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**Biological Control and Biomass Evaluation**  
**of**  
*Botrytis cinerea*

A thesis presented in partial fulfilment of  
the requirements for the degree of  
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## Abstract

The efficacy of biocontrol agents is often judged by symptom development on inoculated plants. This process can involve long delays, as with *Botrytis* infection of kiwifruit and an alternative, quicker approach would be useful. When biocontrol is successful, then pathogen biomass is limited hence a means of measuring the biomass of a pathogen on/in a target substrate (plant material) could be used as a tool for rapid estimation of biocontrol efficiency.

Two yeast (*Enterobacter agglomerans*, *Enterobacter aerogenes*) and two bacteria (*Candida sake*, *Trichosporon pullulans*) with an already identified ability to attach to the surface of *Botrytis cinerea* and to reduce infection in tomato and kiwifruit, were evaluated for control of *B. cinerea* in bean, lettuce and rose in this study. Potential biological control and efficacy was assessed by measuring lesion size and percentage infection by *B. cinerea*.

An investigation of methods of conidial application of *B. cinerea* to these crops tissue showed that disease severity and incidence were increased by a high concentration of wet spore application to bean and dry spore application to lettuce and rose tissue. Each application technique was used as the standard technique for biocontrol experiments on the crop on which it was most efficient.

Three of the potential BCAs (*Enterobacter agglomerans*, *Enterobacter aerogenes*, *Trichosporon pullulans*) were found to reduce lesion size and percentage infection on all three crops at 20<sup>0</sup>C.

Biological control by bacterial BCAs, *Enterobacter agglomerans* and *Enterobacter aerogenes*, were demonstrated by applying them to bean tissue at the

time of inoculation with a suspension of  $1 \times 10^8$  conidia per ml of *B. cinerea*. These two bacteria and the yeast, *Trichosporon pullulans*, showed biological control when applied to lettuce and rose tissue one or two days after inoculation with dry spores of *B. cinerea*.

A potential rapid assessment of biocontrol efficiency of microorganisms has been demonstrated using Laser Scanning Confocal Microscope. A clear image of the fungal hyphae in the host tissue was produced in confocal microscopy by using glutaraldehyde as a fluorescent stain for *B. cinerea* hyphae. Biomass of *B. cinerea* at an early stage of infection in bean and lettuce tissues was successfully measured by computer analysis before and after application of yeast and bacterial biocontrol agents. BCAs application in both tissues prevented development of a large biomass of *B. cinerea*.

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## Chapter One

### General Introduction

#### 1. 1 *Botrytis cinerea*

*Botrytis cinerea* Pers: Fr. is responsible for serious pre- and post-harvest losses on numerous fruits, vegetables, flowers and ornamental plants. It is capable of attacking crops at almost any stage in their growth and in storage, and affecting all plant parts, including the cotyledons, leaves, stems, flowers, fruits and roots (Maude 1980). Although normally regarded as a weak pathogen, it is non-specific in its host range and responsible for considerable economic losses on a global scale (Heale 1992), particularly in temperate areas of the world (Coley-Smith 1980).

##### 1. 1. 1 Economic losses due to *B. cinerea* infection

Postharvest losses due to *B. cinerea* infection have been estimated as approximately 24% of harvested fruits and vegetables in the United States of America (US Dept. of Agriculture 1965) and 50% in developing tropical countries (Coursey and Booth 1972). Jarvis (1980) reported that more than 50% of raspberry and strawberry fruit losses occurred due to *B. cinerea* infection in Louisiana State in USA. *B. cinerea* storage rot is the major cause of post harvest losses to the kiwifruit industry in New Zealand (Hopkirk and Clark 1990; Pak and Manning 1994). Chocolate spot of broad beans caused by *Botrytis cinerea* and *B. fabae* (Weeler 1969) and grey mold of bush snap bean caused by *Botrytis cinerea* (Campbell 1949) are considered the most economically important problems of bean production in the United State. In apple, blossom-end rot caused by *Botrytis cinerea* can lead to considerable economical losses (Lakshminarayan et al. 1987). *Botrytis* blight of rose caused by *Botrytis cinerea* is one of the most serious diseases affecting the production and postharvest quality of cut roses (Redmond et al. 1987). Grey mold caused by *Botrytis cinerea* is a serious disease of lettuce

(Weeler 1969), grapes (Mustonen 1992) and several species of berries such as raspberries (Knight 1980), and strawberries (Jarvis 1962).

### **1. 1. 2 Taxonomy and morphology**

*Botrytis cinerea* is placed in the Division Eumycota, Subdivision Deuteromycotina, class Hyphomycetes, Order Hyphales, Form-order Moniliales, Family Moniliaceae. *Botryotinia fuckeliana* (de Bary) Whetzel is the perfect stage of *Botrytis cinerea* (Agrios 1988). This sexual stage (an apothecium) is seldom found in nature (Polach and Abawi 1975). *Botrytis cinerea* produces abundant grey mycelium and long branched conidiophores with rounded apical cells bearing clusters of colourless or grey, one-celled, ovoid conidia which contain 4-18 nuclei (Jarvis 1980; Agros 1988). The sclerotium is an overwintering structure under natural conditions. It produces hyphae, conidia, or (rarely) an apothecium the following spring and summer (Coley-Smith 1980).

### **1.1.3 General characteristics of *B. cinerea***

*Botrytis cinerea* is a weak, necrotrophic and facultative pathogen. It has a limited ability to infect healthy plant tissue but, an injury to plant tissue could facilitate entry and spread of the pathogen through the affected tissues. This is likely to increase leakage of organic substances on to the surface of the plant due to damage of the cell membrane. The presence of such substances would lead to enhanced spore germination and growth of germ tubes resulting in increased penetration and entry into internal tissues.

Generally, *Botrytis cinerea* lives saprophytically upon the dead leaves and flowers of many plants. Where the substrate is fleshier, such as flower heads or fruits, *Botrytis cinerea* may become the predominant saprophyte in the early stage of the decay of the tissue (Blakeman 1980). It is well fitted for this role since it is

produces large amounts of pectolytic enzymes and is also cellulolytic (Hudson 1968). Many harvested products enter storage with incipient or latent *Botrytis cinerea* infections or contaminated with conidia or saprophytic mycelia (Dennis and Mountford 1975; Goodliffe and Heale 1975). These infections often cannot be detected at harvest, but develop rapidly under the moist conditions encountered during storage and transit (Jarvis 1980).

#### **1.1.4 Specific behaviour of *Botrytis cinerea* on plant surfaces**

##### ***1.1.4.1. Survival of conidia***

*Botrytis cinerea* produces an abundance of conidia, which spread a great distance by wind. These conidia are usually considered to be short-lived propagative spores but Coley-Smith (1980) described evidence that under certain conditions their life can be prolonged especially in winter. Survival of conidia of *B. cinerea* is mostly influenced by temperature and moisture conditions in the atmosphere and survival can be for very much longer when it is on aerial parts of plants (Coley-Smith 1980). Under dry conditions, conidia remain viable for a long time. Brodie and Blakeman (1975) observed that a rapid loss of viability of conidia of *Botrytis* species occurred when they were wetted and dried frequently. Further, they postulated that carbohydrate exhaustion could be the reason for the loss of viability of conidia due to a combination of increased respiration and loss of solutes through leakage.

##### ***1.1.4.2 Germination of conidia***

###### **1.1.4.2.1 Spore concentration**

Spore germination of *Botrytis*, in common with those of other fungi is reduced when the spore concentration is high because of the action of self-inhibitors of germination, but this inhibition can be overcome by added nutrients (Blakeman 1980). Last (1960) noted a decrease of germination associated with an increased

concentration of spores of *B. fabae* and he found that aged conidia were more sensitive to this self-inhibition. Blakeman (1980) considered that the great self-inhibition of old conidia could be associated with a loss of endogenous reserves because nutrients improve the germination percentage of aged conidia.

#### 1.1.4.2.2 Relative humidity and free water

Relative humidity and the presence of free water on plant surfaces are the most important physical factors influencing infections by *B. cinerea* (Blakeman 1980). Contradictory reports have been published on the precise moisture requirements for *B. cinerea* spore germination. Snow (1949) and Nelson (1951) demonstrated that more than 94% humidity with absence of free water could result in infection by *B. cinerea* on grapes. Later, other workers reported that a film of water is required for the germination of *B. cinerea* conidia. Jarvis (1962) reported that dry spores of *B. cinerea* on the surface of strawberry fruits do not readily germinate in a saturated humidity but germinated rapidly in a water film. Hammer and Marois (1989) reported that reduced humidity (50-80%) and absence of free water on cut roses in cold storage significantly reduced *B. cinerea* infection.

#### 1.1.4.2.3 Temperature

Jarvis (1980) summarised from Hennebert and Gilles work that "at an optimum temperature of 20<sup>0</sup>C, germination of conidia on ripe strawberry fruit began within 90 min of inoculation and most of the conidia in the inoculum had germinated in 3-5 h. A second process, germ tube elongation, began somewhat later at about 18 h, and had an optimum temperature of 30<sup>0</sup>C. Only during this second phase were the germ tubes capable of penetration, at about 20 h". *B. cinerea* can germinate at 0<sup>0</sup>C with high moisture condition and cause infection. During storage of vegetables, fruits and flowers *B. cinerea* infection can be enhanced by high

relative humidity and condensed water. Sharrock and Hallet (1991, 1992) stated that stem-end rot of kiwifruit occurred at 0°C during storage.

#### 1.1.4.2.4 Other factors influencing spore germination

Presence of pollen on the plant surface stimulates *B. cinerea* germination and increases infection. Chou and Preece (1968) showed that strawberry pollen stimulated the germination and growth of germ tubes of *B. cinerea* and aggressive formation of lesions on petals and fruits of strawberry and broad bean leaves. Many workers showed that the stimulatory effect of pollen could be associated with increased availability of major nutrients such as carbohydrate, aminoacids, and plant hormones or growth factors such as abscisic acid and gibberelic acid, hormone and with biotin, thiamine, pyridoxine, panthothenic acid, nicotinic acid and inositol growth factors (Blakeman 1980).

Physical injury of plant tissue by mechanical or by climatic conditions such as frost or wind could facilitate spread of *B. cinerea* infection due to increase leakage of organic substances from damaged cells on to the surface. In addition, damage due to frost increases the availability of necrotic tissue in strawberry flowers which provide infection sites that enhance *B. cinerea* infection (Jarvis 1962). In contrast to this observation, Purkayastha and Deverall (1965b) noticed that the removal of the cuticle of bean leaves before inoculation failed to enable *B. cinerea* to cause spreading infections and they explained this as due to substances arising from the damaged leaf cells.

There are factors other than spore concentration that influence the development of *Botrytis* species in detached bean leaves. Purkayastha and Deverall (1965a) and Mansfield and Deverall (1974) reported that the age of *Vicia faba* leaves greatly affects both number of lesions and lesion size with senescing leaves being susceptible to both *B. cinerea* and *B. fabae*.

Some antifungal compounds, such as the phytoalexin wyerone acid (formed by broad bean leaves and pods) accumulate in tissues challenged by *B. cinerea* or *B. fabae* and limited lesion result (Hargreaves et al. 1977). This phytoalexin could be inactive in the presence of pollen (Mansfield and Deverall 1971).

## 1.2 Control of *Botrytis cinerea*

### 1.2.1 Non-Chemical control

Relative humidity and the presence of free water on plant surfaces are considered to be the most important abiotic factors influencing infections by *B. cinerea* (Blakeman, 1980). Disease reduction can be enhanced by control of these factors. Gubler et al (1987) showed that altering microclimate by canopy management (removal of leaves) controlled *Botrytis* bunch rot of grapes. Hammer and Marois, (1989) demonstrated that reduced humidity (50%-80%) and control of free water on rose petals during storage reduced disease incidence and severity in cut roses. Brook (1990) reported that heat treatment by dipping kiwifruit in hot water or by dry air treatments at 44-50<sup>0</sup>C reduced stem-end rots during storage. Hammer and Evensen (1996) observed that decreasing air movement significantly decreasing susceptibility to *B. cinerea* infection in cut roses.

### 1.2.2 Chemical treatment

Fungicide treatments used to be the primary method for controlling post harvest diseases caused by *Botrytis cinerea* (Eckert and Ogawa 1988). Fungicides such as dicarboximides (ipridion, procimidone, vinclozolin) have been used against *Botrytis* diseases in orchard spray programmes but their use all year around increases the prevalence of strains of *Botrytis cinerea* resistant to these fungicides (Denis and Devis 1979; Leroux et al. 1981; Katan 1982; Spotts and Cervantes 1986). Fungicide resistance can become widespread and develop within one season (Fraile et al. 1986; Northover and Matteroni 1986). The other important

problem with fungicide is the environmental hazard and consumer pressure to move away from pesticides as reflected in the increasingly stringent legislation for plant protection products. Due to consideration of these factors, a number of key fungicides are being withdrawn from the market. For example, in New Zealand, seven dithiocarbamate fungicides have already been withdrawn by various manufactures (NZ Apple and Pear Marketing Board, Circular 1990/1991 - cited by Cheah and Hunt 1994). Concern about the above factors has contributed to the realisation that an alternative to chemical control of plant diseases is needed.

### **1.2.3 Biological control of *Botrytis cinerea***

#### ***1.2.3.1 General view on Biological control***

Biological control offers an alternative to fungicide use for the control of plant diseases and it is possible to control plant pathogens biologically by manipulating the epiphytic microflora on plant surfaces (Droby and Chalutz 1991). Some naturally occurring antagonistic microorganisms have been tested by several workers and appear to be exceptionally effective as biological control agents.

