Potter tower at a nozzle pressure of 100 mm Hg. (replicates =3, determinations =3). Parameter values: y=0.96x - 6.03; r^2=0.99; P<0.0001.

These yeast and bacterial cfu densities suggest the deposition process is considerably more inefficient than with *B. cinerea* application. At the highest yeast and bacterial concentrations (2 x 10^{10} cells/ml), cfu density deposited onto the spray table were measured at 24-times and 13-times respectively, higher than *B. cinerea* spore density (300 spores/mm^2) despite a reservoir concentration 400-times higher. Possible causes of this low value may be the method of removing cells from the treated glass coverslips where the glass fragments produced by the action of the glass marble during vortexing, may have killed some of the suspended cells. To remedy this situation, an alternative procedure should be used where the glass marble is omitted during vortexing.

4.4 Section Two: Disease development in tomato stem pieces inoculated with *B. cinerea* using conidial suspensions or aerosol in the Potter Tower.

4.4.1 Experiment Four: Application of B. cinerea *conidia using the Potter Tower or spore suspensions*

4.4.1.1 Objective

To compare the rate of disease development caused by the pathogen *B. cinerea* applied to tomato tissue using pipetted suspension and aerosol.

4.4.1.2 Materials and methods

Suspensions of *B. cinerea* conidia were prepared as detailed in Chapter Two (Section 2.1) and adjusted to $5x10^6$, $1x10^7$ and $5x10^7$ spores/ml for the Potter Tower treatments. Further suspensions were prepared containing $7.4x10^4$, $1.8x10^5$ and $7.6x10^6$ for 30 µl suspension treatments and $3.7x10^4$, $9.2x10^4$ and $3.8x10^6$ spores/ml for 60 µl suspension treatments. Stems from 3 month old tomato plants were grown and prepared as for seedling plants detailed in Chapter Two except that plastic film was wrapped around each stem before cutting into 1 cm lengths.

Seven tomato stem pieces per treatment were inoculated using the Potter Tower at 100 mm Hg nozzle pressure. For the suspension treatments, *B. cinerea* conidia were pipetted onto the tomato stem piece in 30µl or 60µl volumes using the corresponding suspensions detailed in this section. Four replicate inoculations were carried out using the completely randomised block experimental design and after removing the plastic film, the stem pieces were then incubated at 15°C for 10 days in plastic trays containers lined with moist paper towels to provide a humid environment. Stem pieces were checked daily for signs or symptoms of *B. cinerea* infection.

The experiment and data analysis was carried out according to a split plot design where the model was not fully fitted to the data. The residual was then used for calculation of treatment effects, concentration, inoculation method (main plots) and time (split plot).

4.4.1.3 Results and discussion

The 30µl and 60µl suspension volumes used in this experiment represent a 5-fold and 10-fold increase in the volume of SDW landing on the tomato stem surface compared with the aerosol from the Potter Tower. These values were calculated from data in the preliminary experiment using SDW only (Section 4.3.1) and a stem diameter of 10 mm. The concentration of *B. cinerea* conidia in each of these treatments was calculated based on the density data obtained in Experiment Two so that the same number of conidia were delivered by the Potter Tower, in 30 µl and 60 µl SDW volumes. Plastic film was wrapped around the epidermal surface of the stem pieces to minimise spore deposition on this area and thus avoid confounding the comparison.

During the period within 5 days after inoculation, a variety of disease symptoms (Fig. 4.5) were observed in the inoculated tomato stem pieces. Some were sporulating but with no obvious lesion, others exhibited both lesion and sporulation and others with non-sporulating lesion which sporulated after further incubation. Mycelium and spore morphology from some of the lesions was used to check the identity of the causual agent.

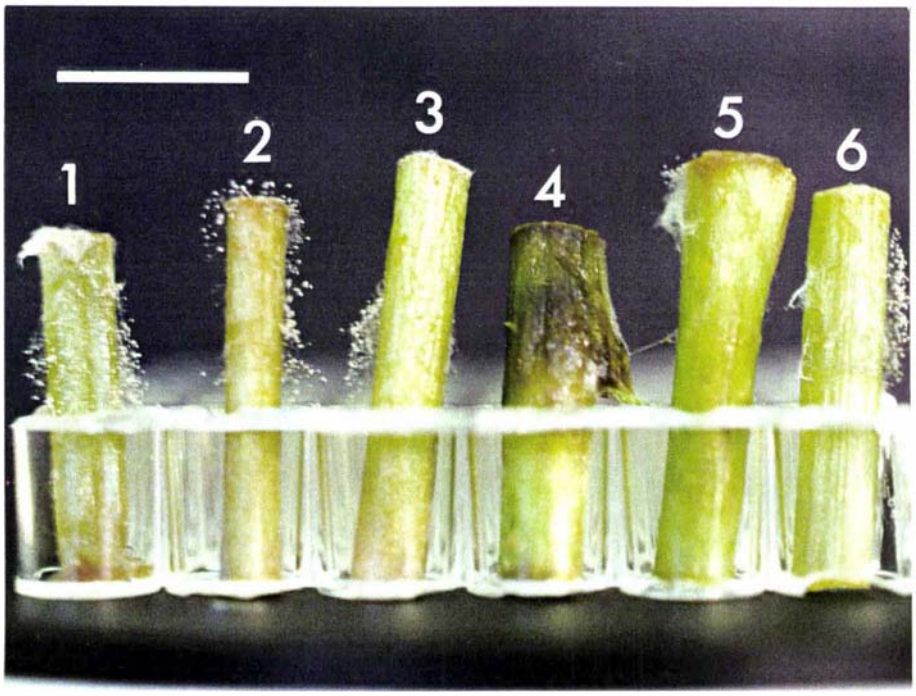


Fig. 4.5. The variable expression of lesion symptoms in tomato stem tissue spray inoculated with *B. cinerea* conidia. Symptoms include sporulation with no apparent lesion (stems 1,2 & 6), stems with lesion but without sporulation (stem 4) and still others with sporulation from mid-way along the stem (3). Bar = 1 cm.

Disease incidence (pathogen signs or disease symptoms) was recorded daily and disease progress curves plotted. At all inoculum densities, disease progress was more advanced in the Potter Tower aerosol treatment than either suspension application (Fig. 4.6). Incidence of diseased stems declined as spore density decreased in all three inoculation methods and there was no disease expression at the lowest dose applied by pipette although stem pieces inoculated with the Potter Tower aerosol were sporulating. Consequently, the interaction between time and concentration or between time and inoculation method were all highly significant ($P<0.0001$). At the highest spore dose (Fig. 4.6) disease expression was observed on day five in the Potter Tower treatment compared to pipetted applications on day six.

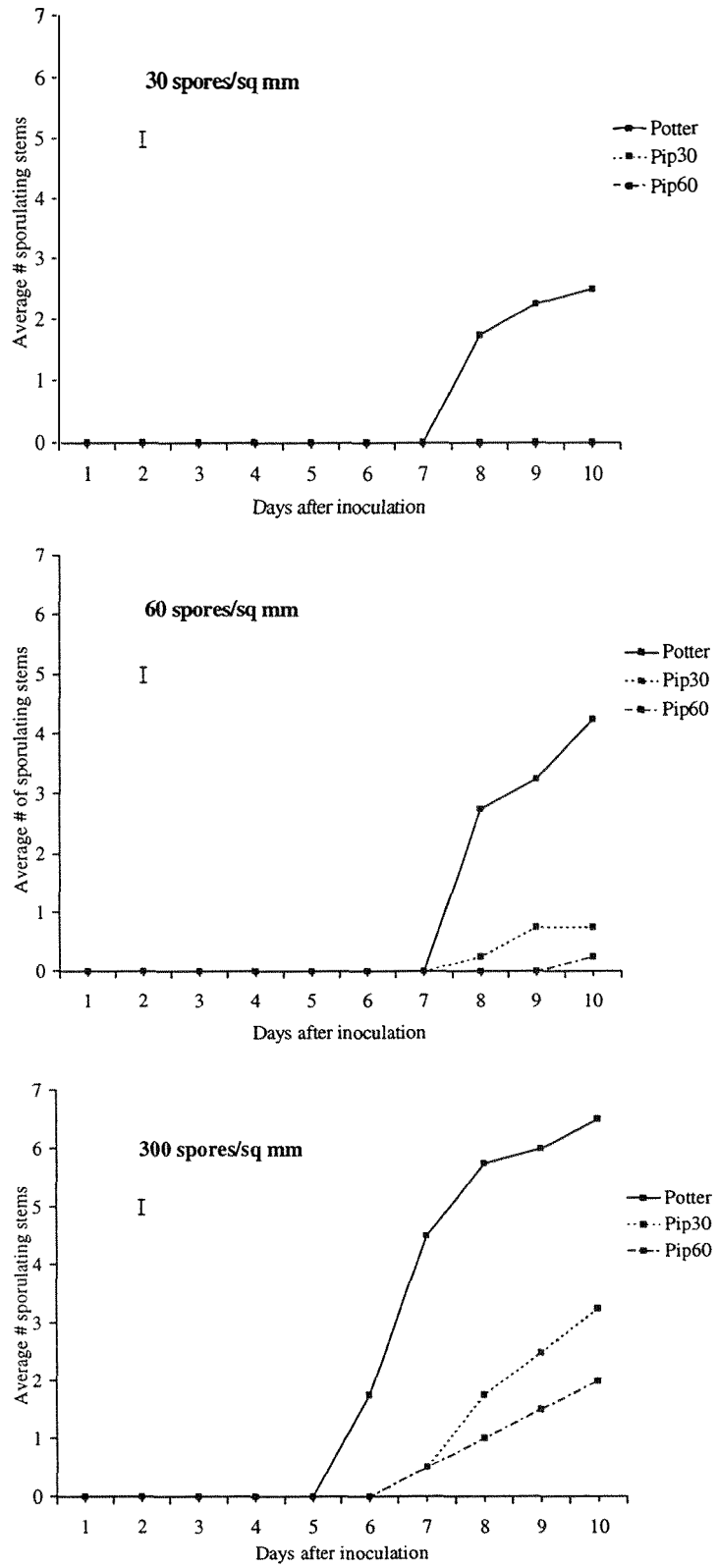


Fig. 4.6. Daily disease incidence in tomato stem pieces inoculated with 30, 60 and 300 spores/mm^2 using either the potter tower or in 30 and 60 µl SDW spore suspensions. Maximum number of stems =7. Vertical bars = LSD (Time x Inoculation method) (α=0.05, df = 289, n=4).

Results of this study show a clear increase in the rate of infection of tomato tissue when inoculated with a fine aerosol than when the same spore loads were applied as droplets of SDW suspensions. The cause of the increased aggression of sprayed *B. cinerea* inoculum is unclear but could be related to the volume of SDW and/or the time it remains on the stem tissue before evaporating.

Sztejnberg & Blakeman (1973) showed evidence that *B. cinerea* conidia were very sensitive to nutrient leaching leading to a decline in germination. The importance of this factor in this experiment is limited as Sztejnberg & Blakeman (1973) applied the leaching treatments continuously over a 24 h period and exudate from the host surface wound would contribute to the nutrient content in the droplet. Further, any nutrient loss from the conidia while in stock suspension prior to application would be similar for all treatments.

The persistence of the bulk liquid applied to tomato stems as spore suspensions may have allowed toxic metabolites to leach from the wounded tissue to partially inhibit spore germination or vigour. Williamson *et al.* (1995) identified this possibility on inoculated rose petals and gave it as the reason for using dry inoculation methods. Alternatively, Cole *et al.* (1996) suggested that the reduced amount of free water limited the spread and dilution of digestive enzymes from *B. cinerea* and this aided more rapid host penetration.

Many have argued that spore attachment is a process fundamental to pathogenesis (Kennedy, 1990; Nicholson & Epstein, 1991; Jones, 1994) and it is possible that those spores that achieve this process quickly will proceed to subsequent phases (pathogenesis) sooner. Such an effect may be evident in this study. Initial attachment of non-germinated *B. cinerea* conidia is very rapid and is dependent on hydrophobic interactions (Doss, Potter, Chastagner & Christian, 1993). The same passive non-specific adhesion characters are exhibited by urediniospores of *Uromyces viciae-fabae* (Beckett, Tatnell & Taylor, 1990). Dry urediniospores incubated at 100% RH formed a pad by capillary condensation with the rapid release of spore surface matrix (Clement, Butt & Beckett, 1993; Clement, Porter, Butt & Beckett, 1994) immediately beneath the spore. Dislodged spores of *B. cinerea* exhibited a flattened area in the region where spore wall contacted the surface (Doss *et al.*, 1993) indicative of a similar pad. This matrix is water soluble and it is plausible that the submerged conidium in the 30µl and 60µl droplets

released adhesins into the surrounding fluid, diluting the concentration of the adhesion pad at the interface and slowing the attachment process. On the basis of the "model" example of , *U. viciae-fabae,* it is reasonable to suggest that the adhesion matrix from *B. cinerea* applied to tomato stems in suspension, migrated into solution (approximately 30 min -1 h) whereas the limited water volume of the aerosol application evaporated rapidly localising the matrix in the pad region and enabling adhesion to proceed quickly. Whether this hypothesis alone is sufficient to create the large differences in infection behaviour requires further study but appears consistent with another similar comparison of application treatments of *B. cinerea* on rose petals. The lowest number of disease lesions was found in the "spraying until runoff" and in the dipping treatments (Pie & Brouwer, 1993). Assuming that spore density applications in each application treatment were the same, spraying to runoff and dipping would have added larger volumes of water than the other treatment (spraying) and this could be responsible for the lower number of lesions.

As results in this experiment show a significant increase in the level of host infection following inoculation by *B. cinerea* spores in an aerosol, this method was used in all biocontrol screening experiments so that BCAs will be selected in an environment where the pathogen is aggressively growing and infecting host tissue.

4.5 Section Three: Confirmation of bacterial and yeast cell deposition in the Potter Tower.

4.5.1 Experiment Six: Yeast and bacteria cell deposition patterns using the Potter Tower.

4.5.1.1 Introduction

There was some doubt over the results of Experiment three where bacterial and yeast populations deposited on the spray table were underestimated. The method of collecting then measuring these densities was critically examined and it was concluded that an alternative method was required.

4.5.1.2 Objective

To examine the deposition patterns of bacteria and yeast on the spray table.

4.5.1.3 Materials and Methods.

Isolates of *S. cerevisae* and *Pseudomonas* sp. were prepared as described in Experiment Three and suspension concentrations were adjusted to 2.5 x 10^6 , 2.5 x 10^7 , 2.5 x 10^8 , 2.5 x 10^9 and 2.5 x 10^{10} cells/ml. Cfu counts from each suspension were determined by plating out a100 µl aliquot of each concentration on three replicate NA or NYDA plates.

One millilitre aliquots of cell suspensions of *S. cerevisae* and *Pseudomonas* sp. were applied using the Tower at 100 mm Hg nozzle pressure to three, 10 ml glass bottles filled with 10 ml SDW which were positioned at marked sites on the spray table. Three replicates of each treatment were carried out in a randomised block design. The inoculated bottles were removed, capped then vortexed for 15 s before a 100 µl aliquot and a ten-fold dilution of this aliquot were plated onto NYDA or NA were appropriate. Plates were incubated at 15°C, total darkness for 48 h before counting colonies.

The experiment and data analysis were carried out according to a completely randomised block design (See Chapter Two, Section 2.6). The relationship between cfu density and cell concentration was modelled using linear regression analysis and 95% confidence intervals.

4.5.1.4 Results and discussion

As suspension concentration increased, bacterial and yeast cfu density on the spray table also increased (Figs 4.7 and 4.8). Values for the coefficient of determination (r^2) were 93% and 97% respectively and indicated that the majority of the variability of the data was explained by this model. All of the data means were within the 95% predictor boundaries but these widened appreciably at low suspension concentrations where the spread in raw data was greatest. When back-transformed, at the highest cell concentration applied , yeast density on the stage was 18,000 cfu/mm^2 and of bacteria was 3,000 cfu/mm^2. The concentration of viable cells (cfu) in each suspension was lower than the total cell count and the proportion of cfu to total cells declined with each succeeding dilution of cell suspension (Table 4.1).

Table 4.1. The concentration of viable cells (cfu) in SDW suspension prior to inoculation through the Potter Tower.

Isolate	**Total cell count (# cells/ml)**	**Cfu determination (cfu/ml) (% of Total cell count)**	**Log cfu**
Saccharomyces cerevisae	2×10^{10}	2×10^{9} (10%)	9.3
	2×10^{9}	7×10^{7} (3%)	7.8
	2×10^{8}	4×10^{6} (2%)	6.6
	2×10^{7}	3×10^{5} (1%)	5.4
Pseudomonas sp.	2×10^{10}	2×10^{8} (1%)	8.3
	2×10^{9}	6×10^{6} (0.3%)	6.7
	2×10^{8}	4×10^{5} (0.2%)	5.6
	2×10^{7}	2×10^{3} (0.001%)	3.3

Numbers of viable yeast and bacteria cells deposited on the spray table were similar to those in Experiment Three and suggest that the assessment protocol used previously did not significantly reduce microbial populations on the table surface. With the close agreement between results of these two experiments, increased confidence could now be placed in the accuracy of the cfu density measurements.

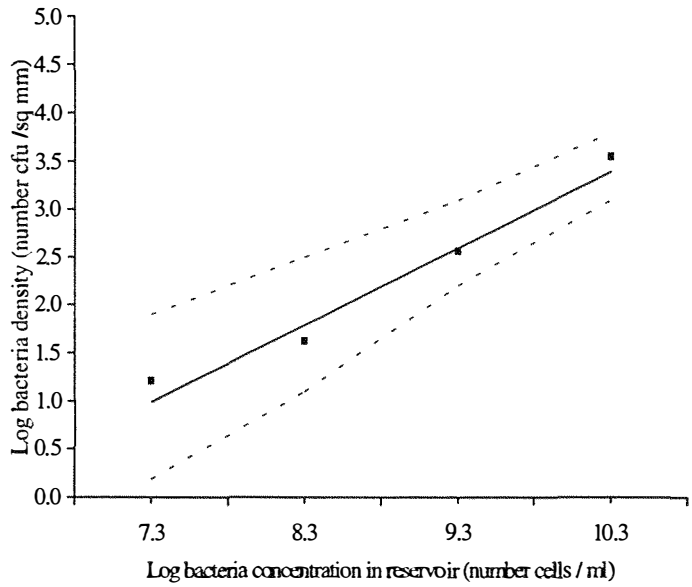


Fig. 4.7. Predictor relationship between $\log_{10}$ of bacterial cfu density and suspension concentration. Dashed lines are 95% predictor bounds for the regression relationship, parameter values: y=0.80x - 4.85 (r^2=0.93; P<0.0001).

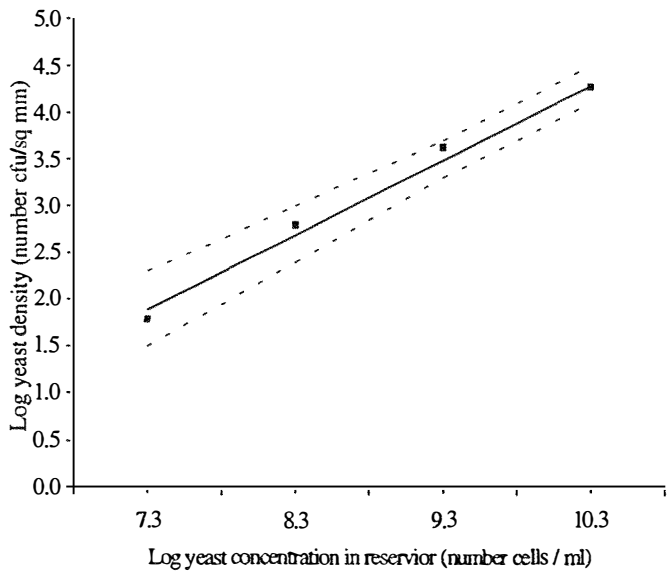


Fig. 4.8. Predictor relationship between $\log_{10}$ of yeast cfu density and suspension concentration. Dashed lines are 95% predictor bounds for the regression relationship parameter values: y=0.80x - 3.95 (r^2=0.97; P<0.0001).

When the proportion of viable cells are taken into account, deposition efficiency can be amended to 4% for yeasts and 6% for bacteria at the highest concentration used in this experiment. This compared with the 2% efficiency calculated for *B. cinerea* and 9% for SDW (Section 4.3.2.3). The closer agreement between efficiency values in this experiment and that of SDW suggest that the later is a better predictor of bacterial and yeast deposition compared to *B. cinerea.* In Experiment Three, the difference between *B. cinerea* deposition and SDW was explained by physicochemical changes in the suspension altering surface tensions (Davis & Evans, 1990) leading to more aerosol landing on the spray tube. This explanation is insufficient to explain the improved efficiencies of bacterial and yeast deposition since the higher surface area to volume ratio and concentration of yeasts and bacteria compared to *B. cinerea* conidia could release more leachate into the SDW possibly leading to a greater change in surface tension.

Alternatively, ranking deposition efficiencies corresponds approximately to the inverse of the cell size of each microbe (*B. cinerea* conidia are 12 μm, yeast are 4 μm and bacteria are 2 μm in diameter). Jones (1994) suggested that fungal propagules were too large to be influenced by electrostatic forces during adhesion and Swinbank, Taggart & Hutchinson (1964) concluded that the charge density on *Merulius lacrymans* spores was insufficient to be biologically important. In contrast, Marshall (1980) stated that bacteria behave as colloid particles due to their size and were influenced by electrostatic processes. It is possible that a small electrical charge was present on the metal construction of the Potter Tower influencing the deposition process. Since bacteria are influenced by surface charge, could be repelled off the interior surface of the spray tube carrying the same polarity and hence disproportionately more land on the spray table. The passage of *B. cinerea* spores remains unchanged as they are not influenced by charge while yeasts of an intermediate size are marginally affected. This hypothesis was tested in a follow-up experiment where the Potter Tower was modified to carry an applied electrical charge. Results were inconclusive (Appendix Two).

4.6 Discussion.

To date, dry inoculation techniques that deliver precise and accurate inoculum doses for bioassays have not been reported. The adaptation of the Potter Tower for the application of *B. cinerea* conidia, bacteria and yeasts in an aerosol is a compromise to the strictly dry nature of pathogen deposition but maintained precise and accurate applications that could be used in bioassays. *B. cinerea* deposition patterns measured after application by the Tower deviated from a theoretical random distribution, particularly at high suspension concentrations where aggregated distributions were observed and there were significant differences in spore density among the various areas on the spray table. However, there were no significant differences between replicate applications.

Elevated levels of pathogen "aggression" were measured in Experiment Four following aerosol application compared with conidial inoculation in suspension. In this comparison, the same number of conidia were applied to each tomato stem piece which is a factor that appears to have been overlooked or to difficult to achieve in previous studies (Bristow *et al.*, 1986; Pie & Brouwer, 1993; O'Neill *et al.*, 1997). This is particularly important as O'Neill *et al.* (1997) showed that pathogenicity varied with *B. cinerea* dose.

Williamson *et al.* (1995) and Cole *et al.* (1996) clearly showed evidence for the etiological 'benefit' of dry inoculation techniques compared to wet on rose petals and bean leaves respectively. In this study spray inoculated pathogen inoculum was more aggressive compared to pipetted suspensions but caution must be exercised before drawing general principles. The tomato tissue used in this study was wounded and there was already water and nutrient from disrupted cells present. In the studies of Williamson *et al.* (1995) and Cole *et al.* (1996), the host tissue was intact and dry prior to inoculation. Nevertheless, the increased infection following aerosol inoculation suggest an infection mechanism worthy of further investigation in the light of the hypotheses proposed in Experiment Four.

The different rates of pathogenesis observed in this study and rapid host penetration by *B. cinerea* (Williamson *et al.*, 1995; Cole *et al.*, 1996) with respect to inoculation technique is critical for screening BCAs. If a pathogen application method is selected that slows pathogenesis then it is questionable how effective the

BCAs selected in the laboratory will perform in the field. This factor may explain past failures of assays to accurately identify agents capable of conferring strong biocontrol when *B. cinerea* is applied in suspension during screening. Spray inoculation could be used in BCA screening but two aspects should be considered:

(1) The inefficient use of inoculum could limit the number of BCA candidates tested.

(2) The balance between relatively precise inoculation dose of a spore suspension using the Potter Tower with a potentially slower infection process and a less precise inoculation dose from dry spores with a more rapid infection process.

A direct comparison of *B. cinerea* infection from aerosol inoculation with dry unwashed spore application remains to be determined but requires a method for the accurate application of dry inoculum. Nevertheless this aerosol method was used for the application of *B. cinerea* spores and BCA inoculum in remaining biocontrol experiments in the laboratory.

Therefore the inoculum application via the Potter Tower became standard technique. To quantify the various populations being applied, the relationship between *B. cinerea* spore suspension concentration and density on the surface in Fig. 4.1 was used. For the BCAs, populations landing on the surfaces was calculated from the regression relationships shown in Figs 4.7 and 4.8.

In vivo screening for biocontrol activity of crude mixtures and pure bacterial and yeast isolates from the attachment assay

5.1 Introduction.

A large number of fungi, bacteria and yeasts have been tested for biocontrol activity against diseases caused by *B. cinerea* (Tables 5.1 and 5.2) and similar lists have been compiled by Wilson & Wisniewski (1989), Dubos (1992) and Droby & Chalutz (1994).

When making a selection for a microbial BCA, the first consideration is the habitat from which candidates are obtained. Some workers contend that the source should closely simulate the environment for which the BCA is to be used (Cook & Baker, 1983) whereas more recent authors argue that source is irrelevant (Andrews, 1992) and illustrate their argument with the soil-borne fungus *Trichoderma* spp. which has been shown to control foliar diseases. Once a decision has been made, the question of how BCAs are identified must be addressed.

Selection techniques have been based on agar plate tests where candidate and pathogen were co-inoculated and presumptive biocontrol identified by reductions or distortions in the radial growth of a fungal pathogen (Dhingra & Sinclair, 1985). Isolates of the *Trichoderma* species group were frequently isolated (Dubos, 1992) as the BCA displays a number of antagonistic mechanisms easily detectable on agar including production of volatile and non-volatile antibiotic compounds (Denis & Webster, 1971, a, b) and mycoparasitism (Dennis & Webster, 1971c; Chet, Harman & Baker, 1981; Elad, Chet, Boyle, & Henis, 1983). This expression *in vitro* could have biased early biocontrol selections resulting in many isolates of *Trichoderma* spp. being tested, despite the antagonist not being a regular component of phylloplane surveys (Dubos, 1992). The *in vitro* assay has been criticised as a selection technique for it is a poor predictor of biocontrol *in vivo* or in the field (Elad, 1990; Andrews, 1990) yet recent reports are still emerging of *in vitro* screening against *B. cinerea* (Leivens, *et al.*,1989; Leifert, Sigee & Epton, 1992a; Franicevic 1993; Sarathchandra, Duganzich &

Burch, 1993) and reflects the practical ease and the low cost with which this assay can screen many candidate isolates.

Attempts to reproduce phylloplane conditions in the laboratory are now more common and *in vivo* tests and their variants, based on excised plant or whole fruit tissue are used for preharvest and postharvest BCA selection (Tables 5.1 and 5.2). However a critical aspect of these techniques, is the maintenance of plant tissue turgor, particularly of leaves and flower petals (Cheah, Wilson & Marshall, 1996) thus such assays are often conducted in environments with high, stable humidities (Sutton & Peng, 1993; Elad Kohl & Fokkema, 1994a, b). Woody stem tissue is more resistant to wilting and this enabled Eden, Hill & Stewart (1996) to incubate large sections of inoculated tomato stem in dry petri dishes. Kohl Molhoek, van der Plas & Fokkema (1995a) used a wet/dry incubation regime in the selection of BCAs on necrotic leaf tissue. An isolate of *Ulocladium atrum* was selected that consistently conferred high biocontrol in the field. The authors proposed that this was due to its capacity to survive the highly variable environment, particularly during periods of low moisture stress.

Other *in vivo* assays have isolated BCAs which suppress pathogen sporulation (Peng & Sutton, 1990; Kohl *et al.*, 1992; Kohl *et al.*, 1995) thereby recognising the important contribution of reproduction in the epidemiology of a polycyclic pathogen (Fokkema, 1993, 1995).

Table 5.1. Isolates of filamentous fungi, yeast and bacteria reported for biocontrol of pre-harvest diseases caused by *B. cinerea*. *In vivo* = excised plant tissue assays, * = *in vivo* screening with wet/dry humidity regime and (1) = sporulation suppression assessed.

Crop	Isolates	Screening method	Reference
Tomato	*Rhodotorula glutinis*	*In vivo*	Elad, Köhl, Fokkema
Bean (*Phaseolus vulgaris*)	*Cryptococcus albidus*	*In vivo*	(1994b)
	Bacillus pumilus	*In vivo*	Elad Köhl & Fokkema
	Xanthomonas maltofilia	*In vivo*	(1994a)
	Pseudomonas sp.	*In vivo*	
	Lactobacillus sp.	*In vivo*	
	Gliocladium catenulatum	*In vivo*	
Lettuce	*Streptomyces griseoviridis*	Unknown	White, Linfield, Lahdenpera & Uoti (1990)
Apples	*Trichoderma* sp.	Unknown	Tronsmo & Ystaas (1980)
Tomatoes	*Cladosporium* sp.	*In vivo*	Eden,Hill & Stewert
	Trichoderma sp.	*In vivo*	(1996)
Onions	*Gliocladium roseum*	*In vivo*	Köhl *et al.*, (1992)
Lilium sp.	*Ulocladium atrum*	*In vivo** (1)	Köhl, Molhoek, van der Plas & Fokkema (1995)
	Penicillium sp.	*In vitro* and *In vivo*	Malathrakis &
	Alternaria alternatum	*In vitro* and *In vivo*	O'Kritsotaki (1992)
	Trichoderma sp.	*In vitro* and *In vivo*	
Strawberry	*Gliocladium roseum*	*in vivo* (1)	Sutton (1995)
	Trichoderma viride	*in vivo* (1)	Sutton & Peng (1993) Peng & Sutton (1990)

The postharvest environment is characterised by the absence of extreme fluctuations in temperature, wetness and radiation that characterise preharvest conditions (Wilson & Wisniewski, 1989; Whipps, 1994) where bacteria are poor survivors (Fokkema, 1993, 1995; Andrews, 1992). Many of the assays (Table 5.2) used wounds in fruit that have relatively high nutrient concentrations and free water compared to the intact phylloplane. Bacteria and yeasts with the ability to rapidly assimilate this resource and survive will be favoured as they have a high surface area to volume ratio compared with filamentous fungi.

Variants of the *in vivo* assay include the method of Wilson, Wisniewski, Droby & Chalutz (1993) and Cheah *et al.* (1996) where crude mixtures of candidate microbes were co-inoculated with the pathogen and the wound incubated. The dominant

colonists were isolated from those sites that exhibited biocontrol. This assay design enables efficient identification of those microbes with an ability to colonise the host in highly competitive environment.

Table 5.2. Isolates of yeast and bacteria reported for biocontrol of post-harvest diseases caused by *B. cinerea*. *In vivo* = assays conducted on excised tissue or wounded fruits of target host. *In vivo** = wounded fruit assay modified by Wilson *et al.* (1993) and *in vitro* =agar plate assays.

Crop	Isolates	Isolation method	Reference
Apples	*Acremonium breve*	*In vivo*	Janisiewicz (1988)
	Pseudomonas sp.	*In vivo*	
	Sporobolomyces roseus	*In vivo*	Janisiewicz, Peterson & Bors (1994)
	Cryptococcus laurentii	*In vivo*	Roberts 1990
	Candida sake	*In vivo**	Wilson, Wisneiwski, Broby & Chalutz (1993)
	C. tenuis	*In vivo**	
	C. oleophila	*In vivo**	
	Bacillus subtilis	*In vivo*	Sholberg, Marchi & Bechard (1995)
	Debaromyces hansenii	*In vivo*	Wisniewski, Wilson, Chalutz, Hershberger (1988)
	Trichosporon sp.	*In vivo*	Gullino *et al.* (1992)
	Candida sp.	*In vivo*	
Apples Pears	*Pseudomonas cepacia*	*In vitro*	Janisiewicz & Roitman (1988)
Pears	*Pseudomonas syringae*	*In vivo*	Janisiewicz (1992)
Grapes / Tomatoes	*D. hansenii*	*In vivo*	Chalutz *et al.* (1988)
Cherry	*Enterobacter sp.*	*In vitro* then *in vivo*	Utkhede & Sholberg (1986)
	Bacillus sp.	*In vitro* then *in vivo*	
Roses	*Exophiala jeanselmei*	*In vivo*	Redmond, Marois & MacDonald (1987)
	Coryneform bacteria	*In vivo*	
	Candida guillermondi	*In vivo*	McLauglin *et al.*(1992)
	Kloeckera apiculata	*In vivo*	
Brassica sp.	*Pseudomonas sp.*	*In vitro* then *in vivo*	Leifert, Sigee & Epton (1992)
	Bacillus sp.	*In vitro* then *in vivo*	
	Serratia sp.	*In vitro* then *in vivo*	

There are other reports of baiting type of techniques designed to isolate mycoparasites (Deacon & Berry, 1993), Dinghra & Sinclair (1985). However the adhesion mechanism as a presumptive test for biocontrol has not been exploited.

5.2 Objectives

To evaluate a collection of attacher bacteria and yeasts obtained from the attachment assay (Chapter Three) for biocontrol activity against *B. cinerea*.

There were three aspects to this study:

(1) The frequency with which attacher microbes were selected from crude field samples using the attachment assay.

(2) Biocontrol activity against *B. cinerea* of attacher mixtures and pure isolates

(3) The identification of the genus and species of those isolates that conferred high levels of biocontrol.

5.3 Materials and Methods

5.3.1 Collection of BCA candidate microbes

Two sample sites were used for the collection of candidate micro-organisms; an apple, nashi, kiwifruit and peach orchard operated under organic management principles was visited twice (January 1995 and March 1995) and a second site, an area of native and introduced flora in the Turitea valley next to the Massey University campus Palmerston North was visited once (February 1995). At each site 4 leaves and 2-3 fruits (where applicable) per plant species were removed and placed into the Plastic Clip Seal bags (150mm x 100mm) ensuring that the material was handled by the petiole or pedicel only. Soil samples were taken from immediately below the target plants by removing surface organic debris and sampling the top 5 cm of the soil, again placed into Clip Seal plastic bags.

Plant species sampled from each location are listed in Table 5.3.

Table 5.3. List of the plant hosts from which leaf, fruit and soil samples were removed to obtain candidate microbes for BCA selection.

Turitea Valley	**Levin Organic Orchards.**
KawaKawa (*Macropiper excelsum)* Hydrangea (*Hydrangea macrophylla*) Convolvulus (*Convolvulus arvensis*)	Apple (*Malus* X *domestica*) Red Delicious Fuji Granny Smith Coxs Orange Pippen Nashi (*Pyrus pyrifolia*) Nijisiki Peach (*Prunus persica*) Kiwifruit (*Actinidia deliciosa*) Shelter belt plants *Acacia* spp. Blue Gum (*Eucalyptus* sp.) Macrocarpa (*Cupressus macrocarpa*) Wattle (*Acacia* sp.) Lombardy poplar (*Poplus nigra*) Maple (*Acer* sp.)

5.3.2 Candidate microbe extraction

In the laboratory, 20 ml of Sterile Distilled Water (SDW) was added to fruit and leaf samples, the bags sealed expelling any air within and were shaken and massaged gently for 2 min. The washings were poured into 25 mm diameter filter holders and vacuum filtered through a 0.2 µm pore filter (Sartorius SM 113 07 025N).

Soil samples were mixed thoroughly with 50 ml SDW and shaken for 2 min before prefiltering (Whatman #1 filter) to remove large soil and organic particles prior to 0.2 µm filtering. Candidate microbes were washed from the filters, resuspended in 10 ml SDW and stored at 4°C until required.

5.3.3 Culture of Botrytis cinerea.

Growth and preparation of *B. cinerea* (isolate BC 20) spore suspension is described in Chapter Two (Section 2.1). Suspension concentration was adjusted to $5x10^7$ spores/ml.

5.3.4 Co-incubation

Dwarfbean seedlings (*Phaseolus vulgaris* cv 'Topcrop') were grown in pumice for 20 days. Fifty millimetre disks were cut from the fully expanded leaves and laid in the bottom of 50 mm diameter glass petri dishes. After autoclaving for 5 min, 500 µl of SDW was pipetted into the petri dish and a sterile washed 50 mm diameter disk of cellophane (Jam & Preserve Covers, Caxton Ltd Christchurch) placed over the leaves.

Four replicate plates of the leaf-cellophane media were prepared from each field sample. They were inoculated with 50 µl *B. cinerea* spore suspension and 950 µl of microbe extract then two plates were incubated at 10°C for 20 h and the remaining two at 15°C for 16 h. At the completion of the incubation period, the microbes and *B. cinerea* on the cellophane leaf disks were suspended in 5 ml McIlvaines Buffer (pH 6.0) (See Chapter Three) and agitated gently with a glass hockey stick to remove the colonies.

5.3.5 Extraction of attacher microbes

Extraction of microbes attached to hyphae and conidia of *B. cinerea* was carried out using the attachment assay procedure (Chapter Three). The crude pathogen / candidate mixture in McIlvaines buffer was poured into the mesh holder fitted with a sterile nylon mesh and washed to remove contaminant microbes (as described in Section 3.4.4). For each leaf, soil or fruit sample, the mesh together with *B. cinerea* hyphae and attached microbes were placed into universal bottles containing 10 ml of either nutrient broth (NB) or nutrient yeast dextrose broth (NYDB), on an orbital shaker (Certomat M, B Braun, Melsungen AG) set at 150 rev/min and returned to 10°C for 72 h or to 15°C for 48 h.

In later collected field samples (1 February and 14 March), the universal bottles were replaced with 50 ml conical flasks filled with 25 ml of broth and stoppered with cotton wool as it was found that conditions became anaerobic in the bottles.

After incubation, the broths were prefiltered through Cell Strainers pore size 70µm (Falcon 2350) to remove *B. cinerea* mycelium then filtered through a 0.2 µm filter to trap bacteria and yeasts. Mixtures from the attachment assay were resuspended in10 % glycerol then stored at -20 °C until required.

5.3.6 In vivo bioassay for biocontrol by attacher mixtures

A 500 µl aliquot from each sample containing the mixture of attacher microbes was pippetted onto NA or NYDA and incubated at either 10°C or 15°C for 3 days. The choice of media and temperature were determined by the conditions used during the extraction process. Cells from the resulting colonies were suspended in SDW and used in the assay for biocontrol.

Seedling tomato plants (*Lycoperiscon esculentum* cv. 'MoneyMaker') ca. 5-6 weeks old were grown in medium term bark potting mix at Plant Growth Unit (Massey University). At harvest, the leaves were removed from the plant and the stems surface sterilised in 0.04M Sodium Hypochlorite plus 1% Tween 20 (pH adjusted to 7.5 with 50% Acetic Acid) for one minute before the residues were washed away under running tap water (see Chapter Two, Section 2.3.2 for details).

The sterilised stems were aseptically cut into 2 cm lengths and randomised by shaking in a sterile container. They were placed vertically in microtitre plates with well bases removed (See Chapter Two, Section 2.4). Eight stem pieces per replicate were used for each BCA candidate mixture with treatments replicated once. Treated stems were incubated at 15°C in tray containers lined with moist paper towels and sealed with plastic film.

One millilitre of crude mixtures of BCA candidates from the attachment assay were applied to the tomato stem pieces using the Potter Tower (See Chapter Four) at a nozzle pressure of 100 mm Hg. Total cell counts of a selection of suspensions showed concentrations of $1x10^{10}$ to $4x10^{10}$ cells/ml. The same tissues were immediately challenged with a 1 ml aliquot of the *B. cinerea* suspension applied in the same way. Control inoculations of water only and *B. cinerea* only were prepared to assess natural infections and confirm the pathogen challenge. Tissues were incubated at high humidity in sealed containers lined with sterilised moistened paper towels and incubated at 15°C for ten days with 12 h D/N regime.

During incubation, the tissues were assessed for signs of *B. cinerea* infection and those with hyphal growth were immediately removed to prevent secondary contamination. Those BCA mixtures that showed high levels of disease control in the

first bioassay were reapplied to a second batch of excised tomato stems using the above inoculation, incubation and assessment protocols.

5.3.7 Confirmation of attachment.

Baited *B. cinerea* hyphae (See section 5.3.4) washed using the attachment assay procedure (Section 3.3.4) were mounted onto microscope slides and observed using dark field or phase contrast microscopy.

5.3.8 Purification of bacteria and yeasts from mixtures

Bacterial mixtures 1 and 27 extracted into NA broth were streaked on NA and incubated at 15°C for 3 days. Individual colonies were examined for colour, colony shape and form and were re-isolated until uniform colonies were consistently obtained.

Yeast mixtures 662,622,572,552,561,532 were similarly treated but colonies were cultured on NYDA (pH 4.5 adjusted using 6 M HCl) and incubated at 15°C for 3 days. Colony colour, form and shape were used as determinants of different species and as an indicator of purity. These cultures were suspended in 10 ml SDW and diluted 100-fold and 10,000-fold then 100 µl aliquots were pipetted onto fresh NYDA and spread using a sterile glass hockey stick. After 3 days at 15°C bacterial contaminants appeared in the yeast cultures but were eliminated by subsequent culture on NYDA amended with four concentrations (0, 50, 100 and 200 mg/ litre) of chloramphenicol (BDH 442042Q) (Hagler & Ahearn, 1987). All bacteria and yeast isolates were stored at -20 C in 25% glycerol until required.

5.3.9 Bioassay for biocontrol of the purified isolates

All twenty BCA isolates (8 yeast and 12 bacterial isolates) were tested for *in vivo* biocontrol activity. Each candidate culture, grown at 15°C for 5 days was suspended in SDW, centrifuged at 12,000 **g** for 5 min, then resuspended in SDW. Suspension concentrations were determined using a Petroff Hauser counter (Chapter Two) and suspensions adjusted to, 5×10^9 cells/ml, 1×10^{10} cells/ml and 2×10^{10} cells/ml. This represented a 100, 200 and 400 times increase in BCA suspension concentration compared to the *B. cinerea* challenge (5×10^7 spores/ml). Mean cfu densities on the inoculated surface

were determined to be 7400, 13,000 and 23,000 yeasts cfu/mm^2 respectively and 900, 1600 and 2700 bacteria cfu/mm^2 respectively for the above cell suspension concentrations while *B. cinerea* challenge was 300 spores/mm^2 (See Chapter Four).

The BCA and *B. cinerea* inoculum were applied to 30 tomato stem pieces held vertically in modified micro well plates using the Potter Tower (Chapter Four). One millilitre aliquots of each concentration of each BCA were pipetted into the reservoir and applied at a nozzle pressure of 100 mm Hg immediately followed by a 1 ml aliquot of *B. cinerea* challenge inoculum.

Four replicate applications of both BCA and *B. cinerea* were made to fresh batches of 30 tomato stems for all 20 BCAs at all three concentrations. After inoculation, the 30 stem pieces were split into three groups of 10 stems and each group was incubated at 1°C ,7°C or 15°C in plastic tray containers lined with moist paper towels and sealed with plastic film. Four pathogen-only inoculations of stem tissue were carried out after every seven BCA treatments during the inoculating phase of this experiment in order to assess the pathogenicity of the *B. cinerea* challenge and how it may vary with time. Stem pieces were assessed daily up to 18 days (1°C), 11 days (7°C) and 8 days (15°C) after inoculation. Diseased stems were removed periodically to reduce the risk of secondary contamination.

5.3.10 BCA candidate identification

Bacterial isolates were identified at the Ministry of Agriculture and Fisheries Plant Protection Laboratory, Lincoln, Canterbury and yeasts at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

5.3.11 Timing of BCA inoculation

Randomly selected bacterial and yeast isolates (ox8a, ox9, 27a 532, 662dia and 662dib) were applied with suspension concentrations of 5 x 10^9 cells/ml and 1 x10^{10} cells/ml; 24 h before, simultaneously, and 24 h or 48 h after pathogen inoculation in the same manner as described in Section 5.3.9. Pathogen challenge remained at 5x10^7 spores/ml. To assess the pathogenicity of *B. cinerea* two control treatments were used: inoculation of the stem piece immediately after tissue preparation or inoculation after a 24 h incubation. All treated stems were incubated at 15°C, 12h D/N for 8 days in plastic containers lined with moist paper towels and sealed with plastic film.

5.3.12 Statistical analysis

Separate statistical analyses were carried out for each purified yeast and bacterial isolate in the *in vivo* biocontrol bioassay using a completely randomised block experimental design. Customised hypothesis tests using linear contrasts were also used to compare percentage of healthy stems in the pathogen-only control with the mean from the three BCA concentration treatments for each individual BCA.

For the timing of BCA application trial, the experiment and data analyses were carried out according to a split plot model to each individual bacterial or yeast isolate tested (main plot= concentration and split plot=time).

5.4 Results

5.4.1 Attachment and biocontrol of crude bacteria and yeast mixtures.

A total of 66 field samples of washings from leaves, fruitlets and soils on or near various host plants were collected. Attachment microbes were detected in 35 of the first 36 field samples taken from the Levin site in January 1995 (Table 5.4a). These mixtures, consistently reduced disease incidence from 100% in the control to between 0% and 33% *in vivo*. Microbes in samples from the second field visit to the Turitea valley (February 1995) did not show strong plant protection activity ranging from 50% to 100% in co-inoculated stems (Table 5.4b).

A further 17 samples were taken from the Levin site (March 1995) and attacher microbes were observed in all of the samples where NA was used as the extractant medium (Table 5.4c) and all but samples 50, 51 and 61 using NYDA as the extractant medium (Table 5.4c). Typical adhesion morphologies from either bacterial or yeast mixtures are shown in Figs 5.1 and 5.2. Fig. 5.3 shows the mats of cells formed in the pathogen-BCA interactions, where yeast or bacterial cells congregated around *B. cinerea* hyphae and conidia, but were absent in the pathogen-only controls. Bacteria from NA extractant at 10°C consistently reduced disease incidence to between zero and two stems out of six but biocontrol of *B. cinerea* conferred by other mixtures varied widely. Thus at 15°C good control was obtained, with the exception of samples 49, 50, 52 and 55. Yeasts from the NYDA broth extractant were more variable in efficacy, for example, at 10°C, samples 49,52 and 55 were less effective than the remainder (Table 5.4c). At the higher incubation temperature (15°C), biocontrol was poor in most samples except for 53 and 56.

Table 5.4a. Samples of leaves, fruitlets and the soil beneath kiwifruit, apple, nashi and peach trees in an organic orchard located at Crop & Food Research Institute, Levin. Sample taken January 1995. Where y=attachment detected.
(1) = Presence of attacher microbes as detected by Dark Field microscopy after samples processed using the attachment assay.
(2) = The number of diseased excised tomato stem pieces with *B. cinerea* infection co-inoculated with BCA mixture (total =6).
Sample numbers in bold type = those from which bacteria were isolated into pure culture for further research in the remainder of this thesis.

(A)

Sample #	**1**	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Attachment[1]	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y
# Diseased Stems[2]	0	1	0	0	0	1	2	0	1	2	0	2	0	0	2	1	0	1
Sample #	19	20	21	22	23	24	25	26	**27**	28	29	30	31	32	33	34	**35**	36
Attachment[1]	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y
# Diseased Stems[2]	0	0	0	0	0	0	0	0	0	1	1	2	1	0	0	1	0	2

Table 5.4b. Samples of leaves from native flora from Turitea Valley located beside Massey University campus (Sample takem February 1995). Where nd=not determined.

(B)

Sample #	37	38	39	40	41	42	43	44	45	46	47	48
Attachment	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
# Diseased Stems	3	6	3	4	4	3	5	1	1	0	nd	nd

Table 5.4c. Samples of leaves (L), fruitlets (F) and soil (S) from beneath kiwifruit, apples, nashi and peach trees in an organic orchard located at Crop & Food Research Institute, Levin. Sample taken March 1995. Where, media= the culture media the candidates were grown, temp=incubation temperature and,
(1) = The number of diseased excised tomato stem pieces with *B. cinerea* infection co-inoculated with bacteria (NA) or yeast (NYDA) BCA mixture (total =6) extracted from bait at 10°C (nd=not determined).
(2) = The number of diseased excised tomato stem pieces with *B. cinerea* infection co-inoculated with bacteria BCA mixture (total =6) extracted from bait incubated at 15°C (nd=not determined).
(3) = Presence of attacher microbes as detected by Dark Field microscopy after bacteria (bact) or yeast samples were processed using the attachment assay (y=attachment detected and n=no attachment detected).

(C)			Peach		Kiwifruit			Apple			Nashi			Orchard Shelter Belt						
Plant Part			F	L	F	L	S	F	L	S	F	L	S	L	L	L	L	L	L	L
Sample #	Microbe	Temp	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
# diseased stems	Bacteria	10[(1)]	1	0	0	0	1	2	2	1	0	0	0	0	0	1	0	0	0	0
		15[(2)]	5	4	nd	1	0	6	1	nd	nd	2	1	3	1	0	1	0	2	0
	Yeast	10[(1)]	4	1	2	5	1	3	5	3	1	2	1	3	3	0	0	0	nd	0
		15[(2)]	3	6	nd	nd	1	5	nd	1	nd	6	5	6	6	6	nd	nd	4	5
Attachment[(3)]		Bact	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y
		Yeast	y	n	n	y	y	y	y	y	y	y	y	y	n	y	y	y	y	y

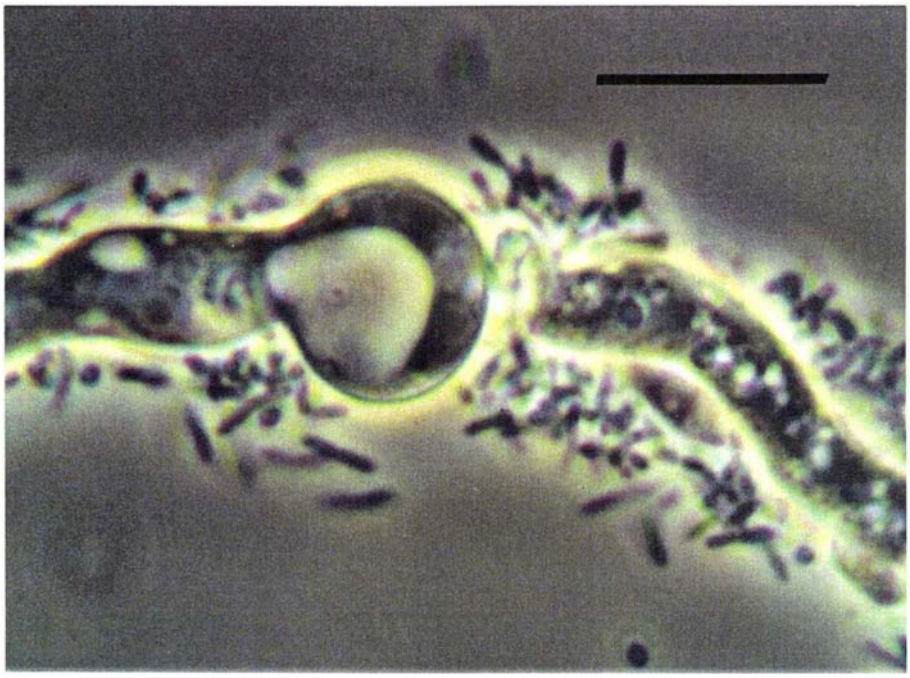


Fig. 5.1 Typical bacterial cell adhesion to *B. cinerea* germlings incubated in SDW obtained from the attachment assay. Bar = 10 µm.

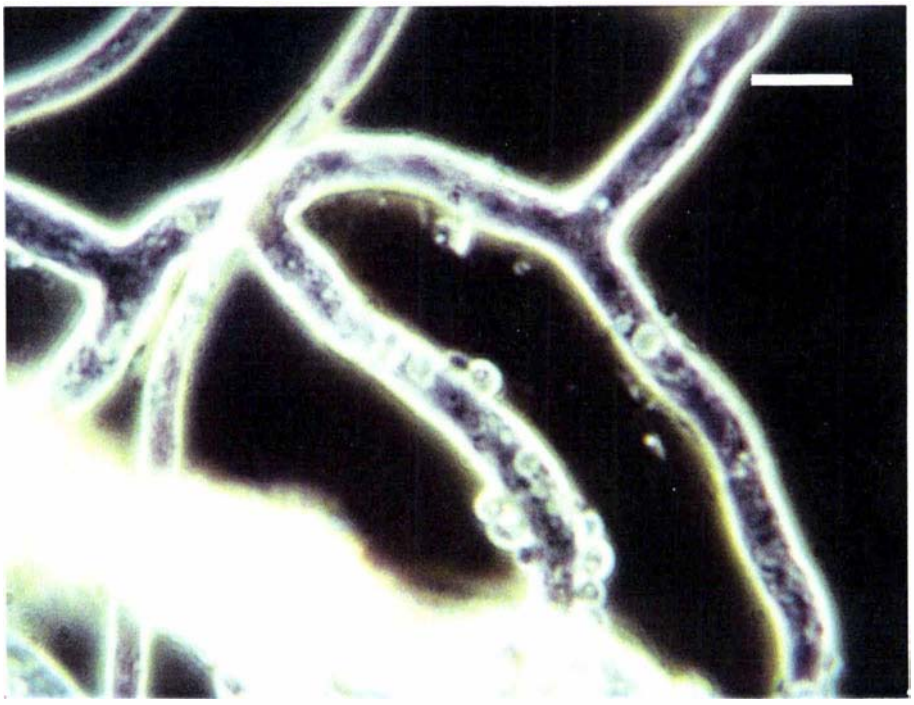


Fig. 5.2. Typical yeast cell adhesion to *B. cinerea* germlings incubated in SDW obtained from the attachment assay. Bar = 10 µm.