A number of antagonists control different postharvest pathogens in various fruits, vegetables and ornamental flowers. A yeast antagonist, *Debaryomyces hansenii* (Wisniewski et al. 1988a) or a mixture of antagonists (Janisiewicz 1988, 1987) were used to control *Botrytis* rot on apple. The same yeast (Chalutz and Wilson 1989) and a bacteria, *Bacillus subtilis* (Singh and Deverall 1984) were used as biocontrol agents against postharvest disease of citrus fruit. Two postharvest cherry diseases were controlled by two antagonistic bacteria, *Bacillus subtilis* and *Enterobacter aerogenes* (Utkhede and Sholberg 1986). *Trichoderma* species were used to control *Botrytis cinerea* on grapevine (Dubos and Bulit 1983) and snap bean (Nelson and Powelson 1988). Chalutz et al. (1988a) used a yeast isolate as a biocontrol agent to control pepper and tomato fruit rot. Nectarine, pear

and apricot fruit brown rot was controlled by antagonistic bacteria (Pusey and Wison 1984); *Botrytis* blight of rose was controlled by antagonistic yeast (Redmond et al. 1987; Hammam and Marois 1989).

An effective biological control agent may inhibit a plant pathogen by producing antibiotics, by successfully competing with the pathogen for nutrients and space, by inducing host resistance, or by interacting directly with the pathogen (Droby and Chalutz 1991). Traditional methods (e.g. dual culture agar plate tests) for identification of potential biocontrol agents tend to select a biocontrol agent that produce antibiotics. Most antagonistic organisms have been selected on this basis in biological control research in many crops. More recently, especially for post harvest diseases, attention is moving to antagonists that have the ability to control pathogens with actions other than antibiotic production because, antibiotic use in the plants could well be subject to the same problems as those of other pesticides resulting in imposition of similar restrictions on future use.

There are several studies, which demonstrate that leaf rust can be controlled by a number of bacterial species. In some studies, the antagonists affecting the rust pathogen were also effective against necrotrophic pathogens, although the mechanism may differ (Kempf and Wolf 1989). Some studies found that antibiotic production was partly or completely responsible for the suppression of the rust. Field experiments by Baker et al. (1985) however, demonstrated that the bacteria as well as their culture filtrate had to be applied three times per week in order to control bean rust to the same extent as a weekly spray application of mancozeb. The researcher's explanation for this observation was that adverse climatic conditions caused a rapid decline in the bacterial population as well as degradation of the antibiotics. In this particular example the same degree of control was obtained with a fungicide as with a biocontrol agent but a fungicide

did not give such a high level of control of coffee leaf rust as a commercial preparation of *Bacillus thuringiensis* which induced host resistance, than with a fungicide (Roveratti et al. 1989).

Some isolates of bacteria and yeast have been identified as antagonists against *Botrytis cinerea* with a mode of action that is not antibiosis but attachment to the hyphae and have given very successful biocontrol (Cook 1997 and Cook et al. 1997a and 1997b). The detailed mode of antagonism needs further investigation. Attachment bacteria have also been observed by Korsten et al. (1995) on avocado post harvest pathogens.

#### ***1.2.3.2 Attachment biocontrol agents***

Microbial attachment of the pathogen to the plant host surfaces is an important process for the subsequent activity for pathogenesis (Beckett et al. 1990; Manocha and Chen 1990; Braun and Howard 1994). In the same way, microbial attachment to fungal plant pathogens could be the first step in suppression. Using light microscopy, researchers have noticed bacterial structures within urediospores (Pon et al. 1954) and within conidia of the baited *Cochliobolus sativas* (Old and Robertson 1970) and observed suppression of the fungi. In later studies with electron microscopy, Old and Roberston (1969, 1970), Old and Patrick (1976), Wong and Old (1974) and Clough and Patrick (1976) attempted to find the causal agents for the cell wall perforations and the bacterium-like structures partially or fully embedded in the wall of *Cochliobolus sativas* conidia and *Thielaviopsis basicola* chlamydospores, but they failed to isolate them into pure culture.

Attachment biocontrol agents differ from other biocontrol agents with a mainly antibiosis mode of action. *Debaromyces hansenii* and *Enterobacter cloacae* are examples of microbes that have recently been selected for testing on the basis of

their ability to attach to fungal hosts (Wisniewsky et al. 1991). Likewise, from an attachment assay Cook et al. (1997a) isolated some bacteria and yeast that attached to *B. cinerea* hyphae did not produce antibiotic, but reduced *B. cinerea* infection of kiwifruit and tomato by more than 80%.

Other studies indicated that a number of plant diseases, including several caused by *Botrytis cinerea*, have been reduced by pre-inoculation or post-inoculation of the phylloplane with epiphytic bacteria, fungi or yeast (Blakeman and Fokkema 1982; Sada 1992; Cook 1997). In one study, three morphologically different stains of yeast effectively reduced incidence and severity of grey mold of pears, and did not show any indication of the production of antibiotic to suppress the causal agent, *Botrytis cinerea* (Chand-Goyal and Spotts 1996). In another study, six of more than 120 yeast isolates reduced Botrytis storage rot incidence in kiwifruit and none of them produced antibiotic (Cheah and Hunt 1994).

### ***1.2.3.3 Utilisation of attachment biocontrol agents in disease control***

Can these attachment biocontrol agents be used to control diseases caused by the same pathogen in ornamental flowers and other vegetable crops? Could their effectiveness and mode of action be similar? Redmond et al (1987) identified four microorganisms, species of yeast and bacteria, with the ability to reduce the number of lesions caused by *Botrytis cinerea* on rose and they found the yeast was the most effective antagonist. Hammer and Marois (1989) reported that two biological control agents, a yeast and a bacterium controlled *B. cinerea* infection in rose during cold storage when applied 0 to 48 h after inoculation with the pathogen. They did not observe whether these antagonists operated through attachment to *B. cinerea* hyphae.

Effective and quick evaluation of antagonists is very important when considering the time, materials and labour involved in basic research. Therefore, estimation of biomass of *B. cinerea* in infected tissue could lead to a rapid assessment of biocontrol effectiveness in experimental work on crops such as kiwifruit where it can take three months to reach a final assessment. Using modern equipment such as Confocal Laser-Scanning Microscopy (CLSM) with new fluorescent stains a new approach can be made to measurement of the biomass of *B. cinerea*.

### **1.3 Confocal microscopy**

#### **1.3.1 Confocal microscope and computer image analysis**

Confocal microscopy is a new modification of fluorescence microscopy that can be used to improve contrast and resolution and to take optical sections. Kwon et al. (1993) compared confocal microscopy with conventional microscopy, and stated that conventional microscopic images of fungal hyphae are most often taken from a single median focal plane to maximise the information content in the image in spite of the out of focus area which detract from the focal plane. This results in images with less than optimal contrast and resolution. In confocal images, such areas are covered with optimal contrast and resolution and allow the viewer to clearly see the details in that plane.

Confocal microscopy not only yields rapid and accurate three-dimensional perspectives, but most images processing software also provides views from many perspectives. In addition to three-dimensional views, confocal microscopy provides an opportunity to quantitatively measure organelle distributions (Kwon et al. 1993).

Since confocal microscope images are acquired digitally, the stored data can be reconfigured in various ways. Any number of individual optical planes of focus

can be added to create a composite image of a desired thickness or planes of interest. Transverse optical cross sections are easily created, providing different information on the vertical distribution of cell components, and three-dimensional views can be constructed. The digitally stored images can be manipulated and quantitatively analysed using an image analysis computer package in appropriate computer system.

### 1.3.2 Staining for confocal microscopy

The confocal laser-scanning microscope has the potential to be a very useful tool for measuring the biomass of *B. cinerea* quantitatively in plant tissue. Several fluorescent dyes have been used for the studies of fungal-plant interaction by confocal microscopy. Cole et al. (1996) used the monoclonal antibody, BC-KH4 and a secondary antibody-FITC conjugate to immuno-fluoresce *Botrytis* sporelings on infected *Vicia fabae* leaves. Wei et al. (1997) obtained clear images of hyphae of *Colletotrichum gloeosporioides* f. sp. *Malvae* in infected mallow leaves from confocal microscope when they stained tissue with 0.05% trypan blue in lactophenol. Glutaraldehyde, a fixative, renders tissue auto-fluorescent (Bacallao et al. 1995) and appears to be useful for confocal microscopic studies to visualise fungal hyphae clearly in plant tissue (Singh et al. 1997).

## 1.4. Objectives of this studies

The objectives of this study were to;

1. Obtain consistent infection of *Botrytis cinerea* in detached plant materials.
2. Test selected Biological Control Agents (BCAs) on crops other than tomato and kiwifruit.
3. Measure the biomass of *Botrytis cinerea* in plant tissue.

## *Chapter Two*

### **General Materials and Methods**

#### **2.1 *Botrytis cinerea* isolates**

##### **2.1.1 culture preparation**

*Botrytis cinerea* isolates were collected from tomato, cucumber and strawberry crops. Spores from the infected tissue were gently tapped on to water agar (WA) plates and grown at 20<sup>0</sup>C for 12-24 h. Germinated single spores were transferred to Malt agar (MA) (Difco, 8g/liter) and incubated at 20<sup>0</sup>C under a 12 h day/night Near Ultra Violet (NUV) regime using a Philips TLD 36W/08, located 40 cm above the cultures, for 5-7 days to induce sporulation. Each culture, isolated from tomato, cucumber and strawberry was given a unique identification (eg. Bc-T1, Bc-C1 and Bc-S1 respectively). Plates with sporulation were stored at 4<sup>0</sup>C for up to 3-4 months for subsequent experiments or spores were stored in silica gel for long term storage (Perkins 1977).

##### **2.1.1.1 Silica gel storage**

Test tubes (100 mm x 12 mm) filled 1/3 with silica gel crystals (grade 123 18-23 mesh, no indicator) were bunged and sterilised at 160<sup>0</sup>C for 2 h or at 100<sup>0</sup>C overnight.

A 20% milk powder solution was prepared by adding 10 g dried skim milk powder in 50 ml Reverse Osmosis (RO) water and was dispensed at the rate of 2 ml per tube. The tubes were sterilised at 113<sup>0</sup> C and 10 psi for 10 min. The sterilised milk powder solution was poured onto sporulating colonies and spores suspended by rubbing the surface of the colony gently with a sterile glass hockey stick. Five hundred micro-litre aliquots of spore suspension were transferred to each tube of silica gel (pre-cooled in ice, bung removed and top flamed) using a 1 ml micro-pipette. All the tubes were kept in a beaker of ice to prevent the heat generated

killing the spores, and were left in the laminar flow cabinet to dry. When dry the silica gel granules, which were stuck together, were broken up by sterile needles and the tubes sealed and then stored in an airtight container with indicator silica gel. To use, a few crystals were tapped out onto agar plates and incubated as usual. All experiments for this project were therefore carried out with cultures only one sub-culture removed from the original isolate.

### **2.1.2 Preparation of spore suspensions**

Fifteen millilitres of sterile distilled water (SDW) was poured over a sporulating colony and spores gently dislodged using a sterilised, bent glass rod. The resulting suspension of conidia and mycelial debris was transferred into a universal bottle and shaken vigorously for a few seconds to disperse clumps of conidia. The contents were transferred to another universal bottle via a sterile 70  $\mu\text{m}$  'Falcon' cell strainer to remove mycelial debris. A 10  $\mu\text{l}$  aliquot was removed from the suspension and pipetted onto a haemocytometer slide to assess the spore concentration. Two spore counts were taken from five of the 25 inner squares, (the corner squares and the central square) each with a volume of 0.1  $\mu\text{l}$ . After counting the spore concentration was adjusted with SDW as required.

## **2.2 Biological Control Agents (BCA); bacteria and yeast**

### **2.2.1 Culture preparation**

Two bacteria, isolate OX2 (*Enterobacter agglomerans*) and isolate OX8a (*Enterobacter aerogenes*), and two yeast, isolate 561 (*Candida sake*) and isolate 622b (*Trichosporon pullulans*) cultures were obtained from Plant Health Section of the Dept. of Plant Science, Massey University. Bacterial isolates were cultured in Nutrient Agar (NA) and yeast in Nutrient Yeast Dextrose Agar (NYDA) at pH 4.5 for 2-3 days in total darkness at 15<sup>0</sup>C.

### 2.2.2 Preparation of cell suspension

After incubation, approximately 3 ml of SDW was used to dislodge cells in the colony by repeated pipetting to make a cell suspension of the isolates. The suspension was transferred to 1.5 ml ependorff tubes and centrifuged at 10,000 rpm for 5 min using a Biofuge A (Heraeus, Spatech) to remove used media. The liquid was decanted off and the microbial cell pellet was re-suspended in 1.5 ml of SDW by repeated pipetting.

### 2.2.3 Cells counts

Bacterial cell counts were taken using a slide counting chamber. A 10 µl aliquot of cell suspension was placed onto a slide counting chamber. Cells count was taken from 100 of the 400 inner squares from alternate rows of a single big square each with a volume of 0.000,05µl. After counting, cell concentration was estimated. Yeast cell concentrations were determined using a haemocytometer.

## 2.3 Source of test materials

### 2.3.1 Bean (*Phaseolus vulgaris* L.)

Bean leaves were collected from beans (*P. vulgaris* cv Top crop) grown in the Plant Growth Unit and Plant Science Glass-house. Seeds received from Watkins New Zealand Ltd. were planted 9 x 14 x 3 inch size of plastic tray contain bark and pumice (2:1)-based medium with 3-4 month osmocote, Dolomite and aglime (50:40:45) fertiliser.

### 2.3.2 Lettuce (*Lactuca sativa* L.)

Lettuce leaves were collected from lettuce (*L. sativa* cv Lollo bionda) grown in the Plant Growth Unit and Plant Science Department Glass-house. Seeds received from the Plant Science laboratory were planted in 15 cm diameter wells in a tray of 36 wells. A mixture of 3-4 month osmocote, FeCl<sub>3</sub>, Dolomite and aglime

(50:5:40:45) mixed with one unit volume of fine bark medium was used as a potting media.

### 2.3.3 Rose (*Rosa hybrida* L.)

Rose flowers from a rose garden at the Faculty of Agricultural and Horticultural Science were harvested when needed for the experiments before winter pruning. In this garden, the rose plants were maintained without any fungicide application.

## 2.4 Fluorescent treatment

### 2.4.1 KH4 antibody fluorescent dye

Monoclonal antibody, BC-KH4, and fluorescein isothiocyanate (FITC) conjugate (Sigma Immune Chemicals - Anti mouse IgG) were used as a fluorescent treatment for *B. cinerea* mycelia in plant tissue to facilitate CLSM image analysis (Cole et al, 1996).

Several pieces of bean leaf tissue (about 4-8 mm<sup>3</sup>) were taken from a *B. cinerea* inoculation site and placed into an ependorff tube. One hundred micro-litres of BC-KH4 antibody was placed into an ependorff tube with the samples and incubated on a micro-shaker at room temperature for about 2 h. After incubation, tissues were washed three times with phosphate buffered saline plus Tween (PBST) then 100 µl of FITC conjugate diluted 1:40 with PBST was added in to the tubes and incubated another 2 h on a micro-shaker at room temperature. After incubation, tissues were washed with PBST three times and mounted on a slide with CITI fluor (glycerol/PBS solution) mounting medium.

### 2.4.2 Trypan blue fluorescent dye

0.05% trypan blue in lactophynol (Wei et al, 1997) was prepared as a fluorescence treatment for *B. cinerea* mycelia in the plant tissue to facilitate confocal microscopic image analysis.

Two or three pieces of (about 4-8 mm<sup>3</sup>) bean leaf tissue were taken from a *B. cinerea* inoculation site and placed on glass slides in a few drops of 0.05% trypan blue in lactophenol and heated over a flame until boiling. Stained leaf pieces were mounted with CITI fluor mounting medium for confocal microscopy examination.

### **2.4.3 Glutaraldehyde fluorescent dye**

Glutaraldehyde fluid (GA) was used as a fluorescent treatment (Singh et al. 1997) for *B. cinerea* mycelia in the plant tissue. GA and buffered sucrose solution were prepared as described by Desbrey and Rack (1970).

#### **2.4.3.1 Glutaraldehyde fluid (GA) preparation**

Phosphate buffer was prepared by mixing 83ml of 1.95% aqueous sodium dihydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>) with 17ml of 2.52% aqueous sodium hydroxide (NaOH) at Ph 7.2 - 7.4. The final solution of glutaraldehyde was obtained by mixing of 16 ml of sterilised 25% glutaraldehyde solution with 84ml of phosphate buffer at pH 7.2 - 7.4 and was stored at 4<sup>0</sup>C for use when ever needed.

#### **2.4.3.2 Buffered sucrose solution preparation**

Buffered sucrose was prepared by dissolving 6.5g. sucrose in 100 ml phosphate buffer at pH 7.2- 7.4 to used as wash solution. The prepared buffered sucrose solution was stored at 4<sup>0</sup>C for future use.

#### **2.4.3.3 Procedure of glutaraldehyde fixation**

Several pieces (4-8 mm<sup>3</sup>) of bean leaf tissues were taken from a *B. cinerea* inoculated site and placed into a universal bottle. About 2 ml of glutaraldehyde solution was placed in the bottle in a fume hood. The bottles were wrapped in aluminium foil and incubated for 24 h at room temperature. After incubation, tissues were washed with buffered sucrose solution to remove glutaraldehyde.

This procedure required at least three hours and the buffered sucrose solution was changed twice during that period. Washed tissues were mounted on a slide with CITI fluor.

## **2.5 Confocal microscopy**

BC-KH4 monoclonal antibody, trypan blue and auto-fluorescent glutaraldehyde treated specimens were examined in a laser scanning confocal microscope (Model Leica TCS/NT) using a different wavelength for each treatment. Confocal images were produced with exciter wavelengths of 488 and 568 nm and with 530 and 590 nm barrier filter for imagining the glutaraldehyde treated specimens (Singh et al. 1997). Wavelength of 568 nm (excitation) and viewed with a broad band green filter at wavelength above 560 nm (imagining) were used for trypan blue treated specimens (Wei et al. 1997). Wavelengths of 490 nm (excitation) and 530 nm (imagining) were used for monoclonal antibody, BC-KH4, labelled with a goat anti-mouse immunoglobulin (IgG ) FITC conjugate (Sigma Chemical Co. Ltd, U.K.) treated specimens (Cole et al. 1996). Each image was created from 15 to 30 optical sections taken 5  $\mu\text{m}$  apart in depth and then recombined to form the final image.

## **2.6 Computer Image Analysis**

Recombined images were transferred to an image analysis computer (Indy Silicon Graphics Computer System) to measure the volume of fungal biomass in the plant tissue using the image analysis programme.

## **2.7 Assessment of *Botrytis cinerea* infection**

### **2.7.1 Data collection**

The number of infected leaf disks, petal or inoculation sites were counted to measure disease incidence (percent infection). A dissecting microscope was used to measure lesion diameter as a record of disease severity. All measurement on all three plant tissue were made on 4<sup>th</sup> or 5<sup>th</sup> day after *B. cinerea* inoculation except on bean leaf tissue where measurements were done every day until lesion covered entire leaf disk in order to observe lasting period of BCA's control ability on pathogen in biocontrol experiment.

### **2.7.2 Observation and recording**

Colour changes of media and of mycelial growth in the media were noted and recorded in *in vitro* experiments. BCA attachment to *B. cinerea* hyphae *in vitro* and *in vivo* were observed and photographed under a conventional light microscope and with the CLSM.

## **2.8 Experimental designs and statistical analysis**

Generally a completely randomised design was used with sub-sampling in appropriate experiments.

Most of the data were analysed by ANOVA using general linear modelling in the SAS system (Version 6.12 PC SAS). Data were transformed into arcsin whenever necessary to satisfy the requirement of ANOVA.

## Chapter Three

### Infection of detached plant materials by *Botrytis cinerea*

#### 3.1 Introduction

It is important to identify and quantify the source of variability for experimental work on infection by pathogenic fungi. Inducing infection by artificial inoculation with spores of *B. cinerea* on plant materials can be a problem. The incidence or severity of disease can vary from one experiment to another. Inherent fungal factors such as isolate variability, nutrient status, age of conidia and inoculum concentration (Lorbeer 1980), environmental factors such as temperature, relative humidity and availability of free water (Blakeman 1980), and condition of plant material such as age and wound can all affect infectivity of *B. cinerea*.

The effect of *B. cinerea* and *B. fabae*, spore concentration and of leaf age on lesion development in bean leaves has been studied by Deverall and Wood (1961). They found that the lesions were produced more rapidly on older than on younger leaves. They also observed that the numbers of lesions increased as the numbers of spores in the inoculum drops increased. Mansfield and Hutson (1980) found a more rapid and linear lesion development on broad bean and tulip leaves when inoculated with a high inoculum concentration (200 conidia/droplet) of suspensions of *B. fabae* and *B. tulipae* than when inoculated with a low inoculum concentration (20 conidia/droplets).

Infectivity of *B. cinerea* may differ with the origin of isolates and different host. Williamson and Jennings (1986), inoculated 31 different *B. cinerea* strains to red raspberry canes (*Rubus idaeus* L.) and found a significant difference in lesion development. Mansfield and Deverall (1974) observed that the rate of lesion development following inoculation with *B. cinerea* spore suspensions varied on

individual plants of broad bean (*Vicia fabae*) and was most rapid in younger leaves.

Relative humidity, availability of free water on the leaf surface, and temperature are very important factors affecting *B. cinerea* spore germination. A majority of workers have demonstrated that *B. cinerea* possess an optimum temperature for growth between 20<sup>0</sup>C – 25<sup>0</sup>C (Blakeman 1980) and high relative humidity of 93 - 100% (Nelson 1951; Snow 1949). Blakeman (1980) and Jarvis (1962) demonstrated the importance of a film of free water even with high moisture conditions for *B. cinerea* infection of the plant surface.

Tissue damage by climatic factors such as frost and windblown sand, or other mechanical agents stimulates development of larger lesions carried by *B. cinerea* on various hosts (Verhoeff 1980). In contrast, Purkayastha and Deverall (1965b) stated that tissue damage failed to enable *B. cinerea* to cause lesions on broad bean leaves.

### 3.2 Objective

Before any evaluation can be made of the efficiency of biocontrol agents, it is essential to have a procedure that gives consistent and reliable infections by the pathogens. Since there is, some disagreement in the literature cited above it was imperative to confirm that a reliable infection system could be developed here. The objective of the work described in this chapter was to obtain consistent infection of *Botrytis cinerea* in detached bean and lettuce leaves, and on rose petals.

### 3.3 Materials and Methods

#### 3.3.1 *B. cinerea* infection on bean and lettuce leaves and rose petals

##### Experiment 1

*Title: Effect of spore concentration, inoculation method and wounding of tissue on B. cinerea infection and lesion development in detached bean and lettuce leaves and in rose petals.*

Spore suspensions of *B. cinerea* isolate Bc-S1 were prepared as described in chapter 2, section 2.1.2.

Bean (*Phaseolus vulgaris L.*) and lettuce (*Lactuca sativa L.*) plants were grown as described in chapter 2, section 2.3.2 and 2.3.3. True fully developed leaves on bean plants that had not reached the flowering stage, 2-3 week old lettuce leaves and petals from rose (*Rosa hybrida L.*) flowers in the Faculty of Agricultural and Horticultural Science garden were collected and brought to the laboratory. Leaf disks (2.5 cm diam.) cut from detached leaves using a cork borer and intact rose petals were used for all inoculations. Tissue culture plates with wells 3.5 cm diameter and 7 or 10 mm deep were used to incubate inoculated plant material, one leaf disk or petal per well. The three host plants (bean, lettuce and rose tissues) were each done as separate experiments.

The fine tip of a glass rod or of a 200µl pipette tip was used to make a wound (the only inoculation site in this experiment) by gently pressing on the centre surface of the leaf disk or petal. The centre of the leaf disk or petal was chosen as. Ten micro-litre of spore suspension (prepared in  $\times 10^8$  and  $\times 10^6$  per ml concentration) was placed on the leaf disk or petal using a micro-pipette for the wet inoculation treatment. Dry inoculation was done by transferring dry spores from 7-10 day old,

well sporulated, cultures by gently picking up clusters of spores and placing them gently on the surface of the leaf disk or petal using a camel hair brush or pipette tip.

Inoculated culture plates were left in the open for 1 or 2 h to dry off the inoculum suspension and were then covered by a wet paper towel. The covered culture plates were kept in a plastic tray (45 cm x 30 cm x 15 cm) with moistened paper tissue underneath the plates. Each tray was inserted into a clear polythene bag, which was then sealed to ensure a high humidity inside the tray during the incubation period of 20<sup>0</sup>C and 12/12 h light and dark condition.

#### ***Statistical analysis and data presentation***

The experiment was a Complete Randomised Design with twelve replications. Data were analysed by the SAS programme for any significant variation at  $P < 0.05$  among treatments and data is presented graphically with letters indicating the significant differences.

#### **Experiment 2**

***Title: Effect of leaf age and position on the plant, inoculum and inoculation time on *B. cinerea* infection and lesion development in detached bean leaves.***

*B. cinerea* isolate Bc-S1 was used in this experiment. A spore suspension was prepared and adjusted to  $2.3 \times 10^7$  spores per ml. as described in chapter 2, section 2.1.2. Infected bean leaf tissue with sporulating *B. cinerea* was obtained from the above experiment 1 to use as another source of inoculum.

Inoculation was done on leaflets (terminal and side) of fully developed 2<sup>nd</sup> and 3<sup>rd</sup> or 3<sup>rd</sup> and 4<sup>th</sup> true leaves (leaves developed after the cotyledons), and the cotyledon leaf of the bean plants, grown in a glass house (Chapter 2). To determine whether

the time of day influenced the success of inoculations, leaves were collected and inoculated at 9 am, 1 pm and 6 pm. A fourth batch of leaves detached 24 h earlier and kept in the dark were inoculated with 9 am batch. Spores suspension and infected plant tissue with sporulating *B. cinerea* were used to determine the effect of source of inoculum on infection. One leaflet or cotyledon was placed in each tissue culture plate (used in experiment 1) cover (13 cm x 8 cm x 4 mm) for incubation.

One treatment was applied to the six inoculation sites (3 each side of the midrib) on each leaf. Each site was wounded with the tip of a glass rod or pipette tip. For the wet inoculation treatments, a micro-pipette was used to place 10 µl of suspension per wound. For the dry inoculation treatment, a portion of *B. cinerea* infected bean leaf tissue (~1mm x 1mm) with spores was placed on the wounded surface of the leaves using a needle.

Culture plate lids with inoculated leaves were kept on a bench for 1 - 2 h to allow the inoculum suspension to dry off. They were then placed in a plastic tray (45 cm x 30 cm x 15 cm) with moistened paper tissue underneath the lids. The tray was covered with moistened blotting paper, placed in a tight polythene bag and sealed to keep a high humidity inside the tray. The leaves were incubated at 20<sup>0</sup>C in 12/12 h light and dark conditions.