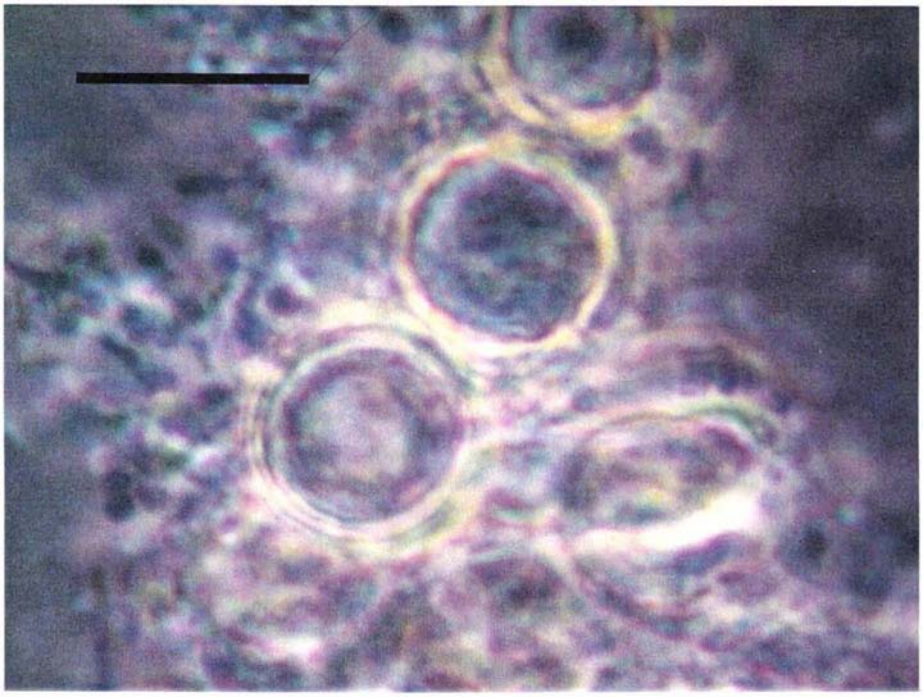


Fig. 5.3. Congregation of bacteria cells around *B. cinerea* hyphae from co-incubated samples from the attachment assay. Bar= 10 μm

5.4.2 Biocontrol by pure isolates of bacteria and yeasts

Biocontrol was recognised by the failure of the pathogen to cause disease. Symptoms of successful infection varied and included pathogen signs and / or soft brown lesions in the tissue (Fig. 5.4). These symptoms were absent in stem pieces when BCA was applied and were replaced with browning of host tissue at the inoculation site, dry surface tissue and the stem remained turgid (Fig. 5.4).

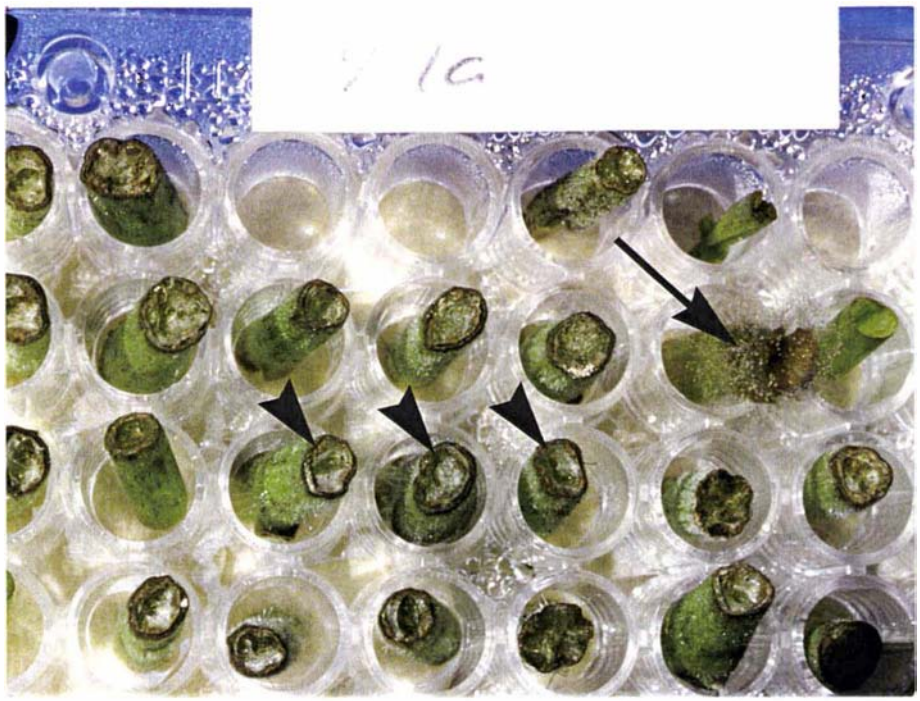


Fig. 5.4. An example of symptoms of diseased tomato stem pieces (Arrow) and those where BCA candidate 27a stopped infection (Arrowhead). Note the brown and dry symptoms of stems with BCA added.

A total of 8 yeast and 12 bacterial isolates were obtained from the selected mixtures and showed biocontrol activity in the tomato stem bioassay at 1°C, 7°C and 15°C (Figs 5.5 - 5.9). At 15°C all of the stem pieces inoculated with isolates ox6, ox7, and ox9 remained healthy at all three application rates compared with 0% to 10% of stems remaining healthy in the pathogen-only control. Isolates ox8a, ox1, 27a, ox4, ox2 and 35a also conferred a strong biocontrol effect with at least 80% of treated stems remaining healthy. Isolates 22b, ox3 and ox5 were less effective with a maximum percentage of healthy stems of 70%, 80% and 70% respectively.

Protection conferred by the bacteria declined as temperature decreased although, isolates 35a and ox4, continued to show high levels of biocontrol activity at all temperatures and application rates. General biocontrol activity is reflected in the statistical comparison of the pathogen-only control (BCA density =0) with the average of the three BCA application rates. There were highly significant differences in disease incidence ($P<0.0001$) at 15°C for all bacterial isolates and at 7°C for all except 22b, ox3 and ox5. Isolates ox4 and 35a were the only bacterial isolates exhibiting biocontrol

activity significantly (P<0.0001) different from the controls at 1°C. There was enhanced *B. cinerea* pathogenicity at 1°C in treatments inoculated with isolates ox7 and ox8a.

Most of the yeast isolates tested gave levels of biocontrol between 90 and 100% in at least one temperature / BCA concentration combination. Yeast isolate 622b was the best performing yeast isolate and gave consistent biocontrol (P<0.0001) at all three BCA application rates and all three incubation temperatures. Yeasts 662dia and 662dib showed similar levels of biocontrol (>90%) at 15°C and 7°C at the two higher cfu densities whereas isolates 552c and 572c exhibited biocontrol activity of 90% to 100% at 15°C (P<0.0001) regardless of application rate but at 7°C this declined to 80% (P=0.0006) and 70% (P=0.023) and at 1°C to 60% (P=0.62) and 40% (P=0.61) respectively. Biocontrol activity of isolate 662e was poor at all temperature and inoculum rates. Biocontrol activity of isolates 561 ox3, ox5 and 22b, was concentration dependent and increased as concentration increased. Yeast 532 gave consistently high biocontrol (P<0.0001) at 15°C and at 1°C (P<0.0001) but declined at 7°C (P=0.027).

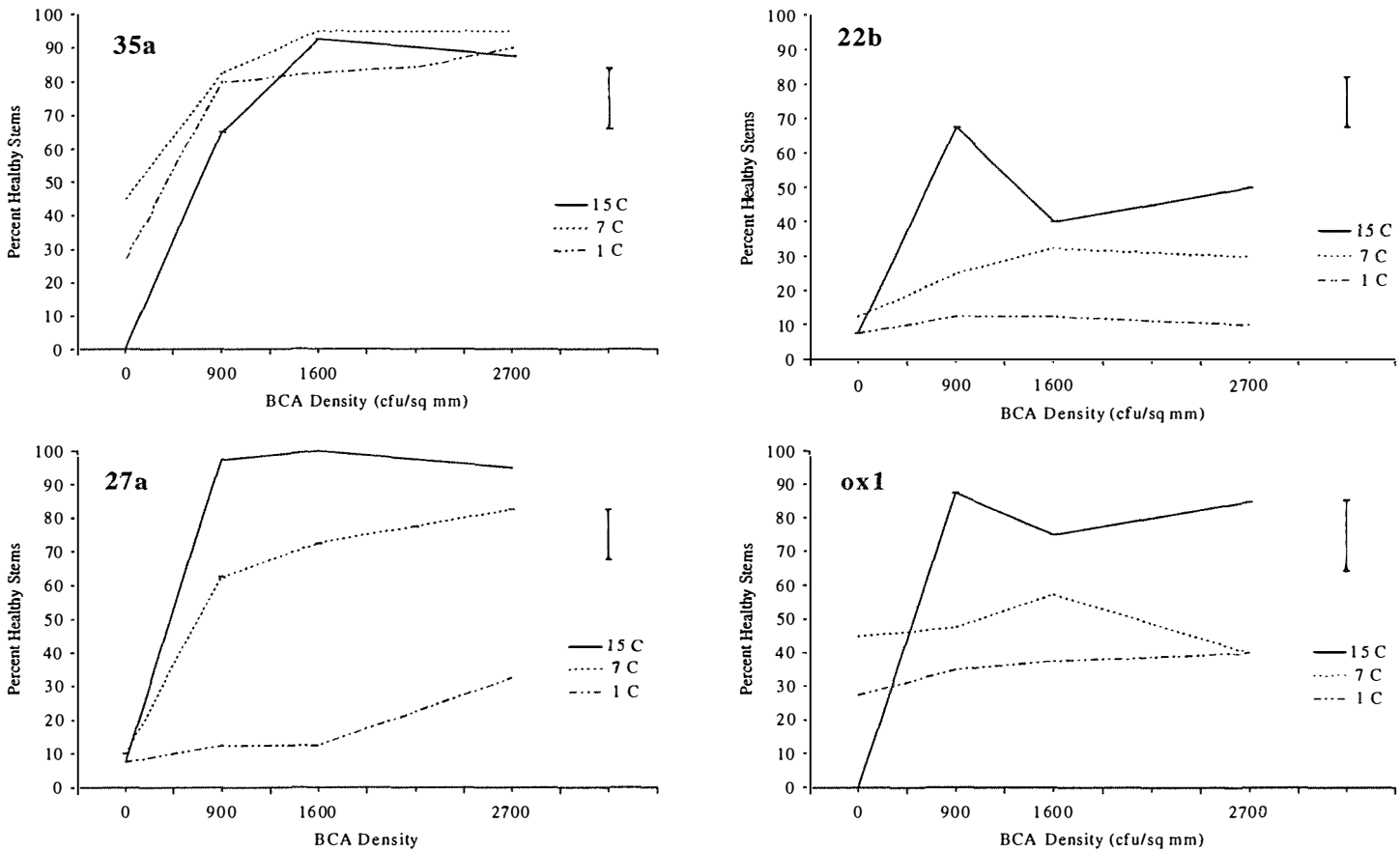


Fig. 5.5. Levels of biocontrol conferred by three concentrations (900, 1600 and 2700 cfu's/sq mm)of bacterial isolate candidates (35a, 22b, 27a and ox1) spray inoculated onto cut tomato stem pieces, incubated at 15°C, 7°C and 1°C and assessed after 8 days, 11 days and 18 days respectively. Pathogen challenge 300 spores / sq mm. Vertical bar represents interaction LSD (concentration x temperature) (df=33, n=4, α=0.05).

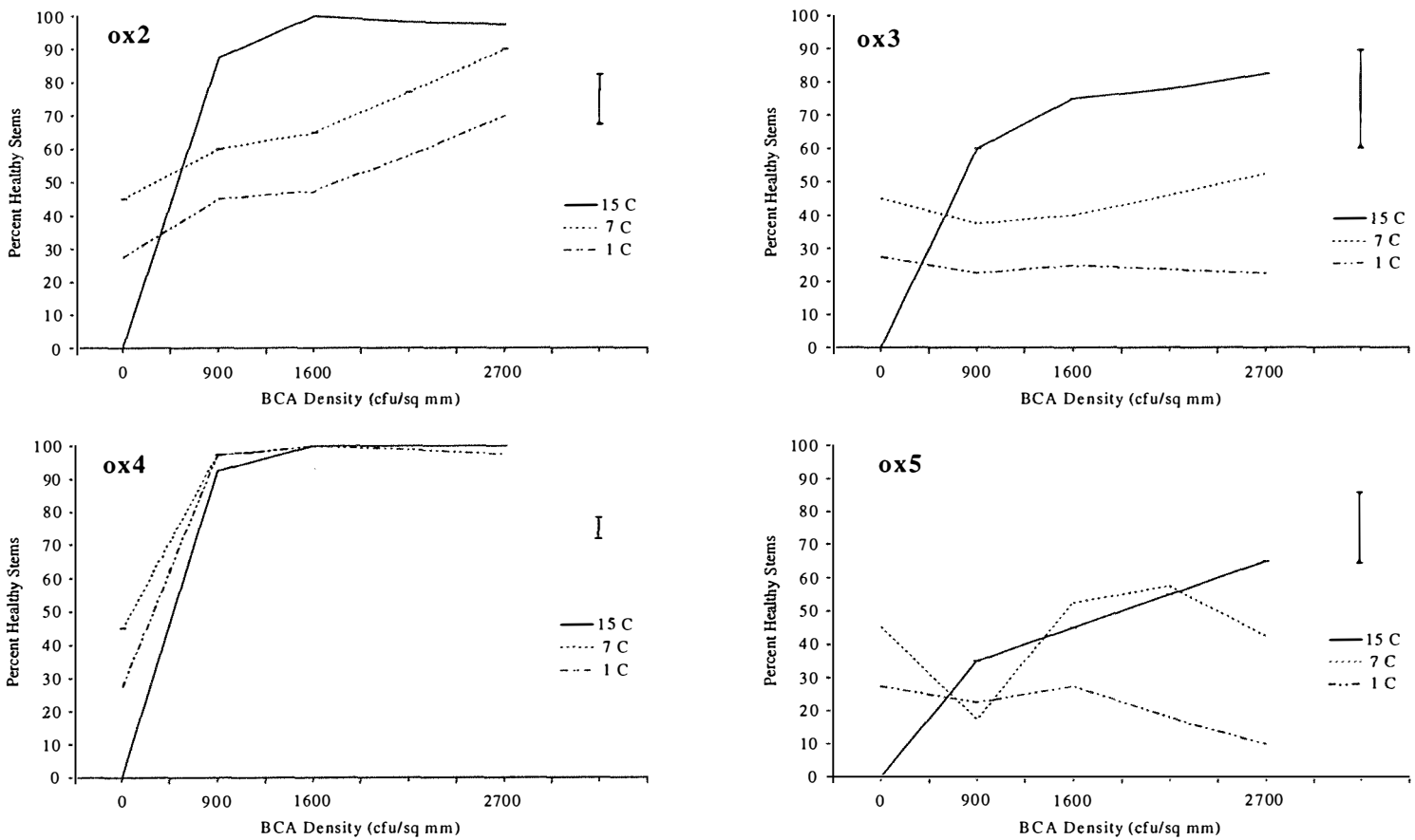


Fig. 5.6. Levels of biocontrol conferred by three concentrations (900, 1600 and 2700 cfu's/sq mm) of bacterial isolate candidates (ox2, ox3, ox4 and ox5) spray inoculated onto cut tomato stem pieces, incubated at 15°C, 7°C and 1°C and assessed after 8 days, 11 days or 18 days respectively. Pathogen challenge 300 spores / sq mm. Vertical bar represents interaction LSD (concentration x temperature) (df=33, n=4, α=0.05).

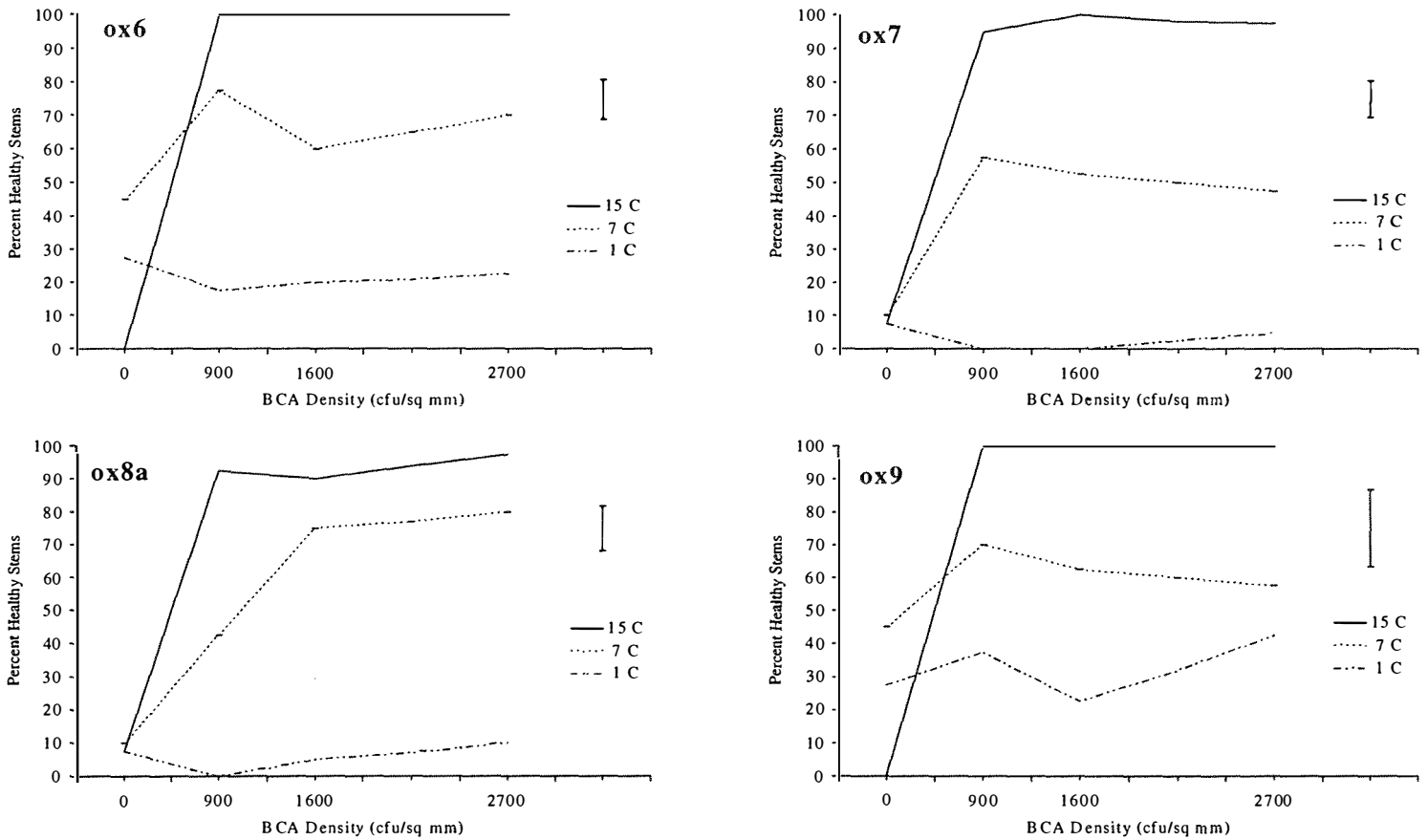


Fig. 5.7. Levels of biocontrol conferred by three concentrations (900, 1600 and 2700 cfu's/sq mm)of bacterial isolate candidates (ox6, 0x7, ox8a and ox9) spray inoculated onto cut tomato stem pieces, incubated at 15°C, 7°C and 1°C and assessed after 8 days, 11 days and 18 days respectively. Pathogen challenge 300 spores / sq mm. Vertical bar represents interaction LSD (concentration x temperature) (df=33, n=4, $\propto$=0.05).

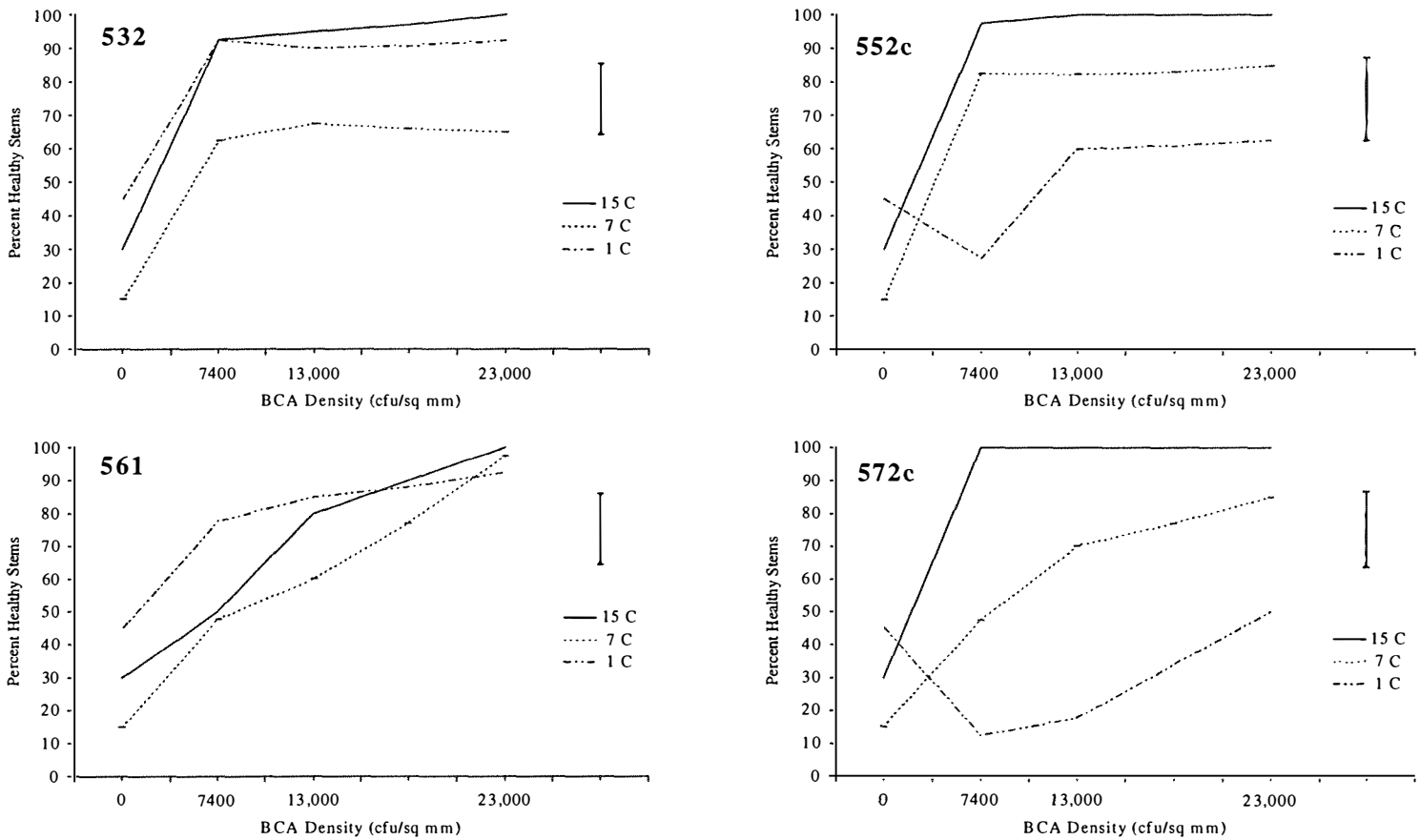


Fig. 5.8. Levels of biocontrol conferred by three concentrations (7400, 13,000 and 23,000 cfu's/sq mm)of yeast isolate candidates (532, 552c, 561 and 572c) spray inoculated onto cut tomato stem pieces, incubated at 15°C, 7°C and 1°C and assessed after 8 days, 11 days and 180 days respectively. Pathogen challenge 300 spores / sq mm Vertical bar represents interaction LSD (concentration x temperature) (df=33, n=4, ∝=0.05).

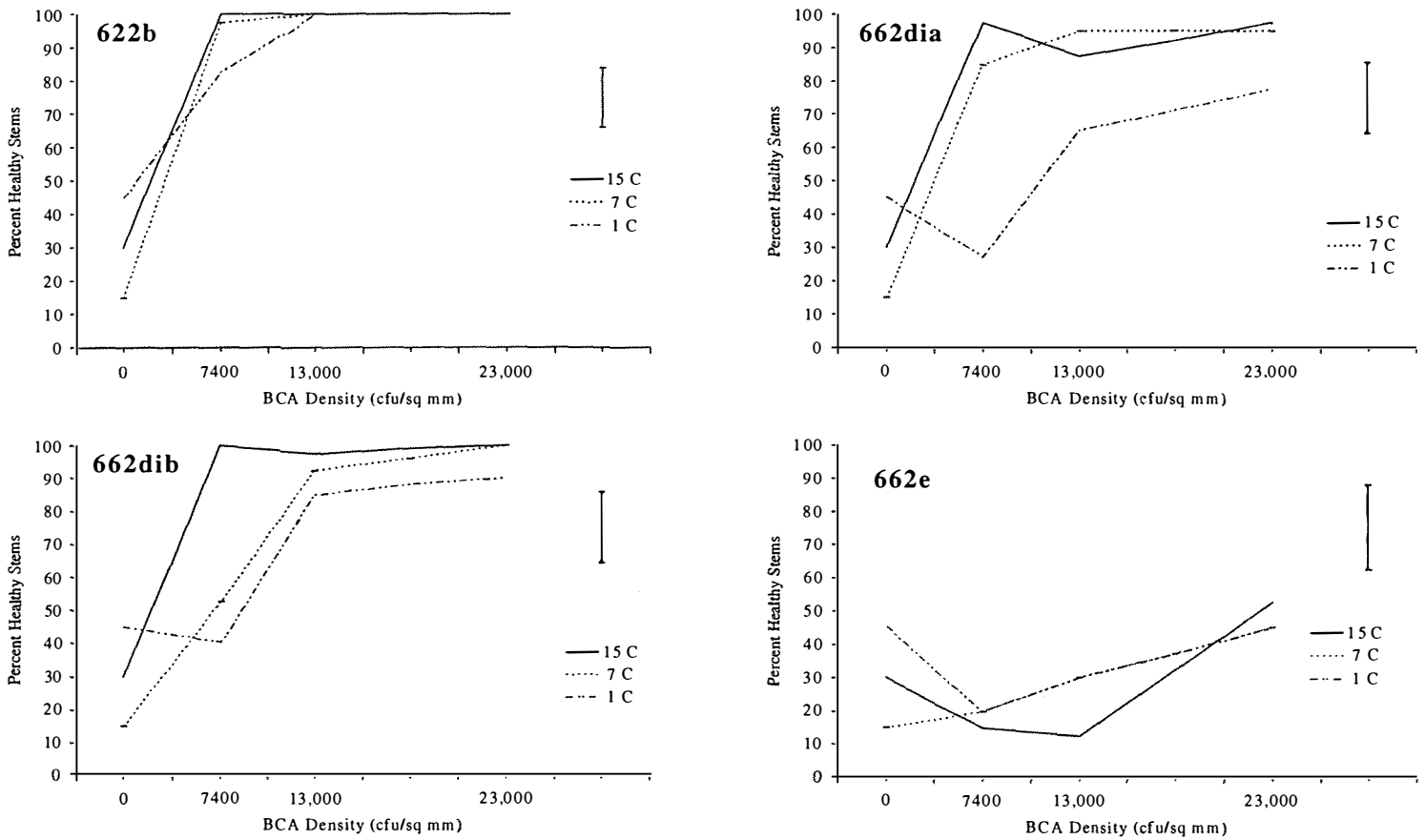


Fig. 5.9. Levels of biocontrol conferred by three concentrations (7400, 13,000 and 23,000 cfu's/sq mm) of yeast isolate candidates (622b, 662dia, 662dib and 662e) spray inoculated onto cut tomato stem pieces, incubated at 15°C, 7°C and 1°C then assessed after 8 days, 11 days and 18 days respectively. Pathogen challenge 300 spores / sq mm. Vertical bar represents interaction LSD (concentration x temperature) (df=33, n=4, α=0.05).

5.4.3 Biocontrol agent identification

Six different species or sub-species of bacteria and five species of yeast were identified among the isolates chosen for their strong biocontrol characteristics (Tables 5.5 and 5.6)

Table 5.5. Identification by the Ministry of Agriculture and Fisheries Plant Protection Laboratory of the bacterial isolates that showed high biocontrol activity.

Isolate	Comment	Bacteria and authorities
27a	most closely resembling	*Enterobacter aerogenes* Hormaeche & Edwards
35a	most closely resembling	*Ochrobactrum anthropi* gen.nov.
ox2	most closely resembling	*Enterobacter agglomerans* (Beijerinck) Ewing & Fife
ox4		*Pseudomonas marginalis* Migula
ox6		*Enterobacter cloacae B* (Jordan) Hormaeche & Edwards
ox7		*Enterobacter cloacae B* (Jordan) Hormaeche & Edwards
ox8a	most closely resembling	*Enterobacter aerogenes* Hormaeche & Edwards
ox9		*Enterobacter cloacae* (Jordan) Hormaeche & Edwards

Table 5.6 Identification by the Centraalbureau voor Schimmelcultures of all yeast candidates tested in the *in vivo* tomato stem piece bioassay.

Isolate Number	Yeast and authorities
532	*Candida sake* (Saito & Ota) van Uden &Buckley
552c	*Galactomyces geotrichum* (E.E. Butler & L.J. Peterson) Redhead & Malloch group A
561	*Candida sake* (Saito & Ota) van Uden &Buckley
572c	*Galactomyces geotrichum* (E.E. Butler & L.J. Peterson) Redhead & Malloch group A
622b	*Trichosporon pullulans* (Lindner) Diddens & Lodder
662dia	*Candida sake* (Saito & Ota) van Uden &Buckley
662dib	*Candida pulcherrima* (Lindner) Windisch
662e	*Rhodotorula fujisanensis* (Soneda) Johnson & Phaff

5.4.4 Timing of BCA application

The percentage of healthy stems increased from 12% when inoculated immediately after tissue cutting (C0) to between 70% and 100% when the stems also received an immediate application of BCA 27a, 662dia, 662dib or 532 (0), or when *B. cinerea* inoculation to the tissue was delayed by 24 h (C-24) (Figs 5.10 and 5.11). Isolates ox9 and ox8a, increased plant protection to between 20% and 50% (Fig. 5.10).

Delayed application of BCA to pathogen inoculated stem pieces generally resulted in a further increase in biocontrol for isolates ox9, ox8a, 27a, a decline for isolates 662dia and 532 or maintained a similar level for isolate 662b in at least one of the BCA densities applied. When the BCA was added to uninoculated stems after a 24 h incubation, the highest level of plant protection (90% to 100%) was observed.

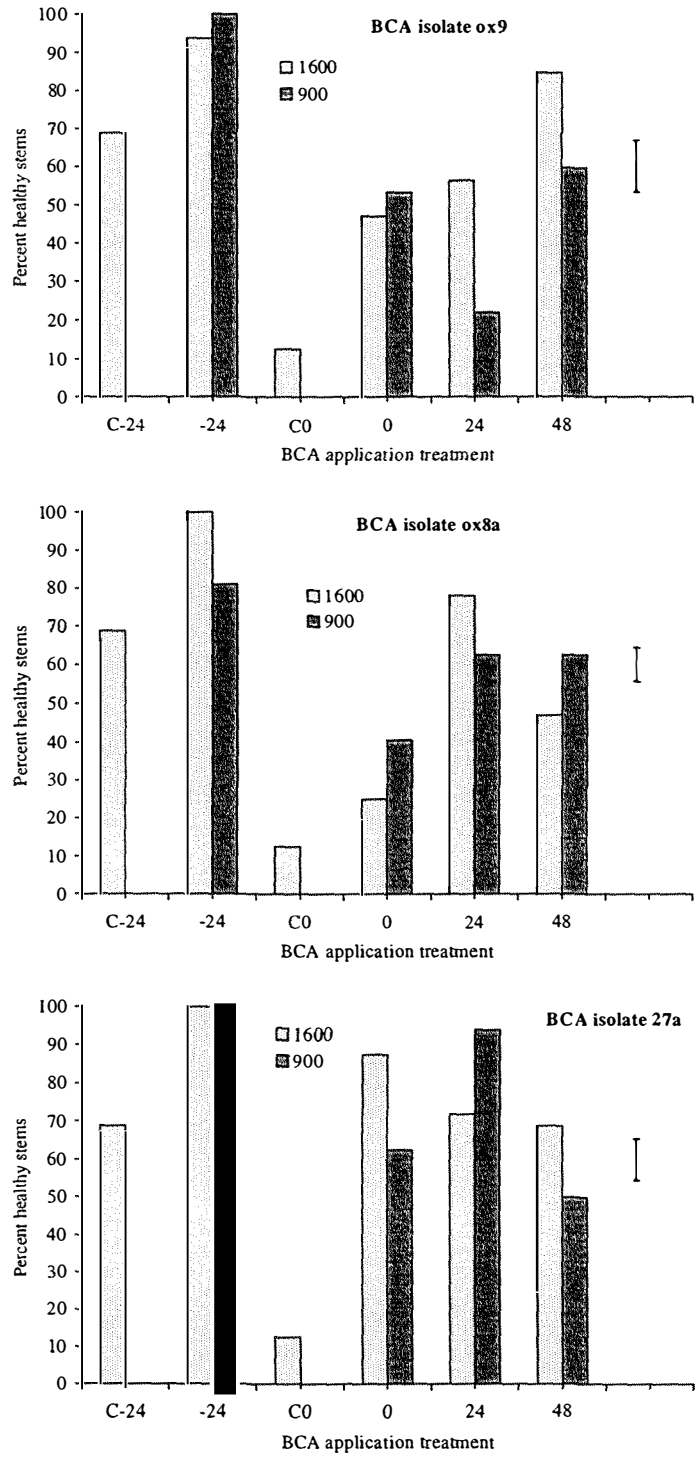


Fig. 5.10. Bacterial isolates (ox7, 0x8a and 27a) applied 24 h before, simultaneously and 24 or 48 h after *B. cinerea* spray inoculation to tomato stem pieces. Bacterial BCAs were applied at two densities; 900 cfu/mm^2 and 1600 cfu/mm^2. Control treatments include stem tissue preparation followed by immediate *B. cinerea* spray inoculation (C0) or 24 h delay (C-24). Treatments were incubated at 15°C. Vertical bars represent LSD (concentration x time (df=27, n=4, α=0.05))

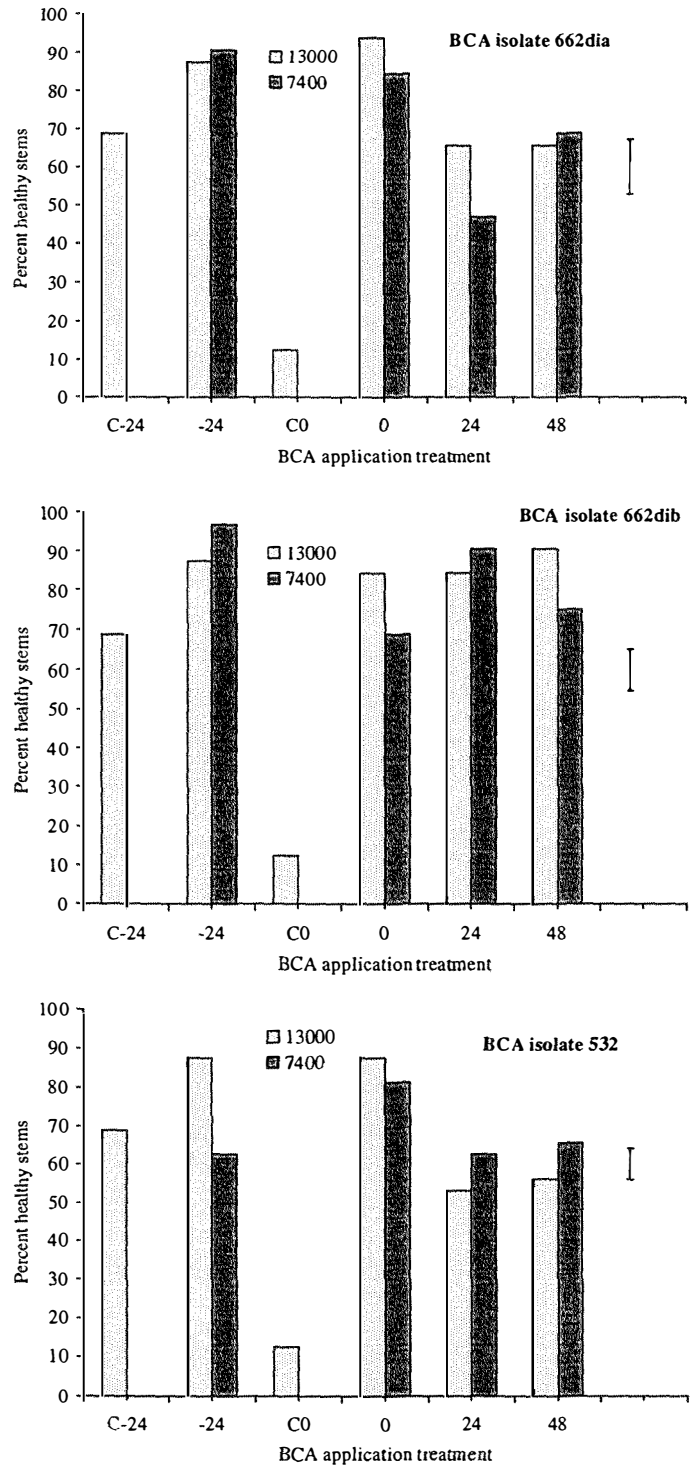


Fig. 5.11. Yeast isolates (662dia, 662dib and 532) applied 24 h before, simultaneously and 24 or 48 h after *B. cinerea* spray inoculation to tomato stem pieces. Yeast BCAs were applied at two densities; 7400 cfu/mm^2 and 13,000 cfu/mm^2. Control treatments include stem tissue preparation followed by immediate *B. cinerea* spray inoculation (C0) or 24 h delay (C-24). Treatments were incubated at 15°C. Vertical bars represent LSD (concentration x time (df= 27, n=4, α=0.05))

5.5 Discussion

Results from biocontrol screening of crude attacher mixtures showed that attacher microbes crowding around hyphae of *B. cinerea* were found in those samples tested. The congregation of the attachers about fungi has been observed previously in similar inter-microbe interactions (Srivastava, Gupta & Arora, 1996) and was used to indicate attachment. However, systematic sampling for microbes capable of attachment to a fungal pathogen has not been previously reported, possibly due to the inability to clearly define these populations from contaminants in previous procedures. Attacher bacteria have been isolated from soil (Malajczuk, Nesbitt & Glenn, 1977; Nesbitt, Malajczuk & Glenn, 1981; Fradkin & Patrick, 1982; Homma, 1984; Malajczuk, Pearce & Litchfield, 1984; Fradkin & Patrick, 1985c; Wilson, Fradkin & Pusey, 1987; Wisniewski, Wilson & Hersberger, 1989; Yang, Menge, Cooksey 1994;), seed surface (Hadar, Harman, Taylor & Norton 1983; Nelson *et al.*, 1986; Nelson, 1988) and manure slurries (Toyota & Kimura, 1993). The yeast *P. guilliermondii* was obtained from the surface of lemon fruits (Wisniewski *et al.*, 1991). With the limited sampling of the current study, bacteria and yeasts with fungal attachment behaviour were found in soil, fruit and phylloplane sites and suggests that such microbes are found in habitats other than soil.

Biocontrol results from attacher mixtures (Table 5.4) showed a definite spatial trend in which strong BCAs were obtained. Although the pathogen was not sought, the Turitea valley site contained plant species that are not reported to be hosts of *B. cinerea* (Pennycook, 1989) whereas the Levin site contained a monoculture of fruit trees and vines that are hosts of *B. cinerea.* This definite pattern is inconsistent with the contention that source is unimportant (Andrews, 1992) and it is conceivable that the pathogen itself is a food source for attacher microbes that derive some benefit (mutualistic, commensal or necrotrophic or biotrophic parasitism) by colonising the fungal host. Therefore, the possible spatial correlation between attacher parasites and the incidence of *B. cinerea* would be an important consideration in future habitat selections.

In the bioassay on excised tomato stem pieces, 7 of the 12 bacterial isolates and 7 of the 8 yeast isolates tested *in vivo* gave 100% disease control on pathogen challenged stems in at least one inoculum concentration/temperature treatment combination. The selection efficiency of the isolation procedure appears considerably higher than that in recent report where good biocontrol was obtained from less than 7% of the 410 candidates tested against *Stemphylium vesicarium* (Montesinos, Bonaterra, Ophir & Beer, 1996) using excised host tissue assays. The results of this study could be the outcome of an exceptional habitat from which the candidates were obtained. However the evidence for a close relationship between attachment ability in *Pseudomonas* sp. (Yang *et al.*, 1994) or *E. cloacae* (Nelson *et al.*, 1986) and biocontrol of the pathogen, suggests that selecting a near-pure population of attacher microbes is likely to substantially increase the probability of selecting BCAs. Furthermore, in the biocontrol bioassay phase of screening the density of BCA cells on the tomato stem tissue relative to the pathogen challenge, are within or below those used previously. Consequently it cannot be argued that the frequency with which BCAs were isolated is due to overwhelming BCA populations.

Much attention has been given to the development of an efficient and accurate technique that will select BCAs whilst minimising resource requirements (Andrews, 1992; Nelson & Craft, 1992; Wilson *et al.*, 1993; Smilanick, 1994). In this study, cell adhesion was used as a putative mode of antagonism that was exploited for identifying biocontrol and resulted in a major improvement in the efficiency of obtaining potential isolates for BCA testing.

Yeast species previously recorded as BCAs to *B. cinerea* feature in this collection including *Candida sake* (Wilson *et al.*, 1993), *Trichosporon pullulans* (Holz, Schmidt & Ferreira, 1995) and the genus *Candida* sp. (Mercier & Wilson, 1995 McLaughlin *et al.*, 1992; Gullino *et al.*, 1992), *Trichosporon* sp. (Gullino *et al.*, 1992) and *Rhodotorula* sp. (Elad *et al.*, 1994b; Kampp, 1994). *Galactomyces geotrichum,* a weak attacher to *B. cinerea,* was not considered a biocontrol agent as this isolate (662e) conferred poor biocontrol activity *in vivo.* Bacterial candidates obtained using this protocol were dominated by *Enterobacter* species including *E. cloacae, E.*

agglomerans and *E. aerogenes*. All have been previously recorded as BCAs of a variety of fungal plant pathogens such as *Rhizopus stolonifer* (Wisniewski *et al.*, 1989), *Rhizoctonia solani* (Chernin, Ismailov, Haran & Chet, 1995), *Sclerotinia homoeocarpa* (Nelson & Craft, 1991), *Fusarium moniliforme in vitro* (Hebbar, Davey & Dart, 1992) and *Phytophthora* sp. (Utkhede & Smith, 1993; Levesque, Holley & Utkhede, 1993). Antagonism by *E aerogenes* or *E. cloacae* against *B. cinerea* were limited to *in vitro* plate trials (Utkhede & Sholberg, 1986; Lorito *et al.*, 1993). *Ochrobacter anthropii* and *Pseudomonas marginalis* have not been previously recorded as biocontrol agents. Whilst many of the isolates in the current collection (Tables 5.5 and 5.6) have been reported as BCAs, none appear to possess the ability to adhere to the pathogen, with the exception of *Enterobacter* sp. (Nelson *et al.*, 1986; Wisniewski *et al.*, 1989).

In this study, BCA populations were applied at rates of three to 80 times *B. cinerea* inoculum densities, which are lower than those used for *B. cinerea* biocontrol *in vivo* on comparable phylloplane sites or wounds. For example on autoclaved cabbage leaves a BCA / pathogen inoculum differential of 400 was used (Leifert *et al.*, 1992; Leifert, Sigee & Epton, 1992), or on detached tomato leaves the differential was 70 - 1000 (Elad *et al.*, 1994a) and on tomato stem pieces 100-1000 (Eden *et al.*, 1996). In addition, four of the six isolates selected for testing maintained strong biocontrol activity despite a 48 hour delay in BCA application after *B. cinerea* inoculation. This too is inconsistent with literature experience where microbial agents were unable to confer protection against *B. cinerea* infection when the pathogen arrival preceded BCA inoculation (Redmond, Marois & MacDonald, 1987; Malathrakis & Kritsotaki, 1992). However, Hammer & Marois (1989) have observed persistent biocontrol by a yeast (*Exophiala jeanselmei*) and a coryneform bacterium similar to that obtained by fungicides when the treatments were applied up to 48 h after *B. cinerea* inoculation. Kaufman & Standard (1988) reported that *E. jeanselmei* was the causal agent of phaeohyphomycosis, (an infection in human patients) and is perhaps a reason why no further reports of this otherwise promising yeast were found.

The frequency with which BCAs were identified, the strong level of biocontrol conferred at the low inoculum density used in this study and the continued biocontrol

regardless of BCA application delay after pathogen inoculation suggest a mode of antagonism other than, or in addition to, competition or antibiosis alone. Microbial adhesion used as the preliminary selection criterion (Chapter Three) is highly likely to be a principle process in biocontrol activity as cells colonising the pathogen hyphae were observed using scanning electron microscopy (SEM) (Chapter Six). Fundamentally, microbial attachment can be considered a vehicle by which antagonistic characteristics can be delivered to highly specific pathogen sites and represents another biocontrol avenue compared with the contemporary biocontrol model. Microbes from this study direct their biocontrol activity primarily at the pathogen target rather than mediated through the phylloplane as with niche competition, antibiosis or induced host resistance. Cook (1993) argued that antagonistic microbes present in the infection court will deliver antagonism more efficiently than blanket applications of fungicides. To extend this argument, attacher microbes theoretically represent a further refinement of the hypothesis because highly specific targeting has enabled lower, but still effective, BCA populations to be applied and delays in application are unimportant to biocontrol success compared with nutrient competition. By colonising the pathogen surface, the activities of such bacteria and yeasts populations have a more immediate effect on the pathogen and considerations such as colonisation of the infection court and rate of population growth to threshold levels for biocontrol are no longer as important. This aspect of site occupation may be the reason why such relative high populations of BCA have had to be applied at the time of pathogen arrival. With the advent of molecular biological techniques, Thomashow and Weller (1996) concluded from their review that the colonisation capabilities of BCAs were often insufficient for good biocontrol activity. Such a conclusion is critical for biocontrol since phylloplane occupation and establishment is necessary for plant protection.

Thus the attacher method for screening candidate microbes provides an additional technique to the growing list of screening methods and provides an alternative environment that appears to favour BCAs with different biocontrol characteristics.

Microscopic studies of the interactions of a range of attacher bacteria and yeasts colonising *B. cinerea*

6.1 Introduction

Microscopic technique has been used extensively for visual verification of fungal attachment or crowding by bacteria and yeasts. Bacteria or bacteria-like structures have been found within spores (Pon *et al.*, 1954; Old & Robertson, 1970) or partially embedded in the wall of *C. sativus* conidia and *Thielaviopsis basicola* chlamydospores (Old & Robertson, 1969; Wong & Old, 1974; Clough & Patrick, 1976; Old & Patrick, 1976). Fungal surface colonisation is another common phenomenon where Fradkin & Patrick (1982) used fluorescence microscopy to reveal unidentified cocci and rod shaped bacterial colonising *C. sativus*. With SEM, bacteria including *Pseudomonas fluorescens* on *Gaeumannomyces graminis* (Rovira & Campbell, 1975), *Pseudomonas* sp., *Bacillus* sp. and *Streptomyces* sp. on *Phytophthora cinnamomi* (Malajczuk, Nesbitt & Glenn, 1977), *Rhizobium* sp. on *P. cinnamomi* (Malajczuk, Pearce & Litchfield, 1984), *Enterobacter cloacae* on *Pythium ultimum* (Nelson *et al.*, 1986), and on *Rhizopus stolonifer* (Wisniewski, Wilson & Hershberger, 1989) and *Pseudomonas stutzeri* on *Fusarium oxysporum* f. sp. *raphani* (Toyota & Kimura, 1993) were reported. In contrast, very few yeasts have been examined on the fungal surfaces. The only examples to date are isolates of *Pichia guilliermondii* (Wisniewski *et al.*, 1991), *Debaromyces hansenii* (Wisniewski, Wilson, Chalutz & Hersberger, 1988), *Cryptococcus laurentii* (C. Wilson, personal communication), *Rhodotorula glutinis* and *Cryptococcus albidus* (Elad, 1996).

Fungal crowding has also been reported in bacteria including *Enterobacter cloacae* on *Pythium* sp. hyphae (Hadar, Harman, Taylor & Norton, 1983), an unidentified bacterium on *Phytophthora cinnamomi* (Nesbitt, Malajczuk & Glenn, 1978) and *Polyangium* sp. *Rhizoctonia solani* spores (Homma, 1984). The later also showed streaming behaviour toward the pathogen *in vitro*.

6.2 Objectives

To determine the type of interaction between the various attacher bacteria and yeasts and *B. cinerea in vitro* and *in vivo.*

6.3 Materials and Methods

6.3.1 Light microscopic examination in vitro.

Samples to be examined by light microscopy were prepared by co-inoculating a sterile cellophane surface overlaying autoclaved bean leaves (See Chapter Five, Section 5.3.4 for details) with 50 µl *B. cinerea* conidial suspension and 950 µl of each of the following bacteria; 27a, 32a, ox1, ox2, ox5, ox6, ox7 and ox9 and yeast candidates 552e, 572c, 561, 532, 662dia and 662dib at $5x10^9$ cells/ml.

The inoculated dishes were incubated at 15°C for 24 h, then stored at 4°C for up to 2 days. Before examination, unattached microbes were removed from the pathogen / BCA mixtures using the wash technique described in Chapter Three. Samples of fungal hyphae were carefully picked from the nylon mesh and mounted in SDW on glass microscope slide, covered with 22mm x 22mm coverslip (Size 2) then examined using phase contrast, nomarski interference and cross polarised light microscopy (Reichert Nr (Austria)). Relevant images were photographed using a Nikon HFX IIA camera and exposure system on Fujichrome 64T film.

6.3.2 Scanning electron microscopy (SEM)

Pathogen-BCA interactions *in vivo* were examined on excised tomato stem pieces using SEM. Tomato stem pieces, grown and prepared as described in Chapter Two (Section 2.3) were spray inoculated with *B. cinerea* spores and BCA inoculum as described in Chapter Four. Suspension concentrations were $5x10^7$ spores/ml for *B.cinerea* and $2x10^{10}$ cells/ml for BCA isolates ox2, ox4, ox6, ox7, ox8a, ox9, 35a, 532, 561, 622b, 662dia, 662dib, 552c and 572c. In addition, a pathogen-only control was applied. The inoculated samples were incubated at 12°C for 70 h in sealed plastic tray containers lined with moist paper towels to provide a high humidity.

Samples were fixed by soaking in primary fixative (3% Glutaformaldehyde, 2% Formaldehyde and 0.1M Phosphate buffer pH 7.2) for 16 h, before residual fixative was

removed in repeated buffer washes. Tissues were dehydrated in a gradient series acetone solutions then critical point dried using liquid CO_2 as the critical point fluid. Samples were mounted onto aluminium SEM stubs using conductive silver paint, sputter coated with gold then viewed using a Cambridge 250 Mk III Scanning Electron Microscope.

6.4 Results

6.4.1 Light microscopy

Many of the yeast and bacterial isolates in this study adhered to hyphae of *B. cinerea in vitro* where the pattern was sporadic and localised. Figs 6.1 and 6.2 show aggressive crowding by bacteria ox3 and yeast 532 respectively. In many candidate-pathogen interactions, crowding of the pathogen hyphae by both yeast and bacterial BCA was evident and an example is shown in Figs 6.3 and 6.4. In contrast, yeast isolates 662e and 572c appeared to be poor attachers *in vitro.*

A regular feature of the *in vitro* microscopy was the presence of bright irregular bodies on the *B. cinerea* hyphae that were larger than the BCA cells and were illuminated in dark field, cross polarised and quarter wave light microscopy (Fig. 6.5). The position or frequency of these bodies, did not correspond with bacteria or yeast adhesion sites, and were not found in the pathogen-only control.

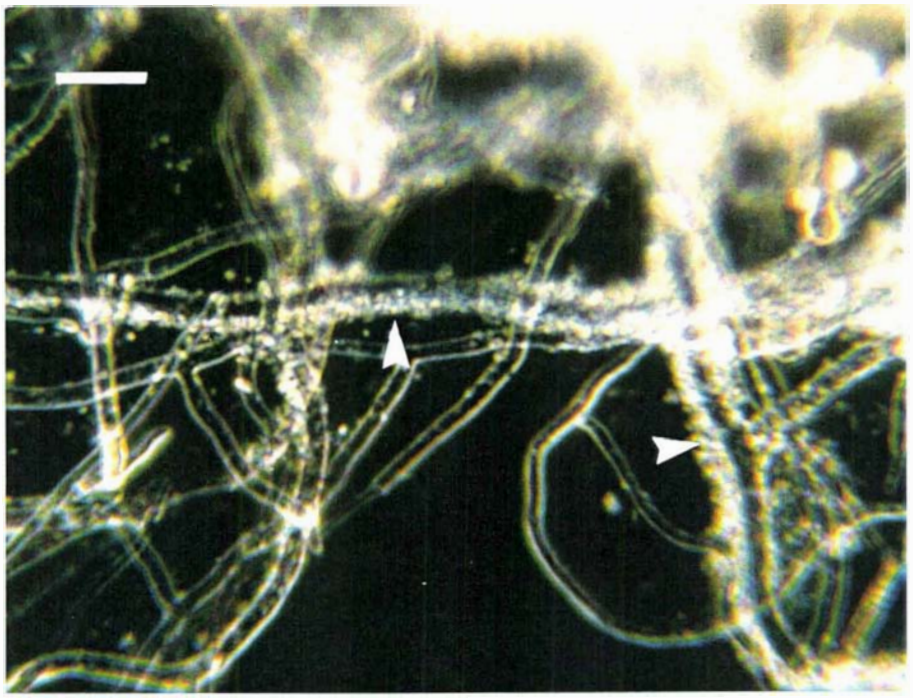


Fig. 6.1. Dark field microscopy of co-inoculated *B. cinerea* and bacterial BCAs on autoclaved bean leaves with a cellophane overlay, highly localised, aggressive crowding (Arrowhead) of bacterial isolate ox3 (Bar=20µm).

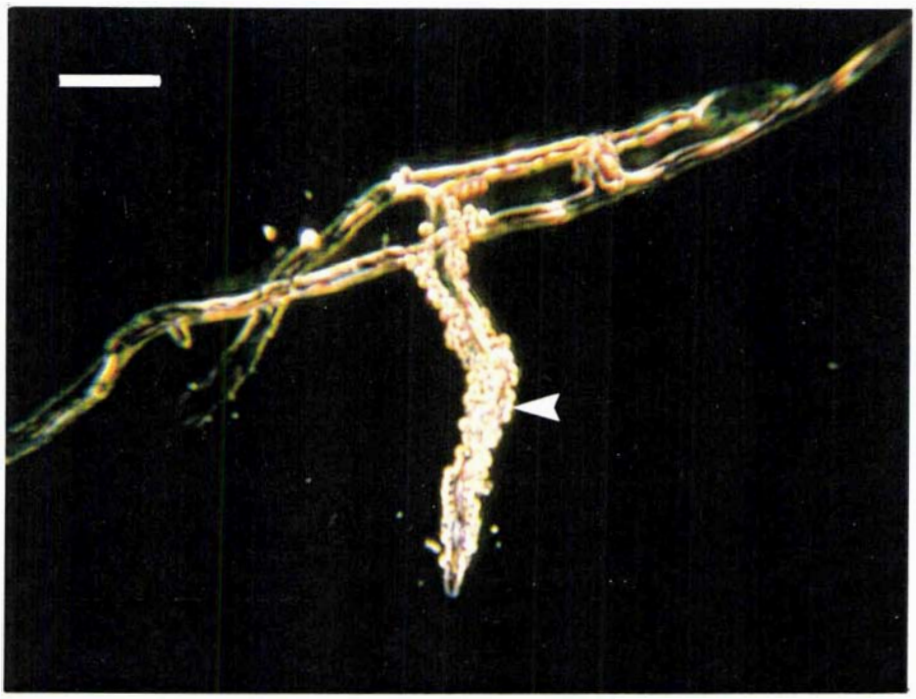


Fig. 6.2. Dark field microscopy of co-inoculated *B. cinerea* and yeast BCAs on autoclaved bean leaves with a cellophane overlay with isolate 532, *Candida sake* (Arrowhead, Bar=20µm).

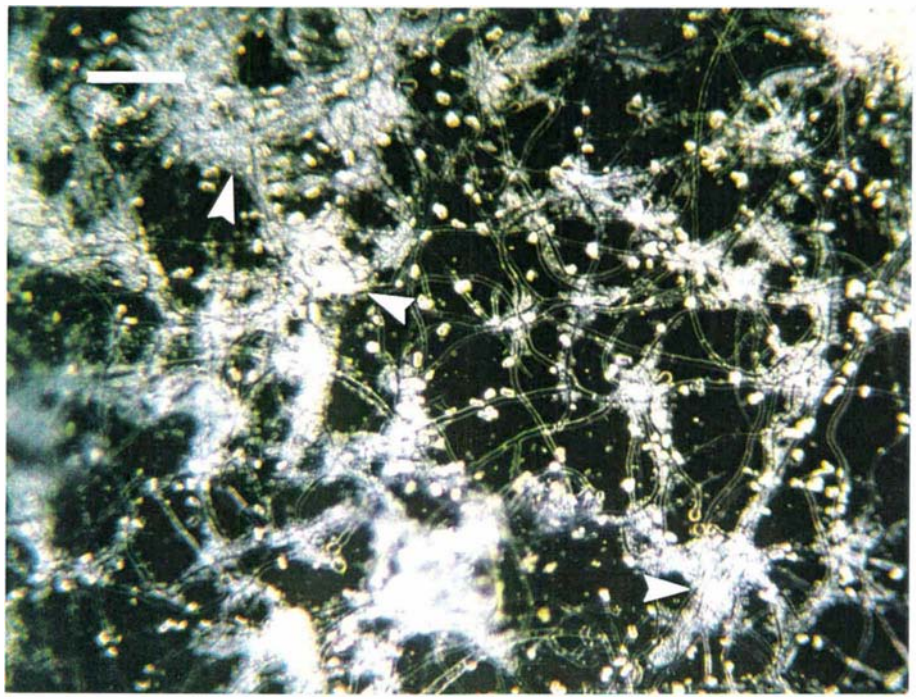


Fig. 6.3. Dark field microscopy of co-inoculated *B. cinerea* and yeast BCAs on autoclaved bean leaves with a cellophane overlay with agglutination by yeast cells of isolate 561, *C. sake* around *B. cinerea* hyphae (Arrowhead, Bar=80µm).

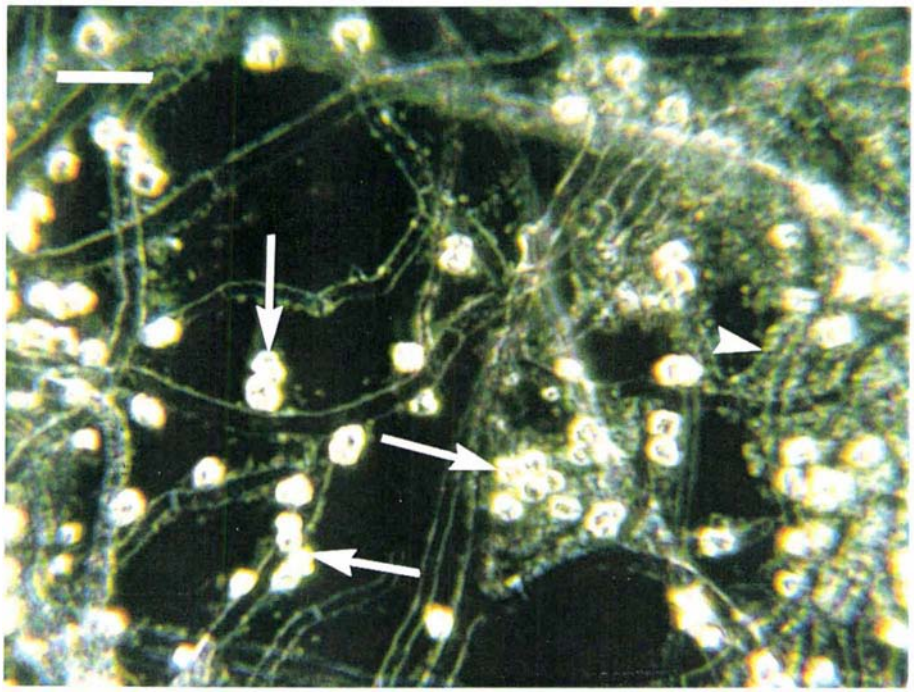


Fig. 6.4. Dark field microscopy of co-inoculated *B. cinerea* and bacterial BCAs on autoclaved bean leaves with a cellophane overlay with isolate ox6, *Enterobacter cloacae* B. (Arrowhead, Bar=20µm) Note the presence of bright white structures on the *B. cinerea* hyphae (arrow).

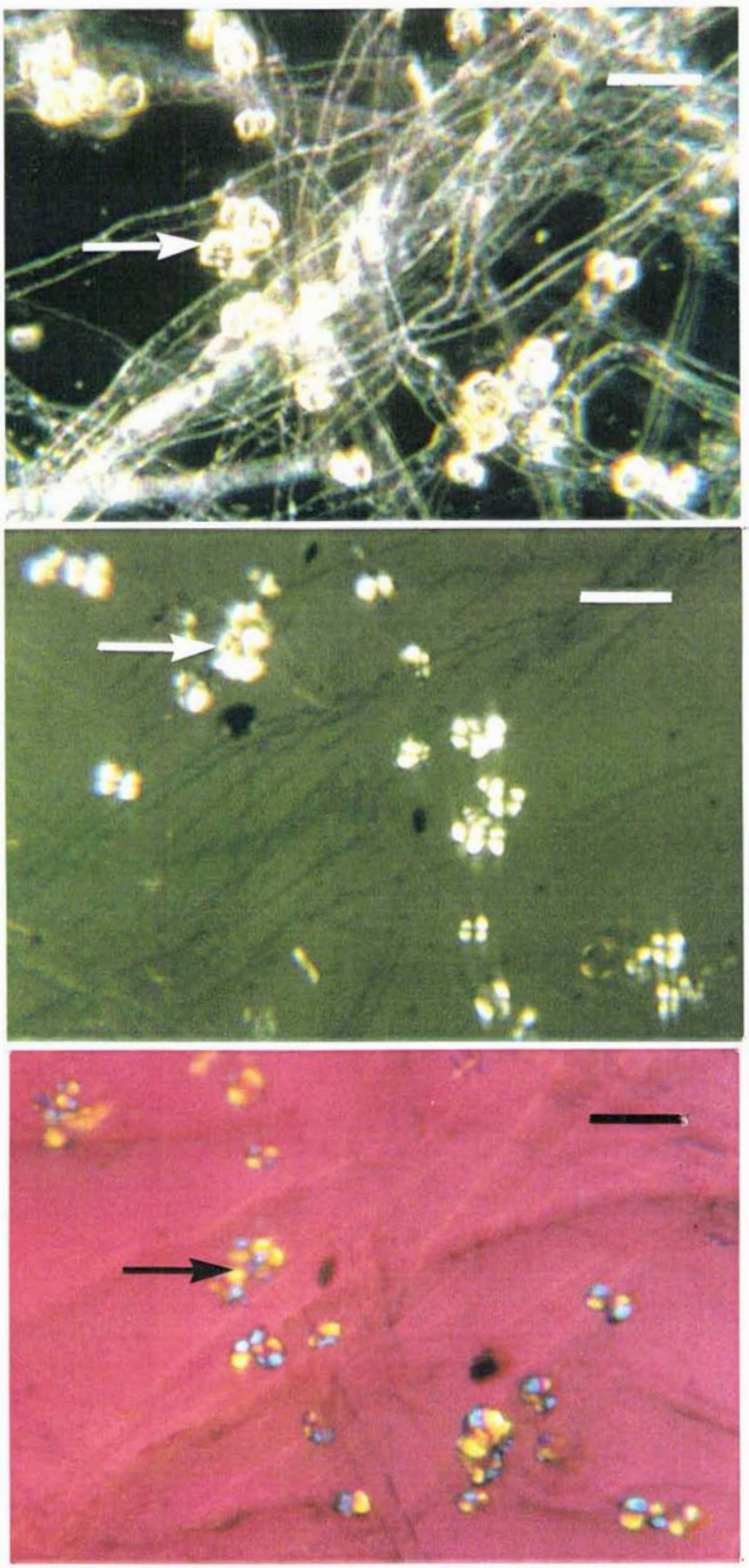


Fig. 6.5. Co-inoculation of bacterial isolate ox9, *E. cloacae* and *B. cinerea in vitro* and viewed by dark field microscopy (Top), polarised light (Middle) and quarter wave light (Bottom). Note the large irregular structures located on the pathogen hyphae (Arrow, Bar=20μm)

6.4.2 Scanning electron microscopy.

A consistent feature of most BCA samples was their close contact with the pathogen hyphae. Examples are shown in Figs 6.7, 6.8, 6.10 and 6.15, although considerable colonisation of the plant host surface was also evident (Figs 6.12, 6.17 and 6.18). No external cell adhesion was evident on hyphae or conidia of the pathogen-only inoculation treatment (Fig. 6.6)

Among the bacterial isolates, colonisation varied widely. Isolate ox8a (Fig. 6.12) and ox 6 (6.10) were particularly aggressive while moderate adhesion was observed for isolates 35a (Fig. 6.7), ox2 (Fig. 6.8), ox4 (Fig. 6.9), and ox7 (Fig. 6.11). Adhesion by cells of isolate ox9 was difficult to establish due to the presence of a large amount of extracellular material on the hyphal surface (Fig. 6.13). Orientation of cell attachment varied with cell shape; rod shaped cells attached end-on (Fig. 6.8), along their long axis (Fig. 6.7) or both (6.9) and there was no specific pattern with spheroid isolates (Figs 6.10 - 6.12).

Adhesion was identified in yeasts 662dia (Fig. 6.15), 662dib (Fig. 6.16) and 561 (Fig. 6.17) by the intimate contact between pathogen and cell and the presence of extracellular adhesion pads (isolate 532 (Fig. 6.18)). Such evidence was not readily observed for isolates 622b (Fig. 6.14), 552c (Fig. 6.19) or 572c (Fig. 6.20).

B cinerea hyphae exhibited a number of different features in the presence of the BCAs ranging from pitting of the hyphal surface in the presence of ox6 (Fig. 6.10), deformed germ tube emergence with ox7 (Fig. 6.11) or the complete collapse of hyphae with yeast isolates 561 (Fig. 6.17), 532 (Fig. 6.18) or bacterial isolate ox8a (Fig. 6.12). Yeast isolates 572c and 552c assumed a hyphal growth form on tomato tissue that may be distinguished from *B. cinerea* mycelium by the very short inter-septal distances. Fragments of hyphae were observed on the host surface (Fig. 6.19 and 6.20) but their origin could not be identified with certainty. Another pathogen feature was the presence of varying amounts of a granular, extracellular material on the hyphae and conidia. The quantity observed on *B. cinerea* hyphae in the BCA treatments was considerably greater than that observed on hypha and conidia on the pathogen-only control (Fig. 6.6). In the presence of bacteria ox9, the entire hyphal surface was covered in this material (Fig. 6.13).

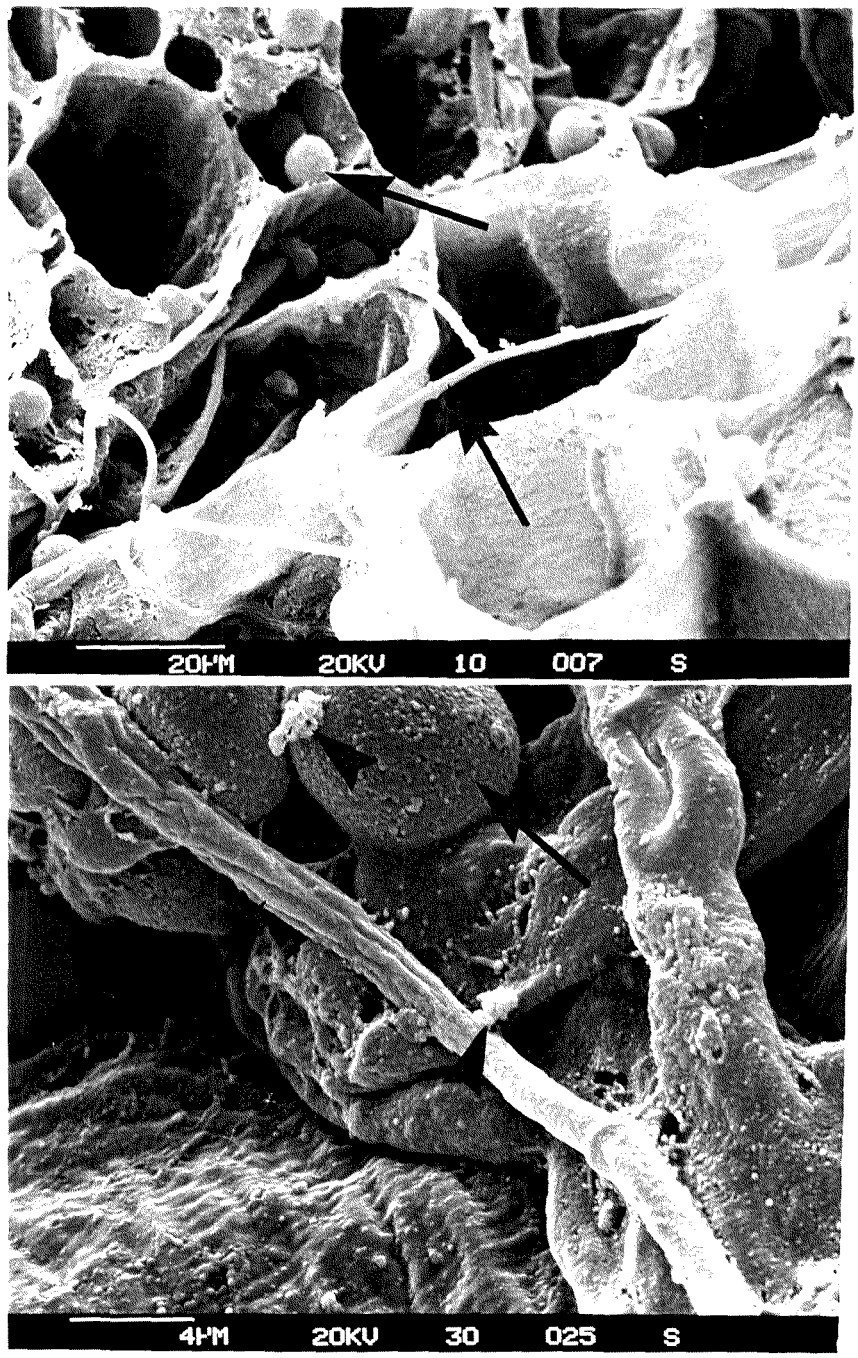


Fig. 6.6. SEM image of pathogen only control, inoculated onto cut surface of tomato stem pieces, incubated at 12°C for 70 h. (Top) Growth of hyphae over the surface of plant tissue (Arrow). (Bottom) The "eruption" of small amounts of amorphous, granular material from hyphae and conidia (Arrowhead) from *B. cinerea* (Arrow).

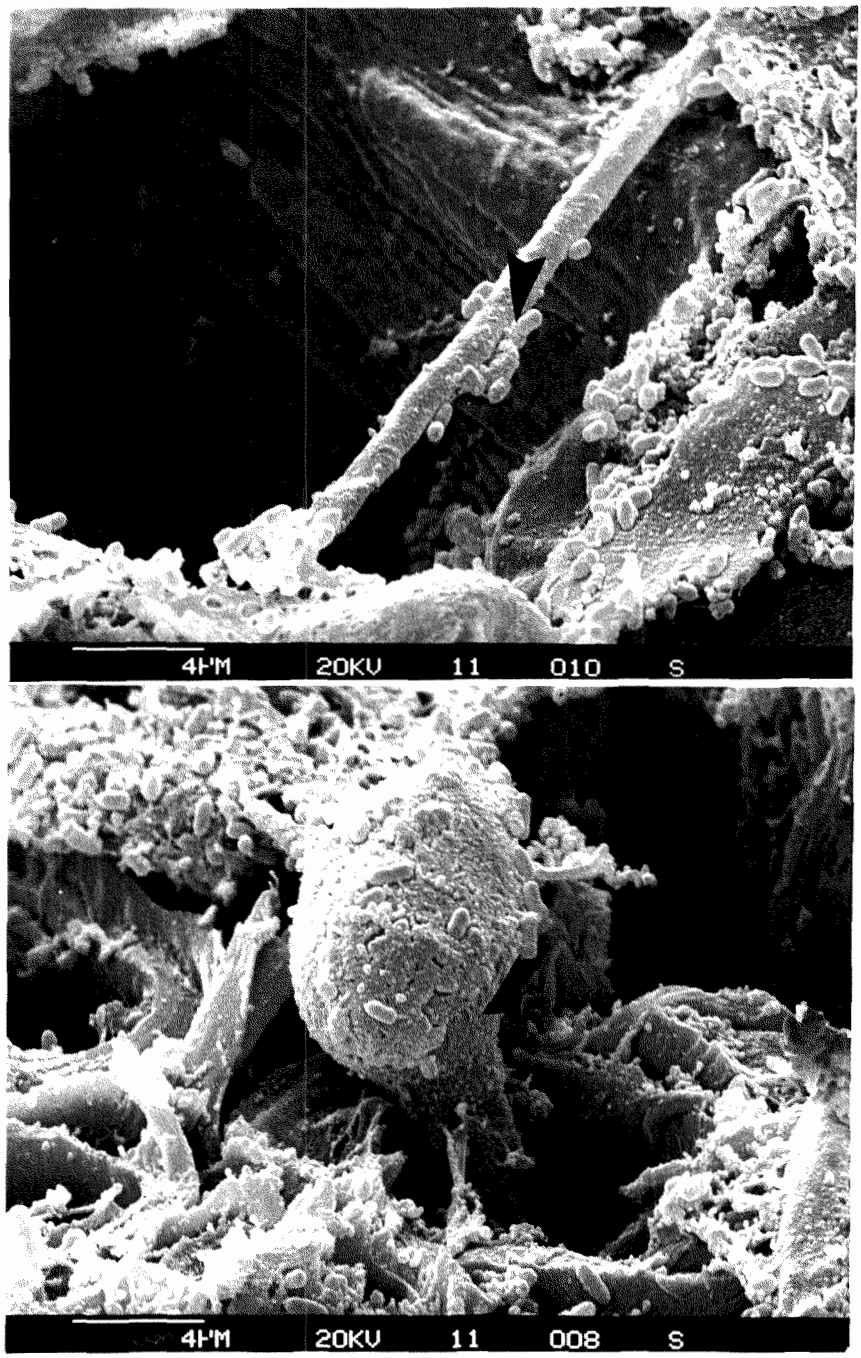


Fig. 6.7. SEM image of hyphal (Top)and conidial (Bottom) colonisation by bacterial isolate 35a (*Ochrobactrum anthropii*) on *B. cinerea* (Arrowhead).

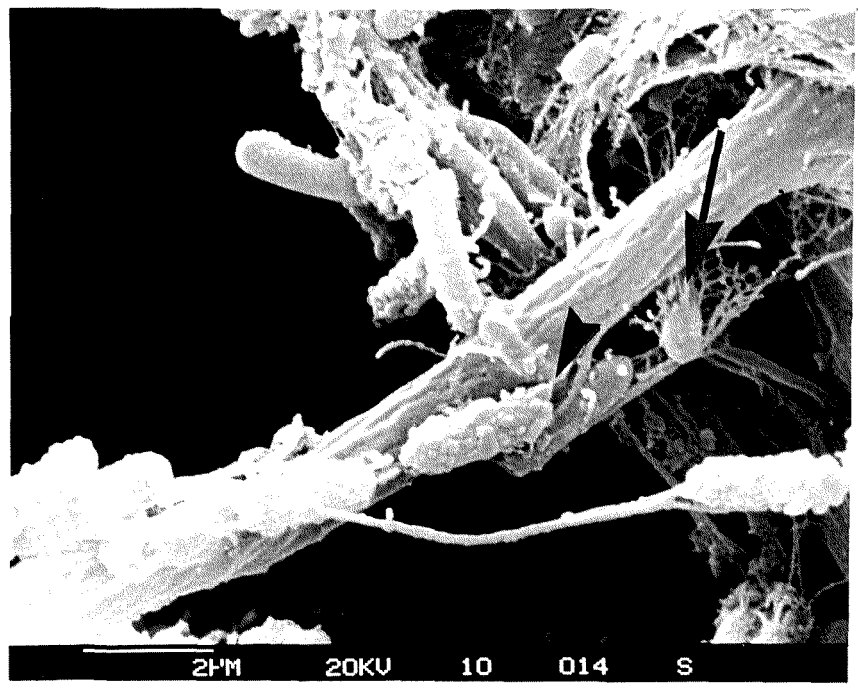


Fig. 6.8. SEM image of end-on adhesion of bacterial isolate ox2 (*Enterobacter agglomerans*) to hyphae of *B. cinerea* . Note the presence of tread-like fimbrae (Arrow)

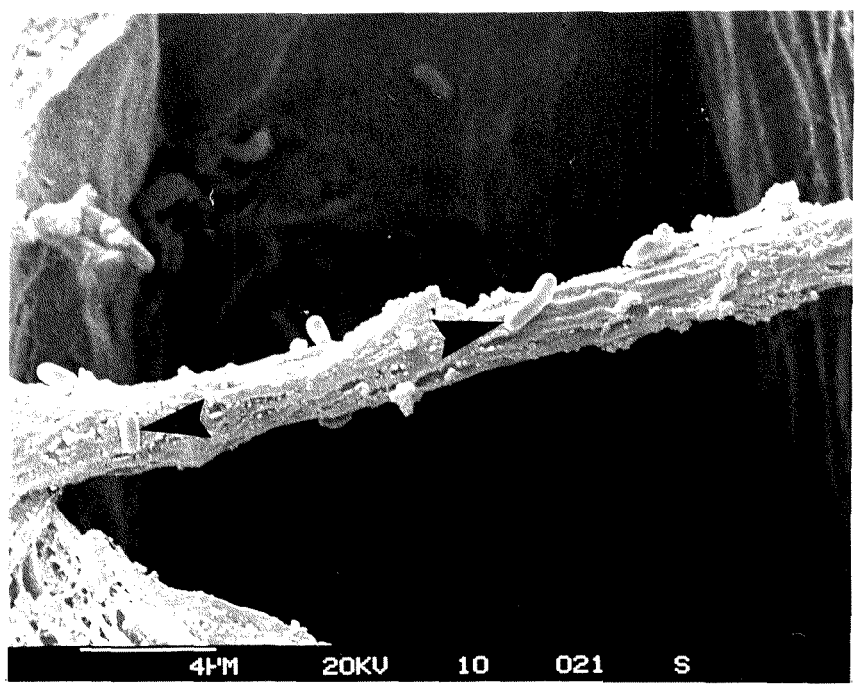


Fig. 6.9. SEM image of sparse colonisation of *B. cinerea* hyphae by bacterial isolate ox4 (*Pseudomonas marginalis*) (Arrowhead).

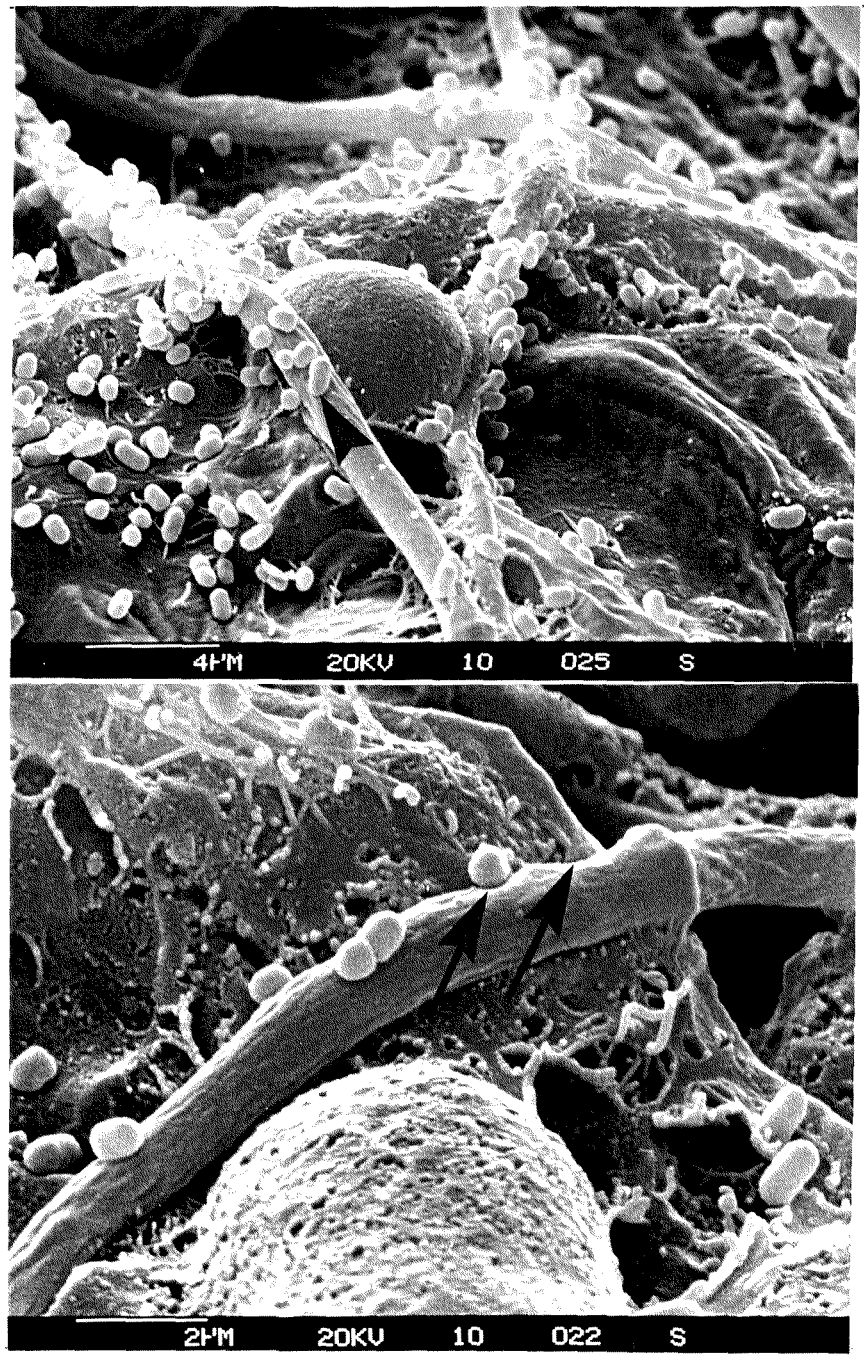


Fig. 6.10. SEM image of bacterial isolate ox6 (*Enterobacter cloacae* B) colonising hyphae of *B. cinerea* (Top) (Arrowhead) and the distortion in the hyphal surface under the bacterial cell (Bottom) (Arrow).

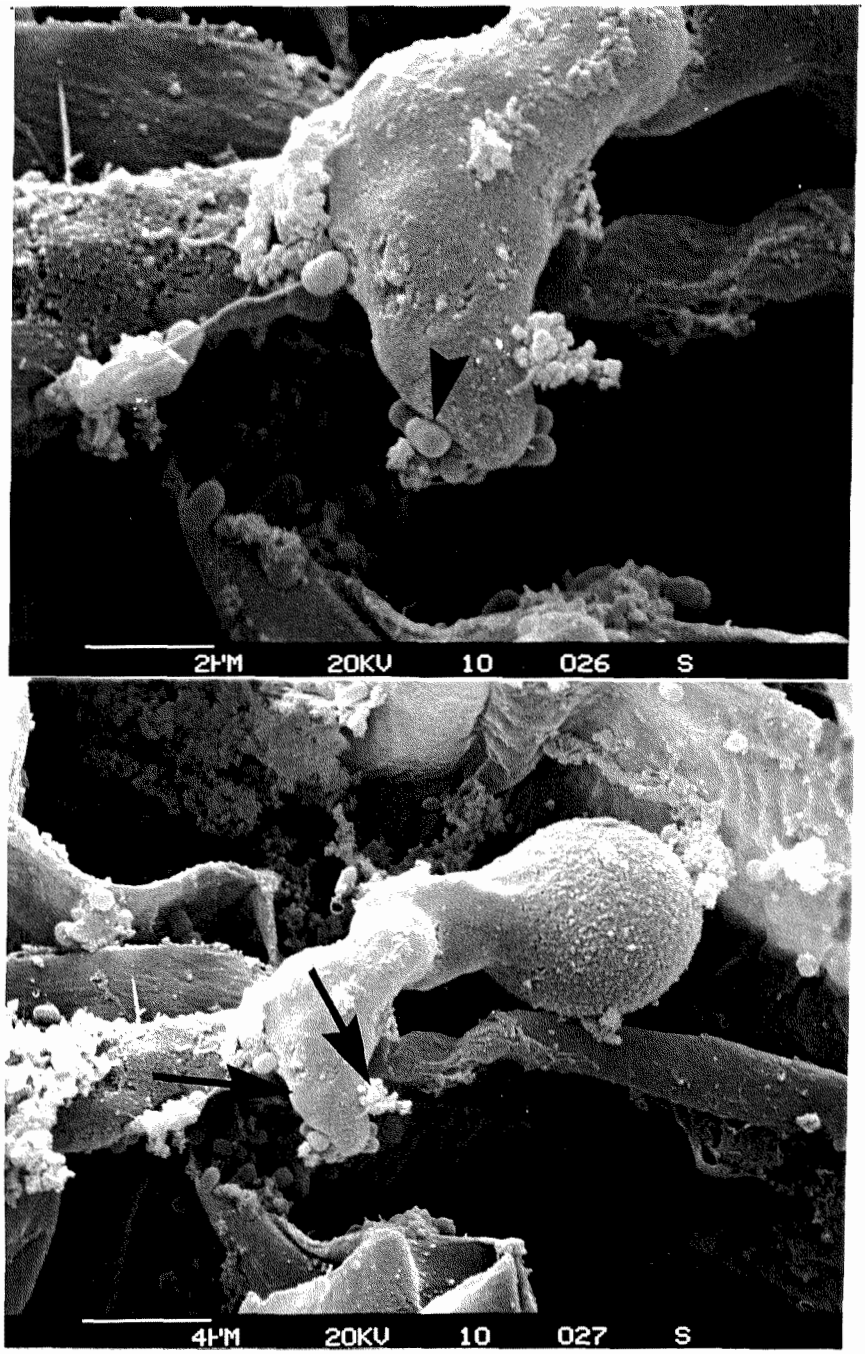


Fig. 6.11. SEM image of bacterial isolate ox7 (*E. cloacae* B) adhering to the tip of an emerging germ tube (Top) (Arrowhead) and distorted pathogen growth (Bottom) (Arrow) with amorphous material "erupting" from the germ tube (Arrow).

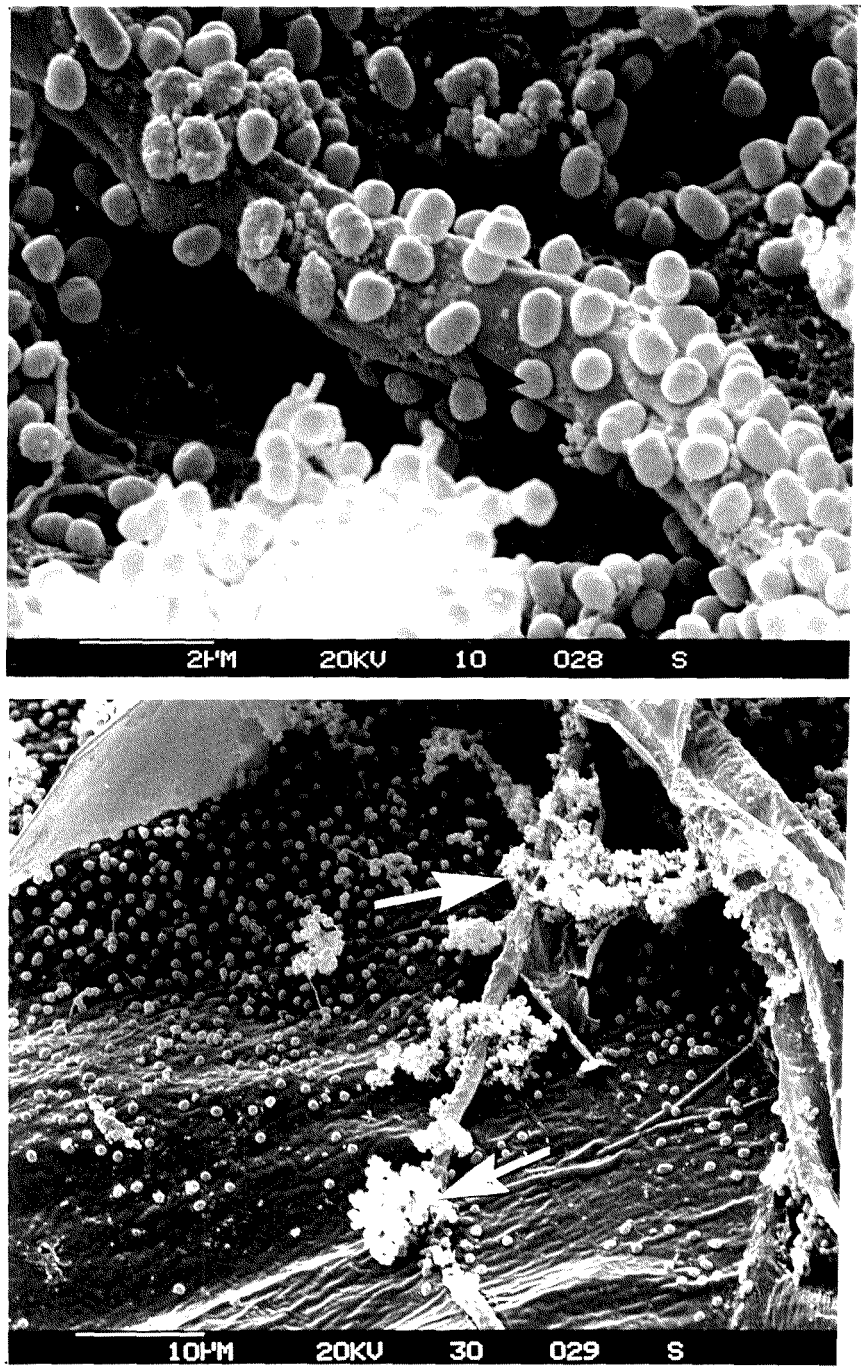


Fig. 6.12. SEM image of aggressive colonisation by bacterial isolate ox8a (*Enterobacter aerogenes*) of the hyphal surface of *B. cinerea* (Top)(Arrowhead) and of the plant host surface (Bottom). Note the presence of the amorphous extracellular material surrounding the hyphae which has been severed (Arrow)

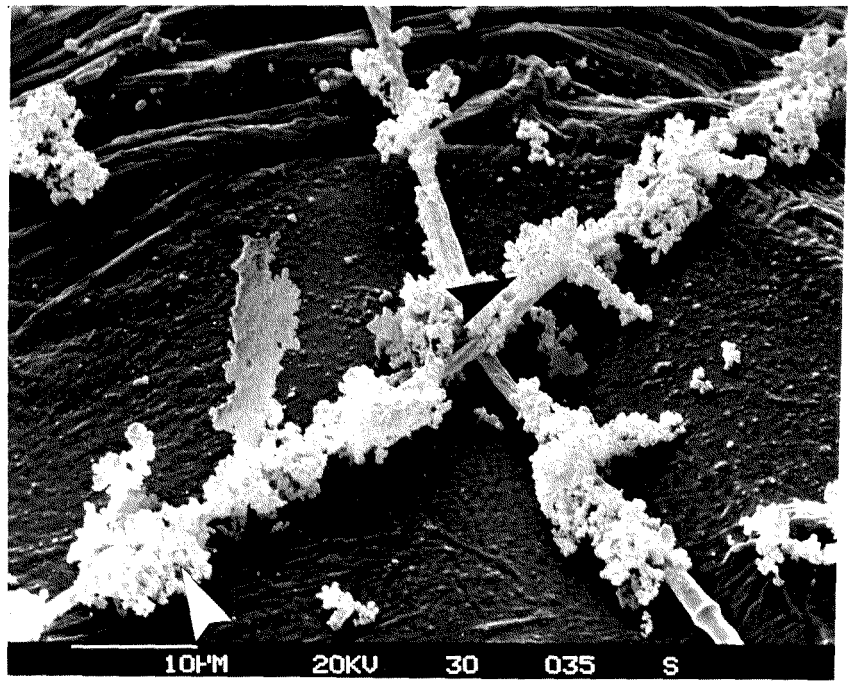


Fig. 6.13. SEM image of bacterial isolate ox9 (*E. cloacae*) cells associated with *B. cinerea* hyphae covered with a large amount of the extracellular material (Arrowhead).

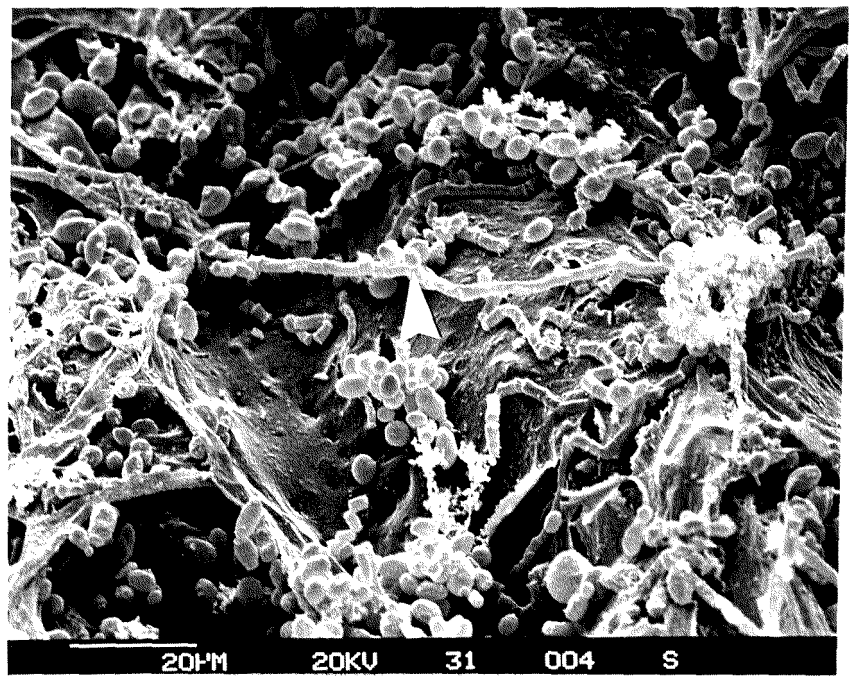


Fig. 6.14. SEM image of yeast isolate 622b (*Trichosporon pullulans*) co-inoculated with *B. cinerea*. Tentative adhesion of yeast cells to *B. cinerea* hyphae is indicated (Arrowhead). Note also the many fragments of hyphae scattered about the plant host surface.

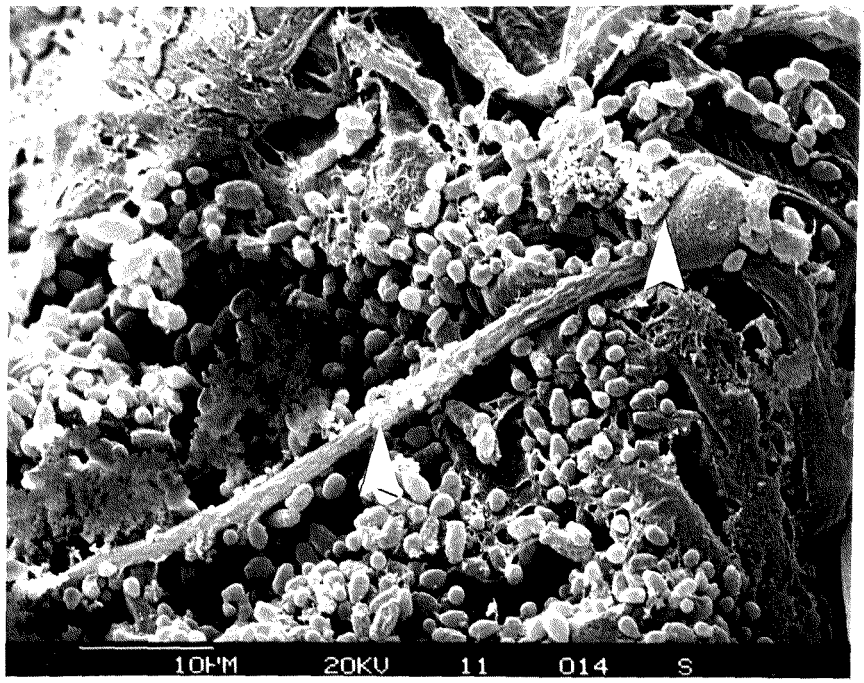


Fig. 6.15. SEM image of yeast isolate 662dia (*Candida sake*) colonising conidia and hyphae of *B. cinerea* (Arrowhead)

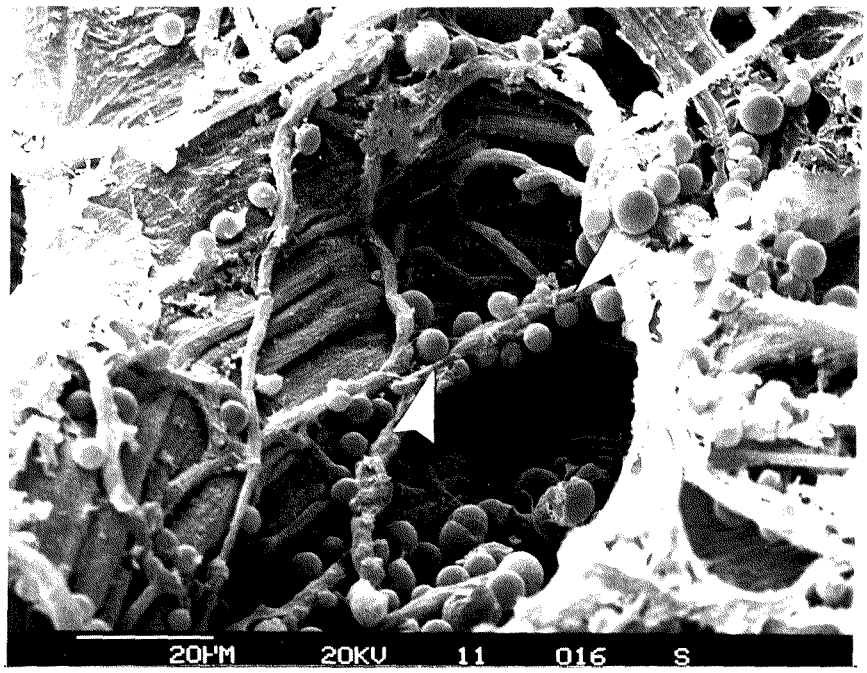


Fig. 6.16. SEM image of yeast isolate 662dib (*Candida pulcherrima*) colonising hyphae of *B. cinerea* (Arrowhead)

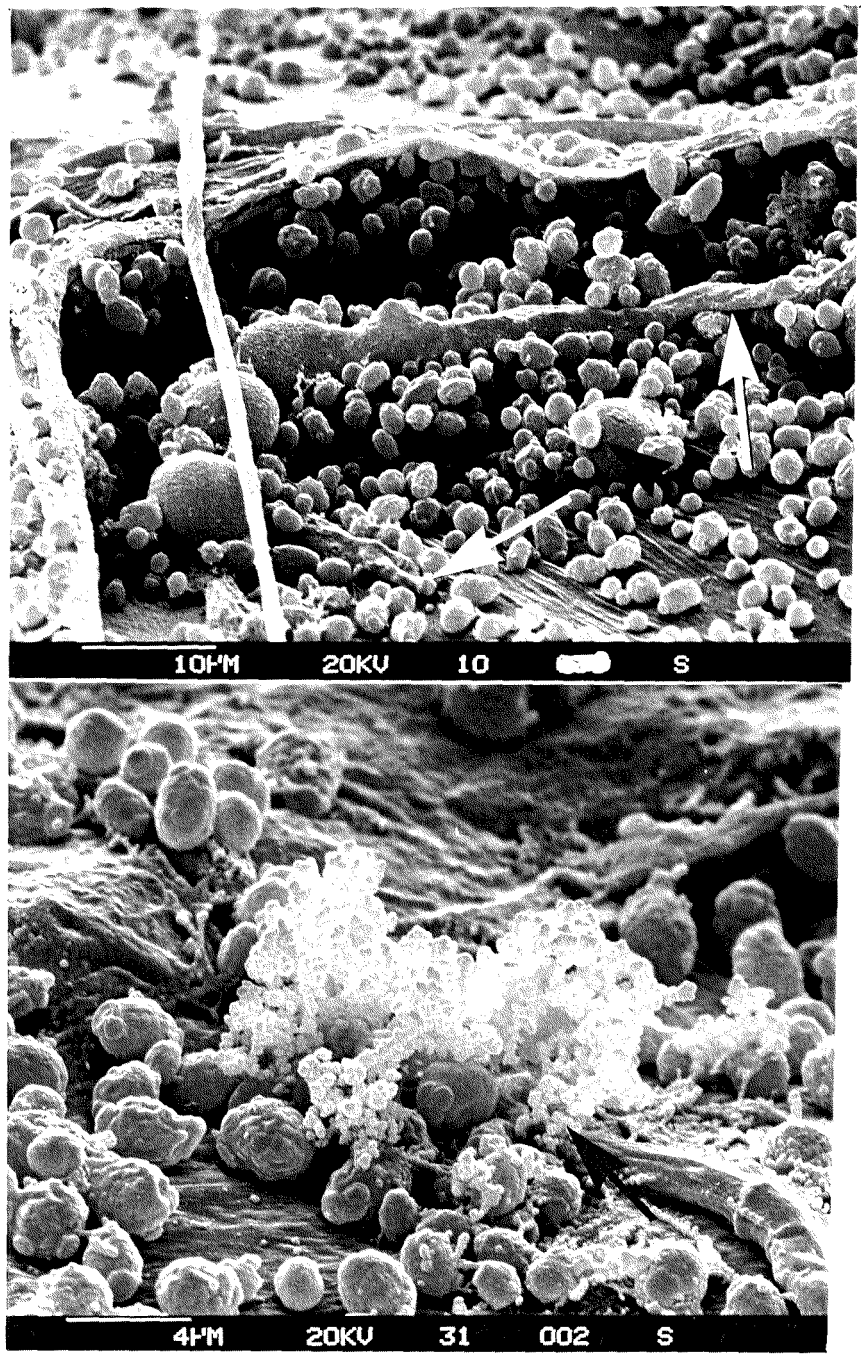


Fig. 6.17. SEM image of colonisation of *B. cinerea* hyphae and plant surface co-inoculated with yeast 561 (*C. sake*) (Top). Note the severed ends of the hyphae (Arrows). (Bottom) eruption of extracellular material from collapsed hyphae.

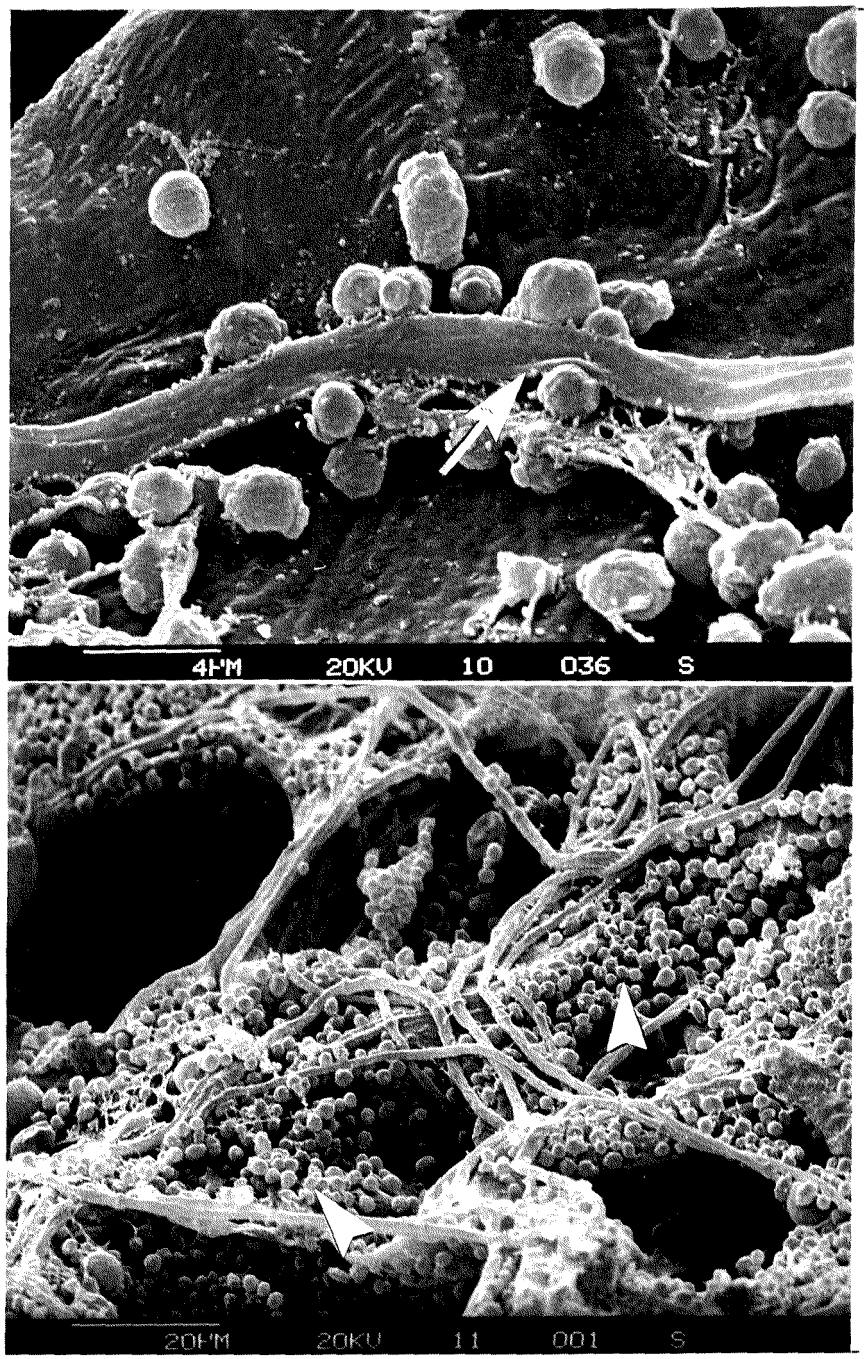


Fig 6.18. SEM image of collapsed and distorted hyphae of *B. cinerea* in the presence of isolate 532 (*C. sake*) (Top) (Arrow) and extensive BCA colonisation of the plant surface (Bottom) (Arrowhead).

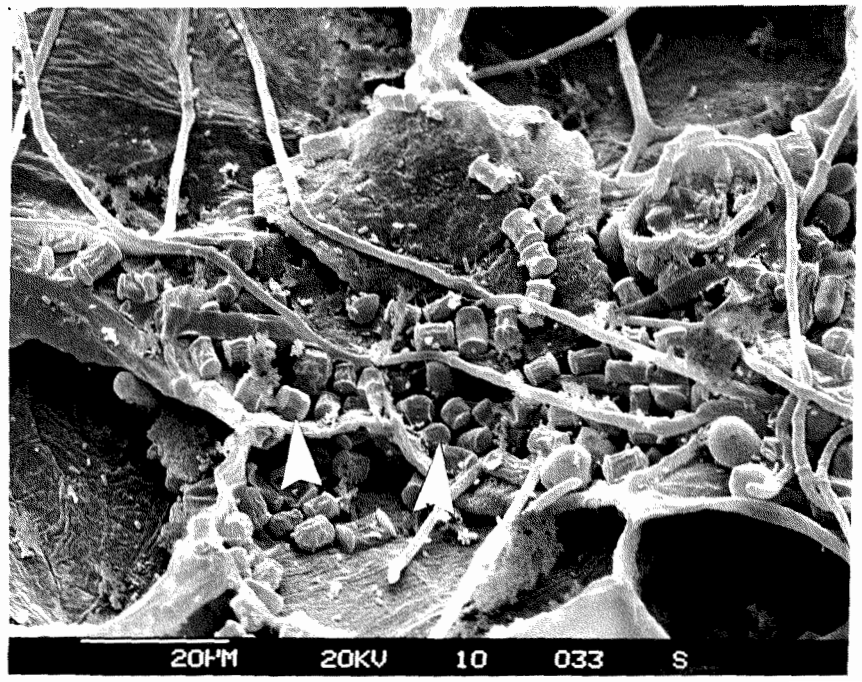


Fig. 6.19. SEM image of the very large cell size of yeast isolate 552c (*Galactomyces geotrichum*) (Arrowhead).