#### ***Statistical analysis and data presentation***

The experiment was designed as a randomised complete block with five replications. Blocking was made with leaf age and its position. Data were analysed by the SAS programme for any significant variation ( $P < 0.05$ ) among treatments and are presented graphically with letters to represent the significant differences using 'Duncans' multiple range analysis .

**Experiment 3**

*Title: Effect of different isolates of B. cinerea on infection in detached bean leaves.*

Three isolates, Bc-C1, Bc-S1 and Bc-T1 of *B. cinerea* were used in this experiment and the cultures prepared as described in chapter 2, section 2.1.1.

Leaf disks were prepared and inoculated with dry spores of three isolates and incubated in tissue culture plate wells as described in experiment 1.

***Statistical analysis and data presentation***

The experiment was designed as a Complete Randomised Design with twelve replications. Data were analysed by the SAS programme for any significant variation ( $P < 0.05$ ) among treatments and are presented graphically with letters to represent the significant differences.

**3.4 Results****Experiment 1**

Wounding of tissue significantly increased lesion size and percent infection on all three plants tissue (Fig 3-1). There were no significant differences in lesion size or percentage infection between inoculum concentrations including dry spore inoculation on lettuce and rose but, differences observed on bean where high inoculum concentration ( $1 \times 10^8$ ) significantly increased lesion size and percentage infection than the other two inoculation (Fig 3-2). The interaction between wound treatment and inoculum concentration including dry spore inoculation on those three crops was not significant. There were no significant differences on lesion size or percentage infection between wounded and unwounded tissue at a high

spore concentration ( $1 \times 10^8$ ) on bean (Fig 3-3). In addition, on bean leaves, dry spores induced more and bigger lesions in wounded tissue than in unwounded tissue although the difference in lesion size was not significant (Fig 3-3).

There were no significant differences on lesion size or percentage infection among inoculation concentration including dry spore inoculation within wounded or unwounded lettuce or rose tissue (Fig 3-4 & 3-5). The difference in lesion size and percentage infection between wounded and unwounded tissue was greater in all three inoculations on rose than on bean and lettuce (fig 3-6).

### **Experiment 2**

Inoculation time of day, source of inoculum of *B. cinerea* and age and position of the leaflet did not significantly influence *B. cinerea* infections on bean (Fig 3-7 and 3-8).

### **Experiment 3**

There was no significant different in aggressiveness between isolates of *B. cinerea* in the three crops but, percentage infection was highest (93 - 100%) in rose petals and the lowest (28 - 30%) in bean leaves (Fig. 3-9).

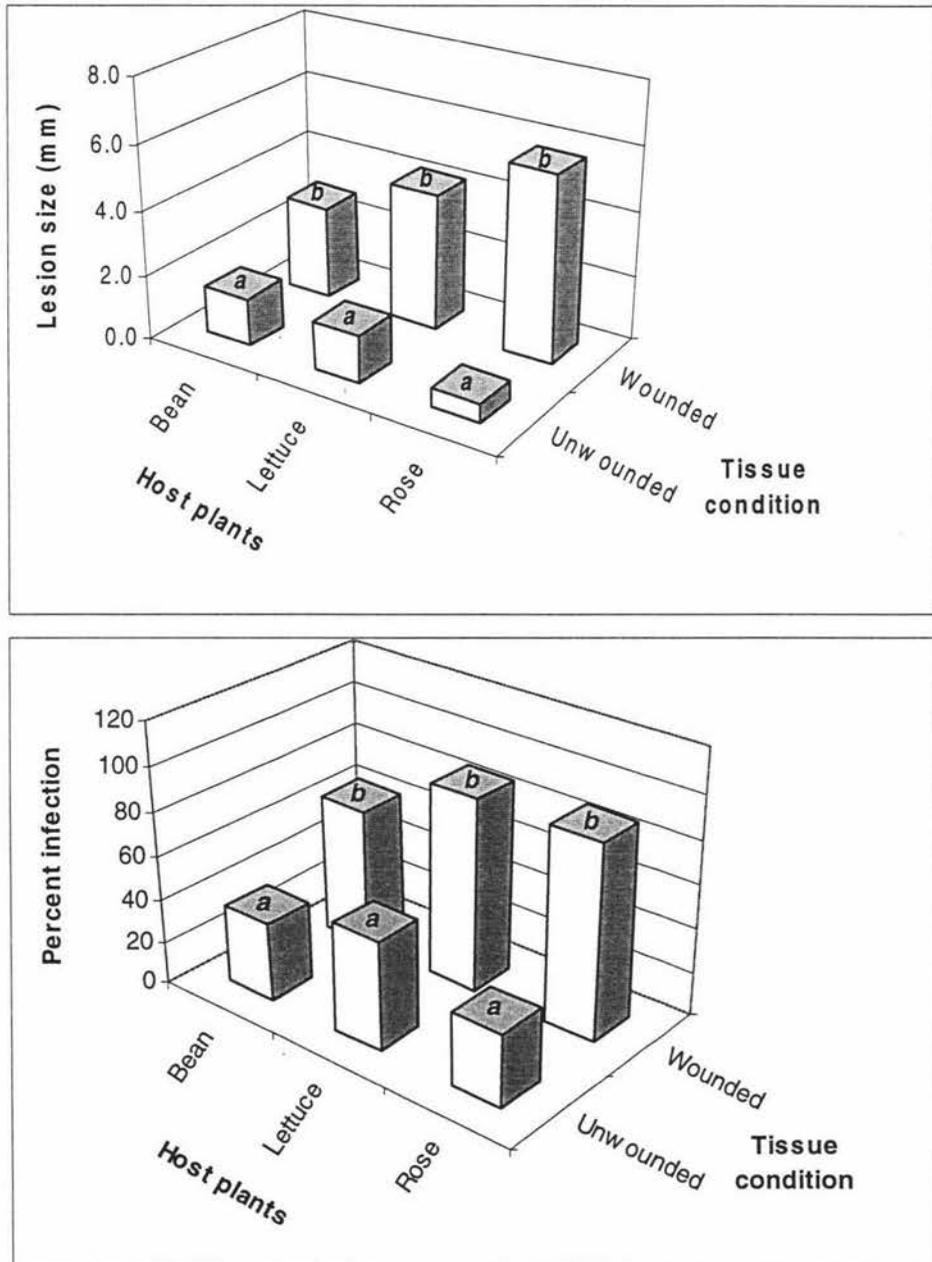


Fig 3-1 Lesion size and percentage infection of wounded and unwounded tissue of bean, lettuce and rose. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

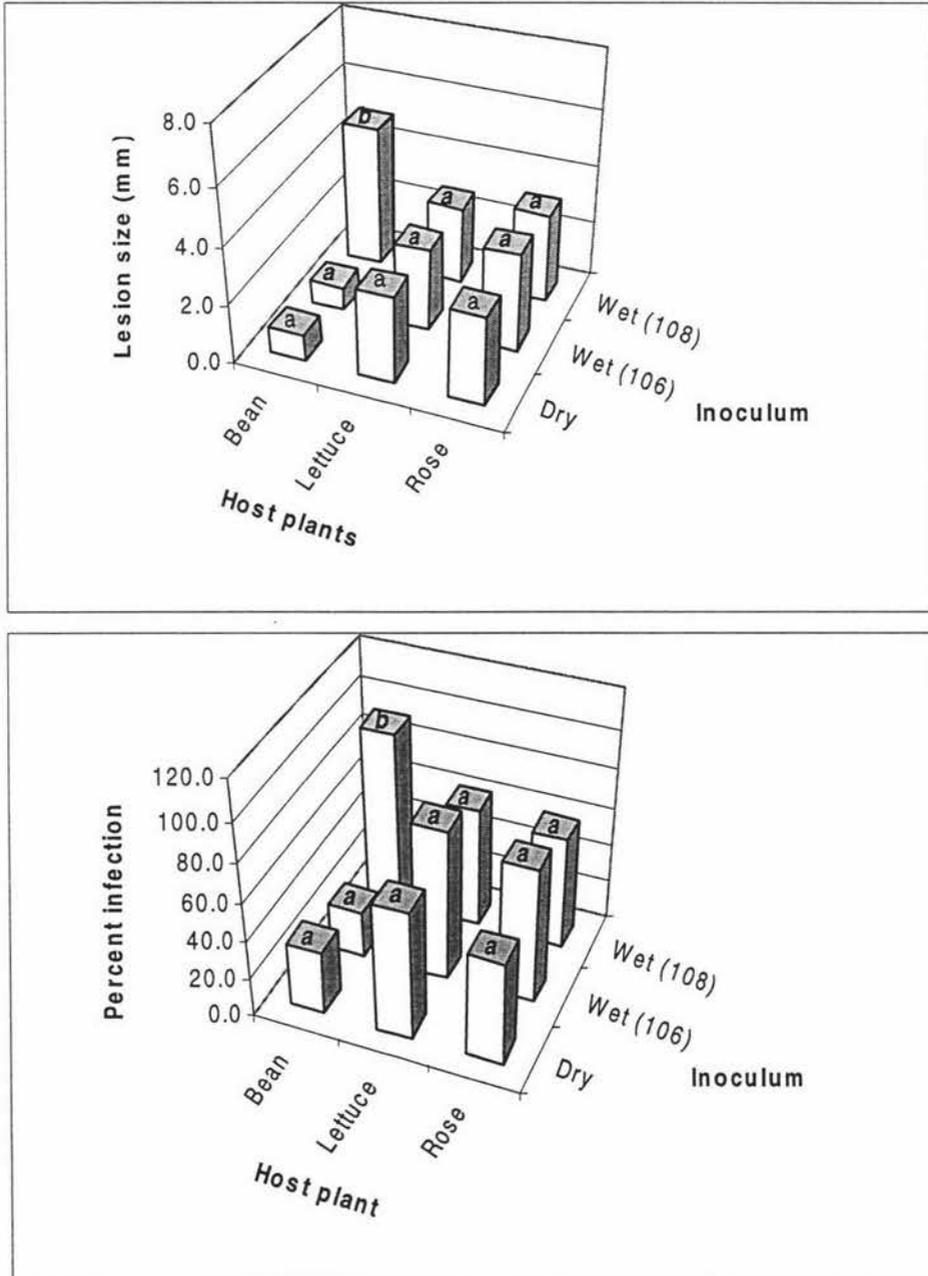


Fig 3-2 Lesion size and percentage infection by dry and wet ( $1 \times 10^6$  and  $1 \times 10^6$  spores per ml.) spore inoculation on bean, lettuce and rose tissue. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

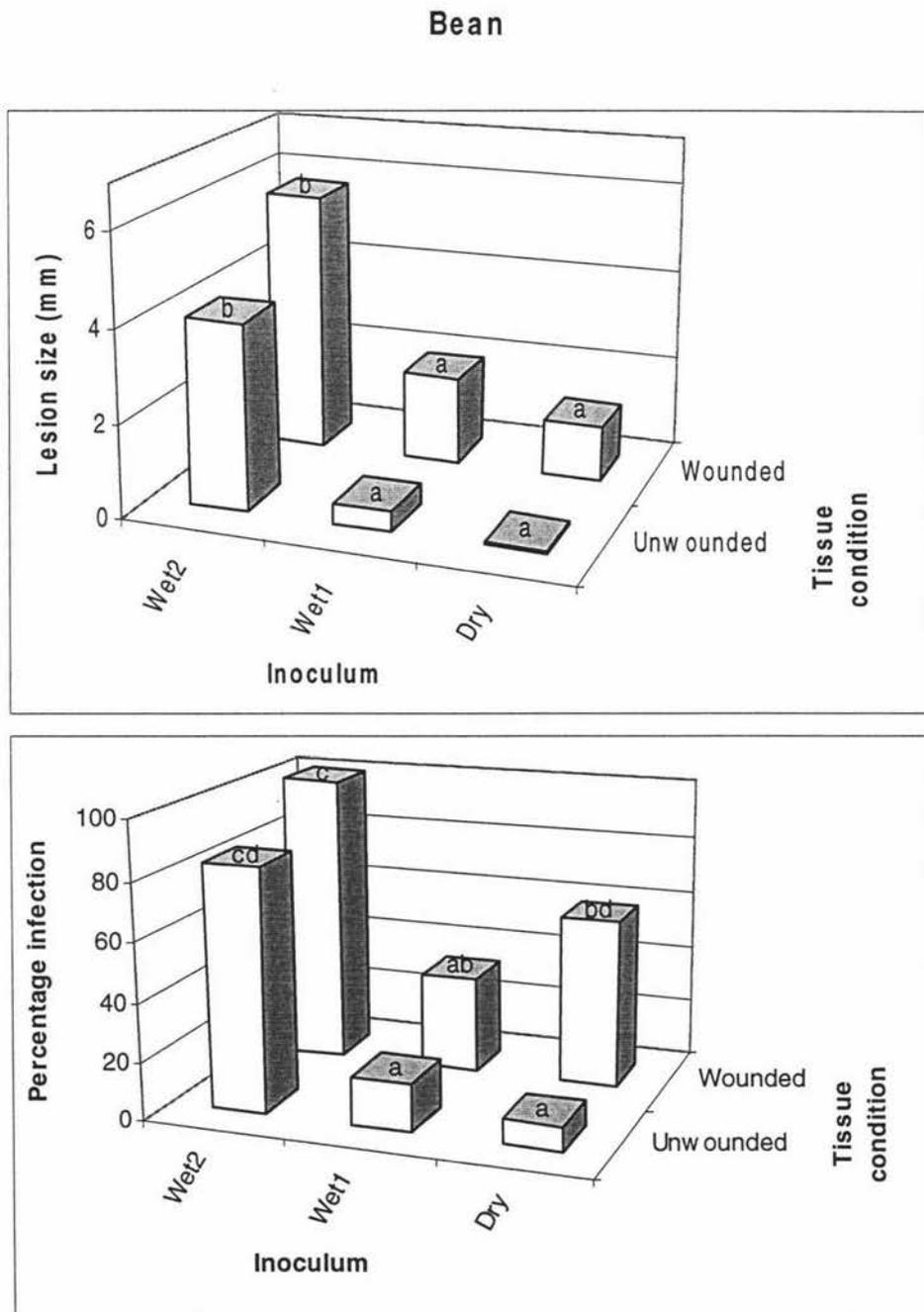


Fig 3-3 Lesion size and percentage infection by *B. cinerea* on bean leaves inoculated with different spore concentration wound and unwounded tissue. Wet1:  $1 \times 10^6$  spores per ml. Wet2 :  $1 \times 10^8$  spores per ml. Dry: Dry spores. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