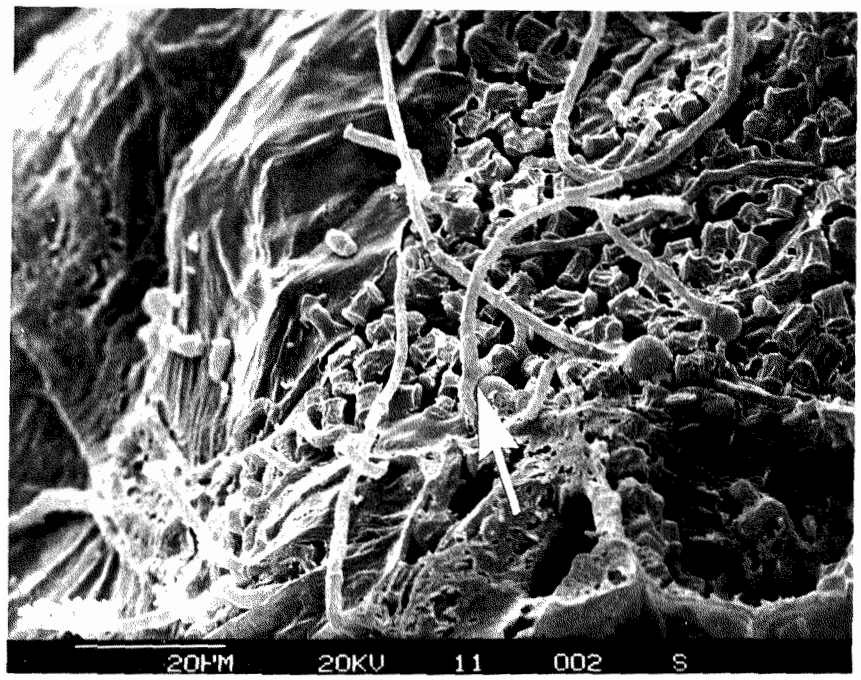


Fig. 6.20. SEM image of distorted growth of *B. cinerea* in the presence of isolate 572c (*G. geotrichum*) (Arrow).

6.5 Discussion

The relatively large number of attacher yeast and bacterial isolates used in this study provided a unique opportunity to examine a range of interactions between their cells and *B. cinerea in vitro* and *in vivo*. In light microscope studies, yeast and bacteria cells closely associated with *B. cinerea* (cell crowding) have been observed in other fungi/bacterial interactions *in vitro* (Malajczuk *et al.*, 1977; Nesbitt *et al.*, 1978; Homma, 1984). Cell crowding favoured young germ tubes but was also observed on random but specific regions on the *B. cinerea* hyphae. This pattern of adhesion is similar to that of immunolabelling by the MAb BC-KH4 (Cole, Dewey & Hawes, 1996) and suggests that the BC-KH4 antigen, may be associated with the BCA adhesion process.

Colonisation of the hyphal surface *in vivo* by the bacteria was also observed using SEM in a similar manner as previously reported (Old & Wong, 1975; Old & Patrick, 1976; Nesbitt, Malajczuk & Glenn, 1981; Fradkin & Patrick, 1982; Malajczuk *et al.*, 1984; Wisniewski *et al.*, 1989; Toyota & Kimura, 1993). Isolates ox8a, ox6, 561 and 532 were particularly fastidious colonisers with many adhesion events along the pathogen hyphae *in vitro* and *in vivo*. In isolate ox4, few adhesion events were observed, but were accompanied by thread-like fimbrae (Wisniewski *et al.*,1989) and these rod shaped cells orientated themselves along their long axis (Malajczuk *et al.*, 1977) compared to the polar adhesion of isolate ox2.

Reports of yeast attachment to *B. cinerea* were limited before this study and is a reflection of the inability of previous workers to efficiently isolate attacher microbes. In the present study, a further five isolates can be added to the list reviewed by Elad (1996).

Adhesion alone should not be viewed as the sole mechanism, through which biocontrol is expressed, as there appears to be no relationship between the frequency of adhesion events and biocontrol. For example, bacterial isolates ox3 and ox5 were moderately antagonistic (Chapter Five) yet extensive fungal crowding was observed *in vitro*. In contrast, isolates ox2, ox4, ox6 and ox8a were all highly antagonistic to *B. cinerea* (Chapter Five) but few adhesion events were observed for isolates ox2 and ox4 *in vivo* whereas isolates ox6 and ox8a were both aggressive colonisers. Conversely,

among the yeast isolates, 662e was a poor BCA and few adhesion events were detected *in vitro* or *in vivo*, but 561 and 532 were both aggressive colonisers and BCAs. Isolates 572c and 552c were both highly antagonistic but adhesion *in vitro* and *in vivo* was doubtful. It is possible that the large yeast cell size itself or their filamentous growth form made it difficult to observe the attachment morphologies. Similarly the destruction of the *B. cinerea* hyphae by enzymic activity from these BCA isolates could have completely destroyed the pathogen and any evidence for attachment.

Changes to mycelium morphology have been previously reported in bacteria or yeast/mycelial fungal interactions and include pitting and holes in the *B. cinerea* hyphal surface by *P. guilliermondii* (Wisniewski *et al.*, 1991) and growth distortions of germ tubes from *C. sativus* (Fradkin & Patrick, 1985a). Many of these phenomenon were observed in this study in the presence of both yeast and bacteria. *B. cinerea* hyphae were pitted, distorted, fractured or had collapsed, indicative of activity by one or more extracellular compounds. Hydrolytic activity was reported from isolates of bacteria obtained from the surface of baited *C. sativus* but there was no correlation between this activity and fungal colonisation (Fradkin & Patrick, 1985c). Another pathogen feature was the appearance of material erupting or surrounding the hyphae and conidia. The identity or exact source of this extracellular material consistently observed *in vitro* and *in vivo* remains unknown. There is correlative evidence to suggest that the material observed *in vitro* is the same as that *in vivo* as it was absent or greatly reduced in pathogen-only controls in both instances. *In vitro*, using cross polarised light, the patterns were similar to starch granules (Moss, 1976; Rawlins, 1992), although stressed and partially degraded cellulose can exhibit birefringence (Singh & Hedley, 1991). "Vesicles" have been reported on *Phytophthora cinnamomi* hyphae from the edge of inhibition zones on co-inoculated agar media (Malajczuk *et al.*, 1977) that could be the result of antibiotic activity. Isolate ox9 appeared to induce a particularly strong response from *B. cinerea* as considerably more material was observed over the entire pathogen. How this response is induced remains unknown, but it appears to be closely linked with the BCAs applied. Plant tissue mediated effects are unlikely as the plant tissue was autoclaved *in vitro* and likely to have disrupted any mechanism.

This study has shown that, adhesion is a common characteristic among the BCAs selected and is confirmation that the target group of microbes have been isolated. However adhesion may be but part of a complex biocontrol mechanism in the majority of BCAs examined here.

Putative modes of antagonism - An *in vitro* investigation

7.1 Abbreviations and terms used in Chapters Seven and Eight

Abbreviations used	Full name/Explanation
Antigen	The general chemical component to which an antibody binds.
BC-KH4	Monoclonal Antibody specific for *Botrytis* spp. Often referred to as the primary antibody.
ELISA	Enzyme Linked Immunosorbant Assay
(PTA-ELISA)	Plate-trapped-antigen-ELISA
(DAS-ELISA)	Double-antibody-sandwich-ELISA
Epitope	The specific part of a chemical molecule on which an antibody binds to an antigen
FBS	Foetal Bovine Serum
IgG	Subclass of monoclonal antibody consisting of a dimer of immunoglobulin molecules
IgM	Subclass of monoclonal antibody consisting of a pentomer of immunoglobulin molecules
MAb	Monoclonal Antibody
PAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PBST	PBS plus Tween 20
RPMI	The product name given to the liquid media in which Monoclonal Antibodies are produced. Used as a control in ELISA experiments.

7.2 Introduction

Accurate identification of the biocontrol function(s) responsible for plant protection is required in order to optimise BCA application conditions (Blakeman & Fokkema, 1982; Wilson & Wisniewski, 1989; Deacon & Berry, 1993), to accurately interpret biocontrol success or failure (Andrews, 1990, 1992; Nelson & Maloney, 1992), to provide further information for appropriate and improved future BCA selections (Wilson & Wisniewski, 1989) and satisfy product registration requirements (Forsyth, 1990; Woodhead, O'Leary, O'Leary & Rabatin, 1990; Klingauf, 1995). Preliminary information is also required to genetically manipulate appropriate BCA morphologies or activities to enhance biocontrol (Nelson & Maloney, 1992; Thomashow & Weller, 1996).

Pathogen antagonism is achieved by a variety of interactions, mediated via the plant or directly upon the pathogen itself. Of the four methods of antagonism frequently studied, antibiosis (the inhibition of one microbe by the extracellular product of another (Cook & Baker 1983), siderophore production (the production of compounds capable of efficient and rapid sequestering of extracellular iron to the benefit of the producer) and predation or parasitism (characterised by an intimate contact between both microbes) are easily observed on agar media *in vitro* using relatively simple methodologies. The fourth, resource competition, cannot be observed *in vitro* and is sometimes assigned to an agent when none of the above are detected. For example, Sutton (1995) suggested the mode of antagonism for an isolate of *Gliocladium roseum* was competition because antibiosis and parasitism were not observed *in vitro* and biocontrol characteristics of mutant isolates did not reveal a specific activity.

There is general acceptance that the *in vitro* technique, for the detection of antibiosis, siderophore production and parasitism is not an accurate method for determining their contribution to plant protection in the field. Antibiosis in particular has come under close scrutiny and there is general agreement that results *in vitro* are equivocal (Fravel, 1988; Thomashow & Weller, 1996) and neglect other important ecological functions, such as, colonisation and survival on the plant surface (Elad, 1990). In addition the exact BCA function may vary with different environment conditions (Dubos, 1987) or several

characteristics may combine to contribute to overall plant protection (Cook & Baker, 1983). These factors are difficult to anticipate *in vitro*.

Induced host resistance, plant growth stimulation, cross protection, physical restriction of pathogen growth (Elad, 1990) alteration of the wettability of the plant surface and interference with the pathogenicity process (Elad, 1996) are more difficult to identify and require more complex methodologies. Molecular biology is becoming a powerful technique to accurately identify other BCA functions and their contribution to the biocontrol interaction. Mutants are created in the laboratory with specific activities, or features removed, attenuated, or added to otherwise non-functional isolates. They are then applied to the disease environment and observed for biocontrol. The molecular biology technique is exploited by either generating random mutants then examining each for changes in biocontrol function and activity (Nelson & Maloney, 1992) or to target in detail a known biocontrol function for its contribution to biocontrol. The former approach has associated risks where non-specific changes in other regions of the genome in the BCA microbe could also contribute to changes in biocontrol activity. The later, requires that a putative mode of antagonism is identified before creating highly specific mutants.

Lytic activity toward entire, or fragments of, fungal mycelium due to either antibiotic or hydrolytic enzyme activities have been previously reported in a number of mycolytic or adherent bacteria (Pon *et al.*, 1954; Carter & Lockwood, 1957a, 1957b; Mitchell & Alexander, 1963; Morgan, 1963; Lloyd, Noveroske & Lockwood, 1965; Mitchell & Hurwitz, 1965; Mitchell & Wirsen, 1968; Rotem, Clare & Carter, 1976) and yeasts (Wisniewski *et al.*, 1991). Therefore in the first part of this study, putative biocontrol functions detectable on agar media such as anti-Botrytis compounds, siderophore production and endochitinase production were investigated.

In the second part, the importance of the adhesion of the BCA to the pathogen hyphae or conidia as a function of biocontrol was examined. In previous studies, a variety of approaches have been used; Yang, Menge & Cooksey (1994) used Tn5 mutation to remove flagella from an isolate of *Pseudomonas* sp. which was a BCA of *Phytophthora parasitica* in citrus. When the mutant was applied to a biocontrol interaction, plant protection failed while the parent continued to be antagonistic. This result was due to the failure of the

bacteria to adhere to the pathogen despite the retention of siderophore production. Nelson *et al.* (1986) proposed that poor biocontrol in high sugar environments by *Enterobacter cloacae* on *Pythium* sp. was due to inhibition of adhesion but Howell Beier & Stipanovic (1988) showed that sugars interfered with anti-fungal ammonia production. Similarly the adhesion of the yeast *Pichia guilliermondii* to *B. cinerea in vitro* was stopped by various salts, chemical agents such as sodium azide and sugars *in vitro* (Wisniewski *et al.,* 1991). However germination, growth or infection by *B. cinerea* could be altered with the addition of many of these agents, for example, sugars (Dowding & Royle, 1972; Blakeman, 1975; Harper & Strange, 1981) which is likely to confound any *in vivo* biocontrol experiments. Treatment effects otherwise recognised by changes in pathogenicity could be due to the inhibition of adhesion, increased pathogen inoculum potential or death of either the pathogen or BCA. An alternative approach would be to use antisera to block binding sites utilised by the adhering microbes. In previous studies, the adhesion of enteropathogenic bacteria to the gut lining of piglets was inhibited *in vitro* (Linggood & Porter, 1980). Toda *et al.* (1984a) and Toda, Tharanathan, Bozzaro & Gerisch (1984b) reported the inhibition of adhesion of the slime mould *Polysphondilium pallidium* with the addition of a monoclonal antibody (MAb). The pattern of binding by the MAb, BC-KH4, raised to surface washings of *B. cinerea* (Bossi & Dewey, 1992) in immunofluorescent studies at the electron microscopic level (Cole , Dewey & Hawes, 1996) appears very similar to the adhesion of BCA bacteria and yeasts observed in Chapter Six. In this study, BC-KH4 was tested for any ability to inhibit BCA adhesion whilst minimising secondary effects on pathogen growth and its effect on biocontrol.

7.3 Objectives

To:

1) detect putative production of endochitinase, antibiotics and siderophore production by the bacterial and yeast BCAs *in vitro*

2) Determine whether the adhesion mechanism itself is important in the biocontrol process

3) Characterise some aspect of the binding site(s) involved in BCA adhesion.

7.4 Materials and Methods

7.4.1 Section One: In vitro detection of endochitinase, siderophores and antibiotics.

7.4.1.1 Micro-organisms and culture media

Cells of bacterial isolates 27a, 35a, ox2, ox4, ox6, ox7, ox8a, ox9, yeast isolates 532, 552c, 561, 572c, 622b, 662dia, and 662dib and conidial suspension of *B. cinerea* spores were prepared as described in Chapter Two (Section 2.2). Suspension concentrations were adjusted to 2 $x10^{10}$ cells/ml.

7.4.1.2 Calcofluor assay for detection of endochitinase

The method used for detecting endochitinase was a modification of the protocol used by Trudel and Asselin (1989). Glycol chitin was prepared by grinding 2.5g glycol chitosan (Sigma G7753) in 50ml of 10% acetic acid. The suspension was left to stand at 20°C for 16h before a further 225 ml of methanol was added and the mixture filtered through Whatman #1 filter. Acetic anhydride (3.75 ml) was added to the filtered mixture, stirred until the magnetic spinning bar ceased to rotate. The resulting gel was cut into pieces, immersed in methanol and homogenised at maximum speed in a Waring blender. The homogenate was centrifuged at 27,000 **g** for 30 min, the pellet resuspended in methanol, homogenised and centrifuged as before. The pellet was then resuspended in 250 ml distilled water plus 0.02% (w/v) sodium azide and the solution stored at 4°C until required.

Media for the assay were prepared by adding 6g bacteriological agar to 396 ml McIlvaines buffer (pH 5.0) and autoclaving for 15 min. The medium was allowed to cool to approximately 40°C before 4 ml of glycol chitin plus azide was added, mixed

thoroughly (not shaken), then 15 ml aliquots dispensed into 90 mm diameter petri dishes.

NA or NYDA media was used to culture bacterial and yeast isolates respectively. In addition, the same two media were amended with a crude *B. cinerea* extract prepared by suspending the mycelium and conidia from two 90 mm diameter plates of MEA incubated for 8 days at 20°C, (See Chapter Two, Section 2.1) in 100 ml SDW, then dividing into two 50 ml aliquots, adding the dry ingredients for NA or NYDA, sterilising and dispensing 15 ml aliquots into 90 mm diameter petri plates. The media was inoculated with 10 µl aliquots of four bacterial and yeast BCA suspensions per plate according to the pattern illustrated in Fig. 7.1A. The plates were incubated at 15°C for 48 h. After the incubation, 13 mm diameter MEA plugs containing each bacterial or yeast colony were cut from the medium using a cork borer and placed in a radial pattern (Fig. 7.1A) on the glycol chitin media, with the BCA culture remaining uppermost. Three replicates of each treatment were inoculated then sealed with plastic film and incubated at 37°C for 24 h. The plugs were removed and the plates were developed by covering the agar surface in 10 ml of freshly prepared 0.01% calcofluor in 500 mM Tris-HCl buffer (pH 8.9) (30.28g Tris base and pH adjusted using 1M HCl), incubating for 10 min at room temperature (R.T.). Each plate was washed in repeated volumes of distilled water then viewed under UV light (UVP, San Gabriel, California).

7.4.1.3 In vitro detection of antibiotics and parasitism

Plates with 15 ml of, NYDA, NA, MEA (Chapter Two, Sections 2.1 and 2.2) or minimal media (D-glucose 10g/litre, Biotin 1 mg/litre, NH_4Cl 1.0 g/litre, $MgSO_4$ 0.2g/litre, KH_2PO_4 4.1g/litre, K_2HPO_4 3.6g/litre and Agar 15 g/litre) were streaked with each BCA yeast and bacterial isolate according to the pattern illustrated in Fig. 7.1B. A 5 mm plug of *B. cinerea* cut from the leading edge of a 3 day old culture on MEA was centrally positioned, facing down. Four replicates of each BCA on each medium were incubated at 15°C for 7 days.

After incubation, the various interactions between the edge of *B. cinerea* colony and the BCA colony were examined and the width of any inhibition zones were

measured. Images of the interactions were recorded using light microscopy and a Nikon HFX IIA camera plus exposure system on Fujichrome 64T film.

7.4.1.4. Siderophore production

The method for the detection of siderophore production was that used by Kloepper, Leong, Teintze and Schroth, (1980). Two batches of Kings B media were prepared with or without 1 μM $FeCl_3$ and inoculated with 10 μl of bacterial or yeast BCA suspensions in a radial pattern illustrated in Fig. 7.1A. The plates were incubated at 15°C for up to 7 days and were examined daily under UV light (UVP, San Gabriel, California) for fluorescent zones around each colony.

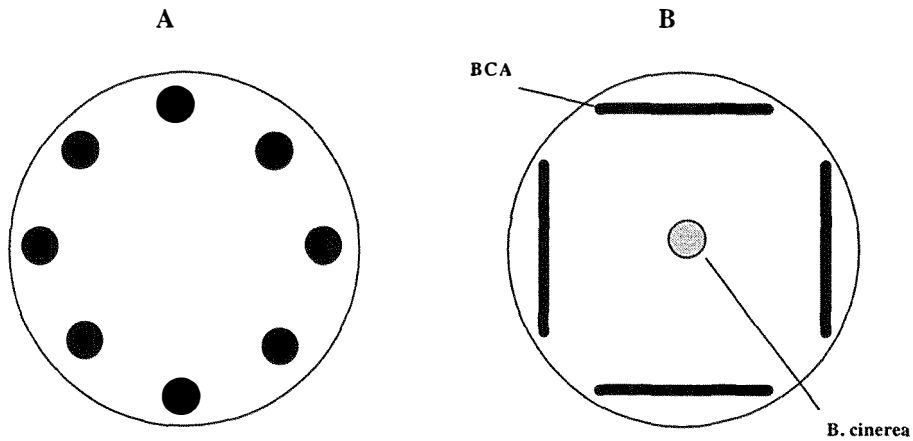


Fig. 7.1. Stylised diagrams of the inoculation pattern used for endochitinase and siderophore detection (A) and for antibiotic detection (B).

7.4.2 Section Two: Inhibition of BCA adhesion to B. cinerea.

7.4.2.1 Preparation of BCA and B. cinerea *suspensions*

All cell or spore suspensions used in this section of the study were prepared from cultures grown on their respective media in conditions described in Chapter Two. In addition to suspending cells or conidia, extracellular matrix material from the cultures was unavoidably suspended. This was removed by centrifuging all BCA suspensions at 8900 **g** and *B. cinerea* suspensions at 1800 **g** for 10 min, discarding the supernatant and resuspending the pellet in SDW.

7.4.2.2 Experiment One

Hybridoma supernatant BC-KH4 (obtained from F.M. Dewey, University of Oxford) was desalted by passing through a PD-10 column (Pharmacia) to remove the azide preservative. The column was equilibrated with 25 ml phosphate buffered saline (PBS) then loaded with 2.5 ml of the supernatant. Three consecutive elution fractions, each of 3.5 ml of PBS were collected, and five 2 ml aliquots of each were stored at 4°C until required.

To test for the effect of BC-KH4 on adhesion *in vitro*, a tomato seedling extract agar was prepared by homogenising fresh seedling stems in a Waring Blender with 1:20 fresh weight:volume SDW then adding 15 g/l agar. Molten agar was poured into 50 mm diameter glass petri dishes and after cooling, 50mm diameter, sterile cellophane disks were laid on top. Onto this cellophane surface 10 µl of a *B. cinerea* conidia suspension (isolate Pezet, adjusted to 5 x 10^5 spores/ml.), 10 µl of cell suspensions ox4, ox5, ox6, ox7, ox9, 561 or 532 and 10 µl of the MAb BC-KH4 from fraction two of the desalting column were applied. SDW replaced the column fraction in the control. After 12 h incubation at 15°C the cellophane was lifted off the MEA and placed on microscope slides. An additional 20 µl SDW was added and the specimens viewed using interference microscopy.

The presence of BC-KH4 antibodies in the column fractions was confirmed by PTA-ELISA after the method of Bossi & Dewey, (1992) (See Chapter Two, Section 2.5.5). One hundred microlitres of a standard *B. cinerea* antigen extract (Chapter Two, Section 2.5.3)

was pipetted into a strip of microtitre wells (ICN Flo Microstrips 9502107, Labsystems Oy Helsinki, Finland) and incubated at 4°C for 16 h. After well washing, 100 µl aliquots from each column sample and untreated BC-KH4 were pipetted separately into micro-titre wells and incubated at R.T. for 1 h.

In a preliminary *in vivo* experiment, 10 µl of the *B. cinerea* conidial suspension (isolate Pezet, adjusted to 5 x 10^5 spores/ml) was pipetted onto tomato stem pieces and incubated for 4 h at 15°C. This was followed by the addition of 10 µl of BCA suspensions ox5, ox9 and 561 and 10 µl of desalted BC-KH4. SDW replaced the dialysate as a control. The stem pieces were returned to the incubator for 8 days unless sporulation was detected, then stem pieces were removed to prevent contamination.

7.4.2.3 Experiment Two

The same BCA isolates and *B. cinerea* were inoculated onto the cellophane overlay tomato extract agar (See Section 7.3.2.2) using 100 µl volumes and 800 µl of either SDW, BC-KH4 or culture fluid (RPMI+FBS). The plates were incubated at 15°C for 12 h then *B. cinerea* germlings were removed and washed using the protocol described in Chapter Three. The washed hyphae were examined for adhesion using interference microscopy.

7.4.2.4 Experiment Three

Adhesion *in vitro* and biocontrol *in vivo* were examined for bacterial isolates ox4, ox7, ox8a and 35a and yeast isolates 532, 561, 622b and 662dib influenced by the addition of BC-KH4, PI-01 and SDW. Twenty millilitres of each MAb or SDW were dialysed against four changes of PBS (1 litre volumes) at 4°C over 48 h.

For the *in vitro* assay, tomato extract agar was prepared as described in Section 7.3.2.1 and 0.5 ml aliquots pipetted into 24 well microtitre plates (Greiner labortechnik # 662160). Cellophane disks, 10 mm diameter, were placed on top of the solid agar then inoculated with 100 µl *B. cinerea* (1x10^7 spores/ml), 100 µl BCA (1x10^9 cells/ml) and 100 µl dialysed MAb or SDW then incubated at 15°C for 48 h.

For the *in vivo* assay for biocontrol, 1 ml of the *B cinerea* conidia suspension was applied to the cut surfaces of tomato stem pieces using the Potter Tower at 100 mm Hg

Nozzle Pressure as described in Chapter Four. These stems were incubated at 15°C for 6 h before 10 µl of BC-KH4, PI-01 or SDW were pipetted on the same cut surfaces. The stem pieces were incubated for 1 h at 15°C, then spray inoculated with 1 ml of 2x 10^{10} cells/ml of each BCA suspension in the same manner as for *B. cinerea.* The inoculated stems were incubated in plastic containers lined with moist paper towels to provide a humid atmosphere at 15°C, 12 h D/N for 8 days. Percent infection was assessed in all treatments by counting the number of infected stems.

7.4.3 Section Three: Characterisation of BC-KH4 epitope

A competitive ELISA format was used to identify the possible epitope BC-KH4 recognised on the *B. cinerea* antigen. Three sets of four microtitre strips (ICN Flo Microstrips, 9502107, Labsystems Oy, Finland) were prepared by pipetting 50 µl of *B. cinerea* antigen stock (Chapter Eight) diluted 600-fold in PBS and incubated at 4°C for 16 h. After the standard PBST, PBS, distilled water wash and plate drying (See Chapter Two, Section 2.5.5), 50 µl of BC-KH4 diluted two-fold in PBST was loaded into the first row of each set of microtitre wells, then serially diluted 1:1 with PBST in successive rows. This was immediately followed by adding another 50 µl aliquot of either 0.5M D(+) galactose in PBST (Sigma G 0750), 0.5 M D(+) mannose in PBST (Sigma M4625) or galactomannose diluted 1000-fold in PBST (prepared from an extract of *Aspergillus niger* obtained from Dr K. Hayes (Royal Postgraduate Medical School, London, UK) to microtitre wells in the first column of each set. This stock was serially diluted 1:1 across the columns of wells in BC-KH4/PBST already present. All plates were incubated at R.T. for 1 h then washed (Chapter Two, Section 2.5.5). The competition-ELISA protocol for the detection of BC-KH4 is detailed in Chapter Two.

7.5 Results

7.5.1 Detection of antibiotics, parasitism siderophores and endochitinase in vitro

Endochitinase was detected from all BCA isolates except bacteria ox7 (*Enterobacter cloacae* B), ox4 (*Pseudomonas marginalis*), ox9 (*E. cloacae*) and 35a (*Ochrobactrum anthropii*) when grown on either NA or NYDA media, and on the same media amended with a crude *B. cinerea* extract (Table 7.1). The largest zones of destained glycol chitin (>20 mm) were observed for bacterial isolate ox8a (*Enterobacter aerogenes*), moderately large zones (12-20 mm) for bacterial isolate 27a (*E. aerogenes*) and all yeast isolates except 662dib (*Candida pulcherrima*), where only one replicate colony produced a small destained zone. Endochitinase production from isolate ox6 (*Enterobacter cloacae* B) was detected only in the presence of autoclaved *B. cinerea* extract.

Siderophore production was detected in isolates ox4 (*P. marginalis*) and 35a (*O. anthropii*) within the first 24 h incubation at 15°C (Table 7.2.). After a further 24 h incubation the chelating agent was detected from isolates ox2 (*E. agglomerans*) and ox6 (*E. cloacae*), and after seven days from yeast isolates 662dib (*C. pulcherrima*) and 622b (*Trichosporon pullulans*). No fluorescent zones were observed around any of the remaining BCAs tested.

Diffusible anti-*Botrytis* compounds were not detected on MEA, PDA or NYDA after 7 days incubation at 15°C (Table 7.3). However on minimal media, there were zones of inhibition between the *B. cinerea* mycelium and isolates ox4 (*P. marginalis*) and 35a (*O. anthropii*) only. For all remaining BCAs tested, *B. cinerea* hyphae grew through the antagonist colony with no apparent alteration to growth or hyphal morphology other than adhesion by unicellular yeast cells (561 and 532 (*Candida sake*)) or coiling by mycelial by yeast 572c (*Galactomyces geotrichum*) on the pathogen (Fig. 7.2). However there were no signs of pathogen growth inhibition in these interactions.

Table 7.1. Endochitinase activity in BCA isolates using the calcofluor assay (modified from Trudel and Asselin 1989). Data is expressed as diameters (mm) of destained zones averaged from two measurements perpendicular to one another (- = missing data).

Media	Rep	Cont	ox2	ox4	ox6	ox7	ox8a	ox9	35a	27a	532	561	552c	572c	662dia	662dib	622b
Std	1	29	11	0	0	0	21	0	0	19	12	13	12	13	15	12	17
	2	27	0	0	0	0	23	0	0	13	13	14	13	0	12	0	17
	3	30	12	0	0	0	23	0	0	19	15	16	0	13	17	0	20
+ *B. cinerea*	1		0	0	11	0	22	0	0	20	17	12	17	14	13	0	16
	2		0	0	13	0	24	0	0	18	15	15	17	0	14	0	17
	3		0	0	0	0	22	0	0	20	-	-	18	-	-	0	0

Table 7.2. The number of days at 15°C before siderophore production was first detected from the yeast and bacterial isolates using the method of Kloepper *et al.* (1980). No siderophore production is indicated as 0.

Media	Rep	ox2	ox4	ox6	ox7	ox8a	ox9	35a	27a	532	561	552c	572c	662dia	662dib	622b
Kings B	1	2	1	2	0	0	0	1	0	0	0	0	0	0	7	7
	2	2	1	2	0	0	0	1	0	0	0	0	0	0	7	7
	3	2	1	2	0	0	0	1	0	0	0	0	0	0	7	7
+ Fe	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7.3. Diameter of zones of inhibition of *B. cinerea* due to anti-fungal metabolite from bacterial and yeast BCA isolates tested on PDA, MEA, NYDA and Minimal media. Measurements recorded seven days after inoculation and incubation at 15°C. Where no inhibition zone was observed (0), *B. cinerea* mycelium grew over the BCA colony.

Media	Rep	ox2	ox4	ox6	ox7	ox8a	ox9	35a	27a	532	561	552c	572c	662dia	662dib	622b
PDA	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MEA	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NYDA	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MM	1	0	7	0	0	0	0	7	0	0	0	0	0	0	0	0
	2	0	7	0	0	0	0	5	0	0	0	0	0	0	0	0
	3	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0

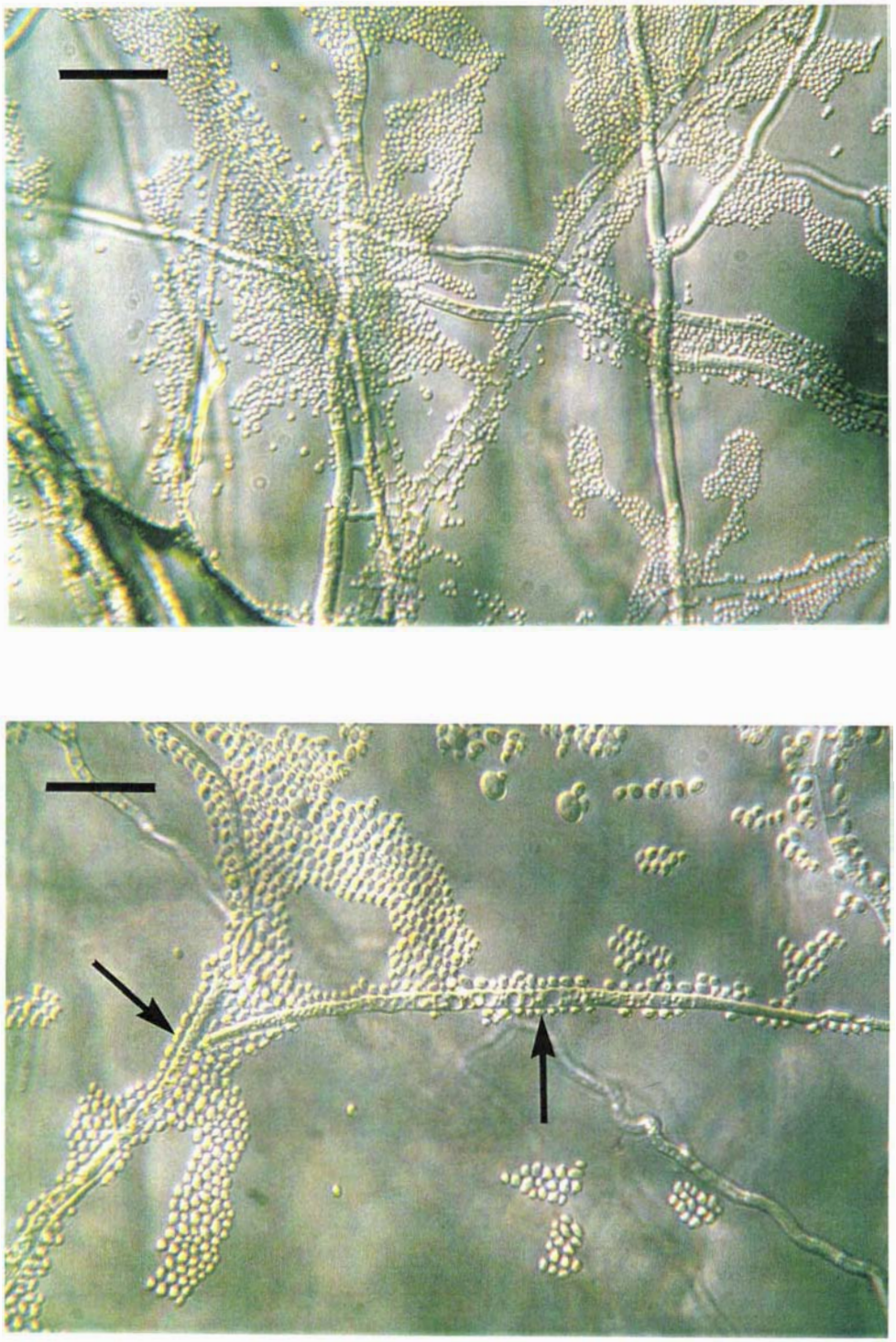


Fig. 7.2. The interaction between BCA yeast cells or mycelium and *B. cinerea* mycelium on minimal media with isolate 561, (*Candida sake*) (Top) and with isolate 622b (*Trichosporon pullulans*) (Bottom). Note the embedded nature of cell contact (Arrow). Bars = 100 µm.

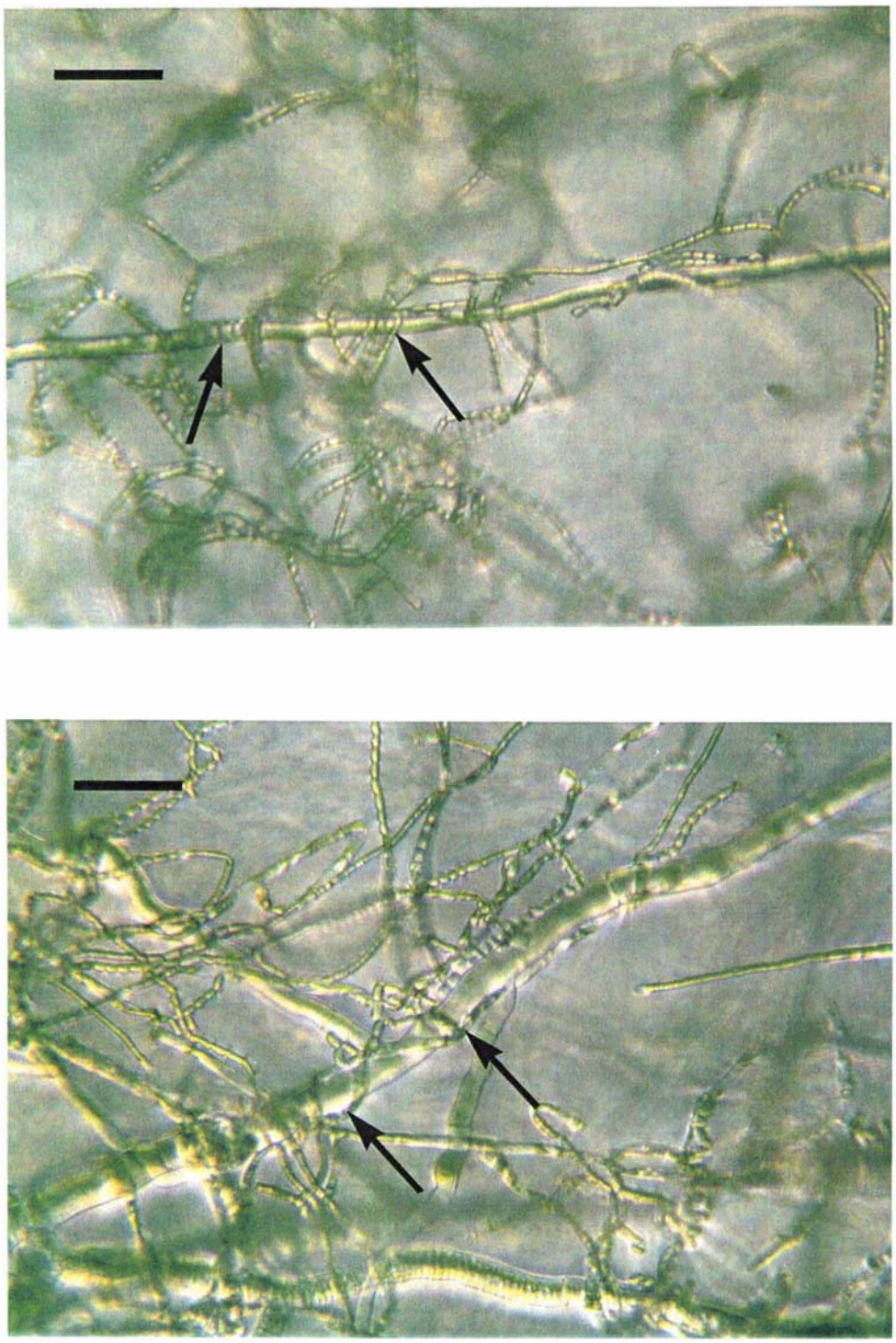


Fig. 7.2 (cont.). The interaction between BCA yeast cells or mycelium and *B. cinerea* mycelium on minimal mediamycelium of isolate 572c (*Galactomyces geotrichum*) (Top and Bottom). Note the coiling and intimate growth habit of the BCA on the pathogen (Arrow). Bar=100µm.

7.5.2 Inhibition of adhesion

7.5.2.1 Experiment One

On tomato extract agar media, all BCA isolates tested, (ox4, ox5, ox6, ox7, ox9, 561 and 532) attached to *B. cinerea* hyphae in SDW. Bacterial attachment, was inhibited by the addition of de-salted BC-KH4 but yeast adhesion was insensitive to this treatment (Table 7.4). The MAb, BC-KH4, was present in column fractions one, two and three as the ELISA absorbance values did not significantly differ (P>0.5) from neat BC-KH4.

Table 7.4 Presence or absence of bacteria (ox4, ox5, ox6, ox7, ox9) and yeast (561, 532) attachment to hyphae of *B cinerea* on tomato tissue extract agar with SDW or BC-KH4 added to the interaction mixture. y = attachment to hyphae. n = no attachment to hyphae.

MEDIA	BCA Isolate						
	ox4	ox5	ox6	ox7	ox9	561	532
WATER	y	y	y	y	y	y	y
PBS	y	y	y	y	y	y	y
FRACTION 1	n	n	y	y	y	y	y
FRACTION 2	n	n	y	y	y	y	y
FRACTION 3	n	n	n	n	n	y	y

B. cinerea germ tube length was not significantly different (P>0.5) when it was grown in SDW or BC-KH4 amended tomato tissue extract agar (Table 7.5). In SDW, growth by the pathogen was significantly less compared to the pathogen-only control when co-inoculated with all BCAs except isolate 561. In the presence of BC-KH4 *B. cinerea* growth was significantly reduced by isolates ox7 and ox6 only.

Table 7.5 Mean germ tube length after 16 h growth at 15°C of *B cinerea* spores germinating in the presence of bacteria or yeast BCAs on a tomato extract agar with SDW or BC-KH4 added. Different letters within the columns indicate significant differences between BCA isolates and control according to LSD (df=112, n=15. α=0.05) in separate analyses (upper and lower case). A third comparison of germ tube growth was conducted of germ tube growth in SDW only and BC-KH4 only as indicated by (*) (df=28, n=15, α=0.05).

BCA	SDW	BC-KH4
Nil	100.95 (A A*)	94.28 (ab A*)
ox4	87.61 (B)	97.00 (ab)
ox5	72.38 (C)	96.19 (ab)
ox6	50.47 (D)	40.95 (c)
ox7	3.81 (E)	40.95 (c)
ox9	62.85 (C)	105.62 (a)
561	100.95 (A)	89.52 (b)
532	89.5 (B)	94.27 (ab)

In the preliminary *in vivo* biocontrol experiment (Fig. 7.3), a similar incidence of healthy stem pieces in *B. cinerea* and *B. cinerea* plus BC-KH4 treatments indicated that the application of BC-KH4 after *B. cinerea* inoculation neither stimulated or inhibited pathogenicity. Thirty percent of stem pieces in the *B. cinerea*-only treatment remained healthy and this increased to 80% and 100% when ox5 and ox9 respectively were applied but declined to 50% when BC-KH4 was added. Yeast isolate 561 conferred strong biocontrol activity with or without BC-KH4.

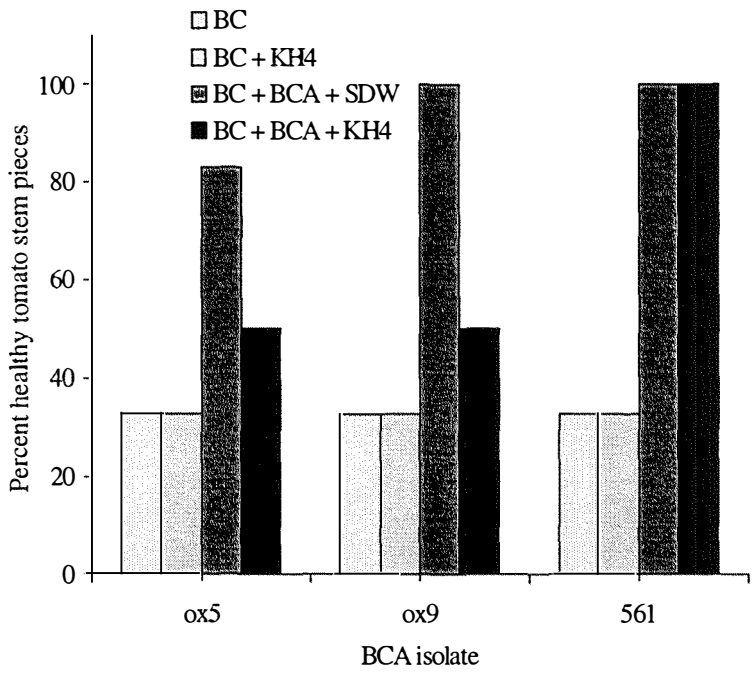


Fig. 7.3 Biocontrol of *B. cinerea* by bacterial isolates ox 5 and ox9 and yeast isolate 561 with and without the addition of BC-KH4 to excised inoculated tomato stem pieces (n=1) where BC=*B. cinerea.*

7.5.2.2 Experiment Two

There was concern that the 10 µl cell suspensions and MAb in Experiment One were insufficient to maintain an intimate contact between all additives on the agar surface consequently volumes in Experiment Two were increased (See Section 7.3.2.3). Bacterial adhesion was again inhibited by de-salted BC-KH4 while yeast adhesion was insensitive (Table 7.6). Fresh hybridoma culture fluid (RPMI+FBS) appeared to be toxic to all microbes present, as *B. cinerea* spores failed to germinate and the bacterial cells were no longer mobile.

Table 7.6 Presence or absence of bacteria (ox4, ox5, ox6, ox7, ox9) or yeast BCA (561, 532) isolates attached to hyphae of *B cinerea* in the presence of RPMI+FBS, where y = attachment, n = no attachment and - = microbes killed.

MEDIA	ox4	ox5	ox6	ox7	ox9	561	532
SDW	y	y	y	y	y	y	y
BC-KH4	n	n	n	n	n	y	y
RPMI + FBS	-	-	-	-	-	-	-

7.5.2.3 Experiment Three

In SDW and in PI-01 environments, bacterial and yeast attached to *B. cinerea* hyphae, with the exception of isolate ox8a where PI-01 inhibited adhesion (Table 7.7). In the presence of BC-KH4, bacterial adhesion was not observed but yeast adhesion was unaltered.

Table 7.7 Adhesion of bacterial and yeast BCA isolates to *B. cinerea* on tomato tissue extract agar with SDW, BC-KH4 or PI-01 added to the interaction. Data is presented from each of the three replicates where y= attachment, n= no attachment.

Isolate	SDW	BC-KH4	PI-01
ox4	y y y	n n n	y y y
ox7	y y y	n n n	y y y
ox8a	y y y	n n n	n n n
35a	y y y	n n n	y y y
561	y y y	y y y	y y y
532	y y y	y y y	y y y
622b	y y y	y y y	y y y
662dib	y y y	y y y	y y y

Variability in the data collected in this experiment was very high and consequently significant treatment differences were not detected using ANOVA even though the incidence of healthy stem pieces (a measure of biocontrol) markedly increased from <10% for the pathogen-only treatments to between 60-80% for all BCA pathogen co-inoculation treatments (Fig. 7.4). However, when the data from all bacterial isolates and yeast isolates were pooled, the effect of additive treatment was significant ($P<0.05$) (Fig. 7.5) where the incidence of healthy tissue was significantly reduced when BC-KH4 or PI-01 were added to stem pieces already inoculated with bacteria and *B. cinerea.* In contrast, only PI-01 significantly reduced the percentage of healthy stem

pieces biocontrol activity when added to tissue already inoculated with the yeasts and *B. cinerea* (Fig. 7.6).

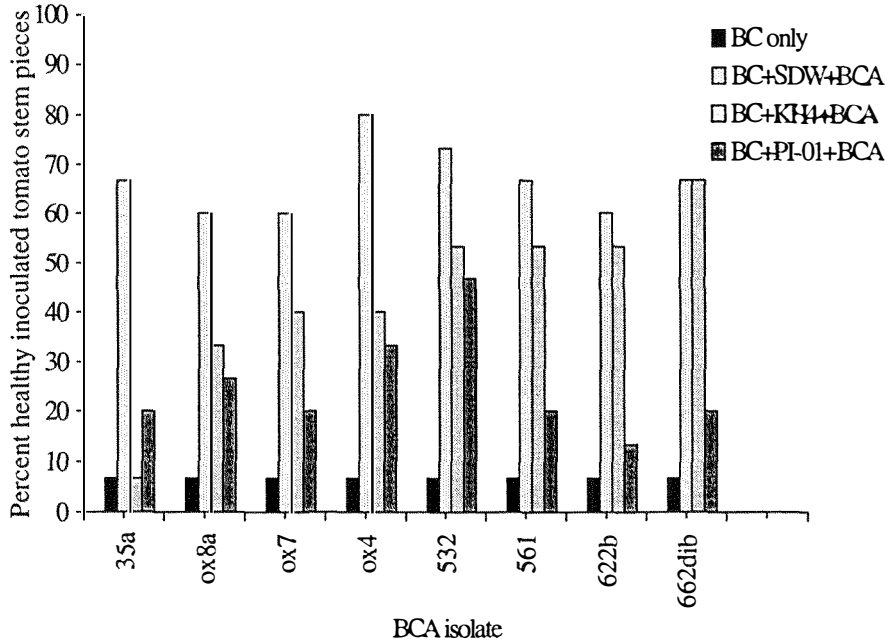


Fig. 7.4. Biocontrol of *B. cinerea* (indicated by percent healthy stems) on tomato stem pieces *in vivo* by each bacterial and yeast isolates in the presence of SDW, BC-KH4 and PI-01.

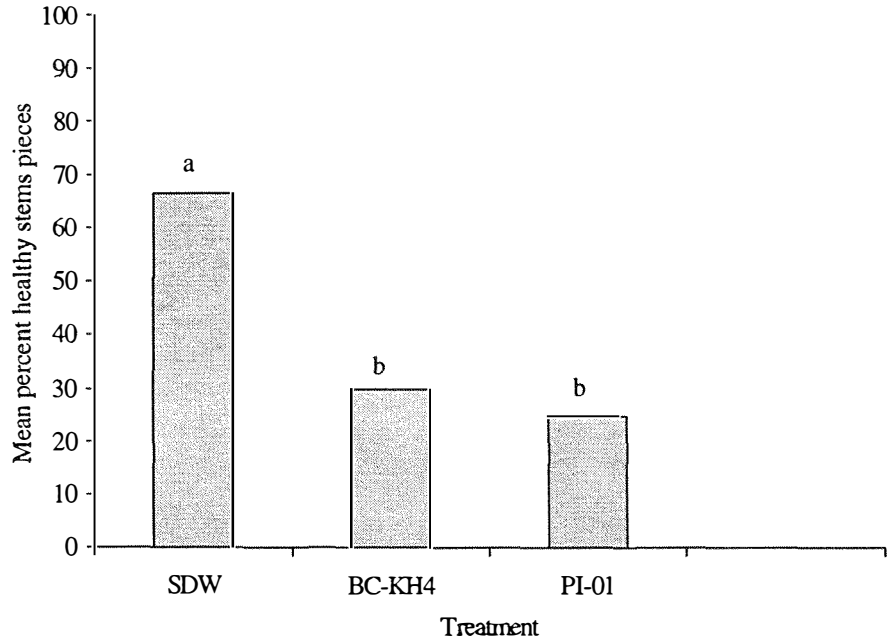


Fig. 7.5. Biocontrol of *B. cinerea* on tomato stem pieces by bacterial BCA isolates in the presence of SDW, BC-KH4 and PI-01. Data from each bacterial isolate has been pooled. Letters indicated significant differences according to Tukeys analysis (df=22, n=3, α=0.05).

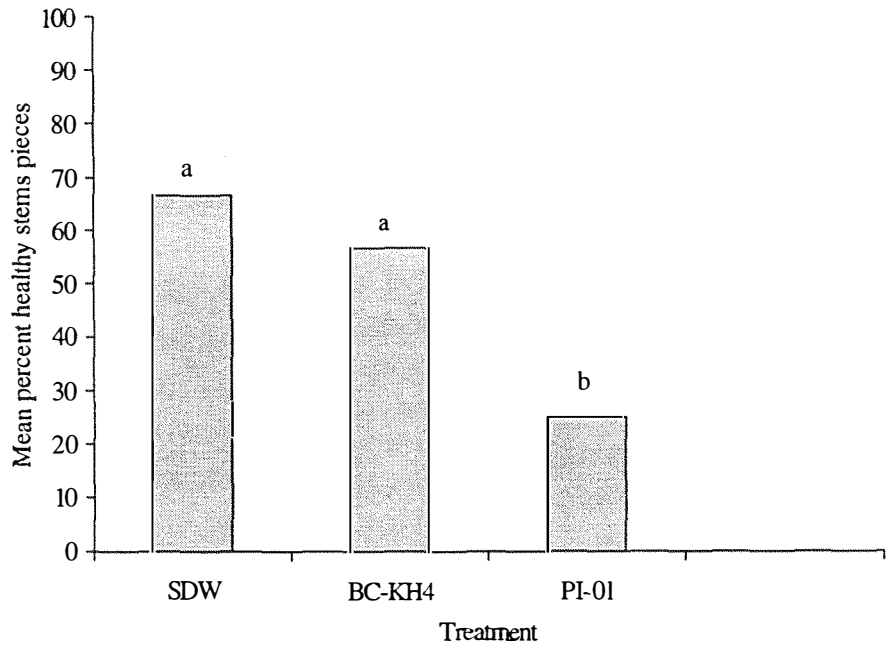


Fig. 7.6. Biocontrol of *B. cinerea* on tomato stem pieces by yeast BCA isolates in the presence of SDW, BC-KH4 and PI-01. Data from each yeast isolate has been pooled. Letters indicated significant differences according to Tukeys analysis (df=22, n=3, α=0.05).

7.5.3 Characterisation of BC-KH4 epitope

Galactomannan extract from *A. niger* exhibited strong inhibition of *B. cinerea* antigen detection in the competitive ELISA (Figs 7.7 to 7.9). Using a 500-fold dilution of BC-KH4, at the highest galactomannan concentration (1000), the ELISA reading was reduced from the 0.55 (control) to 0.15. This suppression was not as strong with increasing dilution of the extract until the dilution 1:32,000 where the ELISA signal from the treatment exceeded the control and peaked at 0.75 at the glactomannan dilution of 1:128,000. After this, signals declined in subsequent dilutions A similar trend was observed when BC-KH4 was diluted 1000-times. Treatment ELISA signals did not exceed control until a galactomannan dilution of 256,000 (Fig. 7.7). For treatments where the BC-KH4 was diluted 2000-fold and 4000-fold, overall, absorbances did not exceed 0.2 and suppression of ELISA signal compared to the control was not strong.

At the highest BC-KH4 concentration (diluted 500-fold) there was a suppression of the ELISA reading with the addition of 500 mM galactose or mannose (Figs 7.8 and

7.9 respectively) from 0.6 (nil sugar control) to 0.3 and from 0.3 to 0.15. However after between three or four serial dilutions, the suppression of ELISA signal diminished and absorbances were similar to the control. At higher dilutions of BC-KH4 (1000-times to 4000-times), the ELISA readings from the serial dilutions were similar to the nil sugar controls with the exception of galactose at 15mM and BC-KH4 was diluted 1000-fold where ELISA signals from the treatment exceeded the control.