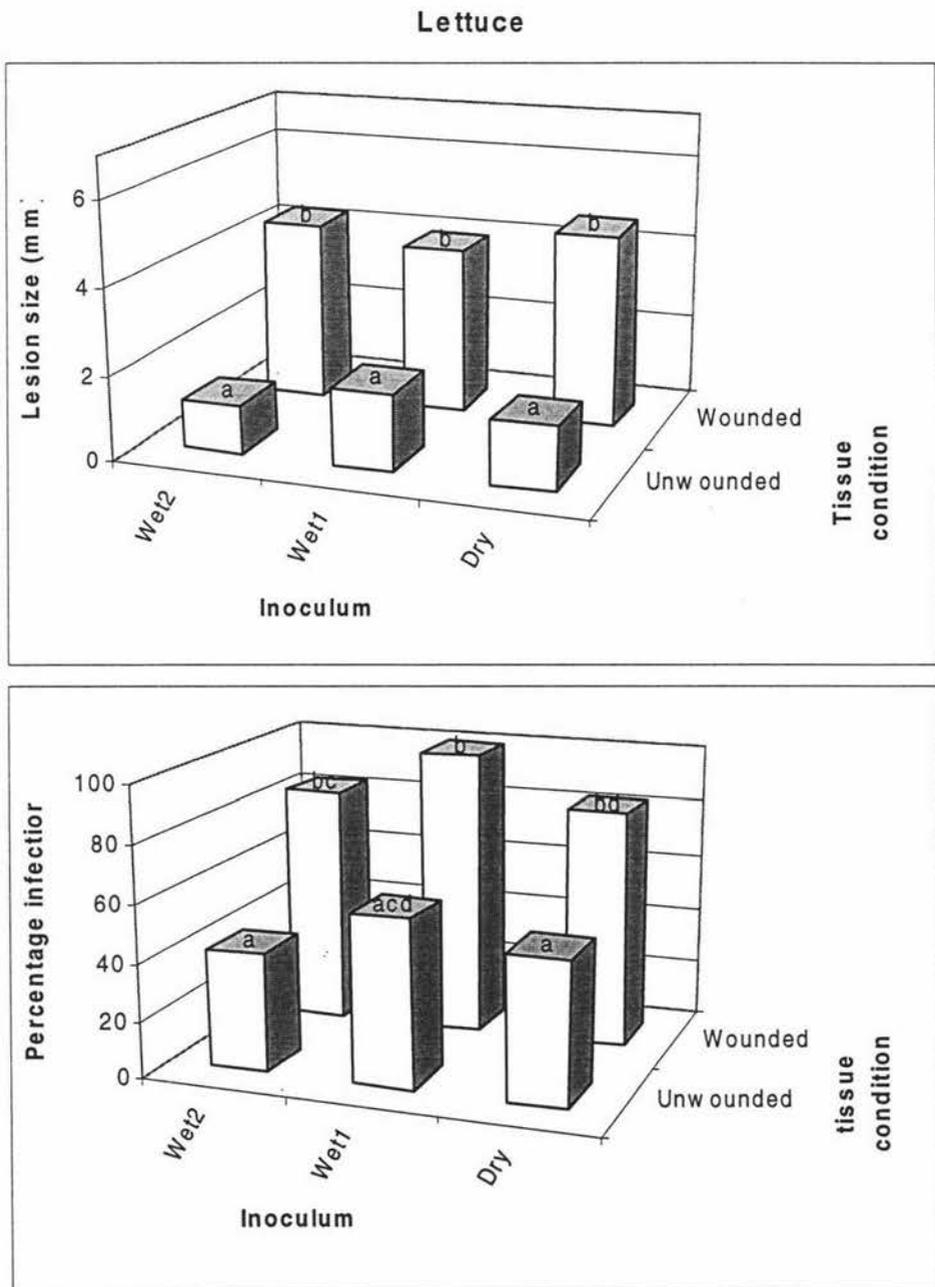


Fig 3-4 Lesion size and percentage infection by *B. cinerea* on lettuce leaves inoculated with different spore concentration on wound and unwounded tissue. Wet1:  $1 \times 10^6$  spores per ml. Wet2 :  $1 \times 10^8$  spores per ml. Dry: Dry spores. Means accompanied by same letters are not significantly different at  $P < 0.05$ .

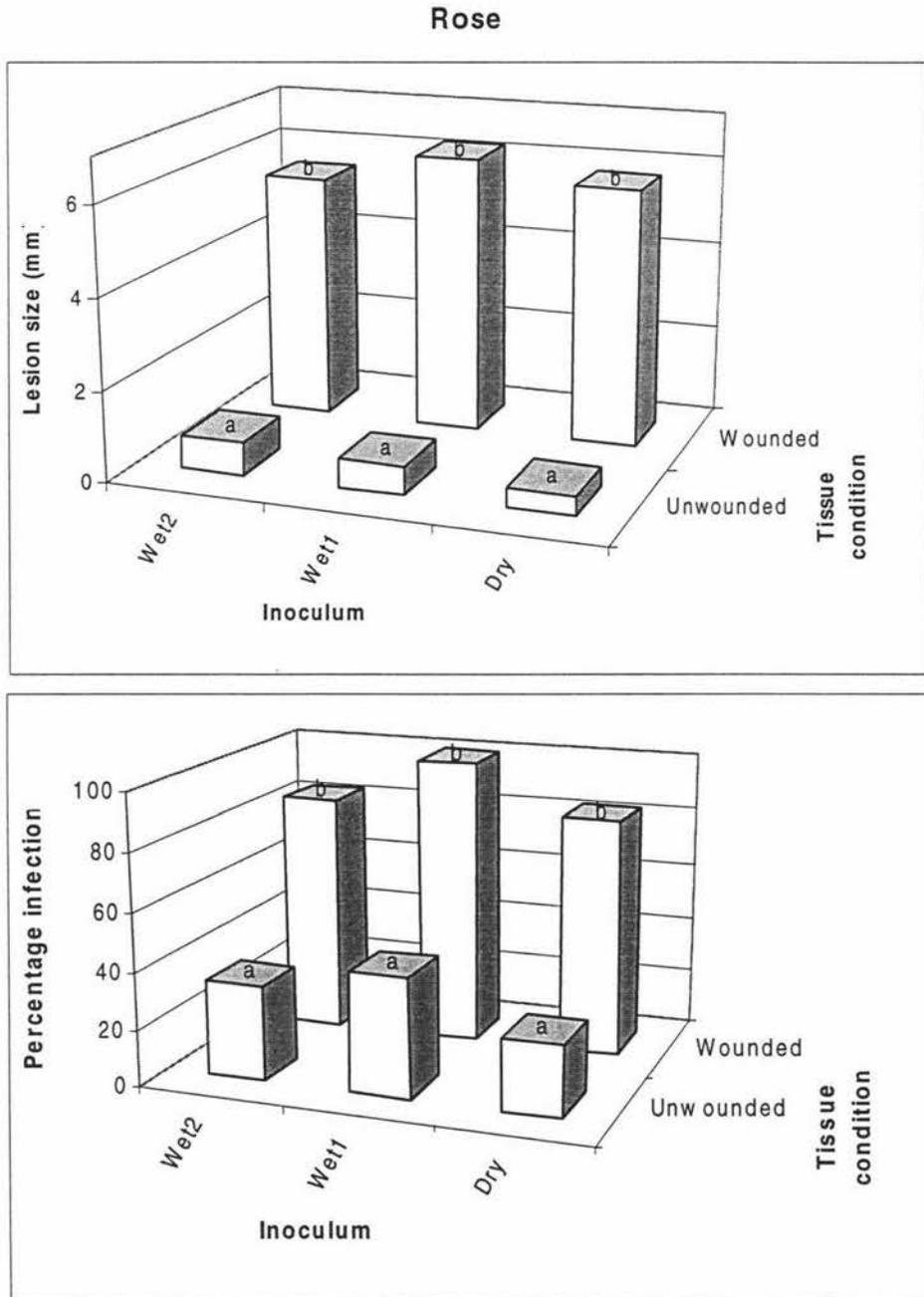


Fig 3-5 Lesion size and percentage infection by *B. cinerea* on rose petals inoculated with different spore concentration on wound and unwounded tissue. Wet1:  $1 \times 10^6$  spores per ml. Wet2 :  $1 \times 10^8$  spores per ml. Dry: Dry spores. Means accompanied by same letters are not significantly different at  $P < 0.05$ .

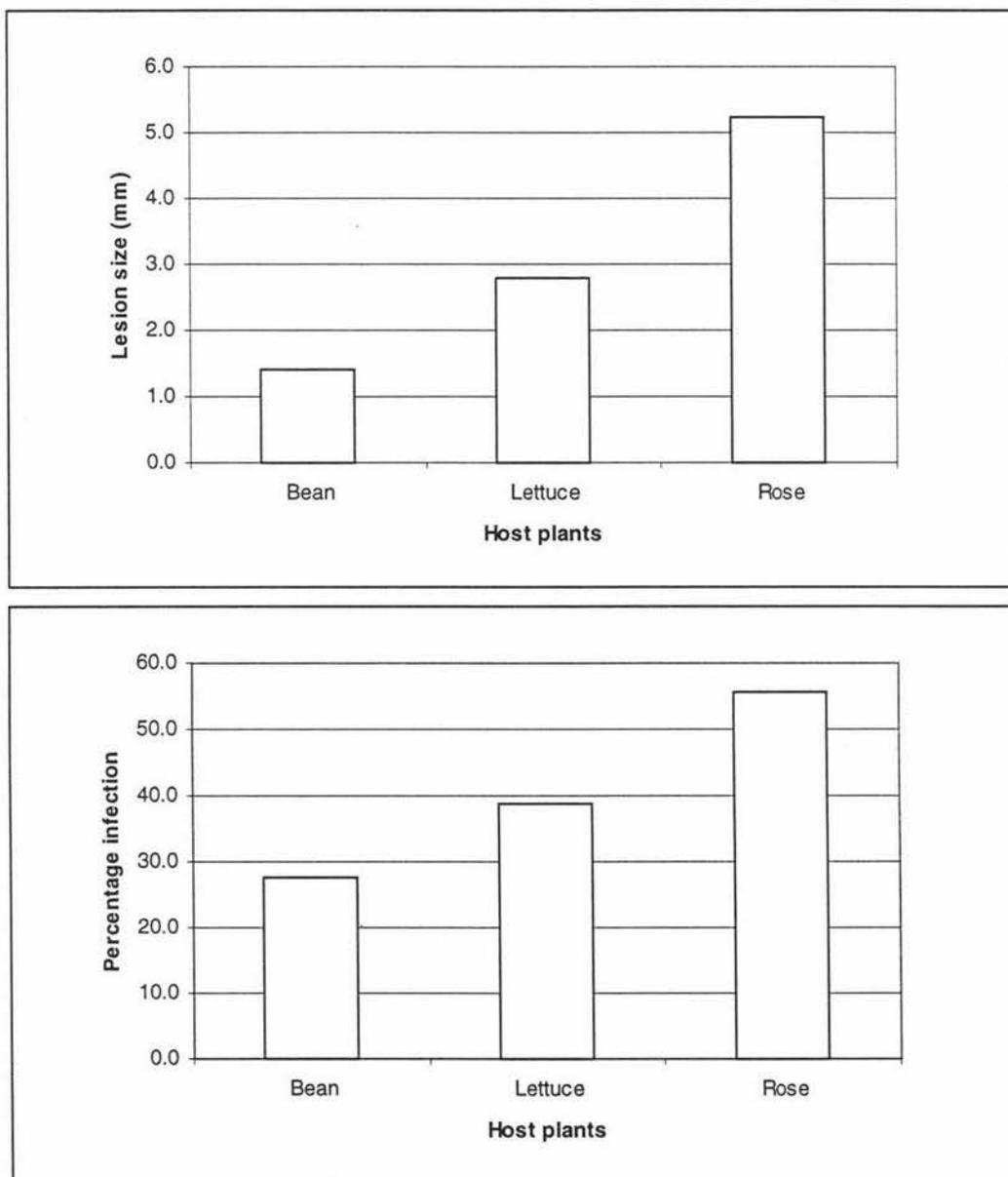


Fig 3-6 Differences of lesion size and percentage infection between wounded and unwounded tissues of bean, lettuce and rose.