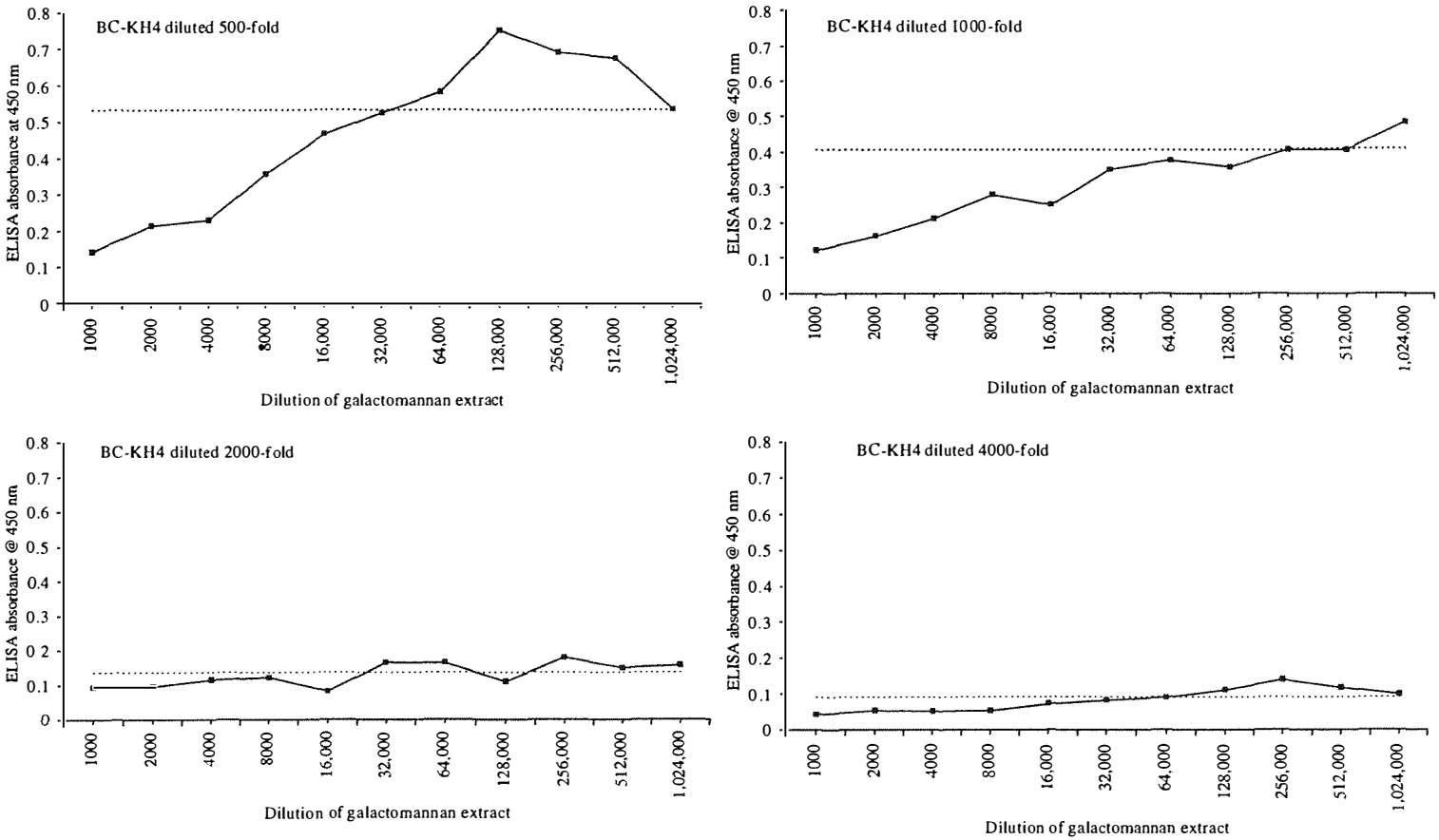


Fig. 7.7. Competitive ELISA for *B. cinerea* antigen detection at various dilutions of BC-KH4 and of galactomannan (extracted from *Aspergillus niger*). Horizontal line represents ELISA absorbance from nil galactomannan added to the microtitre well.

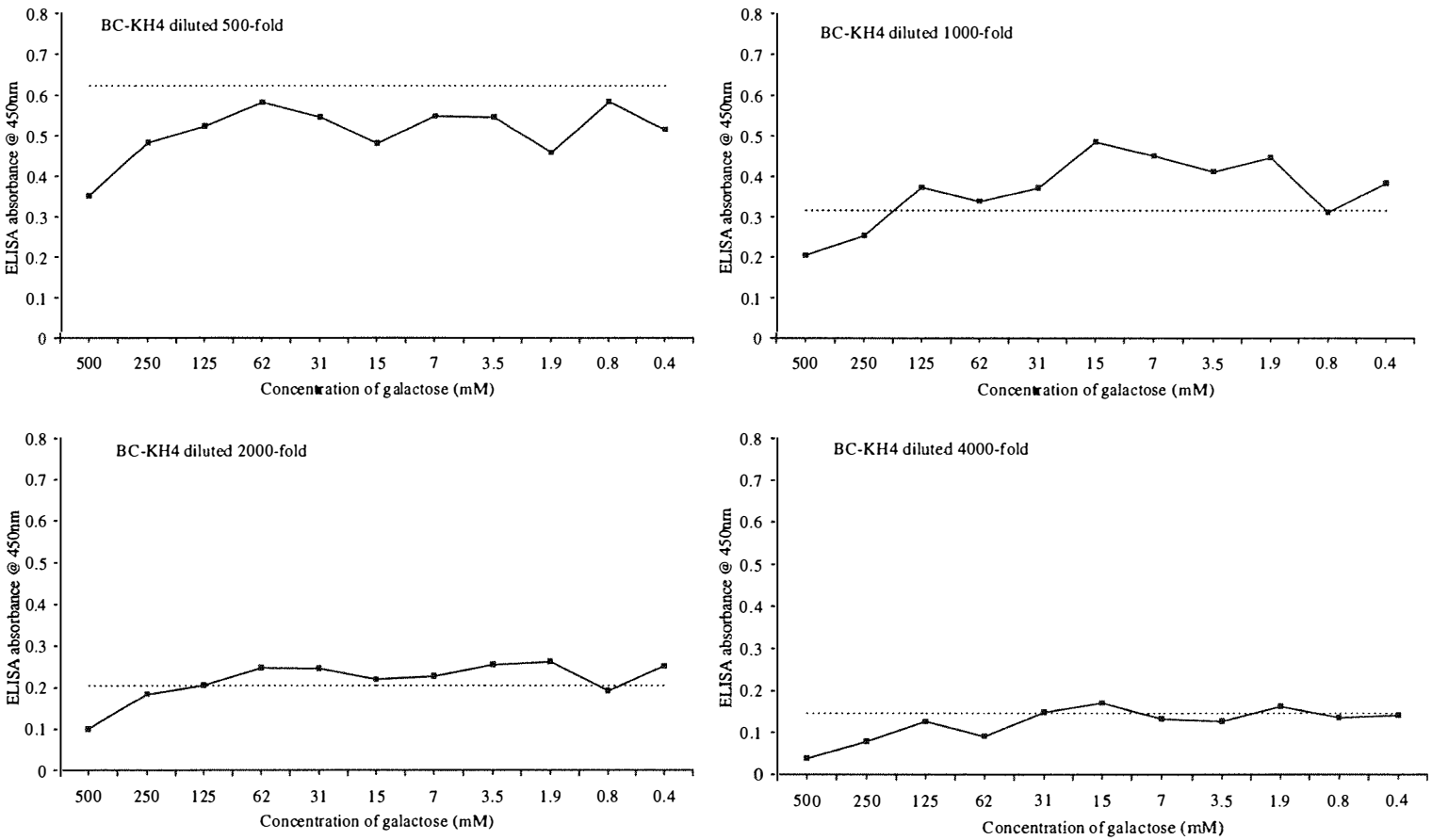


Fig. 7.8. Competitive ELISA for *B. cinerea* antigen detection at various dilutions of BC-KH4 and of galactose. Horizontal line represents ELISA absorbance from nil galactose added to the microtitre well.

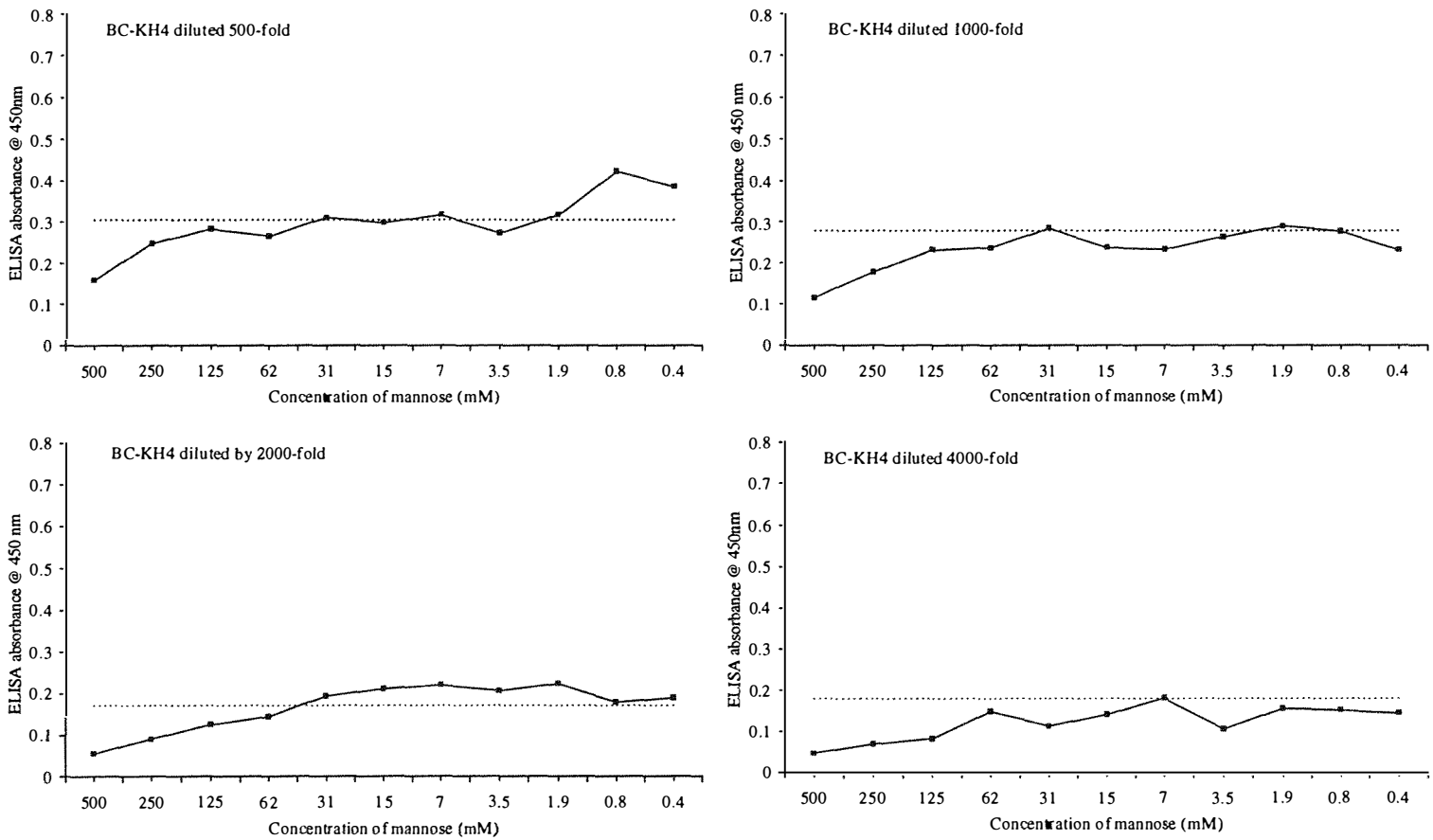


Fig. 7.9. Competitive ELISA for *B. cinerea* antigen detection at various dilutions of BC-KH4 and of mannose. Horizontal line represents ELISA absorbance from nil mannose added to the microtitre well.

7.6 Discussion

Many of the attacher BCA isolates exhibited other features that could be involved in the antagonism of *B. cinerea.* The endochitinase activity detected *in vitro* from many of the BCAs is intuitively consistent with a parasitic-type lifestyle. Microscopic studies in Chapter Six showed evidence for the partial or total destruction of *B. cinerea* hyphae when the pathogen was co-inoculated with some of the BCAs. Chitinase activity has been assumed to be an important mechanism for biocontrol because of the destruction of components in the fungal cell wall (Fridlender, Inbar & Chet, 1993) and has been previously reported in other mycoparasitic interactions, for example from *Pythium nunn* (Elad, Lifshitz & Baker, 1985). Holz, Schmidt & Ferreira (1995) speculated that chitinase from an isolate of *T. pullulans* was possibly involved in the biocontrol mechanism. Early records of fungal colonisation by bacteria also show the presence of lytic activity toward whole hyphae (Morgan, 1963) or fragments of the host (Mitchell & Alexander, 1963). However the general identity of the compounds (i.e. antibiotic or enzyme) was not established and it was questioned whether those isolates were primary colonisers of the hyphal surface (Malajczuk, Nesbitt & Glenn, 1977). In more recent reports, some isolates of *E. cloacae* and *E. agglomerans* exhibited endochitinase activity (Lorito *et al.,* 1993; Chernin, Ismailov, Haran & Chet, 1995) while other *E. cloacae* isolates did not (Nelson *et al.,* 1986). It is possible that these differences are due to isolate or culture conditions. In the current study, only one of three *E. cloacae* isolates tested synthesised extracellular endochitinase *in vitro* demonstrating isolate to isolate variation. This isolate synthesised endochitinase only on medium containing *B. cinerea* extract suggesting that culture conditions incorporating substances from the pathogen could trigger endochitinase production. Endochitinase enzyme activity has not been reported from the remaining bacterial species used in this study possibly because there are few recent reports of bacteria with an attacher lifestyle other than species of Enterobacter

There is debate as to the importance of siderophore production for biocontrol activity. Loper, Ishimaru, Carnegie & Vanavichit (1993) and Costa & Loper (1994) isolated and characterised the genes for biosynthesis for the hydroxymate siderophore, aerobactin, and for the catechol siderophore, enterobactin, from *E. cloacae.* Mutant isolates without siderophore activity continued to confer strong plant protection. In contrast, the mutant created by Yang *et al.* (1994) using a similar genetic approach failed to exhibit strong biocontrol activity although adhesion to *Phytophthora* sp. remained. In this study, bacterial isolates of *P. marginalis*, *O. anthropii E. cloacae* and *E. agglomerans* and yeast isolates *C. pulcherimma* and *T. pullulans* synthesised siderophores *in vitro* but the delay between inoculation and detection suggests this form of antagonism may not be important. No previous reports of siderophore production by yeasts could be found.

The biological significance of antibiotic compounds in biocontrol when results are based on *in vitro* data alone has been thoroughly debated (Thomashow & Weller 1996; Fravel, 1988). Volatile ammonia (Howell *et al.,* 1988) and agar diffusable compounds (Lorito *et al.,* 1993; Kwok, Fahy, Hoitink & Kuter, 1987; Hebbar, Davey & Dart, 1992) have been detected in some isolates of *E. cloacae* while for other isolates none were detected (Wilson, Franklin & Pusey, 1987; Nelson *et al.,* 1986). Sneh, Dupler, Elad & Baker, (1984) did not detect any antibiotic compounds from *E. cloacae in vitro* despite observing germ-tube lysis in soil co-inoculated with *Fusarium oxysporum* f.sp. *cucumerinum.* In this study, no antibiotic compounds were detected from any isolate of *Enterobacter* spp. or from yeasts on any agar media. Anti-Botrytis compounds were detected from isolates of *O. anthropii* and *P. marginalis* on minimal medium suggesting that one or more of the constituents of PDA, MEA or NYDA were interfering with the antibiotic production or expression. For example, the addition of histadine inactivated antibiotic compounds from *Erwinia herbicola* Eh 252 and rendering the compound ineffective for the biocontrol of *Erwinia amylovora in vitro* and *in vivo* (Vanneste, Yu & Beer, 1992) but this amino acid did not interfere with its production (J. Vanneste, personal communication). Antibiotic activity has also been detected from isolates of *E. aerogenes* (Utkhede & Sholberg, 1986; Marchi &

Utkhede, 1994) and *E. agglomerans* (Hebbar *et al.*, 1992) on PDA as well as the fluorescent Pseudomonads (Hebbar *et al.*, 1992) which includes *P. marginalis* (Doudoroff & Palleroni, 1974).

Adhesion by attacher microbes to fungal pathogens has been found to be critical for biocontrol (Nelson *et al.*, 1986; Nelson & Maloney 1992; Yang *et al.*, 1994) and the data from the current study suggests likewise as the MAb BC-KH4 consistently reduced biocontrol by targeting the attachment process. In the preliminary *in vitro* investigation, BC-KH4 inhibited the adhesion of bacterial isolates to *B. cinerea* but not that by yeasts. Germling growth *in vitro* in the presence of some of the bacterial BCAs was significantly less than in the pathogen-only control but was not less when BC-KH4 was added. *B. cinerea* growth in the presence of some of the yeast BCAs was significantly less than the control regardless of additive. In addition, there appeared to be no stimulation or reduction of *B. cinerea* growth *in vitro*. These trends were also found in first *in vivo* biocontrol experiment on tomato stem pieces where biocontrol by bacteria was inhibited but not that by yeasts. In Experiment Two, the role of RPMI + FBS in adhesion could not be defined as the media killed all microbes present despite the absence of the azide preservative. This is possibly due to the presence of a higher concentration of antibodies in the FBS specific to bacteria of yeasts perhaps due to the animal from which FBS was obtained being immunised as it ingested these or similar microbes colonising the grass.

Unexplained activity caused by PI-01 significantly reduced biocontrol activity of all the BCA isolates *in vivo* in Experiment Three but did not inhibit adhesion *in vitro* with the exception of isolate ox8a. PI-01, is an IgG specific for *Penicillium islandicum*, having low binding specificity for *B. cinerea* (Dewey, McDonald, Phillips & Priestley, 1990). The low inhibiting activity *in vitro* is consistent with its low binding to the pathogen and appears unlikely to recognise the BCAs either. The indiscriminate effect of PI-01 in reducing the biocontrol activity of both yeast and bacterial isolate may be related to an inhibition of secondary antagonism functions such as chitinase production or antifungal volatiles (Howell *et al.*, 1988). In contrast, results *in vivo* using BC-KH4 were consistent with those *in vitro* in all experiments indicating that the MAb selectively targets the adhesion mechanism and is

absent in the "antibody effect" as seen with PI-01. BC-KH4 is a MAb of the subclass IgM which could explain these differences.

In vitro, BC-KH4 inhibited bacterial adhesion to a site which was protected by, or in competition with a galactomannan extract. Neither galactose nor mannose expressed levels of competition for binding to *B. cinerea* antigen compared to the galactomannan extract. It may be possible that the high suppression activity in the galactomannan extract was due to an unknown contaminant. As BC-KH4 did not recognise any of the BCAs used in the present studies (See Fig. 8.3, Chapter Eight) it is proposed that the MAb binds to sites on *B. cinerea* that overlap with or are involved in bacterial adhesion. Such sites may be found on the *B. cinerea* fimbrae that facilitate adhesion in a manner similar to the type one fimbrae mediated adhesion by *E. cloacae* and *E. agglomerans* (Nelson & Maloney 1992) which is inhibited by mannose (Sharon & Ofek, 1986; Nelson *et al.*, 1986). As the bacteria in this BCA collection are either species of *Enterobacter*, have polar flagella (*Pseudomonas* spp. (Doudoroff & Palleroni, 1974)) or peritrichous flagella (*O. anthropii* (Holmes, Popoff, Kiredjian & Kersters, 1988)) this is certainly a topic continued research.

In the absence of mutant isolates, the importance of each individual biocontrol mechanism or their interaction in the field is difficult to predict. The creation of specific mutants was beyond the scope of this PhD project. Thus information on the biocontrol by antibiosis, siderophore, endochitinase synthesis and BCA adhesion to *B. cinerea* was limited to the *in vitro* technique. However, given the relatively large number of isolates used, some putative linkages can be made on the basis of the frequency with which a particular mechanism was detected. All of the yeast isolates attached to *B. cinerea* and expressed interactions consistent with colonisation of the pathogen (Chapter Six and this chapter). In addition all produced endochitinase and therefore it is highly likely that their primary mode of antagonism is necrotrophy either by parasitism or predation. It is also worth noting that yeast isolates 552c and 572c (*G. geotrichum*) produced mycelium on Minimal media and interacted with the *B. cinerea* hyphae in a coiling and growth manner similar to that described in other reports (Chambers & Scott, 1995) but this phenomenon was not observed on tomato stem pieces using the SEM (Chapter Six). It was possible that total

degradation of the *B. cinerea* hyphae had taken place during the incubation time of that experiment.

The exact mode of antagonism of bacterial isolates used in this study, is unclear despite the high number of isolates tested. For other isolates of *E. cloacae*, Wisniewski, Wilson & Hershberger (1989), Nelson *et al.* (1986), Nelson & Maloney (1992) proposed that nutrient competition was a potentially important mechanism as the hyphal crowding and intimate contact would position the BCAs to intercept incoming nutrient. Toyota & Kimura (1993) coined the phrase "reduction of nutrient independency" for the reduction of *Fusarium* sp. spore germination by that attacher microbe, *Pseudomonas stutzeri.* This antagonistic activity appears to be important as a mutant strain from this bacterium was created to include chitinase activity (Toyota, Miyashita & Kimura, 1994). The authors capitalised on the colonisation ability of the bacterium but could only achieve a relatively small increase in biocontrol activity compared to the parent. Results from the current study suggest that competition is unlikely to be important compared to pathogen degradation for example. In Chapter Five, biocontrol *in vivo* was exhibited by yeast and bacteria regardless of populations applied or the timing of application. In addition, SEM data in Chapter Six showed varying levels of pathogen colonsiation by the different species of bacteria. Bacterial isolates ox2 and ox8a both have comparable biocontrol functions as shown in this chapter (endochitinase production) but ox2 was an infrequent coloniser compared with the aggressive ox8a. Similarly both ox4 and ox6 produced siderophores and again there were differences in the levels of colonisation. Using the biocontrol model based on phylloplane colonisers that compete for resource, pathogen antagonism is closely related to application timing and populations. Assuming the mechanisms of competition are similar regardless of plant host or fungal host, the differences in coloniser populations observed in Chapter Six or applied to host tissue in Chapter Five do not correlate with differences in biocontrol activity.

Biocontrol mechanisms associated with adhesion including responses to the presence of the pathogen should be addressed in any future research. Fletcher (1984) stated that changes in bacterial physiology are likely to after adhesion occur since conditions at surfaces are different from those in aqueous suspension and attachment could alter the physiology of membrane-associated processes. Some *in vitro* technique where BCA isolates are cultured

alone to detect metabolites assumes that the physiology of the BCA is similar when attached to the pathogen as it is free living and ignores the possibility of communication prompted by physical or biochemical stimuli from *B. cinerea*. For the isolates in the current collection, this consideration is important and is illustrated by the endochitinase production from isolate ox6 when cultured on *B. cinerea*-amended media. Another specific bacterial response not examined in this study was chemotaxis. Directional movement of a number of bacteria toward fungal spores has been reported (Chet, Fogel & Mitchell, 1971; Chet & Mitchell 1976; Lim & Lockwood, 1988; Grewal & Rainey, 1991; Arora & Gupta, 1993) in predatory and pathogenic interactions. It would not be surprising to find such tactile behaviour by bacteria in the present BCA collection and would provide a logical explanation for why such low populations (three-times the number of *B. cinerea* spores) can be applied to plant surfaces and still achieve high levels of biocontrol. There are no reports of chemotaxis by yeast cells which, in the absence of any obvious mechanism of motility, is not surprising.

The capacity for the expression of some microbial biocontrol mechanisms involving antibiotics, siderophores or endochitinase biosynthesis were examined *in vitro*. Their importance in biocontrol in the field, and in combination with adhesion to the pathogen host, remains unknown, but a basis for further research has been prepared by identifying potential mechanisms which physiologists, biochemists and geneticists can target in future research.

An immunoassay using BC-KH4, (a monoclonal antibody specific to *B. cinerea*) as a research tool for the determination of pathogen biomass during biocontrol interactions

8.1 Introduction

Strategies for use of biocontrol of fungal plant pathogens is founded on ecological principles for the manipulation of pathogen populations (Andrews, 1992). Antagonists are introduced or populations augmented to prevent or reduce pathogen establishment, growth or reproduction. It has often been assumed that biocontrol of the pathogen is closely related to reductions in its biomass and both direct and indirect methods have been used to measure this within the biocontrol interaction (Lumsden, Carter, Whipps & Lynch, 1990; Kessel *et al.*, 1996). Direct measurement of the antagonist populations (i.e. the number of colony-forming-units) after application to the infection court are most suitable for unicellular microbes such as bacteria (Weller & Cook, 1983; Janisiewicz, 1992; Elad, Kohl & Fokkema, 1994b) and yeasts (Roberts, 1990; Elad *et al.*, 1994b Mercier & Wilson, 1995). Enumeration methods are reviewed by Herbert (1990) and methods for washing microflora from plant surfaces have been examined (Kloepper & Beauchamp, 1992; Donegan, Matyac, Seidler & Porteous, 1991). Reproductive biomass of the pathogen has been measured by number of spores per unit air volume (Kohl *et al.*, 1992; Kohl, Molhoek, van der Plas, Fokkema, 1995b), conidiophores per lesion (Elad *et al.*, 1994b) or the area of a leaf covered by conidiophores (Kohl, Molhoek, van der Plas, Fokkema, 1995a). Vegetative fungal biomass has been determined indirectly using key biochemical markers including mannan, glucan and trehalose (Whipps, Haselwandter, McGee & Lewis, 1982). Adenosine triphosphate (ATP), fluorescein diacetate staining via esterase activity, chitin, total CO_2 evolution and ergosterol were more recently used in the interaction between *Pythium ultimum* and *Trichoderma harzianum* in potting media (Lumsden *et al.*, 1990). However these markers for biomass require conversion factors that are often based on *in vitro* conditions (Bermingham, Maltby & Cooke, 1995) consequently errors

in estimations are introduced when the same marker is used in the natural environment (Newell, 1992).

Chitin and ergosterol are fungal specific (for microbes), yet there are limitations in their use as biomass indicators that restrict general application. Chitin levels in hyphae vary with mycelium age and growth conditions and it persists after cell death (Sharma, Fisher & Webster, 1977). No correlation was found between ergosterol content and mycelial dry weight in six of nine aquatic hyphomycetes tested by Bermingham *et al.* (1995b) and to date neither method is able to discriminate between a fungal antagonist and pathogen. Measurement of ATP, although a good indicator in soil environments (Prevost, Angers & Nadeau, 1991) is not applicable to phylloplane studies on living host tissue as ATP from the plant will mask treatment differences. Fluorescein diacetate (FDA) staining is highly dependent on the homogeneity of sample preparation in order to release all hyphae from entrapment but in the process damaged mycelium may release cytoplasm which is also stained by FDA while alive (Soderstrom, 1979) but was nevertheless applied to estimates of fungal biomass on the phylloplane (Swisher & Carroll, 1980). Discrimination among the different microbial populations is difficult, therefore only general conclusions regarding overall biological activity can be made.

The introduction of immunology-based technology has increased the range of markers that can be used for microbial biomass measurement. Monoclonal antibodies (MAbs) have been used in preference to Polyclonal (PAbs) as tools to study fungal, bacterial and mycoplasma-like-micro-organisms (MLOs) because of their greater taxonomic specificity (Torrance, 1995). Immunological applications vary from field immunodetection and identification of the target (Lin & Cousin, 1987; Van Vuurde & Roozen, 1990; Dewey *et al.,* 1992; Timmer *et al.,* 1993; Yuen, Craig & Avila, 1993; van de Koppel & Schots, 1995) to laboratory investigations of pathogen-host interactions (Toda *et al.,* 1984; Linggood & Porter, 1980) or immunocytochemical labelling (Hardham, 1989; Cole, Dewey & Hawes, 1996). Furthermore, monoclonal antibody-based techniques have been used to estimate microbial biomass in/on plant tissues. The ELISA format was used for *Verticillium dahliae* in potato (Plasencia, Jemmerson & Banttari, 1996), *Humicola lanuginosa* in rice grains (Dewey *et al.*, 1992) and leaf decomposing aquatic hyphomycete such as *Anguillospora longissima* (Bermingham, Dewey & Maltby, 1995a). Ricker *et al.* (1991) used PAbs to quantify *B.*

cinerea in grapes and expressed juice and reported a detection limit of 0.5 to 0.25% (v/v). Use of MAbs further lowered this to 0.1% in the same host tissue (Bossi & Dewey, 1992). An elegant system for biomass determination of both pathogen and antagonist has been proposed for the *Trichoderma* sp. versus *Rhizoctonia* sp. system (Thornton & Dewey, 1996) where MAbs have been raised to *Trichoderma harzianum* phialoconidia (Thornton & Dewey, 1996) and hyphae (Thornton, Dewey & Gilligan, 1994) and to *Rhizoctonia* hyphae (Thornton, Dewey & Gilligan, 1993). Using the immunofluoresence format, *Pseudomonas stutzeri* populations in a marine environment (Ward & Cockcroft, 1993), sulphate-reducing-bacteria (Lillebaek, 1995), *Erwinia* sp. in cattle manure (Van Vuurde & Roozen, 1990) and *B. cinerea* on cut flowers (Salinas, Schober & Schots, 1994) were immunolabelled for detection. Kessel *et al.* (1996) used image analysis of tissues labelled with the MAb BC-KH4 and immunofluorescence for the spatial analysis and quantification of *B. cinerea* biomass after interacting with the BCA, *Ulocladium atrum,* on necrotic lily leaves.

Recently Bossi & Dewey (1992) reported the development of a suite of MAbs that recognised *Botrytis* spp. Immunocytochemical labelling studies of *Botrytis* spp. have shown large amounts of some of these MAbs, particularly the MAb BC-KH4, binding to the extracellular matrix and fibrillar structures surrounding the young germ tube with a smaller amount on the conidia. Binding was observed both *in vitro* and *in vivo* as the pathogen invades the host tissue (Cole *et al.,* 1996); thus demonstrating the potential of this MAb for quantitative determination of *B. cinerea* biomass. Most immunofluoresence studies are only suitable for examination of surfaces or transverse sections of host tissues therefore it is likely that image analysis of immunolabelled stem pieces would underestimate biomass due to the three dimensional growth of *B. cinerea.* Therefore an ELISA-based method was chosen as the assay format. Results from preliminary experiments showed that currently used extraction buffers was unsuitable (See Appendix Three for details) therefore research emphasis shifted to investigate alternative detection and extraction systems.

8.2 Objective

To develop a MAb-based immunoassay for *B. cinerea* biomass measurement on excised tomato stem pieces.

8.2.1 Experiment One - ELISA optimisation

8.2.1.A Introduction

In preliminary research, a monovalent antibody-conjugate system was used to detect BC-KH4. In the present study, this was changed to the multivalent biotin-avidin detection system where the biotin molecule is responsible for the amplification in ELISA (Durand, 1990). However inter-molecular constraints when using such large molecules as the BC-KH4 IgM and the biotin-streptavidin can still limit this amplification and it was necessary to optimise some of the ELISA conditions

8.2.1.B Materials and Methods

Stock *B. cinerea* antigen extract (Chapter Two, Section 2.5.3) was prepared from germinated *B. cinerea* conidia (5 x 10^6 spores/plate) incubated at 15°C for 60 h on MEA in 5 ml of either PBS or bicarbonate buffer (pH 9.6). Control MEA extracts were prepared in the same way but washing uninoculated plates in the same buffers.

Plate loading was carried out by pipetting 50 µl of each stock suspension in each buffer into a microtitre well and preparing a series of 1:1 dilutions in successive wells with the appropriate buffer. The plates were incubated for 16 h at 4°C . The ELISA procedure used was that described in Chapter Two, Section 2.5.6, except biotinylated antibody (B6649, Sigma, MO, USA) was diluted 1:2500, 1:5000, 1:10,000 and 1:20,000 in PBST and ExtrAvidin (E2886, Sigma, MO, USA) was diluted at 1:2000 in PBST for all treatments.

8.2.1.C Results and Discussion

Maximum ELISA absorbances for the fungal extract in PBS or bicarbonate buffer were 0.2 and >1.0 respectively (Figs 8.1 and 8.2). Small changes in these values (0 - 0.2) were obtained with the different concentrations of the antibody-biotin conjugate in PBS but there were larger changes in ELISA absorbance (0.2 - 1.2) with different antibody-biotin conjugate or *B. cinerea* antigen concentration in bicarbonate buffer. As the antigen was diluted, there was an increase in the absorbance values for all antibody concentrations in bicarbonate buffer (Fig. 8.2).

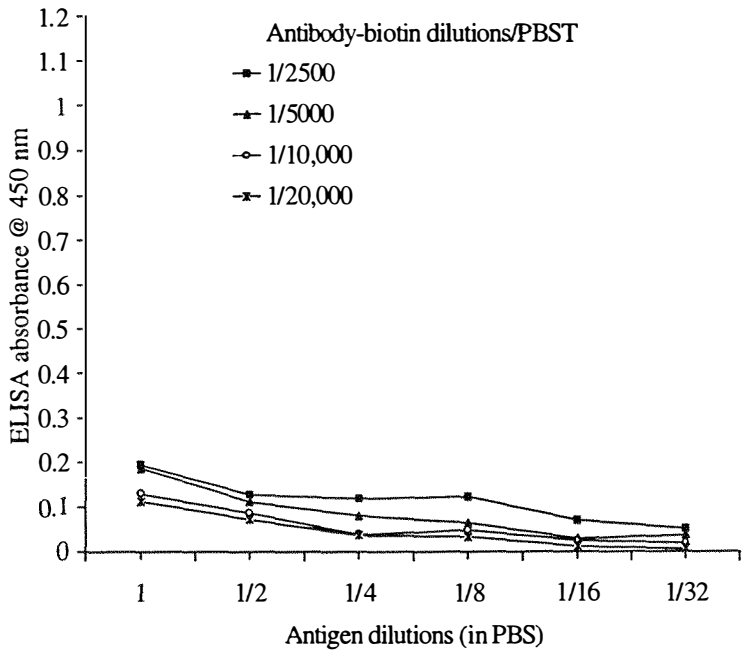


Fig. 8.1. Indirect PTA-ELISA absorbances from serially diluted *B. cinerea* extract in PBS and anti-mouse antibody conjugated to biotin in PBST. The extrAvidin concentration remained constant at 1:1000 in PBST.

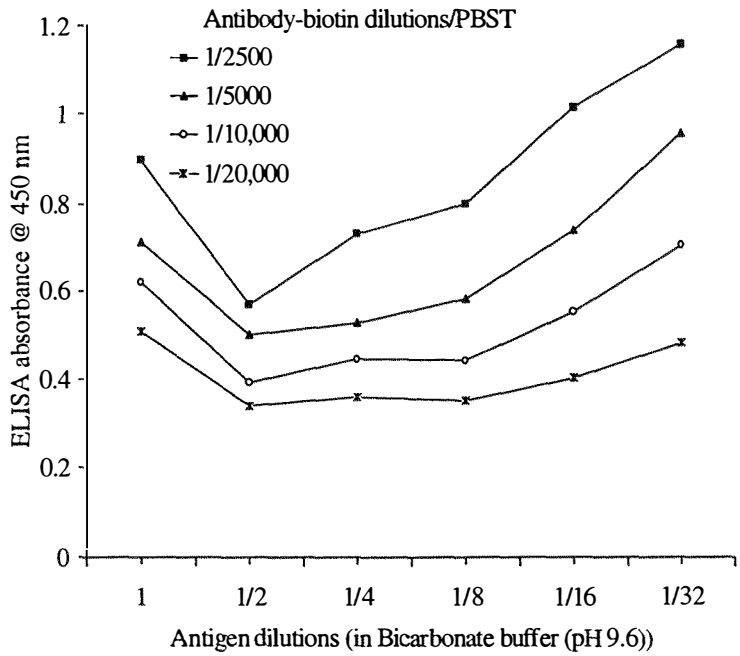


Fig. 8.2. Indirect PTA-ELISA absorbances from serially diluted *B. cinerea* extract in bicarbonate buffer and anti-mouse antibody conjugated to biotin in PBST. The extrAvidin concentration remained constant at 1:1000 in PBST.

The effect of buffer on the detection of the *B. cinerea* antigen *in vitro*, is evident in this experiment where there was an increase in the ELISA sensitivity in bicarbonate buffer compared to PBS. This is in contrast to previous work (Bossi, Cole, Spier & Dewey, 1994) but is consistent with the view that bicarbonate buffer (pH 9.6) does allow the *B. cinerea*

antigen in solution to bind better to micro titre well wall (F.M. Dewey, personal communication). A similar result was obtained in the corresponding buffer comparison using the anti-mouse antibody-peroxidase conjugate in Experiment A3.2 (Appendix Three) but the difference was not as strong probably due to the multivalent binding between the biotin and avidin molecules amplifying the ELISA signals.

Intermolecular factors appear to be responsible for the increase in ELISA absorbance with increasing dilution of the antigen. The MAb BC-KH4 is of the sub-class IgM, the largest antibody of the sub-classes. When high antigen concentrations are bound to the microtitre well wall, a proportion of binding sites on the glycoprotein are not bound by BC-KH4 because intermolecular distances exclude it from some sites. When antigen concentration decreases, inter-molecule space increases and allows BC-KH4 to bind to sites previously unavailable.

8.2.2 Experiment Two - Mechanical methods for antigen release

8.2.2.A Materials and Methods

Tomato stems pieces from seedlings (Chapter Two, Sections 2.3.1 and 2.3.2) were spray inoculated by adding 1 ml of 4 x 10^7 spores/ ml of *B. cinerea* conidia suspension to the reservoir of the Potter Tower (See Chapter Four). A second group of stem pieces remained uninoculated. The tissues were then incubated at 15°C for 5 days by which time, disease symptoms were apparent. Uninoculated control tissues were likewise incubated but separated from the treated stems to prevent contamination.

A slice of 1-2 mm was removed from the treated end of the stem pieces and these subsamples were placed into sterile eppendorf tubes with 50 µl of bicarbonate buffer (pH 9.6) alone or Bicarbonate buffer with 0.02% Azide (final concentration). Subsamples of stems were sonicated for 4 min, mechanically crushed using a sterile glass rod, autoclaved for 5 min at 121°C, frozen for 30 min then thawed or were left untreated and intact. Stock *B. cinerea* antigen suspensions (Chapter Two, Section 2.5.3) were autoclaved, sonicated or remained untreated to serve as controls. The contents of each tube was then transferred to microtitre wells ensuring treatments were randomised within plates before incubating for 16 h at 4°C. The ELISA protocol for the detection of antigen is described in Chapter Two, Section 2.5.6.

8.2.2.B Results and Discussion

None of the mechanical treatments improved the detection of *B. cinerea* antigen between uninoculated and inoculated stem pieces and the addition of azide had no consistent effect (Table 8.1). Non-specific binding with plant material was destroyed with autoclaving but the *B. cinerea* antigen extract was also disrupted by the treatment.

Table 8.1. *B. cinerea* antigen detection by indirect PTA-ELISA using BC-KH4 and biotinylated antibody. Values in parentheses are 5% margins of error for each treatment mean (n=3).

Extractant	Treatment	Inoculated tissue	Uninoculated tissue
Bicarb	Soak	0.17 (0.09)	0.39 (0.08)
	Autoclaved	0.08 (0.07)	0.07 (0.004)
	Freeze	0.43 (0.16)	0.70 (0.51)
	Macerate	0.42 (0.13)	0.35 (0.08)
	Sonicate 4 min	0.46 (0.36)	0.50 (0.17)
Bicarb Az	Soak	0.65 (0.19)	0.62 (0.23)
	Autoclaved	0.08 (0.09)	0.007 (0.006)
	Freeze	0.40 (0.22)	0.55 (0.25)
	Macerate	0.38 (0.39)	0.61 (0.37)
	Sonicate 4 min	0.40 (0.23)	0.53 (0.12)
Control Untreated		0.89 (0.10)	
Control Sonicate 4 min		0.86 (0.06)	
Control Autoclaved		0.06 (0.004)	

The various extraction treatments and buffers used in this study to enhance the detection of *B. cinerea* antigen were ineffective and discrimination between inoculated and uninoculated tissue was poor. This result contrasts with Experiment A3.3, Appendix Three, where treatment differences were observed between fresh uninoculated tissues and inoculated and incubated stem pieces. In the current experiment, the uninoculated tissue was also incubated for the same time period as the inoculated stems. These results suggest that poor inoculation treatment discrimination in Experiment A3.5 (Appendix Three) was the result of a substance in the tomato tissue induced by wounding which increases with incubation.

Reduction of background absorbances by autoclaving the inoculated tissue was considered a treatment with potential as Bossi & Dewey (1992) reported that the *B. cinerea* antigen was heat stable. However this stability is apparently buffer dependent as Bossi & Dewey (1992) used PBS but in bicarbonate buffer the antigen was destroyed.

8.2.3 Experiment Three - an investigation into alternative buffers

8.2.3.A Introduction

In this experiment, the range of extractants examined was based on a literature search for additives to standard buffers and for completely novel extraction buffers. One example is an antigen extraction buffer frequently used in the detection of viruses that incorporates Polyvinylpyrrolidone (PVP MW=44,000) at a concentration of 20.0 g/litre. PVP removes phenols that generally cause problems in antigen extraction. Another is the copper sulphate-based recipes used for the extraction of *Rhizoctonia* sp. antigen from soils (Otten, Gilligan & Thornton, (in press)).

A third approach was to select an extractant capable of quenching the interference from the host tissue. A likely cause of interference was lectins, commonly found in plants from the Solanaceae family including tomato (Kilpatrick, Weston & Urbaniak, 1983; Kilpatrick *et al.,* 1984; Goldstein & Poretz, 1986). Sharon & Lis (1989) stated that some require a metal co-factor as a structural component and if this were removed, the lectin structure would remain incomplete. EDTA is a general chelating agent and could decrease the activity of the interferent. Therefore, copper sulphate-based extractants, PVP additives, EDTA and PBS buffers were compared in this experiment for their ability to enhabce the extraction of *B. cinerea* antigen only from infected tomato tissue.

8.2.3.B Materials and Methods

Tomato stems pieces from seedlings (Chapter Two, Sections 2.3.1 and 2.3.2) were spray inoculated or remained uninoculated then incubated according to the protocol described in Experiment Two. A slice of 1-2 mm was removed from the treated end of the stem pieces and these subsamples were pooled (Three subsamples/treatment) into sterile universal bottles with 3 ml of one of the following extractant:

Bicarbonate buffer (pH 9.6)
Bicarbonate buffer (pH 9.6) + PVP (MW=44,000 (20g/litre))
0.1 M $CuSO_4$ + PVP
0.1 M $CuSO_4$ + KCl + NaCl
0.01M $CuSO_4$ + KCl + NaCl
0.1M EDTA + KCl + NaCl

Subsamples remained in the extractant for 6 h at R.T. before 50 µl was transferred to microtitre wells for incubation at 4°C for 16 h. ELISA procedure for detecting antigen is described in Chapter Two Section 2.5.6. *B. cinerea* antigen alone extracts from pure cultures was also tested as a positive control.

8.2.3.C Results and Discussion

High ELISA signals were obtained from *B. cinerea* antigen regardless of antigen extractant used but varied widely when tomato tissue was used (Table 8.2). The control (bicarbonate buffer pH 9.6 alone) and with added PVP, were poor extractants as ELISA measurements from uninoculated tomato tissue was similar to inoculated. The signal generated from uninoculated tissue exceeded that from inoculated for the extractants, 0.01M $CuSO_4$ + salts, $CuSO_4$ + PVP, and EDTA. The only extractant to give a greater ELISA absorbance in inoculated stem extracts was $CuSO_4$ + salts at 0.1M. This extractant also improved *B. cinerea* antigen detection *in vitro* compared with bicarbonate buffer.

Table 8.2. Indirect PTA-ELISA detection of *B. cinerea* antigen *in vitro* and from inoculated tomato tissue using copper, bicarbonate and EDTA based extractants with and without PVP. Values in parentheses are 5% margins of error (n=3).

Extractant	*B. cinerea* Extract	Inoculated Tissue	Uninoculated Tissue
Bicarbonate (pH9.6)	1.42 (0.12)	0.21 (0.02)	0.26 (0.09)
Bicarbonate + PVP	1.27 (0.13)	0.38 (0.02)	0.43 (0.04)
0.01 M $CuSO_4$ + salts	1.55 (0.03)	0.07 (0.02)	0.23 (0.06)
0.01 M $CuSO_4$ + PVP	1.42 (0.04)	0.01 (0.008)	0.18 (0.11)
0.1 M EDTA	1.29 (0.05)	0.11 (0.02)	0.32 (0.09)
0.1 M $CuSO_4$ + salts	1.50 (0.07)	0.46 (0.06)	0.18 (0.07)

Results of this experiment indicate that a 0.1M copper-based extractant has an application beyond that in soil (C. Thornton, personal communication) and could be equally beneficial to phylloplane studies. However further experiments are desirable to verify that discrimination between inoculated and uninoculated host tissue can be consistently obtained and whether an increase in signal will result from an increased incubation time of the inoculated tissue. The failure of EDTA as a method to quench ELISA background does not exclude the possible role of lectins as the interferent. There is still information required to confirm whether all lectin species found in tomatoes have a divalent metal co-factor requirement and if so, could this be complexed using EDTA.

8.2.4 Experiment Four - Detection of B. cinerea *antigen over time*

8.2.4.A Materials and Methods

Tomato stems pieces from seedlings (Chapter Two, Sections 2.3) were spray inoculated or remained uninoculated as described in Experiment Two and incubated at 15°C for up to 96 h. A total of 36 inoculated and 36 uninoculated stem pieces were removed from incubation at 24 h intervals for fungal biomass and background determination respectively.

Each stem piece was subsampled by cutting slices 1-2 mm thick from the treated end of each stem piece and these subsamples were either pooled (Three samples / treatment) or placed singularly into microtitre wells. One hundred microlitres of extractant (0.1M CuSO4 + 0.1M KCl + 0.1M NaCl) was added.

A second group of stem samples were similarly prepared at each time sample, but during well loading, tissues were macerated by crushing the samples with the end of a sterile washed glass rod. Three replicates of each tissue preparation treatment was prepared at each time interval. ELISA detection for *B. cinerea* antigen is described in Chapter Two, Section 2.5.6.

Each tissue processing treatment was analysed separately using a completely randomised experimental design according to the standard statistical analysis described in Chapter Two, Section 2.6. Preplanned comparisons were carried out between inoculated and uninoculated treatments using customised comparisons.

8.2.4.B Results and Discussion

The copper-based extractant consistently discriminated between inoculated and uninoculated stem pieces in all tissue preparation treatments (Fig. 8.3) except where single tissue samples were loaded per well and soaked. For remaining tissue preparation methods (Fig. 8.3 b, c and d), ELISA values from the inoculated stem samples were significantly greater ($P<0.0001$) than uninoculated at 48 h and generally increased with further incubation (Fig. 8.3 b and c). By pooling and macerating samples (Fig. 8.3d), there was a much smaller but still significant difference ($P<0.0001$) between inoculated and uninoculated tissue over all sample periods. Treatment variability as indicated by LSD was reduced by macerating samples (Fig. 8.3a versus c and b versus d). This ELISA protocol for *B. cinerea* antigen detection appeared promising for testing in the presence of BCAs.

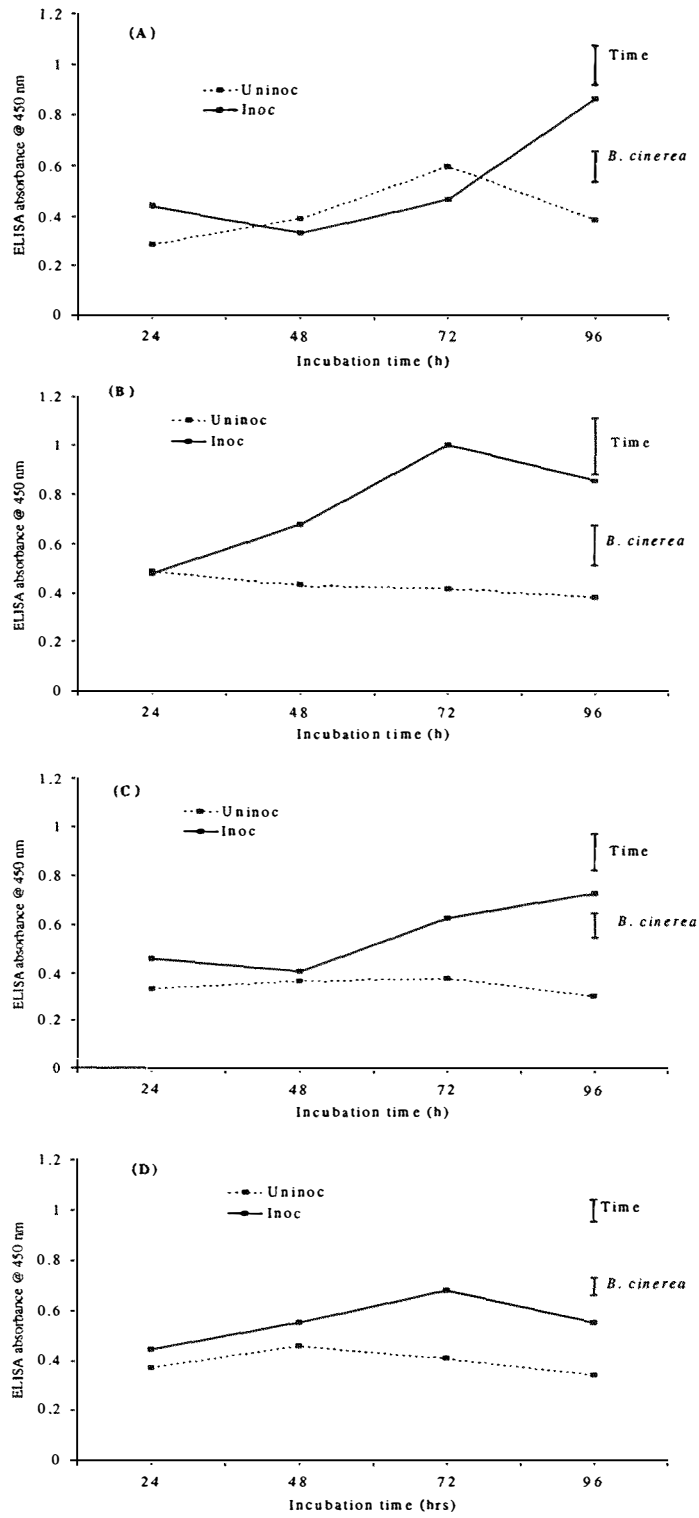


Fig. 8.3. a-d. *B. cinerea* antigen detection by indirect PTA-ELISA using 0.1M $CuSO_4$ + 0.1M KCl + 0.1M NaCl as the antigen extractant from inoculated tomato stem pieces, with BC-KH4 as the primary antibody and biotin-avidin peroxidase detection system. Tissues were prepared by a) one tissue sample per well and soaked in extractant, b) tissue samples pooled (3 / well) and soaked in extractant, c) one tissue sample per well and macerated in extractant and d) pooled tissue samples (3 / well) and macerated in extractant. Vertical bars represent LSD for main plot (*B. cinerea* inoculation (df=43, n=3)) and split plot (Time, (df=43, n=3)).

8.2.5 Experiment Five - Detection of BCAs by the MAb BC-KH4 in the presence of copper extractant

8.2.5.A Materials and Methods

Bacterial and yeast isolates were grown on NA and NYDA respectively for 5 days at 15°C, suspended in 0.1 M $CuSO_4$ + salts extractant and 50 µl pipetted into three replicate wells per isolate. Wells were incubated for 16 h at 4°C and the ELISA procedure for antigen detection is described in Chapter Two, Section 2.5.6.

8.2.5.B Results and Discussion

Table 8.3. Indirect PTA-ELISA detection of *B. cinerea* and BCA isolate antigens using the copper-based extractant and BC-KH4. MOE= 5% (n=3).

Isolate	Mean Absorbance	MOE
B. cinerea	1.30	0.04
ox2	0.03	0.01
ox4	0.03	0.001
ox6	0.02	0.007
ox7	0.03	0.01
ox8a	0.02	0.009
ox9	0.03	0.01
27a	0.02	0.004
35a	0.02	0.005
532	0.01	0.002
561	0.03	0.01
662dia	0.05	0.007
662dib	0.03	0.004
622b	0.05	0.01
552c	0.17	0.06
572c	0.82	0.08
Media	0.03	0.01

Yeast isolates 552c and 572c appear to have some antigenic material (Table 8.3) that can bind with BC-KH4 and consequently they were omitted from further experiments. These yeasts together with the two bacteria noted earlier (Appendix Three) bring to four the total number of bacteria or yeasts found in this study to cross-react with BC-KH4. This is the first report of yeasts to bind to BC-KH4 (F.M.Dewey, personal communication).

8.2.6 Experiment Six - Detection of B. cinerea *antigen with and without BCAs*

8.2.6.A Materials and Methods

Prepared tomato stem pieces (Chapter Two, Section 2.3) were spray inoculated by adding 1 ml of a *B. cinerea* spore suspension ($5x10^7$ spores/ml) to the reservoir on the Potter Tower and immediately followed by an application of 1 ml of yeast BCA isolates 561, 662dib or 622b or bacterial isolates ox2, ox4 or ox 7 ($2x10^{10}$ cells/ml) as described in Chapter Four. Culture and preparation of the *B. cinerea* and BCA cell suspensions are described in Chapter Two. Stem pieces were inoculated with *B. cinerea* only for the positive control, minus KH4 for the ELISA control or left uninoculated for the negative control. Inoculations of all treatments was carried out in a completely randomised block design.

Stem pieces were incubated at 15°C in total darkness for up to 144 h and three stem sub-samples removed from each block of each treatment at 24 h intervals. Slices (1-2 mm thick) were cut from the treated end of each stem piece and pooled in a microtitre well containing 100 µl of 0.1M $CuSO_4$ + KCl + NaCl extractant. The tissues were macerated by crushing with a sterile glass rod, mixed using a sterile needle to lift the tissue compressed against the well walls, then incubated at 4°C for 16 h. Plates were washed in PBST, PBS and water, dried and stored at 4°C until all treatments were completed. The ELISA procedure for the detection of the *B. cinerea* antigen is described in Chapter Two, section 2.5.6.

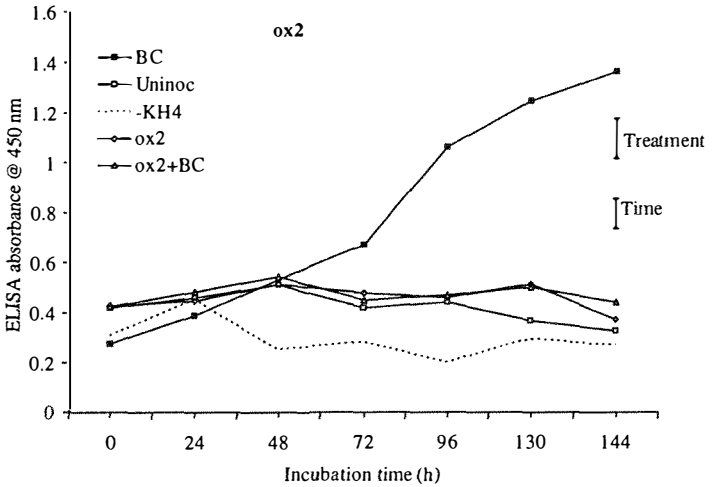
Results were analysed using a completely randomised block design and preplanned comparisons were carried out between pathogen-only treatment versus each BCA co-inoculation treatment and between uninoculated treatment versus each BCA co-inoculated treatment.