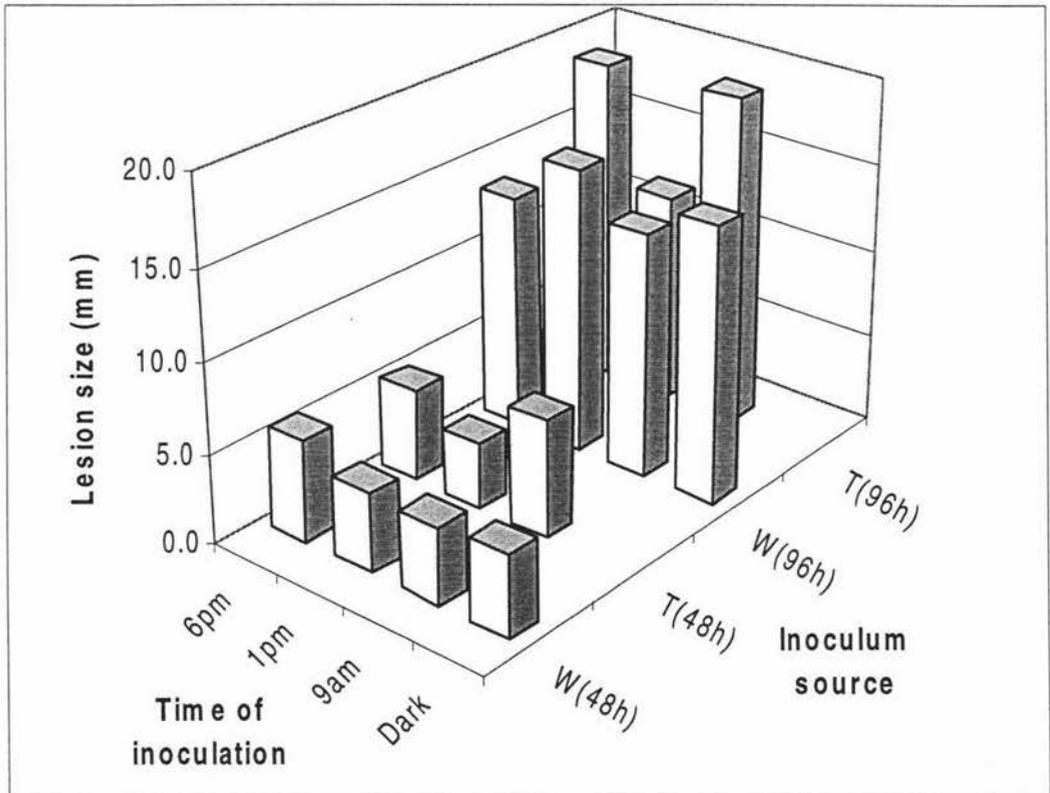


Fig 3-7 Lesion size measured 48 h and 96 h after inoculation of *B. cinerea* on bean leaves. Dark = leaves left 24 h in dark before inoculation; W= Spores in water suspension; T= Infected tissue with spores.

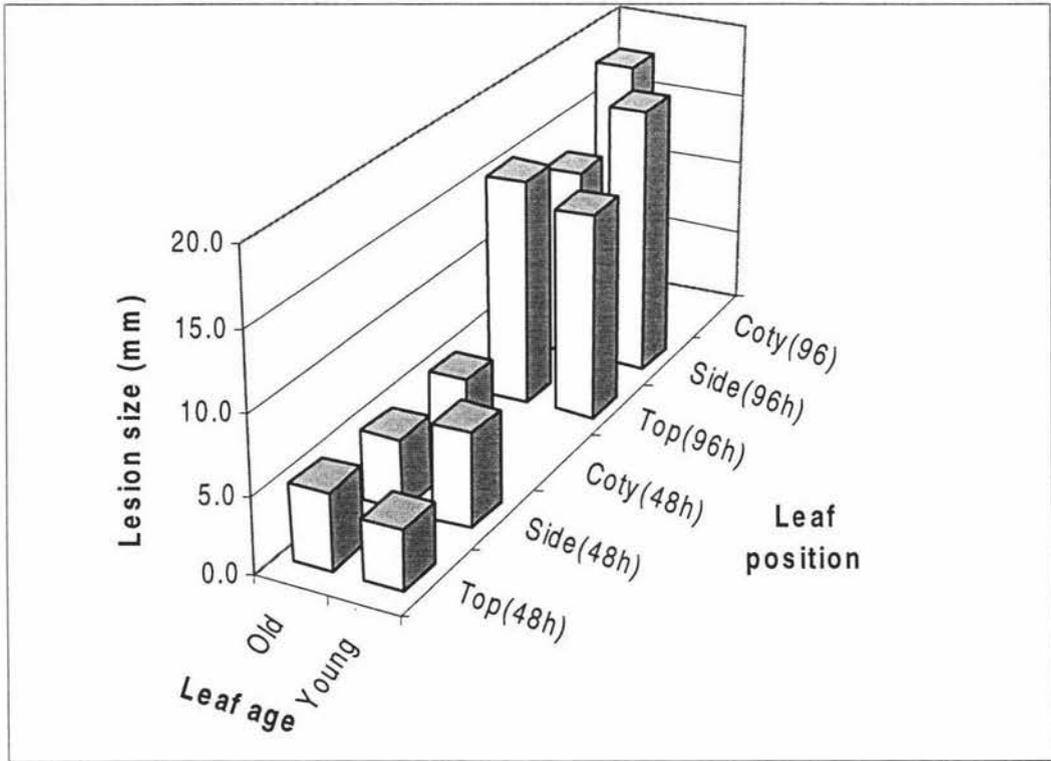


Fig 3.8 Lesion size measured at 48 h and 96 h after *B. cinerea* inoculation on different age and position bean leaves. Coty = cotyledon.

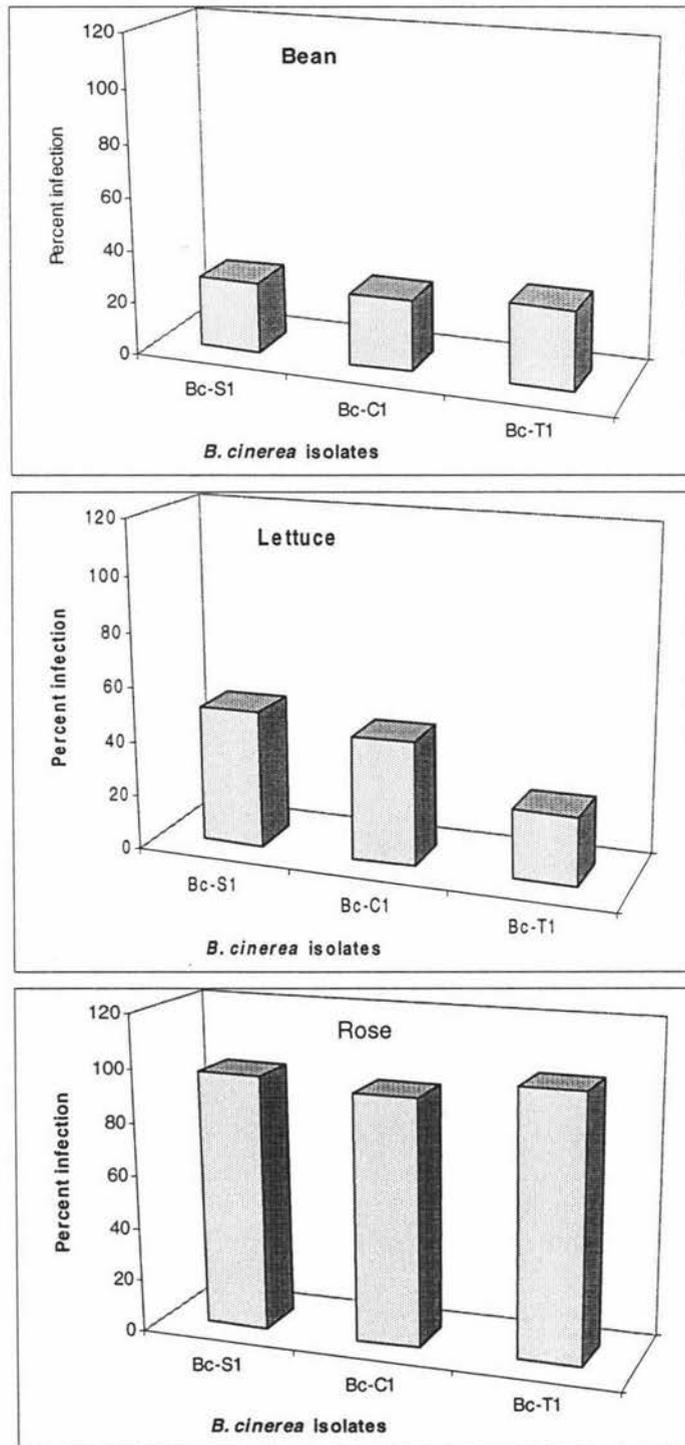


Fig 3-9 Percentage infection measured after different *B. cinerea* isolates were inoculated on bean and lettuce leaves and rose petals

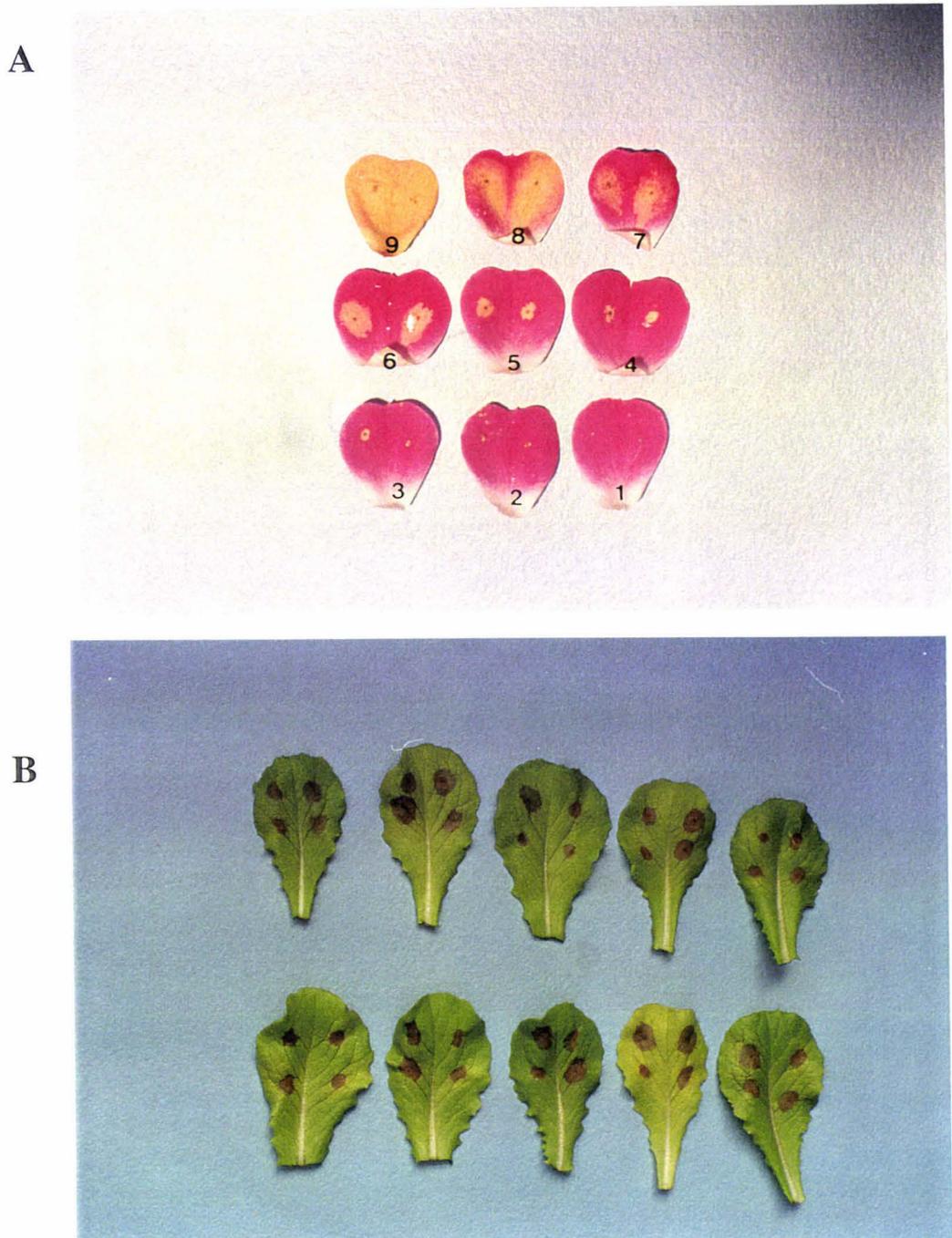


Fig 3-10 Lesion development over time. A) Rose petals inoculated only with *B. cinerea*. Size of lesions after 24 h (1-3), 48 h (4-6) and 72 h (7-9) incubation. B) Lettuce leaves after 72 h incubation.

### 3.5 Discussion

In this study, the inoculated materials were held for two hours in ambient conditions to allow the inoculum suspension (wet inoculation) to dry before incubation under a high humidity to ensure spore germination. In a preliminary study (data not presented) *B. cinerea* spores were incubated in a droplet of water on the surface of a leaf under high humidity and did not germinate until the water droplet had dried to a thin film of free water. Purkayastha and Deverall (1965a) have also observed little growth of *B. cinerea* in water drops on bean leaves. Jarvis (1962) reported a rapid germination of dry *B. cinerea* spores on the surface of strawberry fruits once a film of free water was present with a saturated humidity. Carre (1984) demonstrated that condensation which provides a film of free water is important to *B. cinerea* germination even at high humidity (cited by Hammer and Marois 1989). Thus free water is important but it should be in the form of a thin layer to enhance *B. cinerea* germination and infection development. Later works have shown that germ tubes from *B. cinerea* conidia applied as dry spores, directly penetrate rose petal (Williamson et al. 1995) and bean leaf tissue (Cole et al. 1996) while epiphytic hyphal growth from conidia applied in liquid suspension continued to explore the plant surface. Other factors such as relative humidity and temperatures are also required at an optimum level.

Inoculation of different host plants, tissue injury and inoculum concentration all markedly influenced *B. cinerea* infection but there were no variations in pathogenicity between *B. cinerea* isolates.