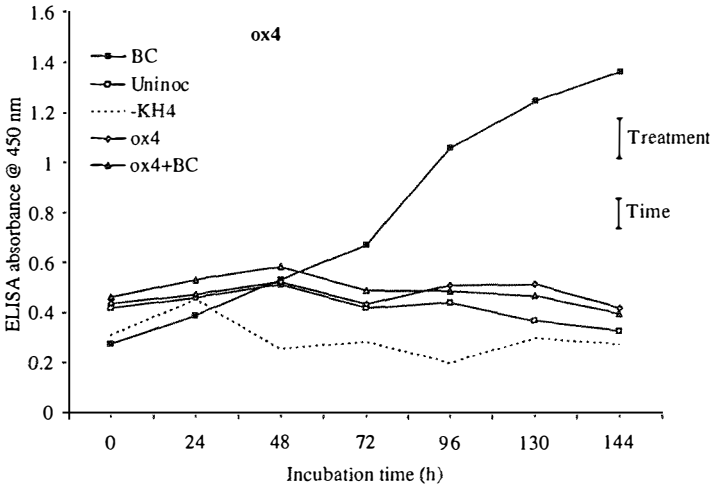
8.2.6.B Results and discussion

There were no differences between any treatments up to 48 h incubation, but from 72 h onwards there was a consistent increase in detection in the pathogen only (BC) treatment (Fig. 8.6). This led to a significant difference ($P<0.0001$) between this treatment and all BCA, uninoculated, co-inoculated and minus BC-KH4 treatments in pairwise comparisons. In all BCA treatments, the levels of *B. cinerea* antigen detected

was maintained at absorbances of 0.4 to 0.6 throughout the incubation period. For BCA isolate 662dia there was an initial elevation in absorbance to 0.8 detected but this steadily declined to about 0.6 at 96 h. Pairwise comparisons of all BCA co-inoculated stems with uninoculated treatments were not significantly different ($P > 0.1$) except for 561 ($P=0.03$) and 662dia ($P=0.0001$). Low ELISA values for the remaining control treatments (minus BC-KH4 and BCA inoculated tissue) indicate that the secondary antibody alone did not bind to the *B. cinerea* antigen nor did the protocol detect BCA antigen.

A cautious approach must be adopted however in interpreting biomass data using biochemical markers (Bermingham *et al.*,1995a) and this study is no exception. The unique nature of the BCAs used in the biomass experiment and the host tissue itself could potentially interfere with biomass determination in binding to or competing with the detection of the *B. cinerea* antigen. Nevertheless, these results suggest that the BCAs had a marked reduction on the levels of pathogen antigen detected.





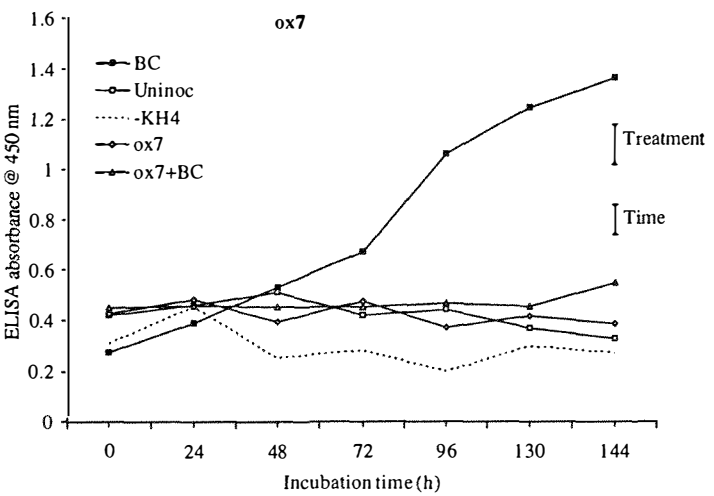
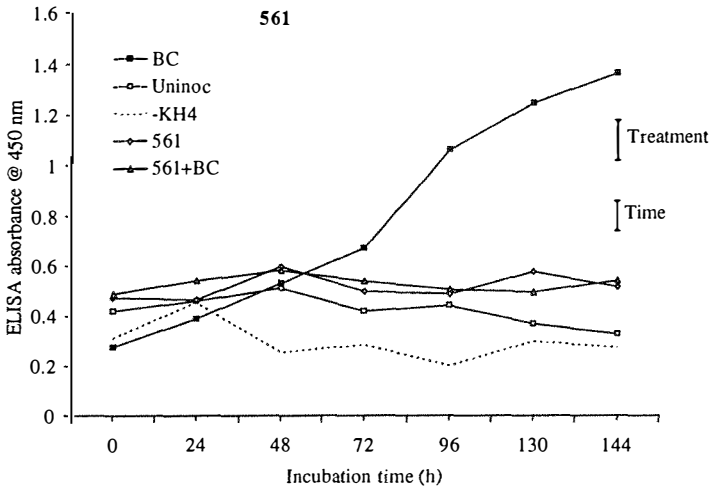
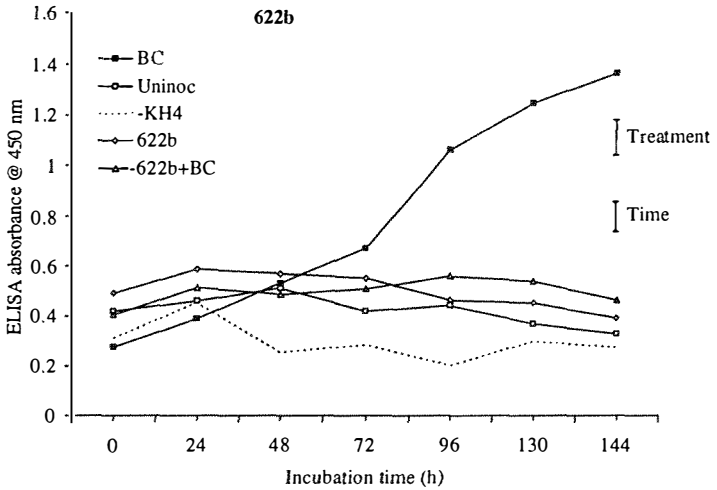


Fig. 8.4. Indirect PTA-ELISA detection of *B. cinerea* antigen in the presence of bacterial BCA's co-inoculated onto tomato stem pieces. Treatments are: *B. cinerea* inoculated stem pieces (BC); Uninoculated stem pieces (Uninoc); *B. cinerea* inoculated stem pieces but without BC-KH4 in the ELISA procedure (-KH4); BCA only inoculated stem pieces (Isolate number) and both *B. cinerea* and BCA inoculated stem pieces (Isolate number + BC). Vertical bars represent overall LSD calculated for the main plot (Treatment (df=42 n=4)) and split plot (Time (df=350 n=7).





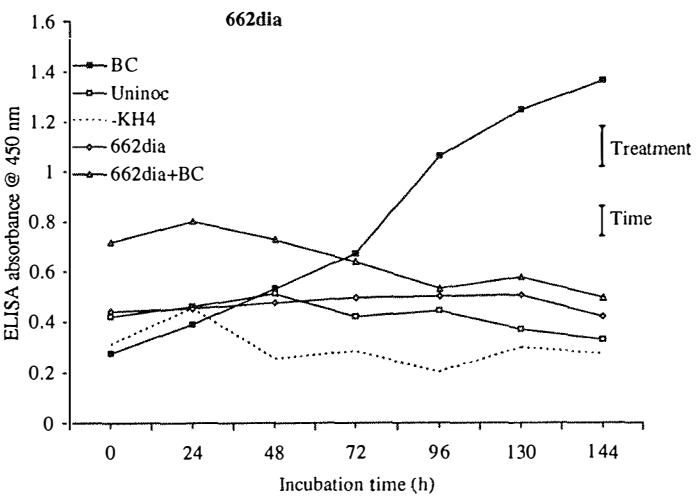


Fig. 8.4. Indirect PTA-ELISA detection of *B. cinerea* antigen in the presence of yeast BCA's co-inoculated onto tomato stem pieces. Treatments are: *B. cinerea* inoculated stem pieces (BC); Uninoculated stem pieces (Uninoc); *B. cinerea* inoculated stem pieces but without BC-KH4 in the ELISA procedure (-KH4); BCA inoculated stem pieces (Isolate number) and both *B. cinerea* and BCA inoculated stem pieces (Isolate number + BC). Vertical bars represent overall LSD calculated for the main plot (Treatment (df=42 n=4)) and split plot (Time (df=350 n=7).

8.2.7 Experiment Seven - Confirmation of biomass determinations

8.2.7.A Introduction

The fate of fungal antigen in the environment is an important consideration when using immunology to detect or measure pathogen biomass (C. Thornton, personal communication). By identifying specific sinks, the influence of these can be reduced with the application of appropriate measures such as modifying extractants, (Thornton *et al.*, 1997) or simplifying the environment (Thornton *et al.*,1996; Thornton *et al.*, 1995)

Two specific processes were considered to be potential interferents in the detection of *B. cinerea* on tomato stem pieces. The first hypothesis is based on the widespread use in fungal immunology of surface molecules from the microbe as immunogens in the creation of MAbs. These same molecules are involved in a variety of interactions between the fungus and the environment, for example in adhesion of the pathogen to the host (Cole *et al.*, 1996; Doss, Potter, Chastagner & Christian, 1993; Doss *et al.*, 1995) or detoxifying plant defence mechanisms (Nicholson, Butler & Asquith, 1986). Similarly the attacher BCAs selected in this study are most likely to possess surface components that are able to bind with compatible molecules on the *B. cinerea* surface. Assuming this lock-and-key model (Douglas, 1987), it is not unreasonable to expect competition for the *B. cinerea* antigen from either of these plant-based or BCA based components during ELISA.

8.2.7.B Objectives.

To examine the effect of tomato host tissue extract and BCA wash extract on *B. cinerea* antigen detection *in vitro* using indirect PTA-ELISA and to observe immunolabelled *B. cinerea* on tomato stems.

8.2.7.C Materials and Methods

Both *B. cinerea* antigen extracts and tomato plant tissue extract were prepared in copper extractant or PBS using the protocol detailed in Chapter Two, Section 2.5. The stock *B. cinerea* antigen extract was diluted ten-fold in the respective extractant then serially diluted further across eight microtitre wells in volumes of 50 µl. A 50 µl aliquot

of the tomato host extract in the same buffer was added to each well and microtitre plates were incubated at 4°C for 16 h. In a second treatment, the same dilution series of *B. cinerea* extract in both extractants was prepared. Cells from BCA isolates ox2, ox4, ox7, 561, 662dib, 622b ($2x10^{10}$ cells/ml) were suspended in copper extractant or PBS then centrifuged at 12,800 **g** for 10 min. The cell free supernatants were added to the *B. cinerea* extract and the microtitre plates were incubated at 4°C for 16 h.

Control comparisons for each experiment were the standard *B. cinerea* dilutions as described and second aliquot containing the corresponding extractant only. Detection of *B. cinerea* antigen was carried out using the ELISA protocol described in Chapter Two, Section 2.5.6.

8.2.7.D Results and Discussion

In three of the four extraction mixtures, ELISA absorbances greater than 1.0 were obtained. The exception was PBS extracted tomato tissue where the reading was 0.65 (Fig. 8.5.). This pattern among the treatments was maintained when *B. cinerea* antigen was diluted. A sharp increase in absorbance from 0.4 to 0.8 was observed when *B. cinerea* antigen was diluted to infinity then mixed with tomato tissue extracted in the copper extractant. The reason for this is unknown. The proposal that the host tissue was contaminated with field infections was dismissed as the data would reflect a constant increase in all treatments as they were all prepared from the same stock extract

The rapid decline in ELISA absorbance of *B. cinerea* antigen in copper extractant compared with PBS is also puzzling and is difficult to explain from this experiment alone. In previous experiments, results showed that antigen detection in copper was as high as that for bicarbonate buffer (pH9.6) in Experiment Three and this in turn was considerably greater than PBS (Experiment One).

Fig. 8.6. shows the detection of *B. cinerea* antigen in mixtures with BCA extracts in PBS or copper extractant. For isolates ox2 and ox7, absorbances of approximately 1.4 were observed for the antigen controls, BCA and *B. cinerea* extracts in copper at the lowest antigen dilution. The absorbances for isolates ox4, 622b and 561 were greater than for the BCA/*B. cinerea* extract mixtures irrespective of extractant. For isolate 662dia, highest absorbance was observed for the controls, followed by the BCA/*B. cinerea* extract mixture in copper extractant then by the mixture in PBS.

With further dilution of the *B. cinerea* antigen, absorbances rapidly declined to very low levels in the presence of BCA extract in PBS and copper. This highlighted the large difference in absorbance values between antigen only in PBS and antigen mixed with BCA extract for all isolates but this difference was much smaller in the copper extractant.

The results of this experiment show that the copper extractant has the ability to reduce the interference on the ELISA detection of *B. cinerea* antigen by substances in the tomato tissue. In addition, the copper extractant appears to perform a similar role in the presence of some BCA extracts (662dia, ox2 and ox7) but only at high *B. cinerea* antigen concentrations.

Despite the beneficial effect of the copper extractant, a cautious approach is still recommended as the protective effect of the copper extractant has been shown to be dependent on BCA extract concentration Thus the relative concentrations of tomato tissue extract, *B. cinerea* antigen and BCA antigen may not be accurately simulated in this experiment.

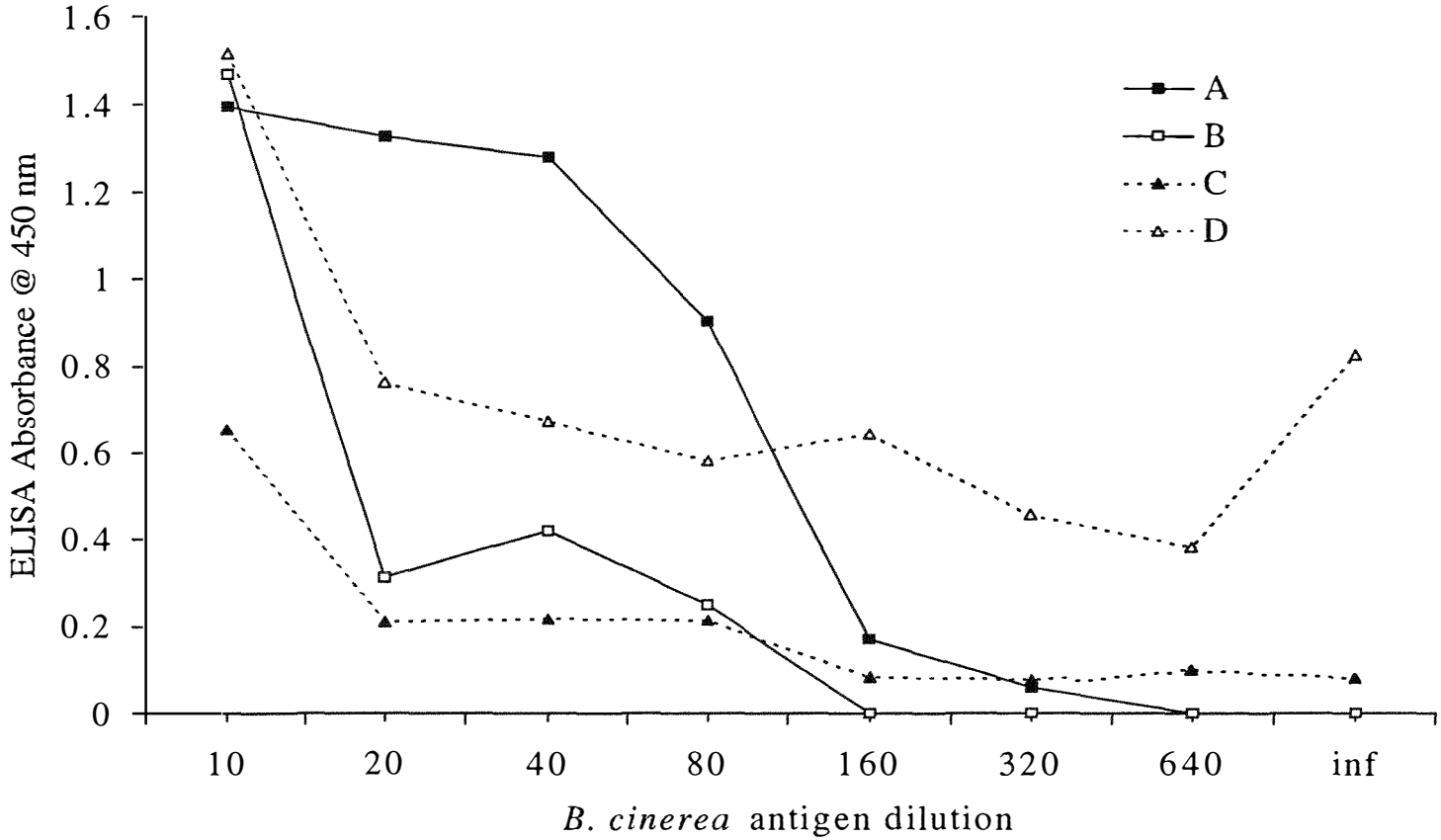
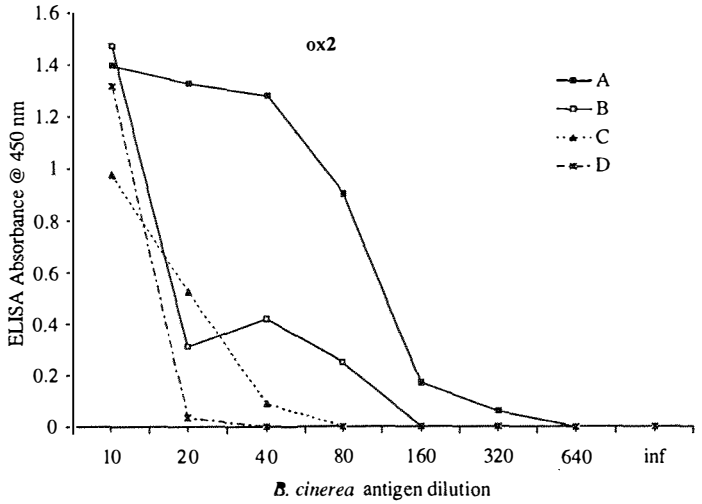
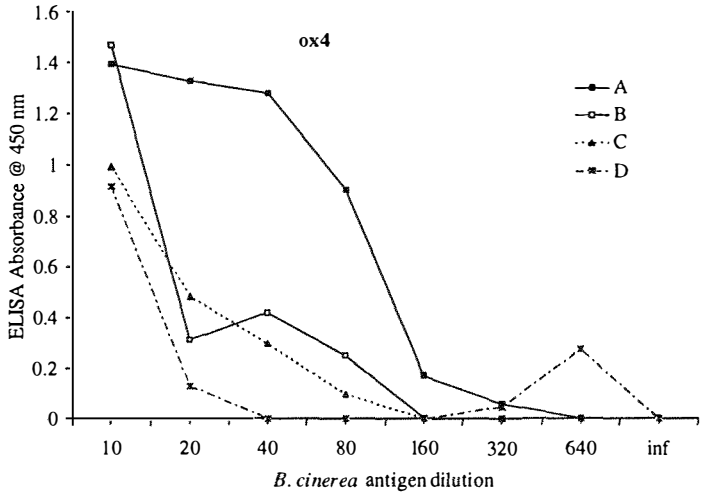


Fig. 8.5. Reduction in *B. cinerea* antigen detection with the addition of copper or PBS extract from tomato tissue in a competitive ELISA format. Wells incubated at 4°C for 16 h and antigen detected using BC-KH4 and biotinylated antimouse conjugate and extrAvidin. Treatments are : (A) = *B. cinerea* antigen suspended in PBS, diluted in PBS; (B) *B. cinerea* antigen suspended in copper extractant and diluted in the same; (C) *B. cinerea* antigen in PBS diluted with a PBS extract of tomato tissue (D) *B. cinerea* antigen in copper extractant diluted with copper extract tomato tissue. Note: "inf" refers to a treatment where *B. cinerea* antigen was diluted to infinity before adding the tissue extract.





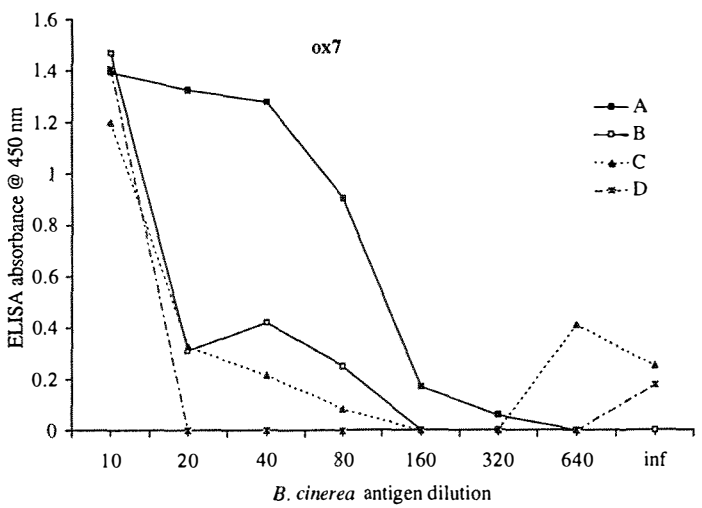
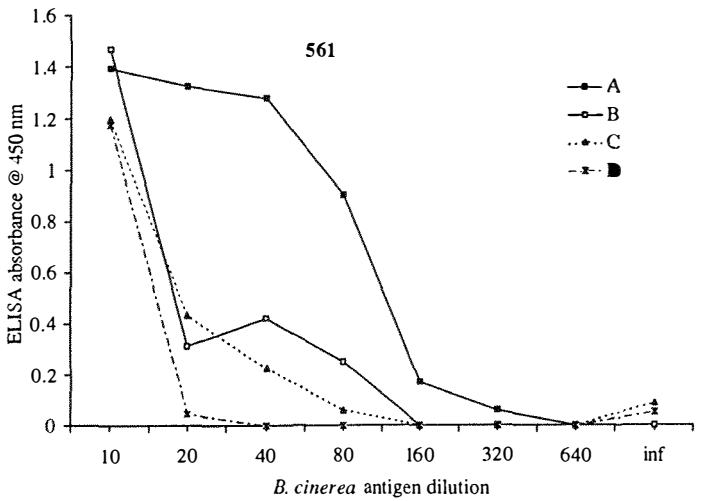
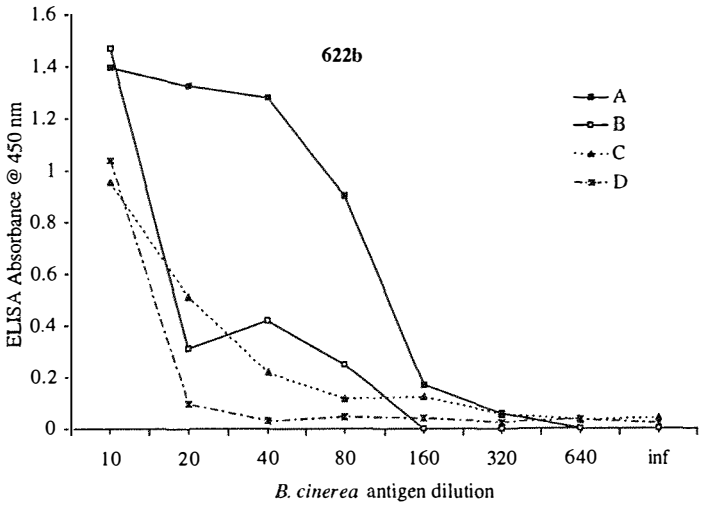


Fig. 8.6. Reduction in *B. cinerea* antigen detection with the addition of bacterial antigen extracted in copper extractant or PBS in a competitive ELISA format. Wells incubated at 4°C for 16 h and antigen detected using BC-KH4 and biotinylated antimouse conjugate and ExtrAvidin. Treatments are : (A) = *B. cinerea* antigen suspended in PBS, diluted in PBS; (B) *B. cinerea* antigen suspended in copper extractant and diluted in the same; (C) *B. cinerea* antigen in PBS diluted by bacterial antigen extracted in PBS and (D) *B. cinerea* antigen in copper extractant diluted by bacterial antigen in copper extractant. Note: "inf" refers to the treatment where *B. cinerea* antigen was diluted to infinity before adding the BCA extract.





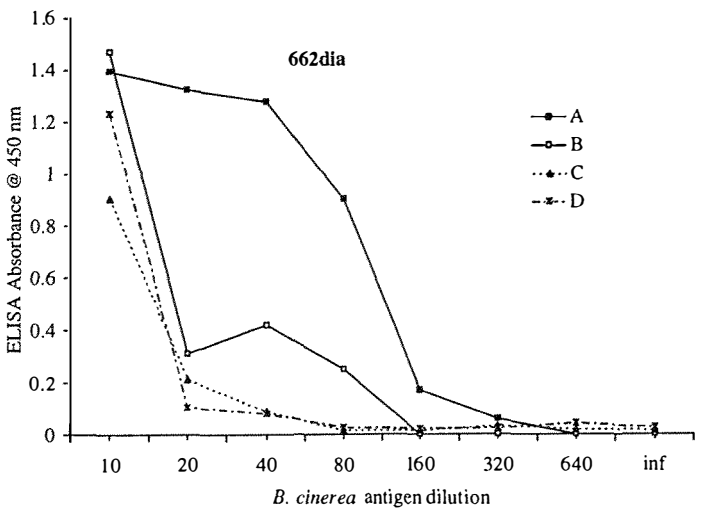


Fig. 8.6. Reduction in *B. cinerea* antigen detection with the addition of yeast antigen extracted in copper extractant or PBS in a competitive ELISA format. Wells incubated at 4°C for 16 h and antigen detected using BC-KH4 and biotinylated antimouse conjugate and extrAvidin. Treatments are : (A) = *B. cinerea* antigen suspended in PBS, diluted in PBS; (B) *B. cinerea* antigen suspended in copper extractant and diluted in the same; (C) *B. cinerea* antigen in PBS diluted by yeast antigen extracted in PBS and (D) *B. cinerea* antigen in copper extractant diluted by yeast antigen in copper extractant. Note: "inf" refers to the treatment where the *B. cinerea* antigen was diluted to infinity before adding the BCA extract.

8.2.8 Experiment Eight - Immunofluorescence examination of B. cinerea *growth on tomato stem pieces*

8.2.8.A Materials and Methods

Prepared tomato stem pieces (Chapter Two, Section 2.3) were spray inoculated by adding 1 ml *B. cinerea* spore suspension ($5x10^7$ spores/ml) into the reservoir of the Potter Tower and the suspension atomised. This was immediately followed by co-inoculation of 1 ml of yeast BCA isolates 532, 561, 662dib or 622b or bacterial isolates ox 7, ox8a, ox9, 35a ($2x10^{10}$ cells/ml) as described in Chapter Four. Culture and preparation of the *B. cinerea* and BCA cell suspensions are described in Chapter Two (Sections 2.1 and 2.2). All treatments were incubated for 4 days at 15°C. At the end of this incubation, 1-2 mm slices were cut from the treated end of each stem piece and immunolabelled by fixing the tissue in 3% paraformaldehyde for 1 h at R.T., washing in five rinses of PBST, soaking the tissue for 5 min each time, then incubating in neat BC-KH4 for 1 h at R.T. After this incubation, the tissues were washed thoroughly in five rinses of PBST as before, incubated in anti-mouse - FITC conjugate diluted 1:40 PBS (Sigma F9006, St Louis MO USA) for 1 h at R.T. then thoroughly washed again in five rinses of PBST before mounting the samples in molten agarose (2% Agarose in 50% glycerol), adding 10 µl citifluor to the top of the tissue and covering with a coverslip.

Samples were observed using a Zeiss (47-3011-9901) epifluorescent microscope.

8.2.8.B Results and Discussion

After 4 days incubation at 15°C immunolabelled *B. cinerea* hyphae were observed on the pathogen-only inoculated control and unlabelled conidia in some of the BCA co-inoculated stem samples. (Figs 8.7 a-d). Intensely labelled *B. cinerea* grew across the host tissue surface in the positive control (Fig. 8.7a) and in treatments co-inoculated with BCA isolates 662dib and ox7 (Data not shown). Unlabelled conidia were observed in the presence of isolates 35a (Fig. 8.7b), 532 (Fig. 8.7c) and 622b (Fig. 8.7d).

The presence of ungerminated, unlabelled *B. cinerea* conidia in co-inoculated treatments suggests BCA antagonism was effective in stopping pathogen germination and growth. The presence of labelled hyphae confirmed earlier observations made in SEM studies (Chapter Six) and suggests a mode of antagonism that does not entirely

eliminate all pathogen growth. Bossi & Dewey (1992) reported that labelling of ungerminated conidia of *B. cinerea* was poor compared with the hyphae, an observation confirmed in this study. Adhesion of the BCA to the pathogen hyphae relative to BC-KH4 antigen labelling could not be determined using the techniques in this experiment. It would be desirable to conduct further studies to examine whether the labelled hyphae observed on the tomato tissue had BCA attached because the possibility that unlabelled hyphae or conidia were heavily infested with BCA cells cannot be discounted. Unlabelled hyphae were tentatively identified in the co-inoculated treatments under light microscopy, however equipment and technique limited the ability to pursue this hypothesis.

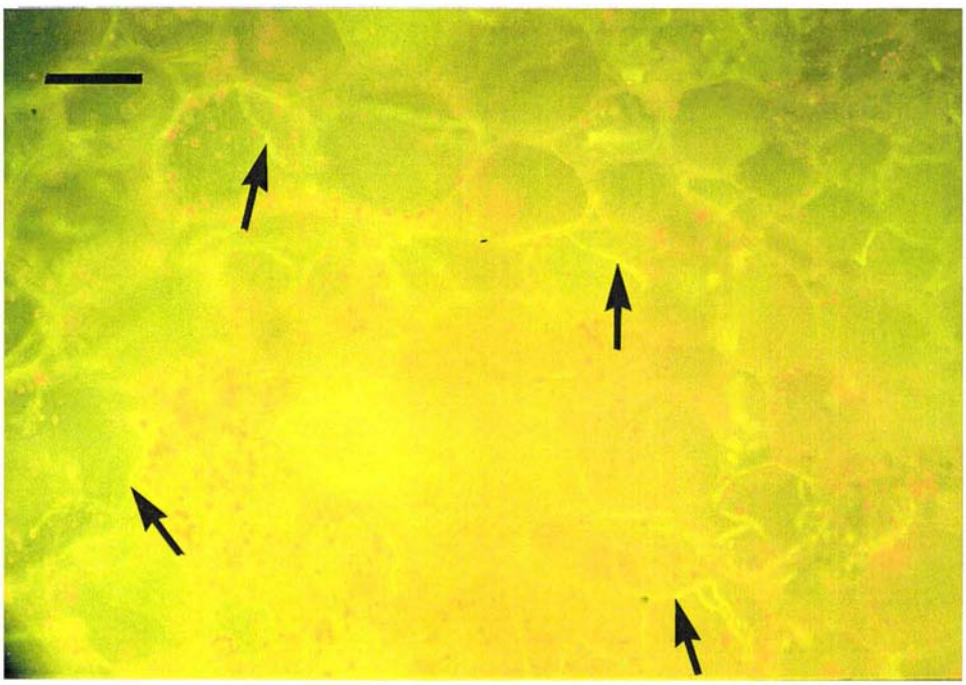


Fig. 8.7a. Immunofluorescent labelling for *B. cinerea* hyphae on pathogen-only inoculated tomato stem pieces showing labelled hyphae (arrow).

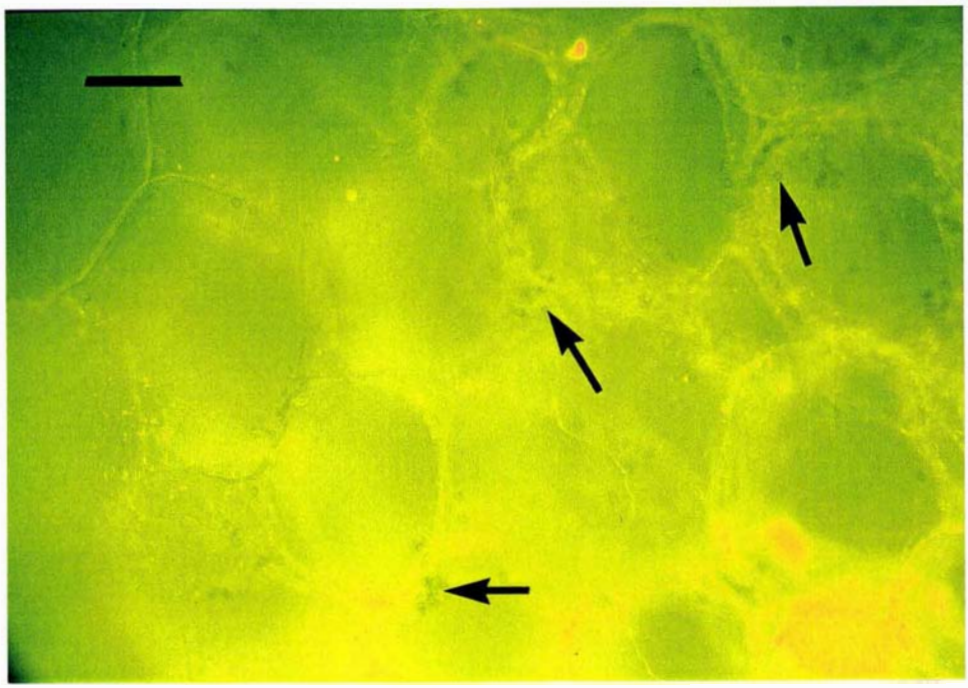


Fig. 8.7b. Immunofluorescent labelling for *B. cinerea* hyphae on tomato stem pieces co-inoculated with isolate 35a showing unlabelled conidia (arrow).

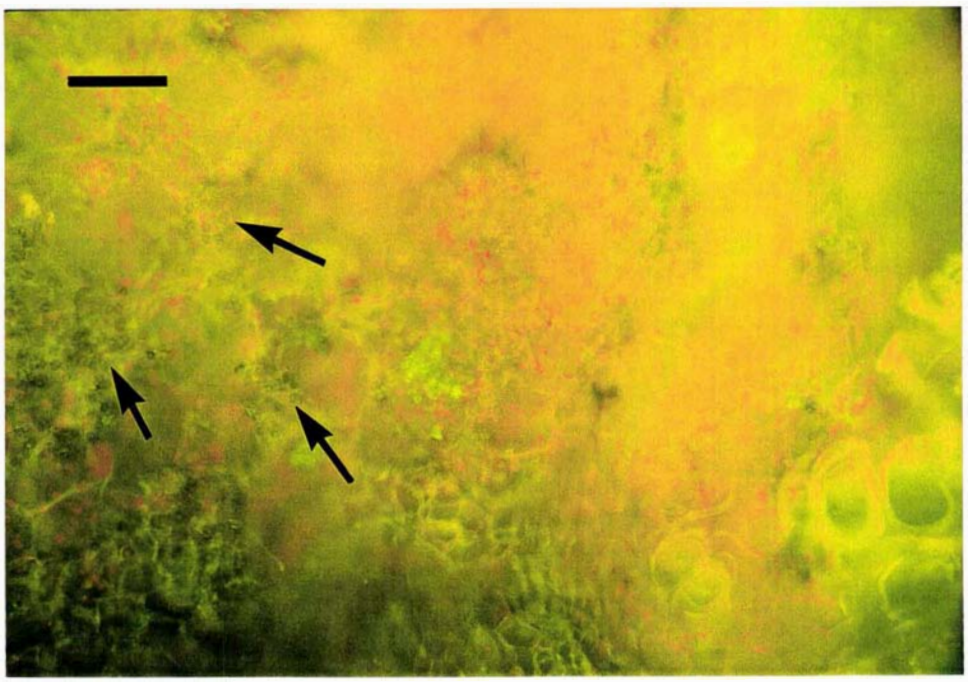


Fig. 8.7c. Immunofluorescent labelling for *B. cinerea* hyphae on tomato stem pieces co-inoculated with isolate 532 showing unlabelled conidia (arrow).

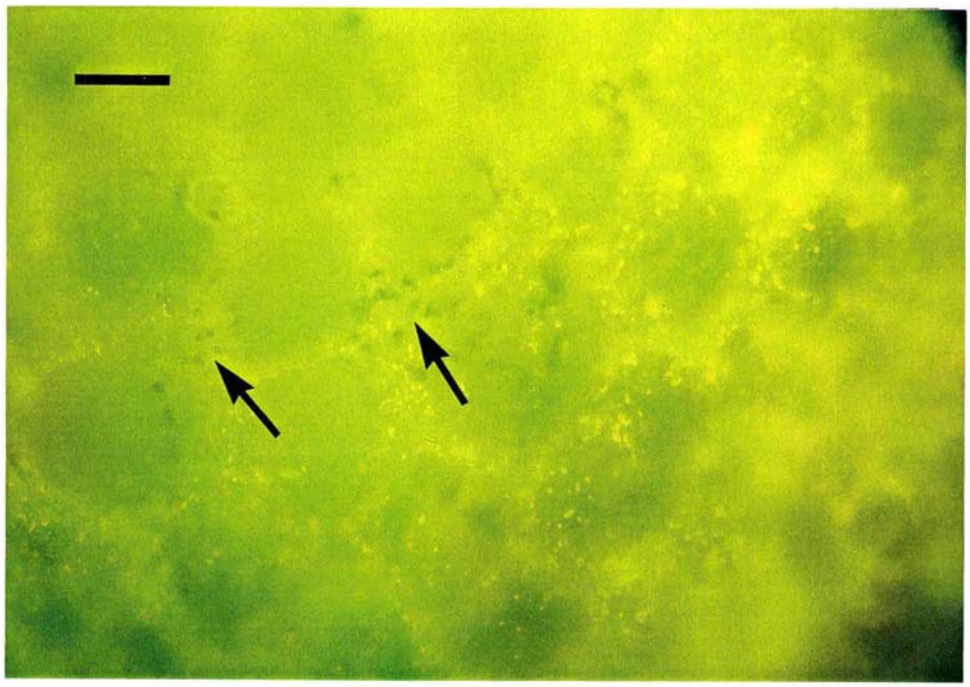


Fig. 8.7d. Immunofluorescent labelling for *B. cinerea* hyphae on tomato stem pieces co-inoculated with isolate 622b showing unlabelled conidia (arrow).

8.3 Discussion

Detection of pathogen antigen from inoculated plant host has been consistently poor (Dewey, 1996) and *B. cinerea* is no exception despite Cole *et al.* (1996) showing in immunolabelling studies at the electron microscopy level that BC-KH4 could clearly label the antigen. Fungal antigen extraction has been described as one of the biggest limitations to the development of immunoassays (Dewey, Thornton & Gilligan, 1997) and has stimulated new developments in extractant selection. Dewey *et al.* (1997) found the simple and most commonly used extractant buffers (for example PBS, bicarbonate and TRIS) are quite unsuitable due to their poor extraction qualities from soil and suggested that pH or the addition of surfactants or cations were important improvements. Results from a comparison of PBS, bicarbonate buffer and the copper based extractant in the current study support this view. However the addition of surfactants to the extractant was not examined because this precludes the use of PTA-ELISA after sample extraction; the antigen will not bind to the micro-titre well wall if surfactants are present (Dewey *et al.*, 1997). In the present study, measurement of *B. cinerea* biomass during infection of tomato stem tissue by way of BC-KH4 antigen detection was obtained after 24 h incubation at 15°C (Experiment Six) but only with the use of a copper-based extractant and the indirect-PTA-ELISA format with the biotin-avidin-peroxidase detection system. A number of other ELISA formats have been successfully used previously, for example, DAS-ELISA (van de Koppel & Schots, 1995) or indirect competitive ELISA (Plasencia *et al.*, 1996) for the detection of *Verticillium* spp. in flowers and potatoes respectively. However the pathogen antigen was extracted using PBS and is in contrast to the results here. High levels of non-specific binding from substances in a wounded, uninoculated tomato stem tissue reduced *B. cinerea* antigen detection possibly due to the various lectins, found in high quantities in many solanaceous plants (Kilpatrick *et al.*, 1983; Kilpatrick *et al.*, 1984; Goldstein & Poretz, 1986). BC-KH4, used in this study recognises a carbohydrate binding site (Bossi & Dewey, 1992) and similar binding sites could also be present on the lectins. Interestingly, Plasencia *et al.*, 1996 successfully used competitive-ELISA for the detection of *Verticillium dahliae* in potatoes using PBS as the extraction buffer but used an IgG1 MAb which may possibly explain the greater specificity.

The fate of fungal antigen and presence of competing substances is an important consideration for its detection by ELISA. Dewey *et al.* (1997) stated that the inability to detect antigen in soil could be the result of either the lack of antigen production by the fungus, adsorption onto soil colloids or its destruction by microbial metabolism. Microbial activity is particularly important for biomass measurement in the current study as the BCAs were microbes capable of adhering to *B. cinerea* and there is initial evidence that the BC-KH4 antigen is involved in BCA adhesion to *B. cinerea* (Chapter Seven). Immunolabelling of *B. cinerea* on tomato tissue showed that antigen was produced by the pathogen on tomato tissue but the inability to simultaneously observe BC-KH4 immunolabelling and BCAs adhesion meant that BCA adhesion and possibly lowering the amount of antigen available to the ELISA, could not be confirmed. *In vitro*, antigen extracted from some bacteria or yeasts BCA isolates tested reduced ELISA absorbances from *B. cinerea* antigen indicating the BCA extracts could bind directly to or compete with *B. cinerea* antigen in either the copper or PBS extractants.

If the interferent is a substance other than lectin-type-adhesion, then other strong candidates include cross-reactive antigens previously reported in many host-pathogen interactions (See DeVay & Adler, 1976, for a review). This has led to the hypothesis that should a common antigen be found, this is an indicator of interaction compatibility and disease susceptibility (DeVay & Adler, 1976; Chakraborty *et al.*, 1995) although Palmerley & Callow (1978) suggested only certain key antigens should be considered indicative. In the present study, the initial problems of cross-reactivity with substances in wounded tomato stem tissue appears to be consistent with this model. Yet it appears that the interferent from tomato tissue (lectin or cross-reactant antigen) and the *B. cinerea* antigen are sufficiently different such that the copper extractant was able to selectively enhance the detection of *B. cinerea.* In Experiment Eight, the copper sulphate and salts extractant increased ELISA absorbances compared to PBS in the presence of tomato tissue extract *in vitro* suggesting that the discrimination between inoculation treatments in Experiments Seven and Six was achieved because of this selectivity. To date use of copper sulphate as an extractant has been limited to antigen extraction from soil (C. Thornton, personal communication). It is of interest to note that this common antigen model and its use to predict compatability appears to be limited to the plant/microbe interactions. Yet cross-reactivity was only found in four of

the 20 bacterial or yeast BCA isolates tested despite the pathogen being a host that is colonised by these BCAs. DeVay & Adler (1976) did include examples of host/microbe interactions where a common antigen was not found but suggested that this inconsistency was due to a number of experimental variables. These factors could explain the absence of a common antigen in the microbe/microbe interactions.

Signal amplification has been used in other studies to biologically amplify the antigen, for example, the addition of nutrients to field samples (Dewey *et al.*, 1992; Thornton *et al.*,1994). In addition to the use of a copper extractant in the present study, resolution between inoculated and uninoculated tomato stems was achieved by pooling and macerating tissue. Presumably these procedures increased the total available antigen present in the well or enhanced release from within the host tissue. Pooling and maceration was carried out in the micro-titre well itself to avoid transfer losses but it was found necessary to stir the contents of the well to lift the crushed sample away from the well bottom and walls suggesting that binding sites were covered by crushed tissue.

The epitope on the glycoprotein to which BC-KH4 binds was found to be heat stable and survived autoclaving for 5 min in PBS (Bossi & Dewey, 1992) thus supporting the view that carbohydrate antigens tended to be longer lived than peptides in soil (Dewey *et al.*, 1997). This characteristic has been exploited for the extraction of antigen from soil (Dewey *et al.*,1997) and was attempted in the current study. However results showed that this stability may be dependent on buffer since heat stability was lost in the current work where bicarbonate buffer (pH 9.6) was used. Interestingly, Dewey *et al.* (1997) stated that binding of proteinaceous fungal antigens to microtitre wells is greatest at high pH (pH 9.6) using bicarbonate buffer. This featurewas observed in the binding of BC-KH4 antigen to microtitre wells using bicarbonate buffer suggesting there is a protein component in BC-KH4 antigen. However this result and the poor heat stability in the same buffer suggest that the protein component may be important for binding of antigen to the microtitre wall or it is immunologically active contrary to previous results (Bossi & Dewey, 1992).

Sharma *et al.* (1977) argued that compounds such as chitin that are stable for prolonged time periods were unsuitable as measures of biomass in the field since there was no discrimination between current and past synthesis of the substance nor the

physiological status of the target microbial population. Fungal components that have been used as immunogens have also been subject to the same scrutiny. Dewey *et al.* (1997) stated the same epitope may not be found throughout the fungal structure at any one time or may change with fungal age. While this provides an opportunity for the creation of two or more MAbs specific for different structures or growth phases of a fungus, for example *Trichoderma* sp. phialoconidia (Thornton & Dewey, 1996) versus hyphae (Thornton, Dewey & Gilligan, 1994), for *B. cinerea,* data to date indicates the BC-KH4 antigen is predominantly located on young hyphae (Cole *et al.,* 1996). Therefore while this MAb would be limited to infection biomass measurements of a "single infection event" where the pathogen is still relatively young, this initial phase can be regarded as vital for initial phases for disease or biological control.

In this study, *B. cinerea* biomass could be measured using an ELISA-based immunoassay, with a copper-based extractant. This requirement is consistent with other reports where the difficulty of obtaining measurable fungal antigen from natural environments has limited immunoassay applications. Mechanisms by which the copper extractant enabled *B. cinerea* antigen to be detected are not clear.

Biocontrol activity in post-harvest coolstored kiwifruit and late sown glasshouse tomato plants

9.1 Introduction.

The biology and biocontrol efficacy of fifteen yeast and bacterial BCA isolates were studied in the laboratory and conferred 80% disease reduction on tomato stem pieces even when pathogen application preceded some BCAs by up to 48 h. Cell adhesion to the host fungal pathogen is highly likely to be important for this activity as SEM studies showed many of the bacterial and yeast isolates attached to *B. cinerea* hyphae and the addition of BC-KH4, inhibiting bacterial adhesion *in vitro,* also reduced biocontrol efficacy *in vivo. In vitro* the BCA isolates synthesised endochitinase, siderophores and two bacterial isolates produced antibiotics which could contribute to *B. cinerea* biocontrol.

However, it is a frequent experience that biocontrol in the field seldom mirrors the promising results obtained in the laboratory where it is generally believed that some aspects of the assay environment poorly simulate field conditions (Andrews, 1992). In preharvest situations, predominantly fungal species, including *Cladosporium* sp. and *Aureobasidium* sp. for disease control in grapes (Bisiach, Minervini, Vercesi & Zerbetto, 1986), *Gliocladium roseum* in strawberries (Peng & Sutton, 1990), *Cladosporium cladosporioides* and *Penicillium* sp. (Eden, Hill & Stewart, 1996) and *Ulocladium atrum* in lilies (Kohl *et al.,* 1995c) have been used for biological control of *B. cinerea. Trichoderma* spp. have received the most attention (Dubos, 1987) and have dominated *B. cinerea* biocontrol research on crops such as apples (Tronsmo & Ystaas, 1980; Tronsmo, 1981), grapes (Dubos, Jailloux & Bulit, 1983; Besselat, 1987), cyclamen (McCain, Sciaroni, Welch and Pierce, 1984), strawberries (Peng & Sutton, 1990), glasshouse tomato (Eden *et al.*, 1996) and kiwifruit (Cheah, Hill & Hunt, 1992; Tate *et al.*, 1992; Pyke *et al.*, 1993). In some early pre-harvest field trials disease control was "insignificant" (Besselat, 1987, abstr) or "variable" (Bisiach *et al.,* 1986, abstr); Blakeman & Fokkema, 1982). Tronsmo & Ystaas (1980) reported biocontrol

levels comparable with those of fungicide treatments on naturally occurring *B. cinerea* infections using *Trichoderma* sp. but only one years results were presented. Agent efficacy has since improved with research concentrating on evaluating the best timing for application, (eg. *Trichoderma* sp. against *B. cinerea* on grapes (Dubos, 1987)), the method of application, (eg. *G. roseum* against *B. cinerea* on strawberries (Peng & Sutton, 1990)) and improved screening assay designs, (eg. *U. atrum* against *B. cinerea* on lilies (Kohl *et al.*, 1995a)).

In the glasshouse environment, *Streptomyces griseoviridis* was effective in increasing yields in lettuce (White, Linfield, Lahdenpara & Uoti, 1990) while an isolate of *Cladosporium cladosporioides* reduced disease in tomato (Eden *et al.*,1996). To date, "Trichodex" based on *Trichoderma harzianum,* isolate T39, is the only commercial product registered against a foliar pathogen (Fokemma, 1995).

Preharvest biocontrol research on New Zealand kiwifruit has achieved little success in trials using *Trichoderma* T39 or local isolates of *Trichoderma* sp., *Bacillus subtillus* or *C. cladosporioides* (Tate *et al.*, 1992; Pyke *et al.*, 1993). Franicevic (1993) tested isolates of *Bacillus* sp. and *Epicoccum purpurascens* applied twice before harvest but stem-end rot incidence increased from 0.9% in the control to 2-3% in fruit from treated vines.

A number of financial and regulatory issues have stimulated research into biocontrol of stored product pest and disease problems. Harvested product is more valuable to the grower as money has been invested in harvesting, grading and packing of the proportion of the crop of highest quality and marketability and the financial consequences of disease in stored product is more immediate. Prophylactic fungicide application can potentially leave unsightly residues particularly on flower crops (Redmond, Marois & MacDonald, 1987; Hammer & Marois, 1989) and more immediate application relative to consumption limits the plant protection products that can be used. Many, predominantly yeasts and bacteria have been tested (See Table 5.2, Chapter Five) while in New Zealand, unidentified yeasts (Cheah *et al.*, 1992) or *Pichia* spp. (Cheah, Wilson & Marshall, 1996) have been evaluated.

To date, yeasts and bacteria appear most suitable for postharvest applications (See Table 5.2) while, in contrast, the filamentous fungi are generally better adapted for preharvest systems (See Table 5.1). However, many of these isolates possess phylloplane-based modes of antagonism and there is no information of this pattern among attacher BCAs. In this study, both preharvest and postharvest systems were selected in which isolates in the current BCA collection were tested.

9.2 Objective

To determine whether bacteria and yeast candidates obtained by the attachment assay (Chapter Three), isolated and evaluated *in vivo* (Chapter Five) show biocontrol activity in post-harvest kiwifruit and late sown glasshouse tomato plants

9.3 Post-harvest kiwifruit experiment: 1995

9.3.1 Materials and Methods

B. cinerea isolate BC 20 was grown as described in Chapter Two (Section 2.1). Dry spores were applied to the stem scar of the kiwifruit using a camel hair paint brush (size =2). Preliminary experiments quantified spore deposition and is described as follows: Disks of PDA, 5 mm diameter were inoculated by gently dipping the tip of the brush hairs into a *B. cinerea* culture then taping once lightly onto the agar disks. After an initial 10 disks were inoculated, a further 10 disks were inoculated, stained with cotton blue in lactophenol and spore density measured as described in Chapter Four. Nine quadrat sites were sampled on each of the ten replicated disks.

Cells of yeast isolates 561 and 332 were cultured in 1 litre NYD broth (pH 4.5) shaken at 125 rpm (Certomat M, B Braun, Melsungen AG) for 4 days at 15°C. As no pure cultures of bacteria were available at the time of the experiment, crude mixtures 1 and 27 were used. All cultures were centrifuged at 12,000 **g** for 10 min to remove the used broth medium, then the pellet was resuspended in 20 ml SDW. Total cell counts were made of all isolates and mixtures (Chapter Two, Section 2.2) and cell concentration was adjusted to 7.6 $x10^8$ cells/ml.

Export quality kiwifruit of size 36 count (c. 102 g/fruit) were harvested from Kuku Pack & Cool Ltd. Hoggs Rd, near Levin on 22 May 1995. Care was taken to ensure the fruit pedicel remained attached to the fruit when picked. A separate sample of 15 fruit harvested from within a radius of 1 m from the vine trunk from a random selection of vines were used for Brix measurement. Juice from subsamples of tissue cut from the top and bottom of each fruit were measured for total soluble sugars using a hand held refractometer (Atago N10, 0-10° Brix) washing the prism between samples with SDW.

At the Plant Growth Unit (Massey University), fruit were laid out in ten trays (replicate blocks) of 25 fruit each by inoculation treatment. Fresh picking wounds were created by removing the pedicels and calyces at the abscission area followed immediately with the dry inoculation of *B. cinerea* with a camel brush and a 17 µl application of BCA suspension. Additional treatments included fruit inoculated with 17

µl of each BCA suspension only and those fruit which received an application of the dry pathogen spores only. All fruit were left to stand for 30-40 min to allow the excess liquid from the BCA suspension application to be absorbed before fruit were packed into plastic fruit cups and placed into trays with polyethylene liners.

Five of the ten trays in each inoculation treatment were cured by placing them at 10°C for 3 days while the remainder went straight into the coolstore (0.0 ±0.3 °C). At the completion of the three days curing period those fruit at 10°C were moved into the coolstore for 12 weeks. Treated fruit were assessed at 28 day intervals for symptoms of *B. cinerea* infection. Diseased fruit were removed to prevent secondary infection to neighbouring fruit. The experiment and data analysis was carried out using a split plot design, where inoculation treatments were the main plots and curing were the split plots.

9.3.2 Results

Mean *B. cinerea* spore density deposited onto the agar plugs by the camel hair brush technique varied from 800 spores /mm^2 to 1600 spores/mm^2 (Fig. 9.1) with an overall average of 1129 spores/mm^2. Total spore load based on a stem plug diameter of 5 mm, was 22,000 spores /stem scar.

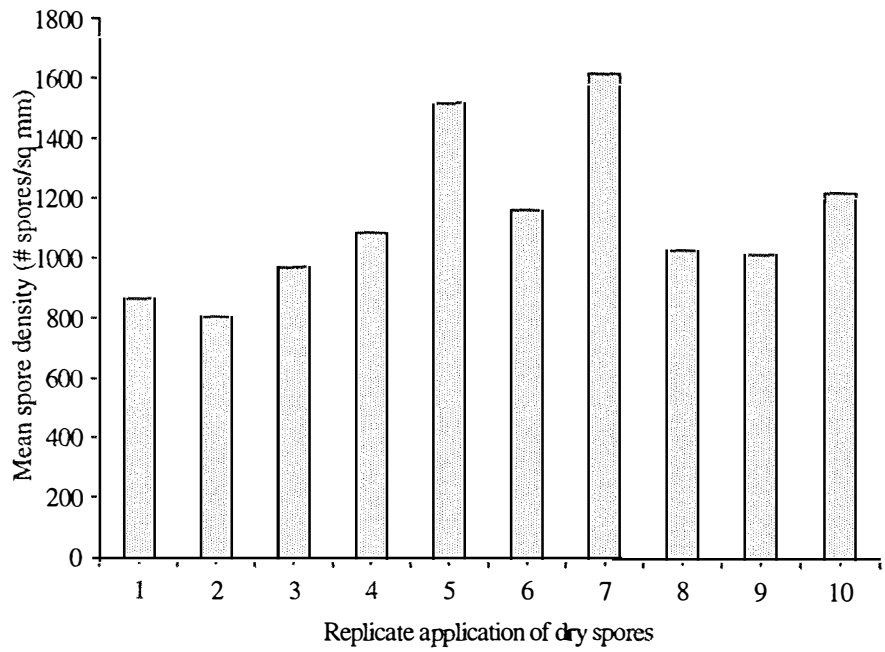


Fig. 9.1. Mean spore density deposited onto 5 mm diameter PDA disks using camel hair brush (size 2).

Mean total soluble solids was 6.9 ± 0.6 (STD) at the proximal end of the fruit and 8.1 ± 1.0 (STD) at the distal end.

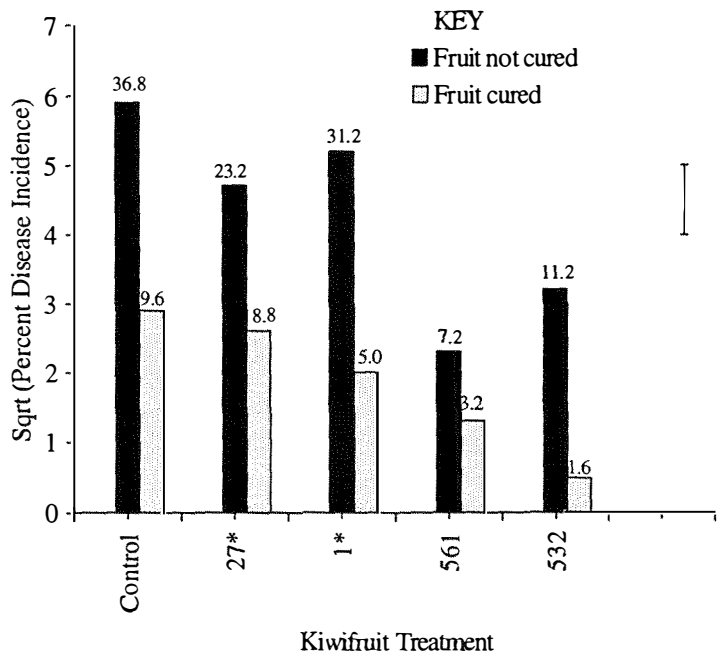


Fig. 9.2. Disease incidence in postharvest coolstored kiwifruit (square-root transformed) inoculated with yeast isolates and bacteria mixtures (*). Post-inoculation treatments included curing fruit at 10°C for 3 days prior to coolstorage at 0°C, or remaining uncured and fruit immediately placed in coolstorage. Percent infection (raw data) are given. Vertical bar represents LSD (curing x BCA) (α=0.05, df=20, n=5).

The square root of disease incidence in inoculated post-harvest kiwifruit was significantly reduced ($P<0.0001$) by the curing treatment but varied with BCA application (Fig. 9.2). There were no significant differences between disease incidence in fruit inoculated with bacterial BCA mixtures and pathogen-only control ($P<0.24$) but disease incidence in fruit inoculated with yeasts 532 and 561 was significantly lower ($P<0.0001$). The interaction of fruit curing and BCA was not significant ($P>0.05$).

Raw data in Fig. 9.2 show 37% of fruit inoculated with *B. cinerea* alone became diseased with stem-end rot. This was reduced to 9% with curing alone, 23% - 31% with bacterial BCA application only and 7% to 11% with yeast BCA application only. A further reduction to between 1% and 3.2% was obtained when both fruit curing and yeast BCA application were combined (Fig. 9.2).

9.4 Post-harvest kiwifruit experiment: 1996.

9.4.1 Materials and Methods

B. cinerea was prepared as described in Section 9.3.1.

Yeast isolates 532, 552c, 561, 572c 622b, 662dia and 662dib were cultured in 100 ml NYD broth shaken at 175 rpm (Certomat M, B Braun, Melsungen AG) and cells prepared as described in Section 9.3.1. Suspension concentrations were adjusted to $1x10^{10}$ cells/ml.

A modeler's air brush (Badger 350 ®, Badger Airbrush Co. Illinois USA) connected to a compressor (GEC 5000 - 11) was used to inoculate BCA cells onto the kiwifruit stem scar region in the 1996 experiment. The air brush handpiece was modified with the addition of a wire guide extending from the nozzle to a distance of 5 cm to ensure an accurate and precise dose with each application. The diameter of the zone created by the spray distribution from the airbrush was measured after SDW was applied to three replicate, 90 mm diameter cardboard targets.

Export quality fruit were harvested, TSS measured then pathogen applied as described in 1995 on May 13 1996. A standardised spray application of yeast BCA suspensions followed immediately after *B. cinerea* inoculation or after a delay of 24 or 48 h during which time, fruit were incubated at 0.0±0.3°C. The total number of cfu's discharged from the spray gun for each yeast isolate was measured by directing a standard spray dose into a sterile universal bottle. Ten millilitres of SDW was then added to suspend the applied cells.

The control treatment included fruit inoculated with dry *B. cinerea* spores only. All fruit were packed into bi-layered Europac ® trays (New Zealand Kiwifruit Marketing Board), each containing 28 fruit per layer. Granules of Purafil ® (Purafil Inc. Norcross, Georgia, USA) were scattered in the box but outside the polyethylene liner to absorb any ethylene and thus prevent premature ripening of other fruit. A total of eight trays were prepared for each inoculation treatment. Four of these were placed into coolstore at 0.0°± 0.3°C for 12 weeks, and the remaining four were cured at 10.0 ± 0.5 °C for 3

days before coolstorage. Treated fruit were examined at 28 day intervals and those with symptoms of stem-end rot were removed to minimise secondary contamination of neighbouring fruit.

The experiment and data analysis were carried out according to a split plot design (See Chapter Two, Section 2.6 for details).

9.4.2 Results

Spray diameters of 28.0 ± 2.0 mm were formed using the Badger Spray gun at a distance of 5 cm away from the target surface resulting in yeast BCA inoculum densities of $2.3x10^5$ to $5.3x10^5$ cfu/mm^2 (Table 9.1).

Table 9.1. Yeast BCA application densities calculated from spray gun total discharge data divided by area of spray zone. MOE = Margin of error (see Chapter Two, Section 2.6) and Pathogen/BCA ratio is the number of BCA cells to one B. cinerea spore applied.

Yeast isolate	Mean cfu density /mm^2	MOE	Pathogen/BCA ratio
532	5.3×10^5	5.4×10^4	300
561	3.8×10^5	2.2×10^4	320
622b	2.6×10^5	5.1×10^4	152
662 dia	5.1×10^5	1.2×10^5	300
662dib	2.3×10^5	8.5×10^4	140

Mean total soluble solids was 6.3 ± 0.6 at the proximal end of the fruit and 7.3 ± 0.8 at the distal end.

There was a significant reduction ($P<0.05$) in the incidence of stem-end rot in fruit treated with all of the yeast isolates compared with fruit inoculated with *B. cinerea* only (Fig. 9.3). The raw data (above each bar) in Fig. 9.3 shows the highest disease incidence (51%) was observed in the pathogen-only, uncured control and was reduced to 13% with fruit curing or to between 16% -2% with yeast BCA alone. When fruit curing was combined with BCA application, there was no significant ($P>0.05$) reduction in disease incidence compared to the same but uncured treatments except for isolates 532 and 622b and the pathogen-only control.

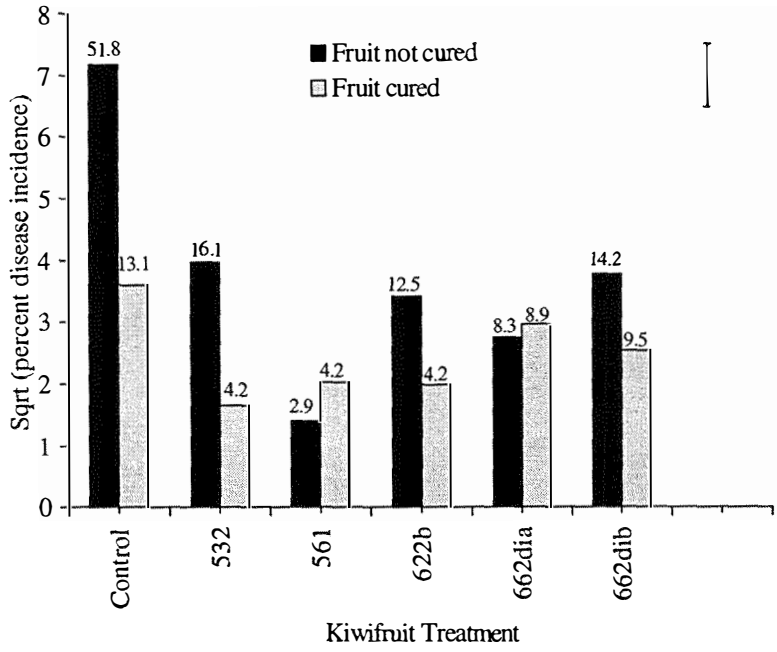


Fig. 9.3. Disease incidence of stem end rot in coolstored kiwifruit treated with yeast BCA isolates and post-inoculation fruit curing (3 days at 10 °C). Raw treatment means are given in parentheses. Bar represents LSD (df=12, n=4, α=0.05)

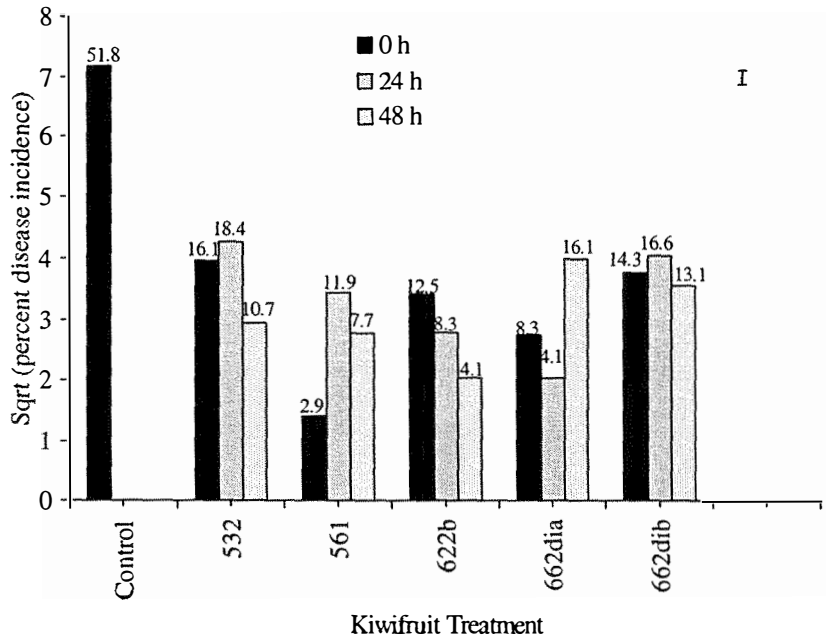


Fig. 9.4. Disease incidence of stem end rot in non-cured, coolstored kiwifruit treated with yeast BCA isolates applied simultaneously (0), 24 h after pathogen inoculation (24h) or 48 h after pathogen inoculation . Fruit were incubated at 0°C during the delayed BCA application. Bar represents LSD (df=12, n=4, α=0.05)

In a second 1996 experiment, there were significant reductions in square root of percent disease incidence ($P<0.0001$) when yeast BCA application was delayed by 24 of 48 h compared with the pathogen-only control (Fig. 9.4). Raw treatment means (Fig. 9.4) show disease incidence was reduced from 51% in the inoculated control to between 16% and 2.9% if the BCA was applied immediately after pathogen inoculation, and between 18% and 4% if yeast BCA application was delayed 24 h or 48 h.

9.5 Glasshouse tomato experiment 1996.

9.5.1 Materials and Methods

Seedling tomato plants cv. 'MoneyMaker' were prepared as described in Chapter Two (Section 2.3) and after 30 days growth, were transplanted into PB12 bags of medium term bark mix in a glasshouse at the Plant Growth Unit (Massey University). Heating and venting setpoint temperatures in the glasshouse were 12°C and 18°C respectively with the sensors centrally located in the glasshouse, mounted 1.5 m from the floor. Pots were arranged on four strips of felt 1 m wide overlaying the concrete floor arranged in 39 rows (19 treatment rows and 20 guard rows) with 3 plants per row. Each plant was watered by hand every second day.

After a further 39 days growth when the plants were at the 4-5 node stage (approximately 1 m high) inoculation treatments were applied. Watering at this time was increased to daily and included flooding the concrete floor to wet the felt mats.

Sixteen isolates of yeast and bacteria BCAs used in this experiment are shown in Table 9.2.

Table 9.2. Yeast and bacterial isolates used for biocontrol experiments on glasshouse tomato plants

Isolate	561 (Y1)	662e (Y2)	662d(i)a (Y3)	662d(i)b (Y4)	572c (Y5)	552c (Y6)	622b (Y7)	532 (Y8)
BACTERIA								
Isolate	ox8a (B1)	ox6 (B2)	27a (B3)	ox2 (B4)	35a (B5)	ox7 (B6)	ox9 (B7)	ox4 (B8)

Note: Numbers in parenthesis correspond to the codes of the various isolate treatments in the glasshouse layout diagram (Fig. 9.5).

All bacterial isolates were cultured in 100 ml nutrient broth and yeasts in NYD broth (pH 4.5), for 5 days at 15°C at 175 rpm (Certomat M, B Braun, Melsungen AG). Used media was removed by centrifuging at 12,000 **g** then the pellet was resuspending in SDW. Total cell counts of each suspension were measured (Chapter Two, Section 2.2) and adjusted to $1x10^{10}$ cells/ml.

Isolate BC20 of *B. cinerea* was prepared and applied as dry inoculum as described in Section 9.3.1. All laterals on the three tomato plants per treatment were cut using secutares to leave a 1 cm stump. This cut surface was inoculated with dry *B. cinerea* spores followed immediately by spray application of the appropriate BCA suspension, or fungicide comparison using the Badger Air Brush (See Section 9.4). Laterals in the guard row plants were removed by hand and remained uninoculated. The wound sites were checked daily for signs and symptoms of *B. cinerea* infection.

The total number of cfu's discharged from the nozzle for each yeast or bacterial isolate dose was measured by directing a standard spray dose into a sterile universal bottle. Three replicates of each isolate was measured. Ten ml of SDW was then added to suspend the applied cells. A 100 µl aliquot was pipetted onto NA or NYDA, incubated at 15°C for 3 days then assessed for colony counts.

G	G	G
Y1	Y1	Y1
G	G	G
B1	B1	B1
G	G	G
Y2	Y2	Y2
G	G	G
B2	B2	B2
G	G	G
B3	B3	B3
G	G	G
Y3	Y3	Y3
G	G	G
C3	C3	C3
G	G	G
Y4	Y4	Y4
G	G	G
Y5	Y5	Y5
G	G	G
B4	B4	B4
G	G	G
Y6	Y6	Y6
G	G	G
C2	C2	C2
G	G	G
B5	B5	B5
G	G	G
C1	C1	C1
G	G	G
B6	B6	B6
G	G	G
B7	B7	B7
G	G	G
Y7	Y7	Y7
G	G	G
B8	B8	B8
G	G	G
Y8	Y8	Y8
G	G	G

G	G	G
Y3	Y3	Y3
G	G	G
C3	C3	C3
G	G	G
Y1	Y1	Y1
G	G	G
B8	B8	B8
G	G	G
B4	B4	B4
G	G	G
Y7	Y7	Y7
G	G	G
B2	B2	B2
G	G	G
B1	B1	B1
G	G	G
B3	B3	B3
G	G	G
C2	C2	C2
G	G	G
Y6	Y6	Y6
G	G	G
B7	B7	B7
G	G	G
B6	B6	B6
G	G	G
Y4	Y4	Y4
G	G	G
Y8	Y8	Y8
G	G	G
Y5	Y5	Y5
G	G	G
B5	B5	B5
G	G	G
Y2	Y2	Y2
G	G	G
C1	C1	C1
G	G	G

G	G	G
B5	B5	B5
G	G	G
B3	B3	B3
G	G	G
Y4	Y4	Y4
G	G	G
B7	B7	B7
G	G	G
B2	B2	B2
G	G	G
B4	B4	B4
G	G	G
B1	B1	B1
G	G	G
Y8	Y8	Y8
G	G	G
Y3	Y3	Y3
G	G	G
B8	B8	B8
G	G	G
Y2	Y2	Y2
G	G	G
Y1	Y1	Y1
G	G	G
C1	C1	C1
G	G	G
Y6	Y6	Y6
G	G	G
C2	C2	C2
G	G	G
Y7	Y7	Y7
G	G	G
Y5	Y5	Y5
G	G	G
B6	B6	B6
G	G	G
C3	C3	C3
G	G	G

G	G	G
B4	B4	B4
G	G	G
Y6	Y6	Y6
G	G	G
B8	B8	B8
G	G	G
Y2	Y2	Y2
G	G	G
B3	B3	B3
G	G	G
B7	B7	B7
G	G	G
Y4	Y4	Y4
G	G	G
B1	B1	B1
G	G	G
Y7	Y7	Y7
G	G	G
Y8	Y8	Y8
G	G	G
Y5	Y5	Y5
G	G	G
B5	B5	B5
G	G	G
C2	C2	C2
G	G	G
C1	C1	C1
G	G	G
B6	B6	B6
G	G	G
Y1	Y1	Y1
G	G	G
C3	C3	C3
G	G	G
B2	B2	B2
G	G	G
Y3	Y3	Y3
G	G	G

Fig. 9.5. Randomised block design - layout plan of tomato plant positions and treatments. G= Guard plants, Y1-8 = yeast isolates, B1-8 = bacteria isolates, C1=Inoculated control, C2=Uninoculated control and C3= fungicide control.

9.5.2 Results

Cfu densities from spray application of yeast and bacterial BCAs range from 6.0 x 10^5 cfu/mm^2 to 1.0 x 10^5 cfu/mm^2 (Table 9.3) which represent a pathogen to BCA application ratio of 60 to 360 cfu to one *B. cinerea* conidium.

Table 9.3. Yeast BCA densities (colony forming units (cfu)) inoculated onto tomato lateral stumps using Badger Airbrush ® and the respective density ratio of these to *B. cinerea* pathogen conidia.

Isolate	Density (cfu/mm^2)	MOE	Pathogen/BCA ratio
ox2	2.3 x 10^5	1.5 x 10^4	130
ox4	5.7 x 10^5	1.3 x 10^5	330
ox6	6.0 x 10^5	1.3 x 10^5	360
ox8a	4.7 x 10^5	2.0 x 10^5	270
ox9	6.7 x 10^5	1.6 x 10^4	390
ox7	7.0 x 10^5	2.9 x 10^5	400
35a	2.4 x 10^5	8.5 x 10^4	130
27a	2.9 x 10^5	5.0 x 10^4	170
561	3.9 x 10^5	2.2 x 10^4	220
532	5.3 x 10^5	5.4 x 10^4	300
662dia	5.2 x 10^5	1.2 x 10^5	300
662dib	2.3 x 10^5	8.5 x 10^4	130
622b	2.6 x 10^5	5.1 x 10^4	150
572c	1.1 x 10^5	2.4 x 10^4	70
552c	1.0 x 10^5	2.6 x 10^4	60

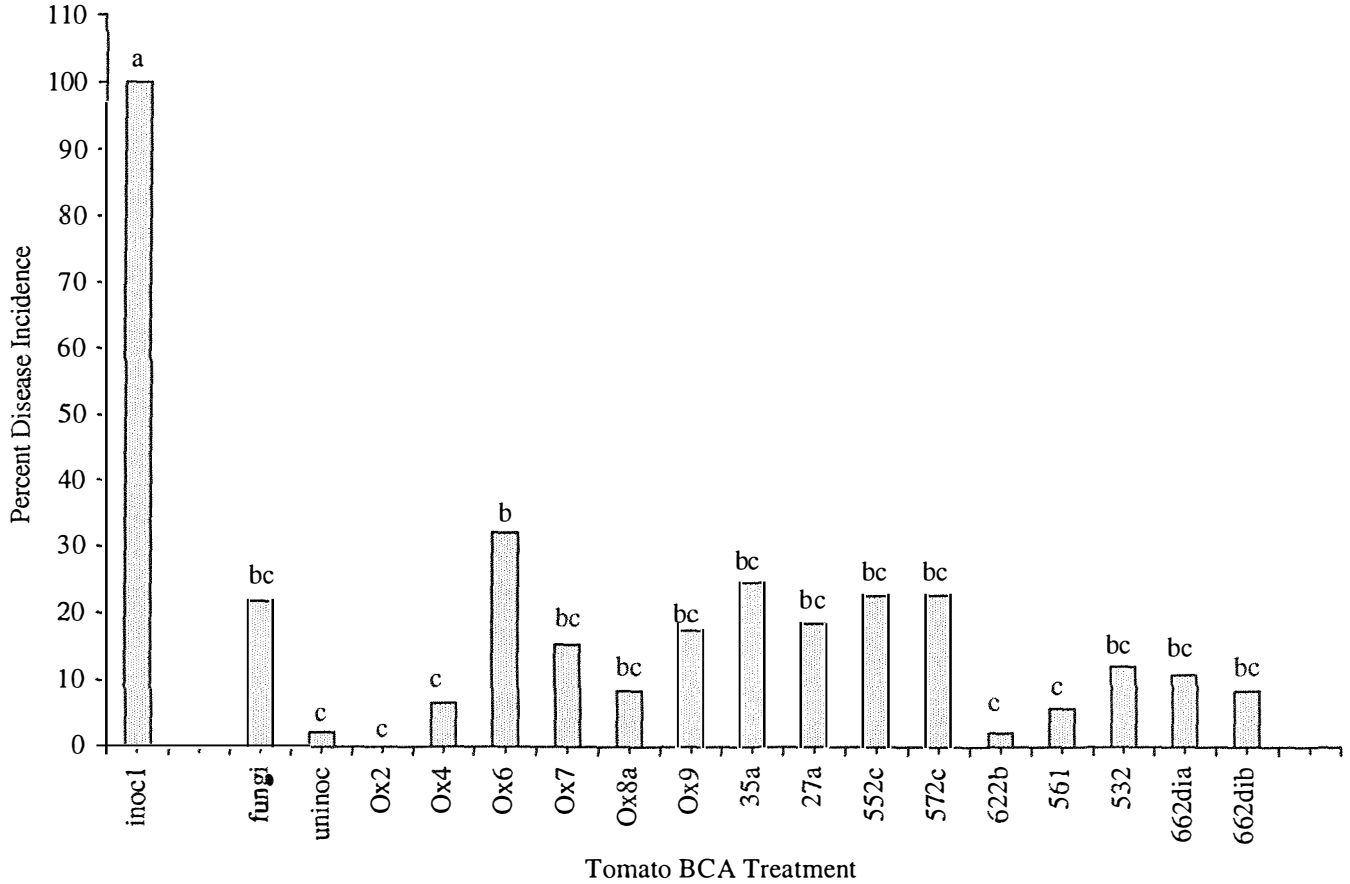


Fig. 9.6. Biocontrol activity of bacterial and yeast isolates against *B. cinerea* inoculated on glasshouse tomato plant lateral wounds, where: inoc = *B. cinerea* only inoculated control, fungi = fungicide (Rovral) and uninoc=uninoculated control. Different letters indicate significant differences determined by Tukeys analysis ($\propto$=0.05).

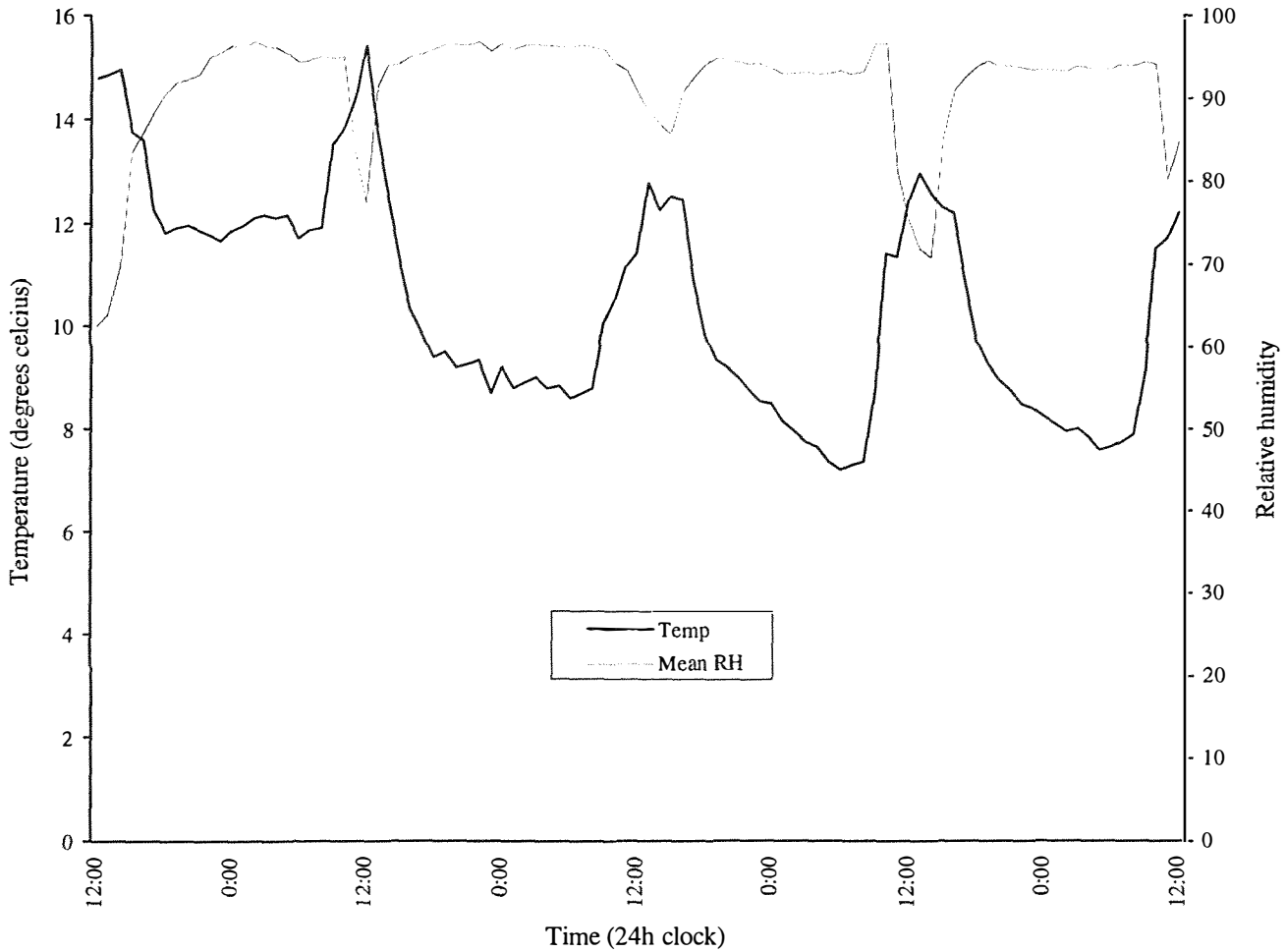


Fig. 9.7. Relative humidity and air temperature within the canopy of the tomato plants.

In the pathogen-only control treatment 100% of the inoculated tomato lateral stumps became infected with *B. cinerea* (Fig. 9.6). Overall, the BCA and fungicide treatments conferred high levels of disease control and incidence was reduced from 100% in the control to between 0% (bacterial isolate ox2) and 30 % (Bacterial isolate ox6) compared with 22% for the fungicide treatment. These differences were significant by Tukeys multiple comparison test

Relative humidity and temperature within the tomato crop canopy showed a distinct diurnal rhythm and wide variation where temperature dipped during the early hours of the morning, with a simultaneous increase in humidity (Fig. 9.7). Maximum temperature during daylight hours coincided with minimum humidity. Due to equipment malfunctions, some environmental measurements had to be taken outside the experimental period, but while the crop remained in the glasshouse. The set point temperatures for the house of 12 °C night minimum and 20 °C daytime maximum and are reflected in the steady rhythm of temperature and humidity measured within the canopy. However during the experiment itself, further equipment failures resulted in these setpoint temperatures being breached on occasions. Maximum air temperature above the canopy was measured at 36°C on two separate occasions and minimum as low as 9 °C using a maximum / minimum thermometer.

There was extensive lesion development in the lateral stumps, extending into the main stem in the pathogen-only treatments (Fig. 9.8 (Top)) but symptoms were limited to discolouration of the wound itself in the BCA treatments (Fig. 9.8 (Bottom)). This was similar to symptoms observed *in vivo* on stem pieces in Chapter Five.

Fig. 9.8. Lesion created in the pathogen only treated lateral stump (Top) and a typical biocontrolled lesion on a lateral stump with an effective BCA added (Bottom) (Bar=1 cm).

9.6 Discussion

All yeast and bacterial isolates which were highly antagonistic to *B. cinerea* in the excised tomato stem bioassay *in vivo* (Chapter Five) conferred strong biocontrol in postharvest kiwifruit, preharvest tomato plants or both.

In the first kiwifruit trial (1995), bacterial mixtures appeared unsuitable for use in the postharvest coolstore environment compared with the yeasts. This result is consistent with that from the *in vivo* bioassay on tomato stem pieces (Chapter Five), where biocontrol from all bacterial isolates except 35a and ox4 were poor at 1°C. Both of these exceptional bacterial isolates came from the mixtures used in the kiwifruit experiment in 1995, and suggests their efficacy was masked by the presence of other microbes in the mixtures. In contrast the yeast isolates significantly reduced disease incidence in both 1995 and 1996 and this was further reduced by fruit curing in 1995 only. Incubating the fruit at 10°C for 3 days is likely to have increased the metabolic activity and hence biocontrol activity of the yeasts themselves in addition to the simultaneous increase in fruit resistance. Bautista-Banos (1995) showed that maximum fruit curing was obtained at 10°C and appears to be mediated through increased activity of a chitinase isoform (Wurms, 1996). In 1996, curing had an inconsistent effect on enhancing biocontrol and the reason for this remains unknown.

In general yeasts appear to hold most promise for postharvest biocontrol in the coolstore. A number of fungal isolates (Tate *et al.,* 1992; Pyke *et al.,* 1993; Franicevic, 1993) and bacteria (Franicevic, 1993) were not strong antagonists of *B. cinerea* at low temperatures however a significant reduction in *B. cinerea* sporulation was obtained using several isolates of *Trichoderma* sp. when co-inoculated into fleshy wounds created in the kiwifruit pericarp (Cheah *et al.,* 1992). The pathological relevance of this to stem end rot is uncertain as the pericarp tissue is quite different from the woody tissue of the picking wound and sporulation is of little economic consequence in postharvest storage of kiwifruit. Yeasts populations 10, 000 times that of the pathogen challenge reduced postharvest stem-end rot (Cheah & Hunt, 1994) and in another study, yeast isolates NZ10 and NZ11 reduced rot incidence in flesh wounds on kiwifruit

from 100% in the control to 20% and 42% respectively at 1000 times the *B. cinerea* challenge (Cheah *et al.*, 1996). In this study of 500 to 60 cfu per *B. cinerea* conidium were used to achieve high biocontrol activity. It is of interest to note that the isolates (NZ10 and NZ11) were obtained from the same orchard as those used in this research programme (L. Cheah, personal communication) using a selection method similar to that of Wilson Wisniewski, Droby & Chalutz (1993). The different yeast BCA species and candidate performance in this study could be the result of the selection criterion used in this thesis.

The wide range of BCA densities from spray application, despite a consistent cell concentration in suspension highlight the issue of the accuracy of spray application. In several studies (eg. Elad *et al.*, 1994b, Hammer & Marois, 1989, Redmond *et al.*, 1987) inoculum was sprayed onto host tissue with no apparent anticipation of differences in the spray pattern from different BCA candidates. The larger number of candidates tested in this study enabled this variability to be observed possibly due to changes in surface tension of water suspension. Fungal spores of *Rhynchosporium secalis*, *Septoria tritici* and *Colletotrichum lindemuthianum* changed the surface tension of the liquid in which they are suspended (Davis & Evans, 1990) and could impart changes in the spray distribution pattern as do other physical parameters on spray performance (Stevens, 1993).

BCA application after pathogen arrival has seldom proved effective yet as Hammer & Marois (1989) argued, it is unlikely that growers would be able to apply BCAs at the time of, or before, pathogen arrival and that material entering the postharvest phase will be carrying pathogen inoculum. Therefore some retrospective control of the pathogen (synonymous to the "reach-back effect" of fungicides) would be highly desirable. Hammer & Marois (1989) found disease reduction was insignificant when BCA application was delayed by 48 h. In the current study, biocontrol activity with delayed BCA application of up to 48 h was the same as for simultaneous application. This result is better than that obtained *in vivo* in Chapter Five where only some of the BCAs tested gave good control with delayed application. The mechanism is likely to be similar to that proposed in Chapter Five. Delayed BCA application would have allowed the

uninhibited germination and growth of pathogen propagules on the host surface thereby creating more substrate for yeast adhesion at the time of the delayed application. Assuming the number of attachment events for a single isolate correlates to a quantitative measure of biocontrol, pathogen control is maintained despite the increased inoculum potential of the pathogen. Fruit incubation during the 24 h or 48 h delay at 0°C was a deliberate selection as any higher temperature was likely to introduce fruit curing effects since maximum curing effects were found at 10°C (Bautista-Banos, 1995) and would confound any effect due to the microbial biocontrol. Alternatively, Elad, Kohl & Fokkema (1994b) isolated yeast BCAs that were applied to existing *B. cinerea* lesions to reduce conidiophore production and argued that biocontrol was due to high yeast populations supported by exogenous nutrient from damaged tissue within the lesion. In this study, a wound was created prior to inoculation of either BCA or *B. cinerea,* in a similar manner to the excised tomato stems in Chapter Five. SEM studies on those tissues showed colonisation of the pathogen but also some colonisation of the plant surface. In the light of adhesion studies in Chapter Seven, and biocontrol data particularly with delayed application in Chapter Five and this chapter, nutrient competition is unlikely.

Yeast used by the food industry have been tested for biocontrol on the basis that these isolates did not produce toxins that would contaminate food product destined for human consumption (Cheah & Hunt, 1994). No antibiotic production was detected from any of the yeast isolates used in this study on NYDA, MEA, PDA and minimal media (Chapter Seven). Thus the risk of rejecting these isolates due to toxicological considerations is unlikely unless any new facts are discovered in future research.

In the glasshouse tomato plant trial, all 15 isolates significantly reduced disease incidence, which is again consistent with *in vivo* data (Chapter Five). The treatment with isolate ox6, recorded the highest disease incidence of all BCAs tested and this was due to a single plant where all lateral sites became diseased. This pattern was quite different from the remaining replicate plants suggesting that these sites did not receive the BCA and in the absence of confirmatory data, these results were retained. Environmental conditions of low overnight temperatures (Morgan, 1984, 1985) and

high humidity (Winspear, Postlethwaite & Cotton, 1970) were maintained to simulate poor management conditions with respect to disease control. The wounds on the tomato plants in this environment were vulnerable to disease as evident by the 100% disease incidence in the pathogen-only control. Despite these conducive conditions and the application of dry conidia (the etiological impact of this has been discussed in Chapter Four), many of the bacterial and yeast BCAs conferred significant levels of biocontrol. Some isolates, for example ox2, 622b, 561, ox8a, ox4 appear to be highly promising for future research as disease incidence was reduced to between 0% and 5%. Eden *et al.* (1996) also obtained good control of *B. cinerea* using *C. cladosporioides* at a concentration 100 times the pathogen challenge while in this study, BCA populations of between 60 and 500 times that of the pathogen were applied.

Results from the postharvest and preharvest trials suggest that all of the BCA isolates merit further field evaluation. Particularly in the light of the application rates, timing and the fact that the yeasts could be applied to both systems. In contrast many BCAs previously tested tended to be specific to either the preharvest or postharvest phases and at relatively high application concentrations.

General Discussion

An improved method for screening micro-organisms for biocontrol agents against *B. cinerea*

10.1 Introduction

As considerable effort and expense is required for development of BCAs for commercial use, the screening and selection process in initial research steps "deserves careful deliberation"(Wilson & Wisniewski, 1989). Whipps (1994) recommended that the issue of screening techniques was one to be addressed if biocontrol was to progress. This thesis commenced with a critical evaluation of the contemporary model for biocontrol of *B. cinerea,* which is predominantly based on those microbes with modes of antagonism is dependent on the host phylloplane (antibiosis, resource competition or induced host resistance) and on the methodologies used to obtain these BCAs (Chapter One).

Microbial colonisation of the plant surface is a complex process whereby the organism becomes established and active in an ecological niche (Andrews, 1990, 1992; Thomashow & Weller, 1996). It is particularly critical for phylloplane competition, antibiosis and induced host resistance because the BCA must establish itself in relatively high populations on the phylloplane to express biocontrol. Thomashow & Weller (1996) concluded that poor biocontrol was the result of poor colonisation and inconsistent antagonist function expression in BCAs used against soil-borne pathogens. The use of agar media for culturing micro-organisms could at least be partially responsible for this since microbial morphologies can be manipulated on agar media and traits essential for colonisation could be unintentially changed.

Consequently, antagonists chosen to date may be fundamentally unsuitable, as their mode of antagonism is inappropriate for the pathogen infection morphology and the problem is compounded by compromised BCA colonisation. For these BCAs, the window of biocontrol opportunity appears to be limited to the period prior to pathogen

arrival and subject to sufficient populations being applied to the target site. This requires a prophylactic and inundative approach to plant protection.

Studies have concluded that extracellular nutrient and moisture are determinants for epiphytic growth of *B. cinerea.* However researchers have often applied conidia in the wet state and it has been shown that epiphytic growth from wet spore application was significantly greater than that from dry spores (Williamson *et al.*, 1995; Cole *et al.*, 1996). This difference has two consequences; firstly, in a screening assay, prolific extracellular pathogen growth is likely to bias towards the selection of competitors or antibiotic producers as the pathogen is heavily dependent on external nutrient and sensitive to anti-fungal agents whilst outside the plant host. Secondly, *B. cinerea* inoculum can be wind borne and the dry deposition events result in direct infection. Thus germination and host entry by *B. cinerea* is less dependent on extracellular nutrient than 'wet' application and BCAs obtained from laboratory tests may be unable to compete or produce sufficient antibiotic to confer biocontrol.

In theory, the empirical approach of *in vivo* testing has the advantage that no preconditions or selection biases have been imposed and therefore the BCAs that are selected are assumed to be those with the most appropriate mechanism for biocontrol of that pathogen. In Chapter One, it was argued that *B. cinerea* morphologies from wet versus dry inoculation was considered responsible for perpetuating this biocontrol model.

These criticisms were guiding principles for the development of an alternative method for the selection of BCAs against *B. cinerea.* Using the attachment assay within the screening programme, a total of 20 bacterial and yeast isolates capable of adhering to *B. cinerea* hyphae or conidia were isolated and 15 showed high antagonistic activity on tomato stem pieces (Chapter Five). This activity was confirmed on glasshouse tomato plants and on postharvest kiwifruit (Chapter Nine) even when BCA inoculation followed after *B. cinerea* arrival (Chapters Five and Nine).

10.2 The attachment assay

The alternative BCA selection criterion was made possible by the successful development of an apparatus and protocol that could selectively and efficiently isolate bacteria and yeasts with the ability to adhere to conidia or hyphae of *B. cinerea* (Chapter Three). There have been a number of previous attempts to isolate colonists of fungal pathogens (Nesbitt, Malajczuk & Glenn, 1978, 1981; Toyota & Kimura, 1993) but they were limited by the small number of isolates obtained and the high contaminant levels in samples (ie. those microbes that did not colonise the target fungi).

The ability to rapidly remove many non-target candidates in the adhesion assay conferred upon the entire biocontrol programme an efficiency that has not been previously reported in biocontrol literature. With their removal, more equipment and resource could be dedicated to testing the 'highly probable' candidates. Consequently, more detailed information about the 20 candidates here was obtained by applying three BCA concentrations and incubating the treated host tissues at three incubation temperatures in the *in vivo* assay. Such an extensive test could not realistically be applied to the many hundreds and sometimes thousands of isolates that are individually assessed for biocontrol in conventional biocontrol assays (Leivens *et al.*, 1989; Montesinos, Bonaterra, Ophir & Beer, 1996).

A key to this successful BCA selection was the identification of an appropriate microbe trait for biocontrol. Andrews (1990) suggested that if a character indicative of successful microbe colonisation could be identified, this would become a powerful selection criterion. James, Suslow & Steinback (1985) used microbial adhesion to radish roots as an indicator of long term colonisation by rhizobacteria but concluded that attachment alone was not an accurate determinant. These findings are in contrast to those of this study. However the focus of James *et al.* (1985) and Andrews (1990) was limited to adhesion to the plant host and phylloplane-based biocontrol functions. The plant surface is biochemically complex and as was the experience in the laboratory at Massey University, there was no method able to discriminate between microbial attachers, non-attachers and those cells that became entrapped. In contrast, the fungal pathogen itself has a surface that was relatively

homogenous biochemically and morphologically, present during the epiphytic phase of an infection event or epidemic, that could be clearly identified and demarcated and is a target of immediate consequence should antifungal activities be delivered. From a practical research perspective, this surface was isolatable and usable away from the plant surface *in vitro*. Furthermore, mycoparasitism, similar to attachment, was regarded as most appropriate for biotrophic pathogens where they are independent of extracellular nutrient and some have infection morphologies similar to *B. cinerea* (Andrews, 1992).

Therefore this type of assay could become an important alternative or addition to the methods currently used for selection of BCAs. Although it does not assess a wide range of mechanisms, its determinative nature, enables selection of a well defined population of microbes by a procedure that appears to be an accurate presumptive test for biocontrol by attachment.

10.3 *B. cinerea* infection

In all biocontrol studies, a thorough knowledge of the biology of the pathogen target is paramount to improve the probability of successful plant protection. In this study, *B. cinerea* infection and the methods of applying conidia to host tissue were examined. Current methods for inoculating targets with dry spores generally falls short of the precision and accuracy required for bioassays as evident in Chapter Nine. This consideration took precedence in the decision to investigate a compromise method based on the low volume spray application using the Potter Tower where inoculum levels could be accurately defined. Disease development from spores applied in aerosol was more advanced and more aggressive compared with the same density inoculum from small droplets of water suspension. This application technique became standard procedure in BCA selection experiments in this programme to ensure that the BCAs were selected against aggressive pathogen infection.

10.4 The biology of the BCAs selected

Microbial attachment as a selection criterion by which the BCAs in this thesis were isolated appears to have been influential or critical in many aspects of BCA biology. These are discussed below.

10.4.1 Habitat and location of candidates

The location from which the candidates were obtained appears to be an important initial consideration in this type of assay as most of the effective candidates came from the Levin organic orchard site. Many site differences between the native bush reserve (Turitea) and the organic orchard site (Levin) would have shaped different microbial populations. The absence of susceptible hosts to *B. cinerea* in the Turitea site compared with Levin suggested that the presence of *B. cinerea* could be a determining factor in the development of a natural 'attacher' populations. Selection of sites within the Levin location appeared unimportant as BCAs were selected from candidates obtained from soil, phylloplane and fruit surfaces. However this study was not designed as a survey and a larger, specific programme would be required to investigate this hypothesis.

10.4.2 Taxonomy

The taxonomic range of the BCAs selected from the attachment assay suggests that the assay exerted a selection criterion sufficiently unique to detect quite different isolates from within the same species or genera that have not been previously reported as attachers or BCAs. Some isolates, for example , *Candida* sp., *Trichosporon* sp. and *Pseudomonas* sp., have been previously reported as BCAs of other plant pathogens, while others have neither been described as BCAs nor attachers to *B. cinerea* (for example, *Ochrobactrum anthropii*). The bacteria were dominated by the genera *Enterobacter* spp. which have been reported to be both a BCA and/or attacher to several plant pathogens, although not to *B. cinerea* beyond the *in vitro* level.

10.4.3 Biocontrol results

The biocontrol results from the 15 isolates tested in the glasshouse or postharvest environments (Chapter Nine) were consistent with data obtained in the laboratory (Chapter Five) which included timing of BCA application and changes in activity with temperature. As a result, only the yeast isolates were selected for the postharvest trials, whilst all candidates were tested in the preharvest tomato trial. In all studies they conferred plant protection as well as, or better than, the fungicide comparisons (Chapter Nine). This strong relationship between field data and laboratory data is likely to be the result of the selection criterion used in the attachment assay. In section 10.2, the role of microbial adhesion as a presumptive test was discussed. If traits for biocontrol had already been selected prior to biocontrol testing, then it is likely that biocontrol will be observed in both the intermediate testing and field testing of the BCA isolates, provided that the trait is not compromised during laboratory culturing.

BCA concentration levels used in experiments outside the laboratory were comparable or lower than those used for other BCA isolates reported in the literature. *In vivo*, biocontrol by some candidates was unaffected by the application rate despite a considerable range in density (3 to 80 -times *B. cinerea* populations applied) (Chapter Five) but this was not examined in either the kiwifruit and tomato trials. Timing of BCA application was also examined *in vivo* and in postharvest kiwifruit only. In the laboratory, a variety of biocontrol responses were observed using a selection of bacterial and yeast candidates, when BCA application was delayed by up to 48 h after pathogen arrival. In contrast, results from the postharvest kiwifruit trial were more consistent where all the yeasts tested continued to provide high wound protection despite arriving 48 h after *B. cinerea* inoculation. This 'reach-back' characteristic desirable in chemical fungicides is seldom seen in BCA isolates whose mode of antagonism is phylloplane-based. Attachment appears to fulfill the role required of a BCA applied to crops entering the postharvest phase which contains pathogen inoculum likely to be resident on the phylloplane. The seemingly lack of biocontrol response with respect to timing of BCA application and BCA rate were discussed in Chapter Five and were related to the direct attacking nature of these BCAs.

10.4.4 Antagonistic function

The alternative selection criterion used in the screening assay and the marked differences in the biocontrol performance by these isolates in the laboratory and in the field suggested mechanisms other than antibiosis, host resistance or competition were important. Using SEM (Chapter Six) and light microscopy (Chapters Six and Seven), all of the isolates were observed attached to, or coiling (by the two filamentous yeasts (552c and 572c (*Galactomyces geotrichum*)) around *B. cinerea in vitro* or *in vivo*. In some instances, there was evidence of pathogen degradation, collapsed hyphae, distorted growth or the possible release of hyphal contents.

Many of the bacteria and all of the yeasts produced varying quantities of endochitinase *in vitro*, some produced siderophores and two bacterial isolates produced anti-*Botrytis* compounds (Chapter Seven). The high number of isolates tested and their common method of isolation enabled some conclusions to be made on the basis of the frequency with which specific functions were observed despite being limited to *in vitro* environments. More definitive results could be obtained using mutation studies but this requires an intensive study, particularly of the attachment mechanism.

Use of monoclonal antibodies (MAbs) as a method to examine the role of adhesion itself as part of the biocontrol mechanism appears to be unique. The inhibition of adhesion by the bacterial BCAs to *B. cinerea in vitro* with BC-KH4 whilst adhesion by yeasts was unaffected, correlated well with *in vivo* biocontrol data by the respective BCAs. Activity by bacterial BCAs was reduced when the MAb was applied to excised tomato tissue while yeast biocontrol was unaffected. This suggests that adhesion is an important mechanism for bacterial biocontrol. Another method was required to determine its importance in yeast biocontrol. In contrast, another unrelated Mab (PI-01), used as a comparison, reduced biocontrol activity of both yeast and bacterial BCAs and highlighted the danger where MAb activity was not limited to adhesion alone.

In Chapter Eight, ELISA and immunofluorescence techniques were used to measure *B. cinerea* biomass in the presence of selected BCAs. The addition of BCAs reduced the amount of *B. cinerea* antigen detected *in vivo* and the presence of ungerminated and unlabelled *B. cinerea* conidia in immunofluoresence experiments suggested that this was due

to reduced pathogen growth rather than materials from the BCAs interfering with *B. cinerea* detection. A key finding in this study that lead to the successful extraction of *B. cinerea* antigen was the use of a copper-based extractant. Other buffers and extractants were examined but the ELISA detection protocol did not detect any *B. cinerea* antigen.

10.5 Towards an alternative biocontrol model

The frequency with which successful BCAs appear to have been isolated using the attachment assay, their different biocontrol abilities compared with conventional isolates, the similarity in antagonistic function among these isolates and effective wound protection of two crops in very diverse production systems presents initial evidence for an alternative model for *B. cinerea* biocontrol. The central tenet to this model is that microbial attachment is used as a presumptive test and function for pathogen antagonism that delivers a suite of biocontrol mechanisms to the pathogen itself. Adhesion, has been shown in previous studies to be critical for subsequent biology, for example in the pathogenesis by plant and animal pathogens and in biocontrol of plant disease. In contrast to previous attempts, attachment has been successfully used in this thesis as a selectable trait for biocontrol but directed at the pathogen itself.

The process of colonisation by an attacher BCA onto *B. cinerea* is arguably the same as for competitor-BCAs on the phylloplane and as a result, biocontrol would suffer the same limitations that have plagued the contemperary model. However in attacher biocontrol, the biochemical and morphological homogeniety of the pathogen surface, the absence of other competing microbial populations on that surface and direct nature of delivering antagonism creates a much simpler biocontrol model. Thus this model differs from the contemporary in one important manner, the degree with which the BCA requires the phylloplane to confer biocontrol and time required to complete biocontrol. Resource competition and antibiosis are examples of the indirect role of the plant surface where a number of plant-controlled factors (nutrient, moisture, topography, pH) will dictate microbial community composition. For optimal biocontrol, a researcher must identify each of these parameters and record how these change temperarily and spatially then reproduce these in an assay capable of seleting a BCA that will survive while aggressively establishing itself on the phylloplane to confer

biocontrol. As this biocontrol is ongoing, a BCA population has to maintain an effective presence to provide biocontrol. These high demands are still beyond current methods and understanding despite significant advances in technique and theory (Andrews, 1992).

In moving away from phylloplane dependence, the survivability of attacher BCAs would be reduced unless other alternative or volunteer microbial hosts are found or possess such traits so that they survive on the phylloplane. As the attachment assay was not designed with the later in mind, the resulting BCAs will probably provide a transient biocontrol, that requires repeated application during a growing season to maintain plant protection. The sole use of adhesion and its importance in biocontrol has revealed the "Achilles heel" of this model. Sole reliance on a single process to deliver a suite of antagonistic mechanisms will increase the probability of biocontrol failure when unsuitable environments prevail. It is critical, therefore, that the parameters of this biocontrol model are substantiated and defined.

Research into attachment on the pathogen itself represents a redirection in research focus in an attempt to suppliment the role of the phylloplane in biocontrol. This in theory should lead to a wider range of plant hosts in which an attacher-BCA will operate and a much reduced emphasis on application concentrations and timing. In this programme, BCAs with these characteristics were isolated and can be considered preliminary evidence for this alternative model. Furthermore, this research represents an attempt to simplify biocontrol and the manner in which it is achieved by using BCAs that attack the pathogen directly. Of necessity, some desirable criteria such as possible persistence of BCA in the infection court were not investigated but if not ideal can be compensated by increasing the application frequencies.

10.6 Future research

In this thesis aspects of *B. cinerea* infection biology, screening procedures for isolating BCAs and microbial biocontrol have been addressed. However some investigations remain preliminary and further research is warrented.

10.6.1 B. cinerea *aetiology*

Investigations into the biology of *B. cinerea* were limited to examining the effect of different application techniques of *B. cinerea* on subsequent host infection. In Chapter Four, it was shown that spray application of *B. cinerea* conidia as an aerosol significantly advanced the infection process compared to conidia in water suspension. Further research is required to investigate the exact mechanisms that are involved and how they are influenced by the manner of conidial application. To fulfil this objective, it is recommended that the aerosol inoculation technique is applied to other plant hosts to provide more information about the phenomenon and mechanisms.

10.6.2 The attachment assay

In Chapter Five, the adhesion assay was used to select isolates from two habitat sites only, consequently, conclusions about the effectiveness of the method as a selection criterion are limited because of the small data base. The high biocontrol activity of the isolates obtained and the efficiency with which BCAs were identified could be the result of an exceptional site in Levin. Additional selections from other habitats within New Zealand and overseas using the attachment assay are required to assess biocontrol activity of isolates in relation to the habitats from which they were obtained.

10.6.3 Biocontrol studies

Studies on the biocontrol activity of the isolates obtained in this programme have been limited to wound protection against artificially inoculated *B. cinerea* in preharvest glasshouse tomatoes and postharvest kiwifruit. As these two environments and hosts represent quite different conditions and indicate that these isolates are capable of

biocontrol on a wider spectrum of *B. cinerea* plant hosts, further work, for example, on preharvest and postharvest cut flowers is required for confirmation.

Regardless of crop selection, extensive field trials replicated in time and space would be required to gather a comprehensive database from which the consistency of the biocontrol activity of these isolates in semi-commercial environments could be evaluated. In these studies a variety of BCA formulations should be tested as they are important for the success of biocontrol (Harman, 1991; Sutton & Peng, 1993). Thought should also be given as to the likely supporting technology required to apply these isolates to the target site, for example, to the stem scar region of postharvest kiwifruit.

10.6.4 Mode of antagonism

For future registration purposes and scientific endeavour, the exact mode of antagonism among these isolates requires intensive research particularly as attachment as a mode of antagonism represents a relatively new field of study. Such studies could be conducted with three aims:

(1) To study the adhesion mechanism itself and its importance to the biocontrol event. Clones of these isolates deficient in some biochemical or morphological feature critical for the attachment event, but identical in all remaining aspects could be created in the laboratory and tested for biocontrol in controlled environments. The process of identifying the exact mechanisms involved in adhesion itself in these bacteria and yeasts could be scientifically profitable as the common manner in which all of the current BCA collection were isolated would enable comparisons to be made, particularly between bacterial and yeast adhesion.