Most postharvest infection of vegetable, ornamental flowers and fruits occurs through wounds on surface tissue. The results of these experiments indicated that wounds on rose petals and, bean and lettuce leaves are the preferred entry points for Botrytis infection.

There were fewer and smaller infections on bean than on rose and lettuce. Bean plants are known to have inhibiting factors such as the phytoalexin phaseolin (Rahe and Arnold 1975), which inhibits *B. cinerea* germination and growth. Bean plants have been studied by many workers for antifungal materials. Thus Hargreaves et al. (1977) and, Deverall and Vessey (1969) observed that the phytoalexin wyerone acid was formed by broad bean leaves upon infection by *B. cinerea* and that its development was markedly reduced. A high infection rate and large lesion were observed in the high inoculum concentration ( $1 \times 10^8$ ) treatments in bean leaves and indicated that an increased inoculum load can successfully challenge bean tissue and overcome its resistance.

Rapid lesion development in rose petals indicated that they were more susceptible to *B. cinerea* infection than lettuce leaves as showed in Fig 3-10 (A and B).

The results of experiment 2 did not support the hypothesis that bean leaf age and a time period to expose to sunlight before inoculation could affect the course of infection on bean. Inconsistent infections found in earlier experiments that must have been caused by other factors.

The most consistent and high levels of infection were obtained on wounded plant tissue so all subsequent work was done using wounded tissue. Both wet and dry inoculum gave consistent results on lettuce and rose tissue, but in bean tissue, only wet inoculum at a high concentration consistently gave infections. Therefore, a high concentration in the form of wet inoculum was used in subsequent work on bean but dry inoculation was used for subsequent work on lettuce and rose due to the greater ease of handling.

## *Chapter Four*

### **Biological control of infection of rose, bean and lettuce by *Botrytis cinerea***

#### **4.1 Introduction**

*Botrytis* blight in roses and grey mould diseases in bean and lettuce seriously affect the production and post harvest quality of these crops. *Botrytis* blight first appears as small flecks or blisters on petals of roses. Such infections are not always visible at harvest but they develop rapidly in transit to market under the conditions of high humidity maintained in shipping containers (Redmond et al. 1987). Grey mould disease in bean and lettuce varies in symptom development, with the age and parts of the plants, but the distinctive feature of grey mould is the formation on the surface of lesions in a moist atmosphere of greyish powdery fungus growth consisting chiefly of the conidiophores and conidia of the pathogen. In bean plant, grey mould can be found on all aerial portions (Campbell 1949).

At present, these grey mould diseases are managed primarily by application of fungicides. In some cases, however, the application of a fungicide may actually increase the severity of *Botrytis*-caused diseases (Bollen and Scholten 1971; Zalewski and Johnson 1977). This increase is presumably due to the presence of fungicide-resistance strains of *Botrytis* that can develop within one season (Fraile et al. 1986; Northover and Matteroni 1986) and to suppression of natural antagonists by the fungicide. The development of resistant strains of *B. cinerea* to fungicides (Bollen and Scholten 1971; Zalewski and Johnson 1977; Denis and Devis 1979; Pappas et al. 1979; Katan 1982; Leroux et al. 1981) has increased the focus on alternate control measures particularly on biological control.

A number of plant diseases, including several caused by *Botrytis cinerea*, have been reduced by pre-inoculation or post-inoculation of the phylloplane with

epiphytic bacteria, fungi or yeast (Blakeman and Fokkema 1982; Blakeman and Fraser 1971; Cook 1997; Sada 1992). Special attention has been given by several researchers to identification of a biocontrol agent (BCA) with no antibiotic production. For example, three morphologically different strains of yeast effectively reduced incidence and severity of grey mould of pears, and there was no indication of any production of antibiotic to suppress the causal agent, *Botrytis cinerea* (Chand-Goyal and Spotts 1996). Cheah and Hunt (1994) and Cook (1997) demonstrated that some yeast and bacteria did not produce antibiotic but did reduce *Botrytis* storage rot incidence in kiwifruit. Further, Cook (1997) showed that these biocontrol agents had the ability to attach to hyphae of the pathogen.

Can these attachment biocontrol agents be used to control diseases caused by *B. cinerea* on ornamental flowers and vegetables with a similar level of effectiveness and the mode of action? Several species of yeast and bacteria have been shown to control *Botrytis cinerea* on rose (Redmond et al. 1987; Hammer and Marois 1989).

## 4.2 Objective

To test the effectiveness against *Botrytis cinerea* infection of rose, bean and lettuce of four attachment biocontrol agents (two each of bacteria and yeast) used by Cook et al. (1997b) against *Botrytis cinerea* on kiwifruit and tomato.

### 4.3 Materials and Methods

Three experiments were carried out, one on each on bean, lettuce and rose.

#### Experiment 1

##### *Title: Evaluation of BCAs on bean*

Bean leaves were obtained and prepared for the experiment as described in chapter 2 and chapter 3 section 3.3 respectively.

Based on the experiments in chapter 3, a spore suspension of  $1 \times 10^8$  spores per ml. was selected as the inoculum for bean leaf disks in this experiment.

A cell suspension of the bacteria (isolates OX2 and OX8a) and yeast (isolates 561 and 622b) were made as described in chapter 2. The cell concentration of each isolate was estimated using a slide counting chamber for bacteria and a haemocytometer for yeast. Concentrations were calculated as  $1.5\text{-}2.4 \times 10^9$  per ml of bacterial isolate OX2,  $1.8\text{-}2.3 \times 10^9$  per ml of bacterial isolate OX8a,  $1.0\text{-}5.0 \times 10^8$  per ml of yeast isolate 561, and  $6.2 \times 10^7 - 1.4 \times 10^8$  per ml of yeast isolate 622b.

All bean leaf disks were placed in culture plate wells and inoculated with *B. cinerea* suspension by placing the suspension onto two sites on the leaf disk using a micro-pipette. Each site received 5 $\mu$ l of suspension. Inoculated sites were wounded by gently pressing with the micro-pipette tip during inoculation. All four BCA suspensions were applied by micro sprayer on to the inoculation sites at three different times after *B. cinerea* inoculation: 0 h, 24 h, 48 h. SDW was applied as a control. The experiment was a split-plot design where time of BCA application was used as a main treatment and the BCAs the sub-treatments. One culture plate

(six leaf disks) was used as the experimental unit and was replicated three times. The culture plates of leaf disks were incubated as described in chapter 3.

***Assessment:***

The lesion size at the wound sites was measured as the diameter of the lesion across the wound site using an eyepiece micrometer in a dissecting microscope. Lesion size was measured every day till lesion covered entire leaf disk in order to observe BCA's control ability over the time. Data was analysed by a split-plot model using the SAS statistical package.

**Experiment 2**

***Title: Evaluation of BCAs on Lettuce***

Lettuce leaves were obtained and prepared for the experiment as described in chapter 2 and chapter 3 section 3.3 respectively.

Based on the experiments in chapter 3, dry inoculation was selected to inoculate lettuce leaves in this experiment and *B. cinerea* cultures were established in petri-plates as described in chapter 3 for the dry inoculation.

All four biocontrol agents were used in this experiment and were prepared as described in the previous experiment. Concentration were  $1.5-2.4 \times 10^8$  per ml of bacterial isolate OX2,  $1.7-2.9 \times 10^8$  per ml of bacterial isolate OX8a,  $3.8 \times 10^6 - 2.0 \times 10^7$  per ml of yeast isolate 561 and  $1.8-2.9 \times 10^7$  per ml of yeast isolate 622b.

Lettuce leaves were placed in culture plate covers (13 cm x 8 cm x 4 mm) and inoculated with *B. cinerea* spores on two sites per leaf using a micro- pipette tip. The tip was gently touched on a 7-10 days old sporulating colony of *B. cinerea* in a petri-plate. The attached spores were transferred to the leaf tissue by pressing

with the tip to make a slight wound. All four BCA suspension were applied by micro sprayer on to the inoculated leaves at three different times after *B. cinerea* inoculation: 0 h, 24 h, 48 h. SDW was applied as a control. The experiment was set-up and incubated as described in experiment 2 under section 3.3.1 in chapter 3.

***Assessment:***

Same as experiment 1 in this chapter.

**Experiment 3**

***Title: Evaluation of BCAs on Rose***

Rose flowers were obtained and prepared for the experiment as described in chapter 2 and section 3.3 in chapter 3 respectively.

The dry inoculation method was selected and rose petals were inoculated as in the above lettuce experiment (Fig 4.1).

All four biocontrol agents were used in this experiment and were prepared as described in the above bean and lettuce experiments. Concentration were  $1.8-2.7 \times 10^7$  per ml of bacterial isolate OX2,  $1.9-2.5 \times 10^7$  per ml of bacterial isolate OX8a,  $1.4-1.7 \times 10^6$  per ml of yeast isolate 561, and  $1.6-2.9 \times 10^7$  per ml of yeast isolate 622b.

Inoculated petals were maintained and incubated in culture plate wells, and the full experiment was set-up as in the above bean experiment.

***Assessment:***

Same as previous experiments in this chapter.

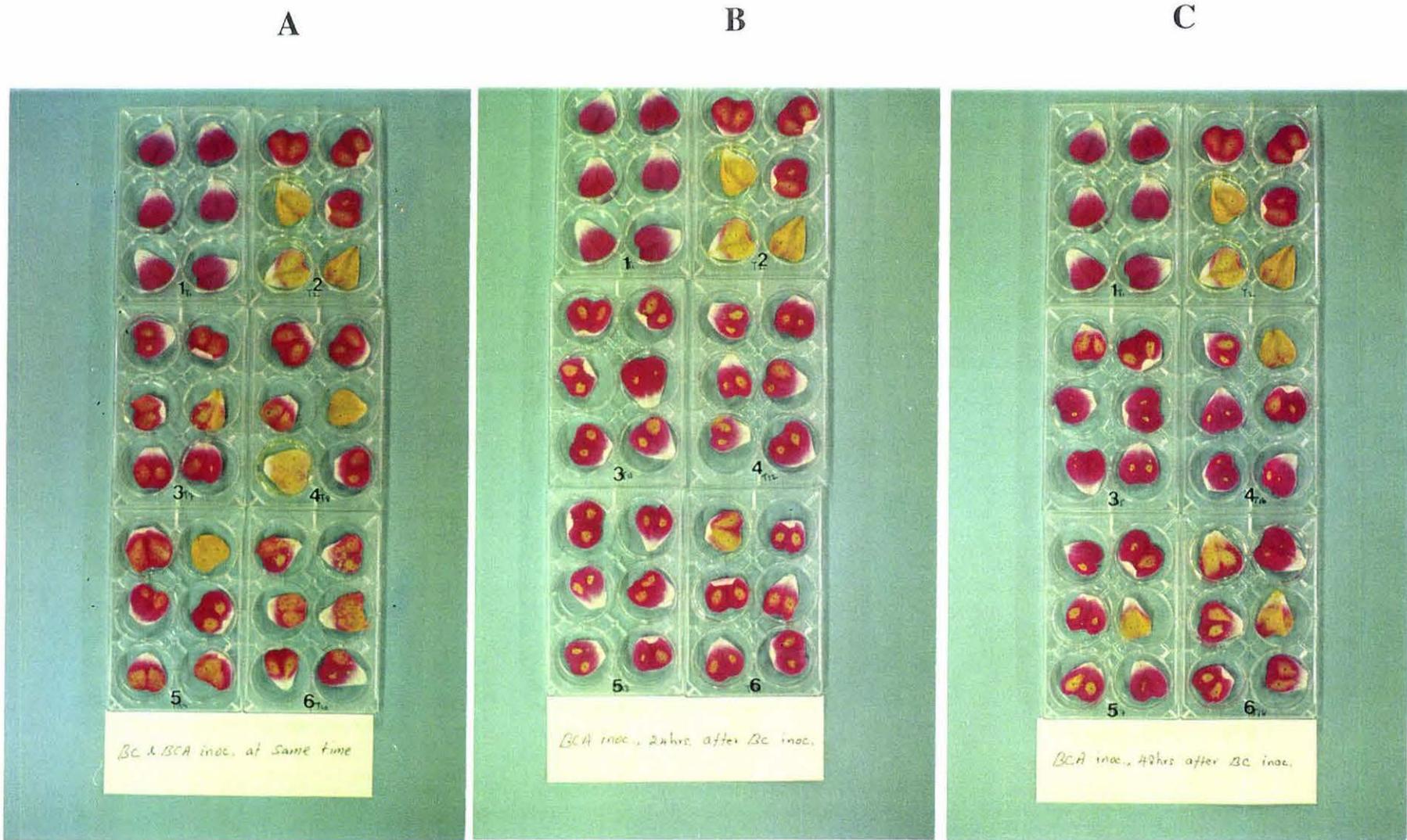


Fig 4-1 BCAs added at 0h (A), 24h (B) and 48 h (C) after inoculation of rose petals by *B.cinerea*. Control 1: no BCAs or *B. cinerea* inoculated 2) Control2: only *B. cinerea* was inoculated 3) OX2 4) OX8a 5) 561 6) 622b.

## 4.4 Results

### Bean leaf tissue

Bacterial isolate OX2 applied to bean leaf tissue simultaneously (0 h) with, or delayed by 24 h or 48 h after *B. cinerea* inoculation significantly controlled lesion development and percentage infection when compared with the water control (Fig 4-2, 4-3 & 4-4). Control of lesion development and of disease incidence (percentage infection) was maintained longer by early (0 h) application (Fig 4-2A) and 48 h delayed application (fig 4-4B) respectively. Early application significantly reduced lesion size and maintained control throughout the observation time when compared with other two applications but, no significant difference was observed among these application times on disease incidence (Fig 4-5).