(2) To investigate associated mechanisms that cause the death or destruction of the *B. cinerea* hyphae following attachment. Again, molecular technique could be used to create mutants deficient in specific traits, for example, chitinase production.

(3) To investigate possible chemotactic characteristics in this group of isolates. There have been previous reports of such behaviour in predatory (Chet, Fogel & Mitchell, 1971) and in phytopathogenic bacteria (Lim & Lockwood, 1988) and other bacteria toward fungal spores (Arora & Gupta, 1993). This area of study was reviewed by Chet and Mitchell (1976). If chemotaxis was found, it would contribute to the understanding of the biocontrol mechanism.

10.6.5 Commercialisation

Preliminary results from the studies in this thesis programme merit the continuation of this biocontrol research. If the above recommended field trials and research into the mode of antagonism are completed and the isolates continue to show potential as a plant protection tool, then commercial release would be the next obvious step. Already, in this programme, two aspects of BCA biology have been identified that are likely to be influential in any future release. In Chapter Six, there was evidence that the BCA isolates are not limited to adhesion to *B. cinerea* and are capable of adhering to other microbes. The biocontrol potential of this could be exploited in studies designed to investigated whether these isolates are capable of biocontrol activity against other fungal plant pathogens. If this is the case, the commercial spin-off could be considerable as a single product could be registered for a number of diseases in a range of crops thereby widening the market for such products. However, this same phenomenon could also exclude the use of biocontrol mixtures, either within a single product, or the mixing of two products together in the field. This aspect of BCA biology and that of the influence on non-target organisms would require careful research.

Another consideration is the current practice of adding surfactants and wetters to pesticides to obtain more efficient application, residency and efficacy. The addition of detergents inhibits microbial adhesion which under the current hypothesis is critical for the BCA mode of antagonism. Thus the obvious consequence of adding surfactants to a biocontrol product made from these isolates requires careful research to establish compatible combinations between existing products and the BCA based product to avoid the situation where the additive inhibits adhesion and therefore biocontrol efficacy.

To fulfil any future registration requirements, human health and environmental risks need to be addressed. One of the isolates *Ochrobactrum anthropi* obtained in this study, has been previously reported as an opportunistic human pathogens (Holmes, Popoff, Kiredjian & Kersters, 1988). The current approach to minimise the risk of isolating such potential problems has been to avoid using incubation temperatures at or near mammalian blood temperature (37°C) and to discard those candidates that are able to grow at this temperature *in vitro*. Isolates from this study were isolated at 10-15°C but have not been incubated at higher temperatures. Therefore any future commercial use of these isolates would be subjected to this consideration.

10.7 Blue skies research

The successful development of the attachment assay has created the opportunity to apply this general approach of screening BCAs to other pests and diseases in crop plants. Such assays could become additional selection tools in the efforts to find better agents for biological control. Some possible examples include:

10.7.1 Variants of B. cinerea *biocontrol*

Manocha & Campbell (1983) showed that mycoparasitism by specific fungal isolates differed according to the age of the pathogen mycelium. In this study, isolates were obtained for the control of germinating *B. cinerea* conidia in the infection court. However it is conceivable that a different range of BCAs are selected for biocontrol of older hyphae of *B. cinerea* that are present during sporulation in the field reducing the reproductive capacity of the pathogen. This model has already been successfully developed using other competition mediated BCAs (Elad, Kohl & Fokkema, 1994a, 1994b; Fokkema, 1993, 1995; Kohl, Molhoek, van der Plas & Fokkema, 1995a, 1995b; Kohl, van der Plas, Molhoek & Fokkema, 1995c).

Alternatively, the process of attachment as a mechanism of delivering antagonistic mechanisms to *B. cinerea* can be exploited to deliver viruses or viroid particles. Virus-like particles have been found in *B. cinerea* (Howitt, Beever, Pearson & Forster, 1995) and the BCAs in the current collection could be used as carriers, transporting the virus to the target and delivering it into the pathogen when attached.

10.7.2 Other fungal plant pathogens

The adhesion criterion was used successfully for biocontrol against a plant pathogen with an epiphytic growth phase, yet Andrews (1992) stated that mycoparasitic BCAs were most appropriate for the biocontrol of biotrophic pathogens. Thus BCA isolates from the attachment assay could be applied to a wide range of foliar fungal pathogens with a variety of infection processes. Further, it may also be possible to isolate BCAs against bacterial and yeast plant pathogens. Appropriate modifications to the protocol would be necessary to adjust for the obligate growth of some pathogens, such as the mildews and retain bacteria in the apparatus during the washing to remove contaminant microbes.

10.7.3 Insect pests

A variety of entomopathogenic microbes have been investigated for the biocontrol of insect pests. Bacteria have been most successfully brought into commercial use with products based on the cells and protein products of *Bacillus thuringiensis*, *B. sphaericus* and *Serratia* spp. Fungi belonging to the family Entomophthoraceae have also been examined for the control of glasshouse and soil dwelling stages of pests. A common thread in the mode of action of some of these agents is adhesion where either pathogenicity is initiated when the insect target ingests the agent and the bacterium becomes established within the insect (for example *B. theringiensis* or *Serratia* spp. (Van Driesche & Bellows, 1996)) or the agent binds to a weakness in the exoskeleton (for example, *Verticillium lecanii*, infect the target by direct penetration through the cuticle, natural orifices or between body segments of aphid or whitefly (Samson & Rombach, 1985)) . The attachment assay could be modified by replacing the *B. cinerea* with an appropriate target could offer a more efficient approach of screening mixed microbial populations for isolates with antagonistic properties against insects.

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Appendix 1 - Example files submitted to The SAS System

A1.1 ANOVA assumptions and reapplying model after transforming data. Experimental design = Completely Randomised Block Design - Factorial.

COMMAND FILE

```
/* file H:\bcau.dat text file */

options ls=78 ps=63 nocenter nodate;
data healthy;
infile 'bcau.dat';
 do temp = '15','7','1';
do isol = '3a','2b','2a','1','2','3','4','5','6','7','8a','9','72c','52c','6b','dia','dib','62e','32','61';
do block = 1 to 4;
do conc = '0','100','200','400';
input stem @@;output;
end;end;end;end;
run;
data dd2;set healthy;
pcstem = (stem/10)*100;
pstem =stem/10;
run;
proc means data=dd2 noprint;
class temp isol conc;
var stem;
output out=dd3 mean=mstm std=sstm
run;
proc print data=dd3;
run;
data dd4;set dd3; where _type_=3;
msstm=sstm/mstm;
mssstm=sstm/sqrt(mstm);
lmstm=log10(mstm);
lsstm=log10(sstm);
run;
proc print data=dd4 uniform;
run;
options ps=30;
proc plot data=dd4;
plot lsstm*lmstm;
run;
proc glm data=dd4;
model lsstm=lmstm;
run;
data dd5;set dd2;
logstm=log10(pstem +1);
sqrstm=sqrt(pstem +1);
arstm=arsin(pstem);
run;
```

```
title2 'Analysis raw data';
proc glm data=dd2 order=data;
class temp isol block conc;
model pcstem=temp isol conc block temp*isol temp*conc temp*isol*conc/ss1;
means temp block/duncan tukey lsd;
output out=dd6 p=pstm r=rstm student=ststm;
run;
proc plot data=dd6;
plot ststm*pstm/vref=0 2 -2;
run;
title2 'Analysis of log data';
proc glm data=dd5 order=data;
class temp isol block conc;
model logstm=temp isol conc block temp*isol temp*conc temp*isol*conc/ss1;
means temp block/duncan tukey lsd;
output out=dd7 p=plstm r=rlstm student=stlstm;
run;
proc plot data=dd7;
plot stlstm*plstm/vref=0 2 -2;
run;
title2 'Analysis of sqrt data';
proc glm data=dd5 order=data;
class temp isol block conc;
model sqrstm=temp isol conc block temp*isol temp*conc temp*isol*conc/ss1;
means temp block/duncan tukey lsd;
output out=dd8 p=psstm r=rsstm student=stsstm;
run;
proc plot data=dd8;
plot stsstm*psstm/vref=0 2 -2;
run;
title2'Analysis by arcsin transormation';
proc glm data=dd5 order=data;
class temp isol block conc;
model arstm=temp isol conc block temp*isol temp*conc temp*isol*conc/ss1;
means temp block/duncan tukey lsd;
output out=dd9 p=parstm r=rarstm student=starstm;
run;
proc plot data=dd9;
plot starstm*parstm/vref=0 2 -2;
run;
```

A1.2 Complex SAS command file fully fitted and partially fitted models. Experimental design =nested.

COMMAND FILE

```
/* file final.dat text input */

options ls=78 ps=63 nocenter nodate;
data spores;
title 'Analysis of spore dispersion using potter tower -nested
design unbalanced';
infile 'final.dat';
 do load = '5^6','7.5^6','1^7','2.5^7','5^7';
  do rep = 1 to 3;
   do sector = 1 to 9;
  do site = 1 to 6;
input counts @@;
output;
end;end;end;end;
run;

title 'Check for assumptions';

proc means noprint data=spores;
class load rep sector;
var counts;
output out=mspores mean=mspr std=sspr;
run;
data mspores; set mspores;where _type_ =6;
  mscnt = sspr/mspr;
  msscnt =sspr/sqrt(mspr);
  lmcnt =log(mspr);
  lscnt =log(sspr);
run;
proc print data=mspores uniform;
run;
proc plot data=mspores;
plot lscnt*lmcnt;
run;
proc glm data=mspores;
model lscnt=lmcnt;
run;

/*transforming data*/
data dd1; set spores;
logcnt=log(counts);
sqrcnt=sqrt(counts);
pwcnt=counts**-0.09;
if sector=9 then logcnt = logcnt*0.1;
run;

title 'analysis raw data - fitting partially nested design';
```

```
proc glm data=spores order=data;
class load rep sector site;
model counts=load rep(load) sector(load rep)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
test h=sector(load rep) e=site(load rep sector)/htype=1
etype=1;
means sector/duncan tukey lsd e=site(load rep sector);
output out=dd6 p=pcnt r=rcnt;
run;
options ps=30;
title 'Plot of residuals - raw data partially fitted model ';
proc plot data=dd6;
plot rcnt*pcnt=sector/vref=0;
plot rcnt*pcnt=site/vref=0;
run;

title 'anaysis raw data using fully fitted nested model';

proc glm data=spores order=data;
class load rep sector site;
model counts=load rep(load) sector(load rep) site(load rep
sector)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
test h=sector(load rep) e=site(load rep sector)/htype=1
etype=1;
output out=dd6 p=pcnt r=rcnt;
run;
options ps=30;
title 'Plot of residuals - raw data fitted full model';
proc plot data=dd6;
plot rcnt*pcnt=sector/vref=0;
plot rcnt*pcnt=site/vref=0;
run;

title 'analysis of log data using partially fitted nested model';

proc glm data=dd1 order=data;
class load rep sector site;
model logcnt=load rep(load) sector(load rep)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
output out=dd3 p= plgcnt r= rlgcnt;
run;
proc plot data=dd3;
plot rlgcnt*plgcnt=sector /vref=0;
plot rlgcnt*plgcnt=site/vref=0;
run;

title 'Analysis of log data using fully fitted nested model';

proc glm data=dd1 order=data;
```

```
class load rep sector site;
model logcnt=load rep(load) sector(load rep) site(load rep
sector)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
test h=sector(load rep) e=site(load rep sector)/htype=1
etype =1;
output out=dd3 p= plgcnt r= rlgcnt;
run;
proc plot data=dd3;
plot rlgcnt*plgcnt=sector /vref=0;
plot rlgcnt*plgcnt=site/vref=0;
run;

title 'Analysis of square root data partially fitted model';

proc glm data=dd1 order=data;
class load rep sector site;
model sqrcnt=load rep(load) sector(load rep)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
output out=dd4 p= pscnt r= rscnt;
run;
proc plot data=dd4;
plot rscnt*pscnt=sector/vref=0;
run;

title 'Analysis of square root data fully fitted model';

proc glm data=dd1 order=data;
class load rep sector site;
model sqrcnt=load rep(load) sector(load rep) site(load rep
sector)/ss1 e1;
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
test h=sector(load rep) e=site(load rep sector)/htype=1
etype =1;
output out=dd4 p= plgcnt r= rlgcnt;
run;
proc plot data=dd4;
plot rlgcnt*plgcnt=sector /vref=0;
plot rlgcnt*plgcnt=site/vref=0;
run;

title 'Analysis of power (-0.09) data fitted partially model';

proc glm data=dd1 order=data;
class load rep sector site;
model pwcnt=load rep(load) sector(load rep)/ss1 e1;
random site(load rep sector);
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
output out=dd5 p=ppwcnt r=rpwcnt;
run;
```

```
proc plot data=dd5;
plot rpwcnt*ppwcnt=sector/vref=0;
run;

title 'Analysis of power (-0.09) data fitted full model';

proc glm data=dd1 order=data;
class load rep sector site;
model pwcnt=load rep(load) sector(load rep) site(load rep
sector)/ss1 e1;
random site(load rep sector);
test h=load e=rep(load)/htype=1 etype=1;
test h=rep(load) e=sector(load rep)/htype=1 etype=1;
test h=sector(load rep) e=site(load rep sector)/htype=1
etype=1;
output out=dd5 p=ppwcnt r=rpwcnt;
run;
proc plot data=dd5;
plot rpwcnt*ppwcnt=sector/vref=0;
run;
endsas
```

A1.3 Complex SAS command file. Experimental design = Split Plot/ Completely Randomised Block/Factorial with partially fitted models.

COMMAND FILE

```
/* file h:iu.dat text file */

options ls=78 ps=30 nocenter nodate;
data inoc;
infile 'iu.dat';
do conc ='5x10^7','1x10^7','5x10^6';
do inoc ='Pot','30P','60P';
do rep =1 to 4;
do time= 1 to 10;
input infect @@;
output;end;end;end;end;
run;
title 'Analysis of inoc data on tomato tissues';
proc glm data=inoc order=data;
class conc inoc rep time;
model infect = rep conc inoc conc*inoc rep*conc*inoc time
time*conc time*inoc/ss1;
title 'main plot';
test h= rep conc inoc e=rep*conc*inoc/htype=1 etype=1;
contrast 'pot vs 3p'inoc 1 -1 0/e=rep*conc*inoc;
contrast 'pot vs 6p'inoc 1 0 -1/e=rep*inoc*conc;
contrast '3p vs 6p' inoc 0 1 -1/e=rep*inoc*conc;
output out=dd1 p=pinf r=rinf;
run;
```

```
proc plot data=dd1;
plot rinf*pinf/vref=0;
run;
proc means data=inoc noprint;
class conc inoc;
var infect;
output out=dd2 mean= minf std= sinf;
run;
data dd2;set dd2;
msinf=sinf/minf;
mssinf=sinf/sqrt(minf);
lminf=log(minf);
lsinf=log(sinf);
run;
proc print data=dd2 uniform;
run;
options ps=30;
proc plot data=dd2;
plot lsinf*lminf;
run;
proc glm data=dd2;
model lsinf=lminf;
run;
data dd3;set inoc;
linf=log10(infect+1);
crinf=infect**0.33;
sqinf=sqrt(infect);
run;
proc glm data=dd3 order=data;
class conc inoc rep time;
model linf = rep conc inoc conc*inoc rep*conc*inoc time
time*conc time*inoc/ss1;
title 'main plot';
test h= rep conc inoc e=rep*conc*inoc/htype=1 etype=1;
contrast 'pot vs 3p'inoc 1 -1 0/e=rep*conc*inoc;
contrast 'pot vs 6p'inoc 1 0 -1/e=rep*inoc*conc;
contrast '3p vs 6p' inoc 0 1 -1/e=rep*inoc*conc;
output out=dd4 p=pinf r=rinf;
run;
proc plot data=dd4;
plot rinf*pinf/vref=0;
run;
proc glm data=dd3 order=data;
class conc inoc rep time;
model crinf = rep conc inoc conc*inoc rep*conc*inoc time
time*conc time*inoc/ss1;
title 'main plot';
test h= rep conc inoc e=rep*conc*inoc/htype=1 etype=1;
contrast 'pot vs 3p'inoc 1 -1 0/e=rep*conc*inoc;
contrast 'pot vs 6p'inoc 1 0 -1/e=rep*inoc*conc;
contrast '3p vs 6p' inoc 0 1 -1/e=rep*inoc*conc;
output out=dd5 p=pinf r=rinf;
run;
proc plot data=dd5;
```

```
plot rinf*pinf/vref=0;
run;
proc glm data=dd3 order=data;
class conc inoc rep time;
model sqinf = rep conc inoc conc*inoc rep*conc*inoc time
time*conc time*inoc/ss1;
title 'main plot';
test h= rep conc inoc e=rep*conc*inoc/htype=1 etype=1;
contrast 'pot vs 3p'inoc 1 -1 0/e=rep*conc*inoc;
contrast 'pot vs 6p'inoc 1 0 -1/e=rep*inoc*conc;
contrast '3p vs 6p' inoc 0 1 -1/e=rep*inoc*conc;
output out=dd6 p=pinf r=rinf;
run;
proc plot data=dd6;
plot rinf*pinf/vref=0;
run;
```

Appendix Two - Calibration of the Potter Tower

The apparatus.

The Potter Precision Laboratory Spray Tower (Burkard Manufacturing Co. Ltd. Rickmansworth, UK) was connected to a compressor (GEC 5000-11). The complete assembly as set up in the laboratory is shown in Fig. A2.1. A sectioned view of the tower alone is shown in Fig. A2.2.

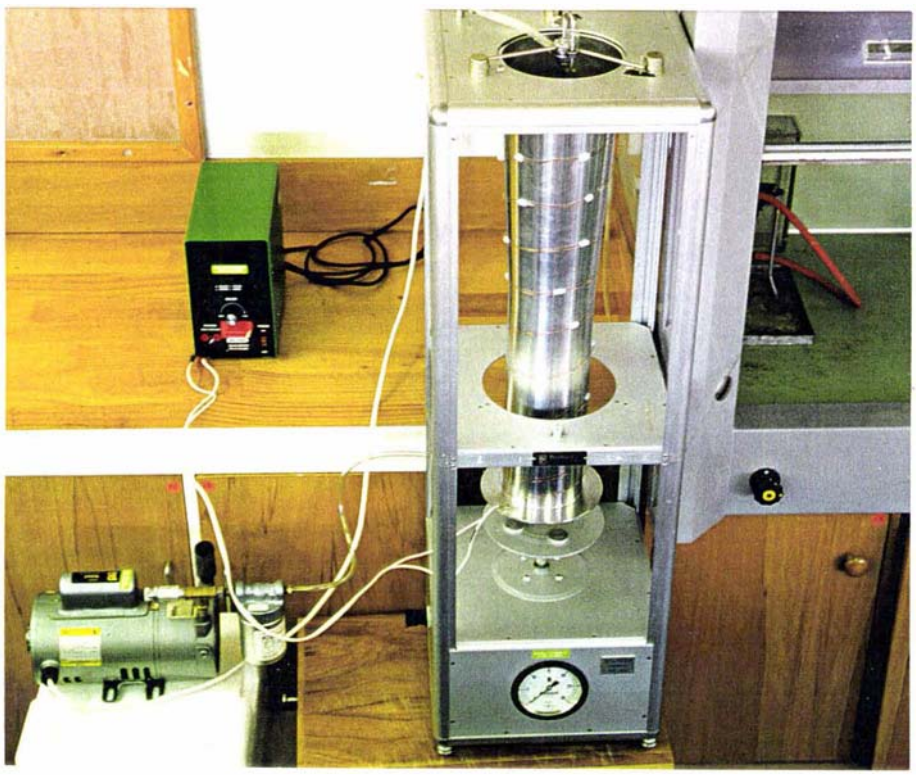

Fig. A2.1. The complete assembly of the Potter Tower and compressor used in the laboratory.

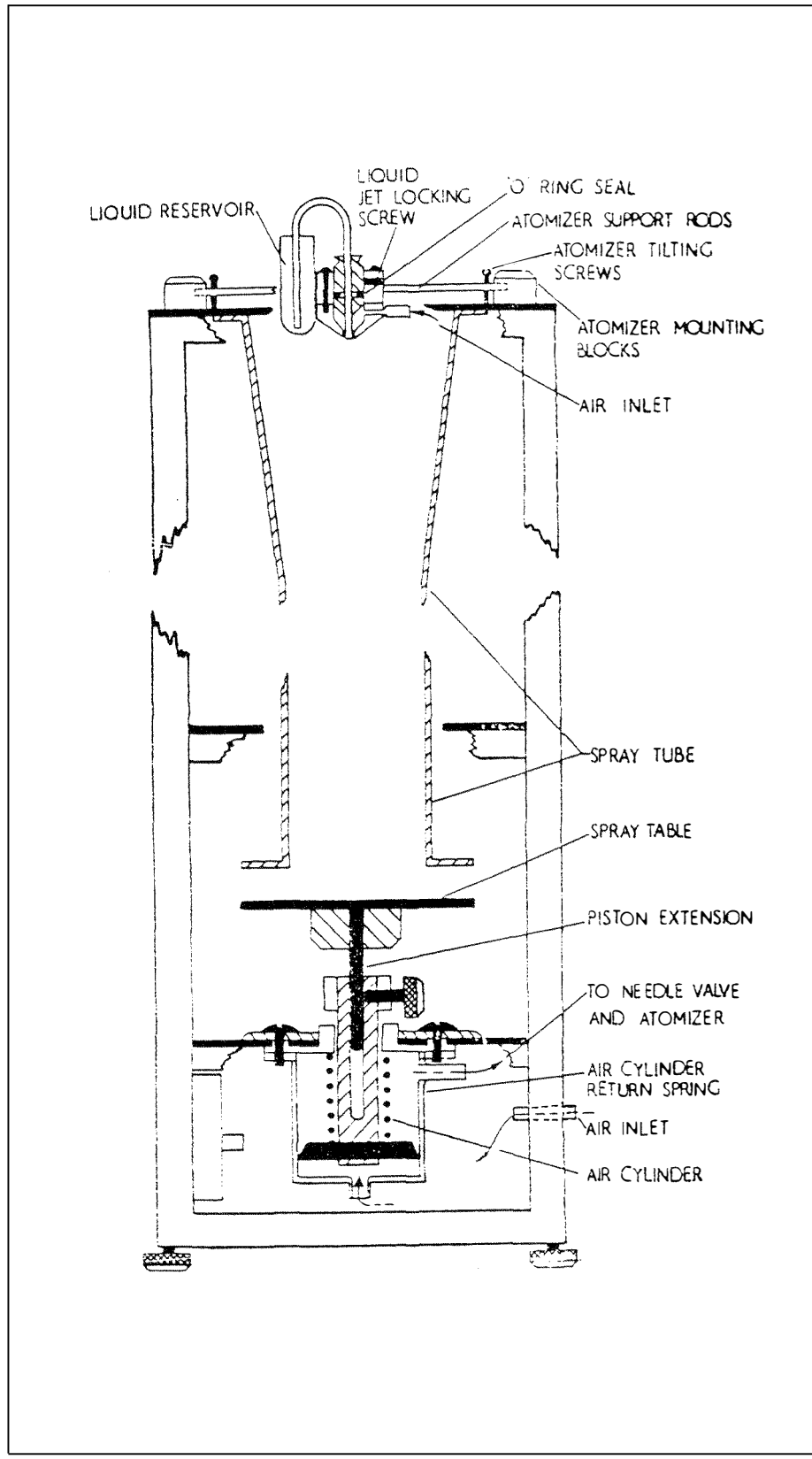

Fig. A2.2. Vertical section view of the Potter Tower.

Optimisation of the Potter Tower

The spray pattern created on the spray table in the Potter Tower is controlled by two elements, nozzle pressure and nozzle tilt (Refer to Fig. A2.2) controlling droplet size and spray pattern respectively. Optimisation of these elements was carried out according to the instruction manual (Burkard Manufacturing Co. Ltd) that accompanied the equipment.

Objective

To optimise deposition pattern of SDW in the Potter Tower.

Materials and Methods.

Nine pre-weighed glass coverslips (22 mm x 22mm, size 2) were positioned in a 3 x 3 grid on the spray table (Refer to Fig. A2.2). Two millilitres of SDW was pipetted into the liquid reservoir and atomised into the tower at a nozzle pressure of 100 mm Hg (2 PSI). Net coverslip weight was used to measure volume of water deposited and where gradients were evident among these coverslips, the nozzle tilt was adjusted using the mounting screws (Refer to Fig. A2.2). Iterations of this procedure were carried out until an even coverage was obtained.

Four replicates of same volume of SDW were applied at nozzle pressures 100 mm Hg, 225 mm Hg and 450 mm Hg to the coverslips and deposition patterns measured as described above. As there was greater evaporative loss from the coverslips treated with SDW delivered at 450 mm Hg, an evaporative loss curve was created. This curve was obtained by re-weighing three replicate coverslips sprayed with two ml SDW at 450 mm Hg at 30 sec intervals. Thereafter, gross weights of each of the nine coverslips was appropriately adjusted using this curve according to the time delay when weights were taken.

The consistency of spray flows using bacterial and yeast suspensions were tested by loading one ml of *Pseudomonas* sp. and *D. hansenii* suspensions (concentration undetermined) into the reservoir (Refer to Fig. A2.2). The spray cone and nozzle were closely watched for any obvious breaks in output.

The experiment and data analysis was carried out according to a completely randomised design (See Chapter Two for details).

Results and discussion

Optimisation of spray patterns using nozzle tilt adjustment required five iterations before obtaining consistent distributions. There was a highly significant decrease ($P<0.0001$) in SDW volume delivered to the spray table at each of the nozzle pressure settings (Fig. A2.3). As nozzle pressure was increased the spray cone became wider, delivering greater volumes of SDW to the interior surface of the spray tube (Fig. A2.2). Spray droplet size decreased with increasing nozzle pressure and showed higher evaporative losses.

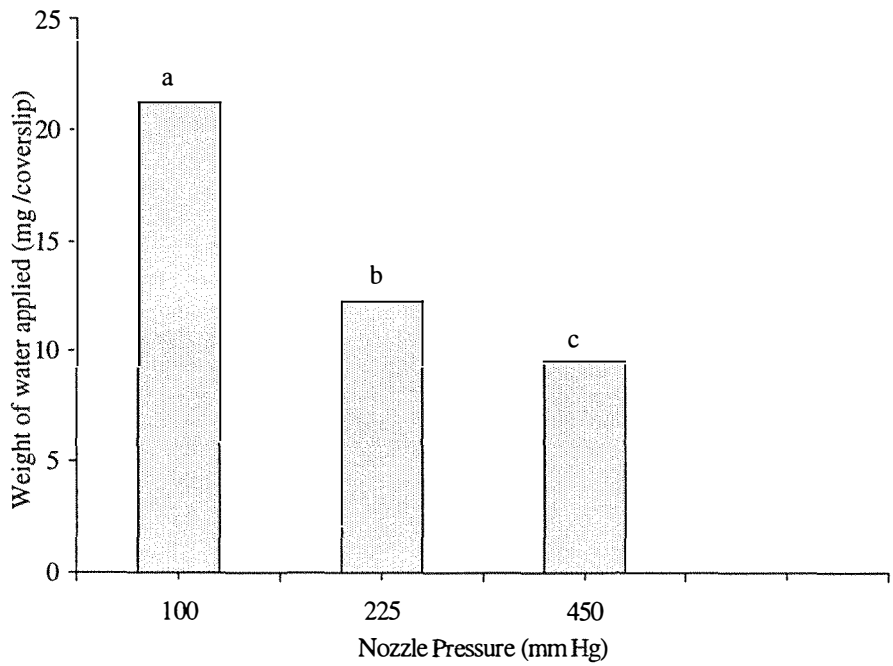

Fig. A2.3. The deposition of SDW onto nine coverslips (22mm x 22mm) arranged into a 3x3 grid on the spray table in the Potter Tower using three nozzle pressure settings. Significant differences are indicated by different letters using Tukeys multiple comparison test ($\alpha=0.05$, df =24,n=3).

Repeated applications of yeast *D. hansenii* and the bacterium *Pseudomonas* sp. suspensions at 100 mm Hg did not cause any breaks or interruptions in the consistency of spray output.

The relationship between nozzle pressure and volume of SDW deposited on the spray table was predictable given the wider nozzle angle at higher pressures directing more liquid onto the interior walls of the spray tube. However efficiency of SDW use was low with only 9% of the SDW loaded into the reservoir landing on the area covered by the nine coverslips at 100 mm Hg. The 100 mm Hg was selected as the nozzle pressure was high enough to atomise the more viscous suspensions of yeast or bacteria cells and maximise the amount of liquid reaching the spray table.

Electrostatic factors influencing yeast and bacterial cell deposition in the Potter Tower

Materials and Methods

Isolates of *S. cerevisae* and *Pseudomonas* sp. were prepared as described in Experiment Three and suspension concentrations were adjusted to 2.5×10^6 , 2.5×10^7 , $2,5 \times 10^8$, 2.5×10^9 and 2.5×10^{10} cells/ml.

The Potter Tower (Appendix Two) was modified by coiling bare copper wire (0.5 mm diameter) about the external surface of the spray tube ensuring a gap of 0.5 cm was maintained between tower surface and wire with a complete revolution every 7 cm. The spray tube itself and the spray nozzle were insulated from the remaining structures of the Potter Tower by rubber matting at the junction of the spray tube and potter tower frame or under the screw mounts of the nozzle assembly respectively.

An electrical potential was applied the wire coil and spray tube using a Bio-Rad 250/2.5 electrophoresis power supply. Voltages (5, 20 and 40 V) were applied to the apparatus or wire before yeast cells were sprayed onto a 90 mm diameter NYDA surface. A positive charge was applied to the wire followed by the application of yeast cells. The entire apparatus was then earthed, a negative charge of the same voltage was

applied to the wire and the spray application repeated. The density of resulting yeast colonies was examined after incubating the NYDA plates at 15°C for 48 h.

The experiment was repeated using three standard Potter Tower treatments, earthed and applying a 5V potential with either polarity. One millilitre aliquots of cell suspensions of *S. cerevisae* and *Pseudomonas* sp. were applied using the Tower under these electrostatic conditions at 100 mm Hg nozzle pressure to three, 10 ml glass bottles filled with 10 ml SDW which were positioned at marked sites on the spray table. Three replicates of each treatment were carried out in a randomised block design. The inoculated bottles were removed, capped then vortexed for 15 sec before a 100 µl aliquot and a ten-fold dilution of this aliquot were plated onto NYDA or NA where appropriate. Plates were incubated at 15°C, total darkness for 48 h before counting colonies.

The experiment and data analysis were carried out according to a completely randomised block design (See Chapter Two, Section 2.6). Secondary data analysis consisted of calculation 95% confidence intervals for the slopes of each linear regression equation and a comparison of the same equations for slope and intercept for both the bacterial and yeast treatment.

Results amd discussion

Preliminary electrostatic experiments indicated that potentials -5, -20 or -40 V notably increased cfu deposition (Fig. A2.4), although the effects of this treatment on deposited cfu density were not significant ($P>0.4$) in the main experiment. Figs A2.5 and A2.6 show a weighted linear regression relationship between the $\log_{10}$ of cfu density and increasing $\log_{10}$ cell concentration. Comparison of the regression equations for slope and intercept showed that when an electrostatic charge was applied, regardless of polarity, cfu density did not significantly differ ($P>0.4$) from the earthed control for both the test bacteria and yeast respectively.

Fig. A2.4. Yeast cell density (*S. cerevisae*) applied to NYDA using the Potter Tower electrostatically charged. '+x V'= Positive terminal to copper wire and '-x V'= negative terminal to copper wire.

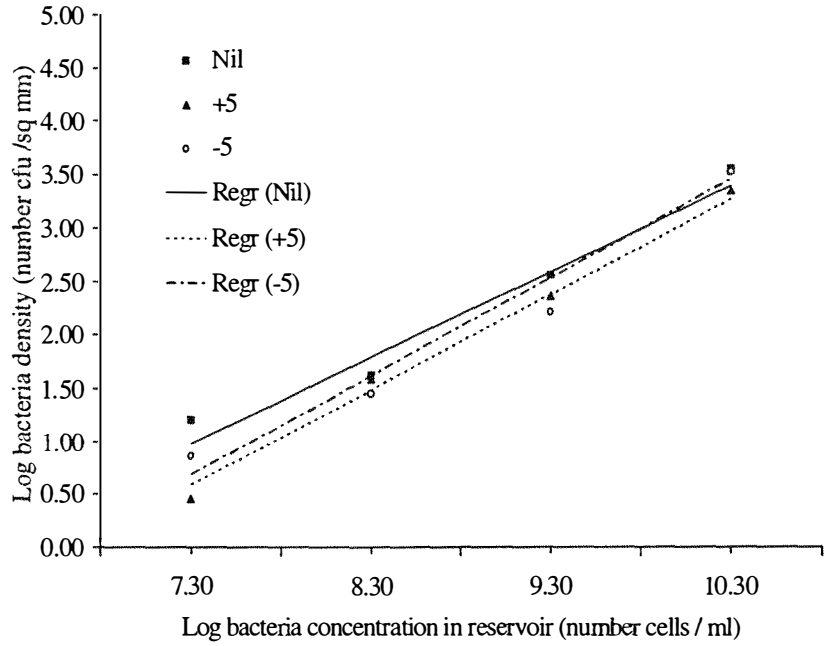

Fig. A2.5. Log_{10} of bacterial (*Pseudomonas* sp.) cfu density applied to spray table using the Potter Tower, earthed (Nil) or charged with + 5V (positive output connected to the copper wire) or - 5V (positive output connected to the insulated spray tube). Parameter values: Nil, y=0.80x - 4.85 (r^2=0.93; P<0.0001): +5V, y=0.92x - 6.02 (r^2=0.91; P<0.0001): -5V, y=0.89x - 5.9(r^2=0.93; P<0.0001).

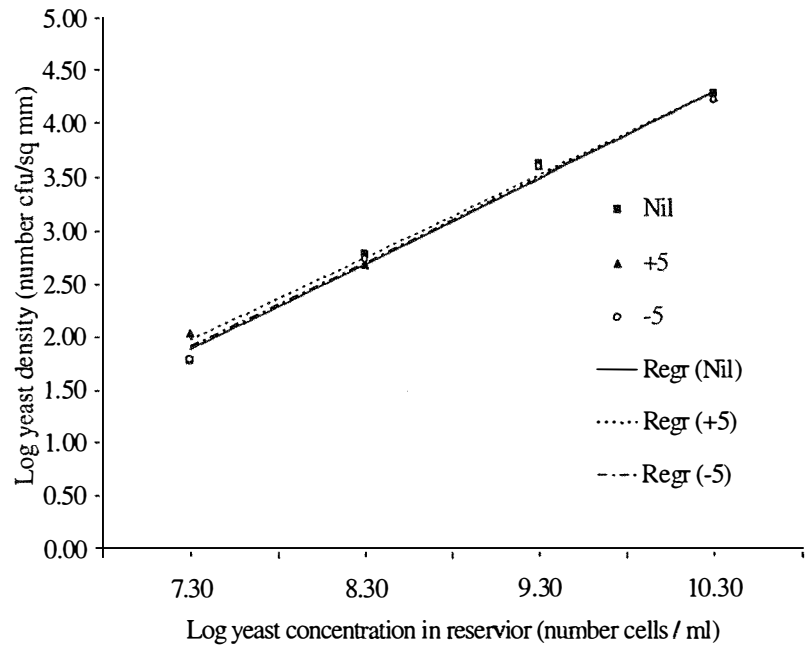

Fig. A2.6. Log_{10} of yeast (*S. cerevisae*) cfu density applied to the spray table using the Potter Tower, earthed (Nil) or charged with + 5V (positive output connected to the copper wire) or - 5V (positive output connected to the insulated spray tube). Parameter values: Nil, y=0.80x - 3.95 (r^2=0.97; P<0.0001): +5V, y=0.79x - 3.85 (r^2=0.98; P<0.0001): -5V, y=0.77x - 3.64 (r^2=0.90; P<0.0001).

The inconsistency of the electrostatic effect between the preliminary and main experiments is unclear. One possibility is that residual liquid from cleaning was present during the preliminary study, breaching the insulation of the spray tube and consequently charging the spray table. Another is that insufficient contact was made in the wiring of the tower such that no charge was applied. However the tower was re-wired many times during the experiment and inconsistent contacts would be reflected in variable density data assuming electrostatic charge was influential. Potter (1952) applied an electrostatic charge to the entire Tower in an attempt to alter the deposition patterns of liquid sprays but concluded that this was unimportant in the deposition process. A slightly different approach was adopted in this study but with similar conclusions.

Appendix Three - Optimisation and use of an ELISA protocol based on an anti-mouse conjugate secondary antibody to detect BC-KH4 bound to *B. cinerea* antigen.

Introduction.

A number of indirect-ELISA formats have been developed (see Dewey & Thornton, 1996; Dewey, Thornton, Gilligan, 1997 for reviews). For experiments in this preliminary research, an indirect plate-trapped-antigen-ELISA (PTA-ELISA) was selected where the primary antibody (BC-KH4) binding to the *B. cinerea* antigen was itself detected by an anti-mouse antibody conjugated to a peroxidase enzyme (Sigma A0412). BC-KH4 is a pentameric immunoglobulin of the subclass IgM (Bossi & Dewey, 1992) with a potential maximum of ten binding sites (Mernaugh, Mernaugh & Kovacs, 1990) assuming there are no intermolecular interferences.

Five experiments to optimise assay parameters were carried out. The first experiment was designed to investigate whether *B. cinerea* conidia were detectable when mixed with tomato tissue extract using an indirect-PTA-ELISA. In Experiments A2 and A3, two buffers were examined for their effect on extraction *Botrytis* antigen *in vitro* and *in vivo* respectively and binding to micro-titre wells. The potential for cross-reactivity by the ELISA detection procedure of the various yeast and bacterial BCAs was examined in the fourth experiment while in the fifth, the ELISA procedure was used to measure *B. cinerea* antigen production following co-inoculation with bacterial and yeast BCAs on excised tomato stem pieces.

Experiment A1

Materials and Methods

To 25 µl of homogenised tomato tissue extract (Chapter 2.5.4) or sterile distilled water (SDW) a further 25 µl of a *B cinerea* (isolate Pezet) spore suspension was added. Concentrations were adjusted such that 0, 156, 312, 625, 1250, 2500, 5000 or 10,000 spores were added to each microtitre well. The plates were incubated at 15°C for 16 h.

Two different ELISA controls were used in all immunological experiments: the first, omitting BC-KH4, and the second, replace BC-KH4 with RPMI+5%FBS in the ELISA procedure. All treatments and controls were replicated four times and the ELISA protocol for the detection of fungal antigen is detailed in Chapter 2.5.5.

Results and Discussion

B. cinerea spores germinated in the tissue extract (>70%) and germ tube growth ranged from 40-50 µm long compared with a very low germination (<10%) in SDW and germ tubes 5-10 µm long. Routine checks during the ELISA procedure showed that germlings remained in place on the well bottom and did not physically interfere with absorbance readings; absorbance values for wells without conidia was 0.292±0.001 and for wells in which 10,000 conidia per well had been allowed to germinate but were not incubated with the MAb was 0.293±0.001.

Antigen levels detected by ELISA increased steadily with increasing spore concentration and were similar in both SDW and tissue extract despite the poorer germination and growth in the former (Fig. A3.1). This suggests that material in the tomato tissue extract reduced the ability of ELISA to detect *B. cinerea* antigen. Possible mechanisms include, direct interaction with the antigen or competition for binding sites on the microtitre well wall and could possibly be overcome with the use of other extraction buffers.

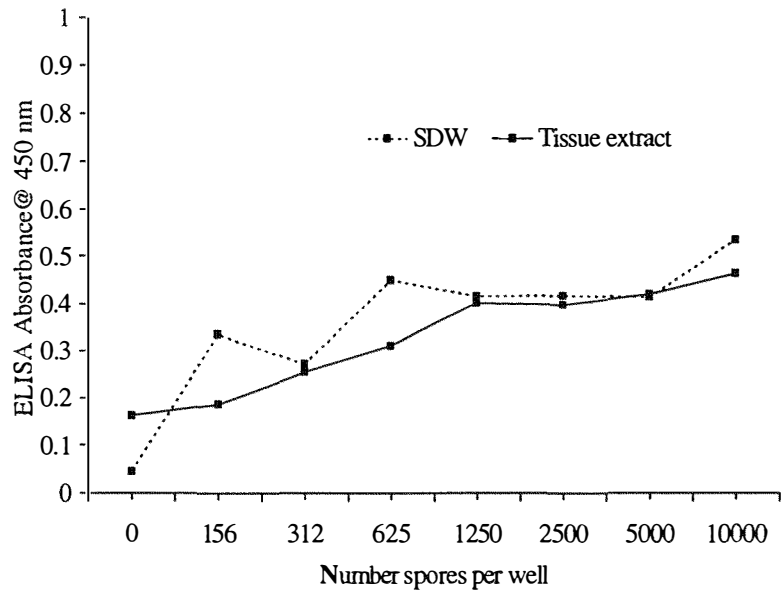

Fig. A3.1. *B. cinerea* antigen detection by indirect PTA-ELISA using BC-KH4 Mab of *B. cinerea* conidia germinated in microtitre wells containing tomato tissue extract or Sterile Distilled Water (SDW) at 15°C for 16h.

Experiment A2

Materials and Methods

Some difficulty was experienced in establishing infection in the tomato stems using *B. cinerea* (isolate Pezet) so 5 mm diameter disks of double strength PDA were each inoculated with 10 µl containing 20,000 spores of *B. cinerea.* The disks were incubated for 10 h or 24 h and then examined microscopically for pathogen growth. A 2 mm disk from the inoculated end of the agar piece was cut off and incubated in the wells containing 100 µl of coating buffer (SDW, PBS or bicarbonate) for 16 h at 4°C . *B. cinerea* antigen was detected using the ELISA procedure detailed in Chapter 2.5.5.

Results and Discussion

More than 90% of the spores applied to PDA disks had germinated after 10 h at 15°C. The ELISA procedure detected a two-fold higher level of *B. cinerea* antigen from

both 10 h and 24 h samples when subsamples of the agar disks were soaked in bicarbonate buffer in the microtitre wells compared with SDW or PBS extractants (Table A3.1).

This result *in vitro* suggested that bicarbonate buffer was the better of the three extractants tested to date.

Table A3.1. Indirect PTA-ELISA detection of *B. cinerea* antigen from inoculated PDA disks incubated at 15°C for 10 h or 24 h. Agar subsamples were soaked in 100 µl of ; Sterile distilled water (SDW), Phosphate buffered saline (PBS) and bicarbonate buffer, pH 9.6 (Bicarb) to extract the antigen. (MOE = Margin of error n=6).

Incubation	Extractant	Mean Absorbance	MOE
10 h	SDW	0.08	0.03
	PBS	0.06	0.03
	Bicarb	0.12	0.03
24 h	SDW	0.13	0.03
	PBS	0.18	0.07
	Bicarb	0.37	0.09

Experiment A3

Materials and Methods

Tomato tissues were grown and prepared as described in Chapter 2.3.1 & 2.3.2 and inoculated with 5 µl *B. cinerea* spore suspensions ($5x10^5$ spores/ml) of isolates, Pezet and IPO 700. The stems were then incubated for 24 h at 15°C.

Microtitre wells were loaded by soaking subsamples (1-2 mm disk cut from the inoculated end of each incubated stem piece) in 100 µl of either PBS or bicarbonate buffer for 16 h at 4°C. Fresh tissue controls were cut from healthy tomato plants and loaded into wells. Six replicates of each treatment were examined and the ELISA procedure for the detection of fungal antigens is described in Chapter 2.5.5.

Results and Discussion

Antigen detection by ELISA from intact, inoculated host stem subsamples did not vary with the three buffers tested (Table A3.2) and the higher ELISA signal when bicarbonate buffer was used to extract *B. cinerea* antigen *in vitro* (Experiment A2) was not repeated in this experiment. However there was a two-three-fold difference between uninoculated and inoculated tissue treatments using both buffers regardless of *B. cinerea* isolate.

Table A3.2 Indirect PTA-ELISA detection of *B. cinerea* isolate Pezet or IPO 700 applied to tomato stem pieces and incubated for 24 h at 15°C followed by antigen exteraction in either PBS or bicarbonate buffer at pH 9.6 (Bicarb). (MOE=Margin of error, n=6).

Extractant / *B. cinerea* isolate		Mean absorbance	MOE
Inoculated tissue (IPO)	PBS	0.71	0.08
	Bicarb	0.74	0.16
Inoculated tissue (Pezet)	PBS	0.68	0.15
	Bicarb	0.79	0.03
Fresh uninoculated tissue	PBS	0.26	0.01
	Bicarb	0.26	0.02

The absence of any difference in antigen extraction qualities with buffer is contrary to that observed in Experiment A2 and could be a host effect. At this stage of ELISA assay development, the reason for this contradiction was unknown. However inoculation treatment differences were apparent in this experiment after 24 h incubation. By using either of these buffers; bicarbonate or PBS and soaking an intact subsample of host tissue much of the cross-reactant material released into solution in Experiment A1 remained within the host subsample and thus enabled host treatment differences to be detected. This procedure was considered sufficient to attempt the full experiment; determination of relative *B. cinerea* biomass in the presence of BCAs.

Experiment A4

Materials and Methods

Fifty microlitres of a *B. cinerea* spore suspension (5 x 10^5 spores/ml in 0.02% Azide in SDW) was added to three microtitre wells. Cell suspensions in SDW of bacterial isolates ox1, ox2, ox3, ox4, ox5, ox6, ox7, ox8a, ox9, ox12 and ox22 and yeasts 561 and 532 were prepared as described in Chapter Two. Ten microlitres of each suspension was pipetted into three replicate microtitre wells and diluted to 100 µl with SDW + 0.02% Azide. Wells were incubated at 4°C for 16 h. The ELISA procedure described in Chapter 2.5.5 was used for the detection of *B. cinerea* or similar antigens.

Results and Discussion

In this experiment each of the BCAs to be used in the full experiment were examined for any cross-reactivity. BC-KH4 did not cross-react by ELISA with most of the bacterial and yeast BCAs but did cross-react with bacterial isolates ox3 and ox22. Absorbances from these isolates were 2.4 and 3.5-times higher than with *B. cinerea* (Table A3.3).

It appears that these two BCA isolates have an epitope similar to that recognised on the *B. cinerea* glycoprotein. No other bacteria have been found by other workers to cross-react with BC-KH4 but other species of *Botrytis* and a few unrelated fungi have been found to cross-react (F.M. Dewey, personal communication). Only those isolates with absorbance readings below that for *B. cinerea* were considered for future experiments.

Table A3.3. Indirect PTA-ELISA detection of *B. cinerea* conidia and bacterial or yeast BCA cells in SDW (n=3).

Isolate / Treatment	Mean absorbance	MOE
B. cinerea conidia alone	0.18	0.02
ox1	0.09	0.03
ox2	0.14	0.04
ox3	0.64	0.003
ox4	0.07	0.03
ox5	0.07	0.01
ox6	0.08	0.02
ox7	0.07	0.02
ox8a	0.09	0.02
ox9	0.08	0.01
ox12	0.09	0.02
ox22	0.44	0.02
561	0.09	0.02
532	0.10	0.03

Experiment A5

Materials and Methods

Excised tomato stem pieces were prepared as detailed in Chapter Two and inoculated with 5 µl of *B. cinerea* spore suspension ($5x10^5$ spores/ml of isolates Pezet and IPO 700). After a 2 h incubation at Room Temperature (R.T.), 5 µl of BCA yeast suspensions of isolates 561 and 532 or bacterial BCA suspensions, isolates ox4, ox5, ox6, ox7 and ox9 ($2x10^8$ cfu/ml) were applied to stem pieces. Uninoculated stem pieces were used as the

negative and pathogen-only application as the positive control. All stems were incubated at 15°C in a sealed plastic container lined with saturated paper towels to provide a humid atmosphere.

A total of 30 stem pieces were prepared for each inoculation treatment according to the format for a completely randomised block design. From this pool, three stem pieces were taken at random and subsampled by removing a 1-2 mm slice from the treated end of each stem piece. This slice was placed into a microtitre well containing 100 µl of PBS + 0.02% Azide. Microtitre plates containing the samples were incubated for 16 h at 4°C then washed in PBST (four-times), PBS (once) and water (once) before storing dry at 4°C. The sampling procedure was repeated ten times at 8 h intervals. The ELISA procedure for the detection of *B. cinerea* antigen is described in Chapter 2.5.5. The experiment and data analysis was carried out using the completely randomised block experimental design. Secondary analyses included contrasts between uninoculated and BCA co-inoculated treatments and between pathogen-only and BCA co-inoculated treatments.

Results and Discussion

ELISA absorbances for all inoculated, co-inoculated and uninoculated tissue treatments were highly variable with values between 0.15 and 0.6. There was a general upward trend in absorbances as incubation times increased (Fig. A3.2) but individual treatment effects could not be distinguished despite visual observations confirming pathogenicity of the *B. cinerea* and biocontrol in the appropriate treatments. Pairwise comparisons between the uninoculated control and the various BCA/pathogen coinoculated treatments were not significant ($P>0.05$) with the exception of isolate 532 ($P=0.0001$). Similarly the comparison between pathogen-only control and the various coinoculated treatments were significant only for isolates 532 ($P=0.0001$), ox7 ($P=0.02$) and ox9 ($P=0.02$).

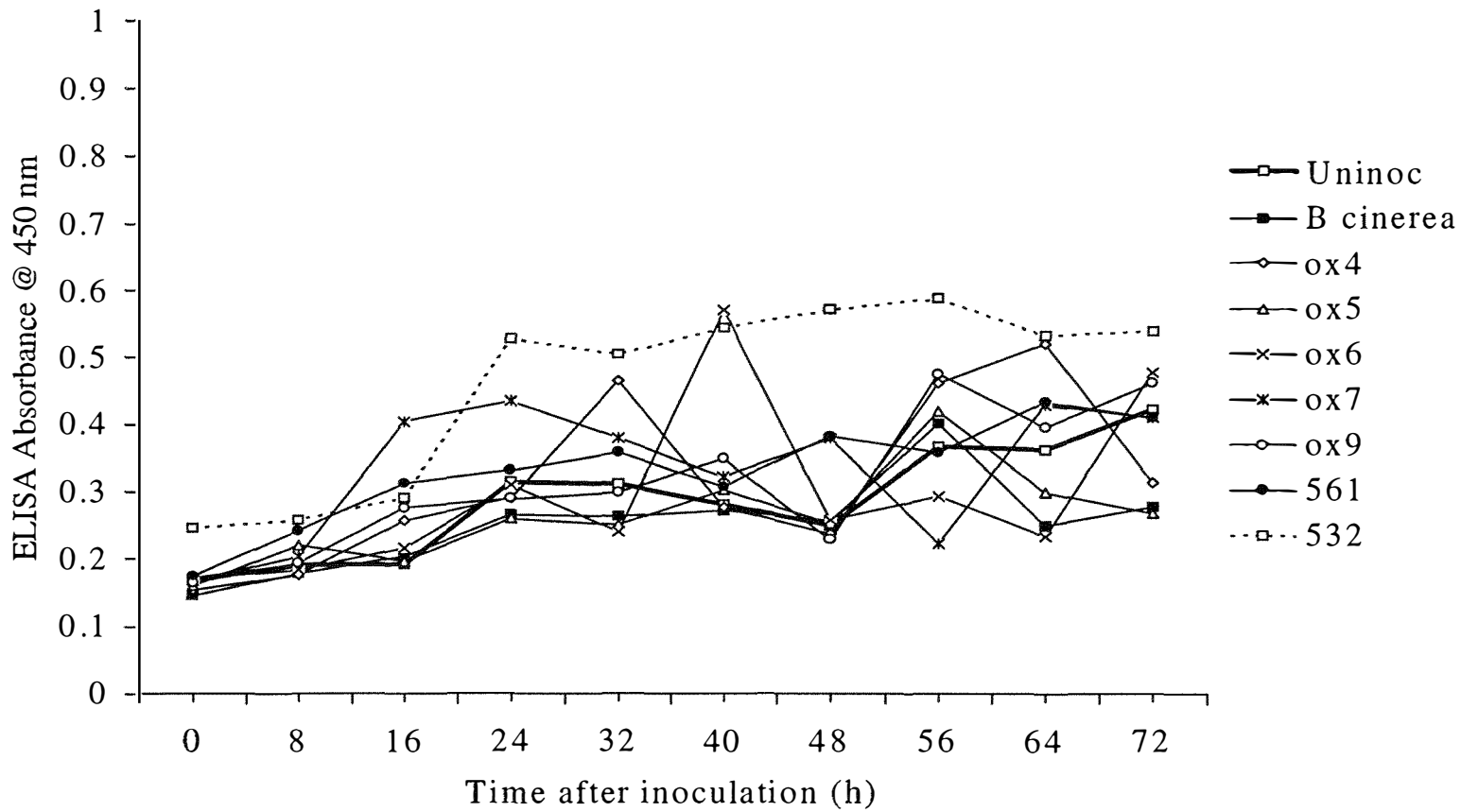

Fig.A3.2. Indirect PTA-ELISA detection of *B. cinerea* antigen as a measure of pathogen biomass in the presence of yeast and bacteria BCA's on excised tomato stem pieces. BC-KH4 was used as the primary antibody and inoculated tissues were extracted in PBS + 0.02% Az.

It was clear that the current tissue preparation, well loading or ELISA protocols were insensitive and inadequate to distinguish treatment effects. Non-specific binding to material from uninoculated symptomless tomato tissue was as great as that from the pathogen-only treatments. Results from Experiment A1 showed that material from the tomato tissue interfered with ELISA detection of *B. cinerea* antigen and was sufficient to render the protocol ineffective.

A number of hypotheses were proposed as a result:

(1) Material from the plant host tissue was competing with the pathogen antigen for binding sites on the well wall

(2) A substance from tomato tissue was directly binding to the BC-KH4 MAb. Recently, Cole *et al.*, (1997) carried out immunolabelling studies at the light and electron microscopy level and showed that BC-KH4 bound to the extracellular matrix of the fungus and the adhesion pad. The antigen may have a role in adhesion and assuming the docking model for adhesion which is mediated by complimentary molecules on both surfaces (Douglas 1987), then the antigen compliment may have been released from host tissue during extraction and these molecules could directly bind with the antigen *in vitro*.

Three lines of investigation were initiated in an attempt to overcome these problems. These were :

(1) To investigate alternative indirect PTA-ELISA protocols for detecting the antigen.

(2) To seek alternative extractant buffers that could selectively enhance the detection of the *B. cinerea* antigen.

(3) To enhance the release of *B. cinerea* antigen from infected plant tissue.