Bacterial isolate OX8a, gave control of lesion development when applied together (early) with *B. cinerea* and a 24 h delayed application but no significant control was observed with a 48 h delayed application. Early application kept control for a longer period than the 24 h delay application (Fig4-2A & 4-3A). A significant reduction of disease incidence was observed for eight to nine days at all three times of applications (Fig 4-2B, 4-3B & 4-4B). A significant reduction of lesion size development and disease incidence was observed from an early application compared with a 24 h delay application through out the observation period (Fig 4-6).

Yeast isolate 622b significantly controlled lesion development with a 24 h or 48 delay application after *B. cinerea* inoculation (Fig 4-3A & 4-4A). No significant reduction occurred when the isolate was applied together with *B. cinerea*. The isolate significantly controlled disease incidence throughout the observation period at all three applications (Fig 4-2B, 4-3B & 4-4B). There was no significant

difference between a 24 h and a 48 h delay application on lesion development and on percentage infection except at day 3 where lesion size was significantly reduced by the 24 h delay application treatment (Fig 4-7).

Yeast isolate 561 did not show any significant control on lesion development except on day 4 from an early application with *B. cinerea* (Fig 4-2A). A significant reduction of disease incident was observed following an early application with *B. cinerea* (Fig 4-2B).

### **Lettuce leaf tissue**

All four isolates applied together with *B. cinerea* to lettuce leaf tissue significantly reduced lesion size compared with the water control, but percentage infection was significantly reduced only by isolate OX2 (Fig 4-8), this isolate also reduced lesion size with a 24 h delay application (Fig 4-9). By comparison, isolate OX2 significantly reduced lesion size and percentage infection with the early application (Fig 4-10).

### **Rose petal tissue**

All four isolates, except isolate 561 applied with a 48 h delayed application to rose petal tissue significantly reduced lesion size compared with the water control but percentage infection was not reduced by any of these isolates (Fig 4-11).

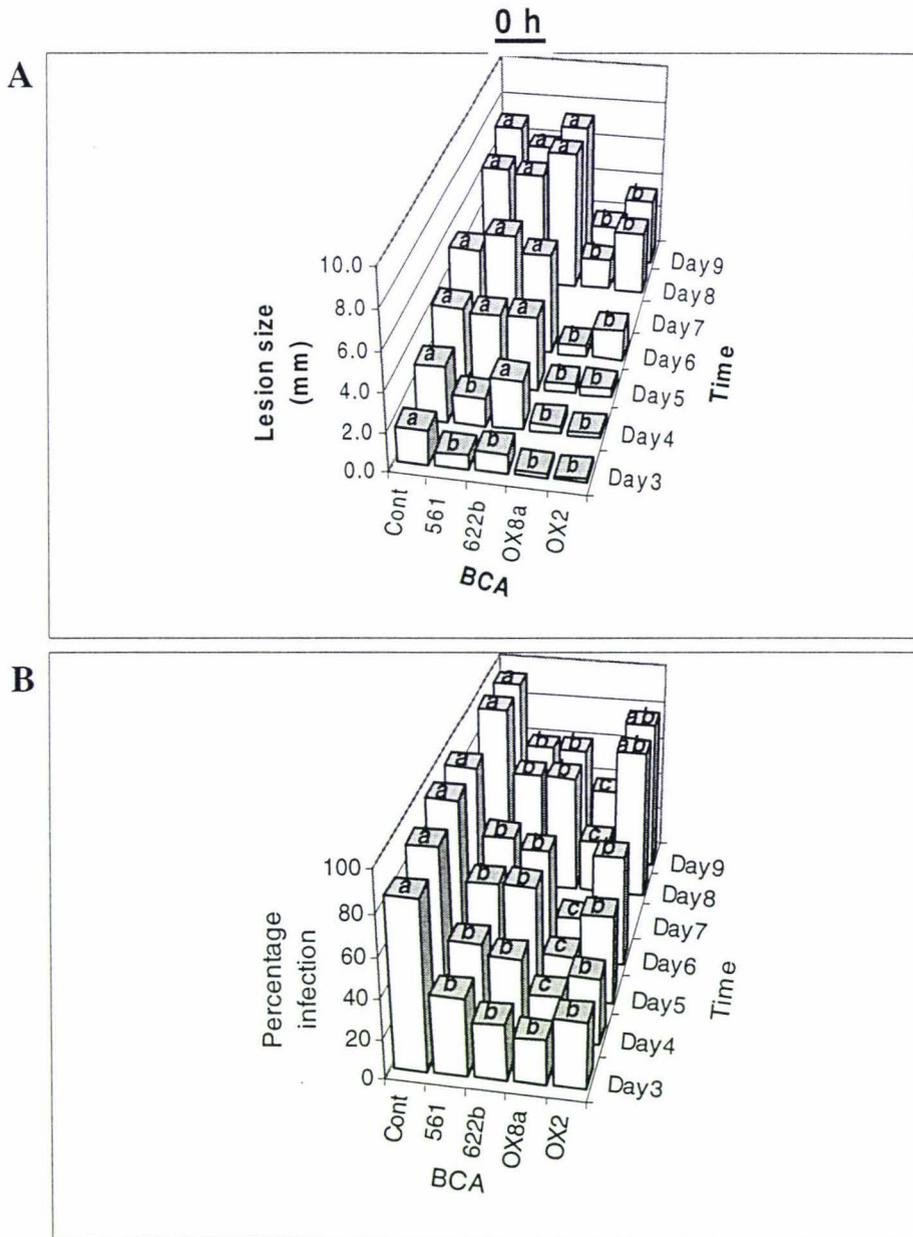


Fig 4-2 Lesion size (A) and percentage infection (B) measured every day from 3<sup>rd</sup> to 9<sup>th</sup> day (except 7<sup>th</sup> day) of inoculation on bean leaf tissue. BCAs added at 0 h after *B. cinerea* inoculation. Bars with same letter in a day is not significantly different ( $P < 0.05$ ).

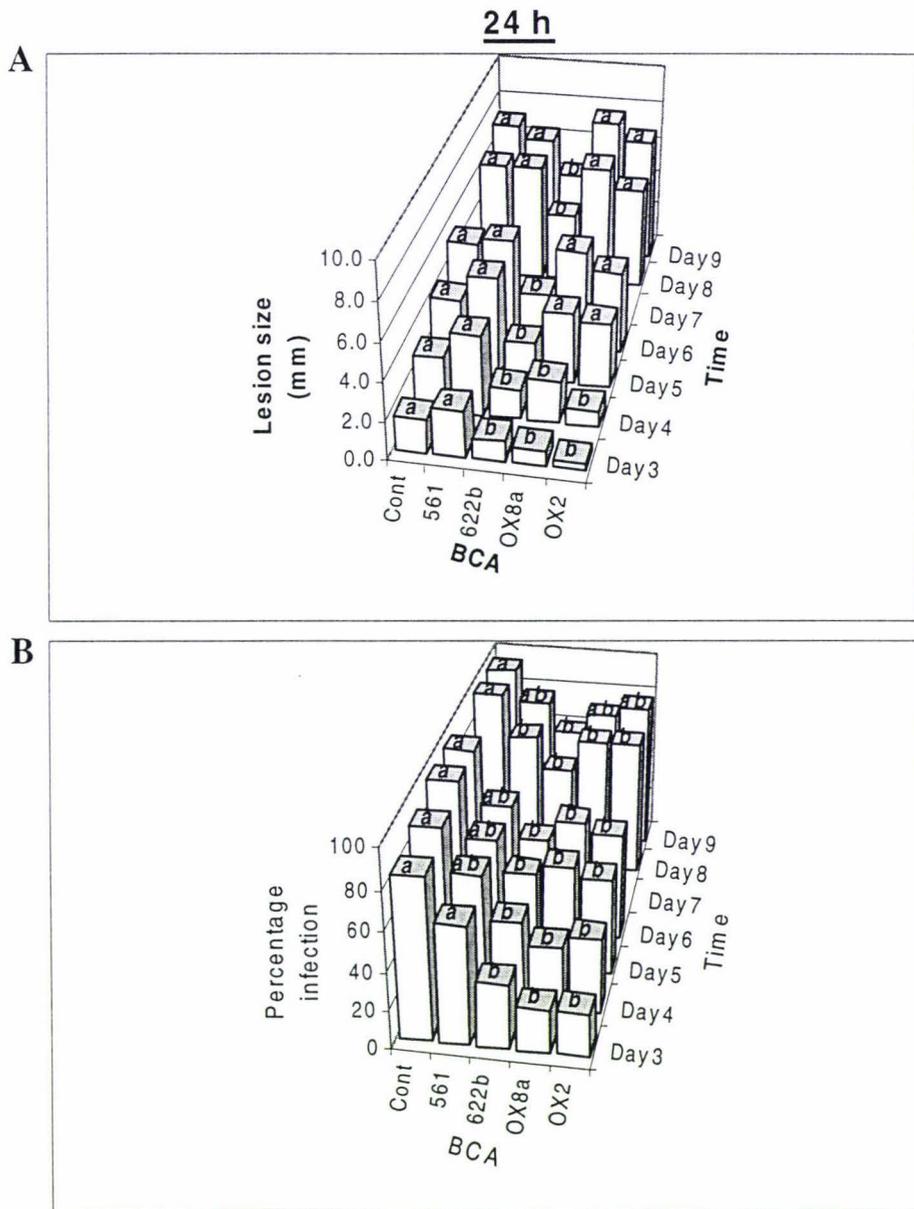


Fig 4-3 Lesion size (A) and percentage infection (B) measured every day from 3<sup>rd</sup> to 9<sup>th</sup> day (except 7<sup>th</sup> day) of inoculation on bean leaf tissue. BCAs added at 24 h after *B. cinerea* inoculation. Bars with same letter in a day is not significantly different ( $P < 0.05$ ).

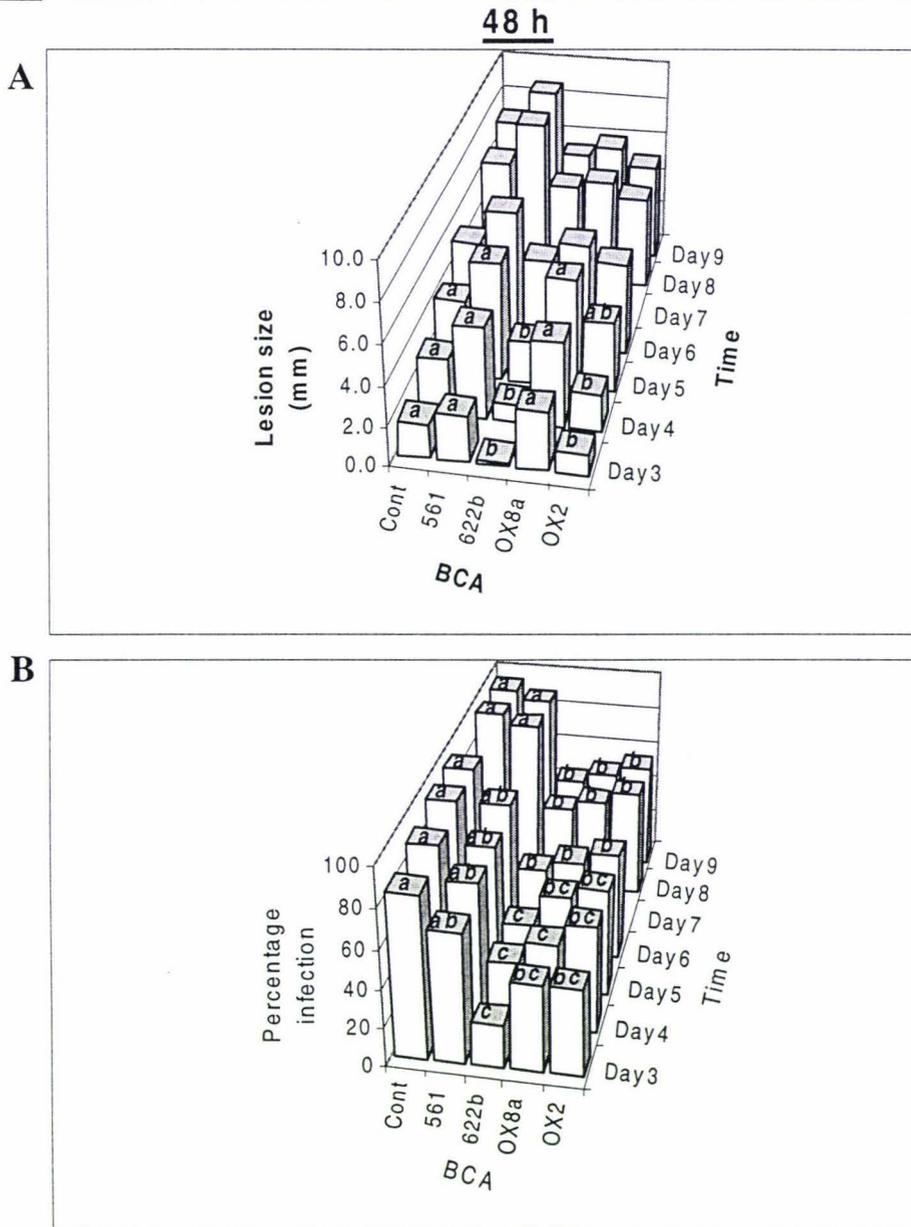


Fig 4-4 Lesion size (A) and percentage infection (B) measured every day from 3<sup>rd</sup> to 9<sup>th</sup> day (except 7<sup>th</sup> day) of inoculation on bean leaf tissue. BCAs added at 48 h after *B. cinerea* inoculation. Bars with same letter in a day is not significantly different (P<0.05).

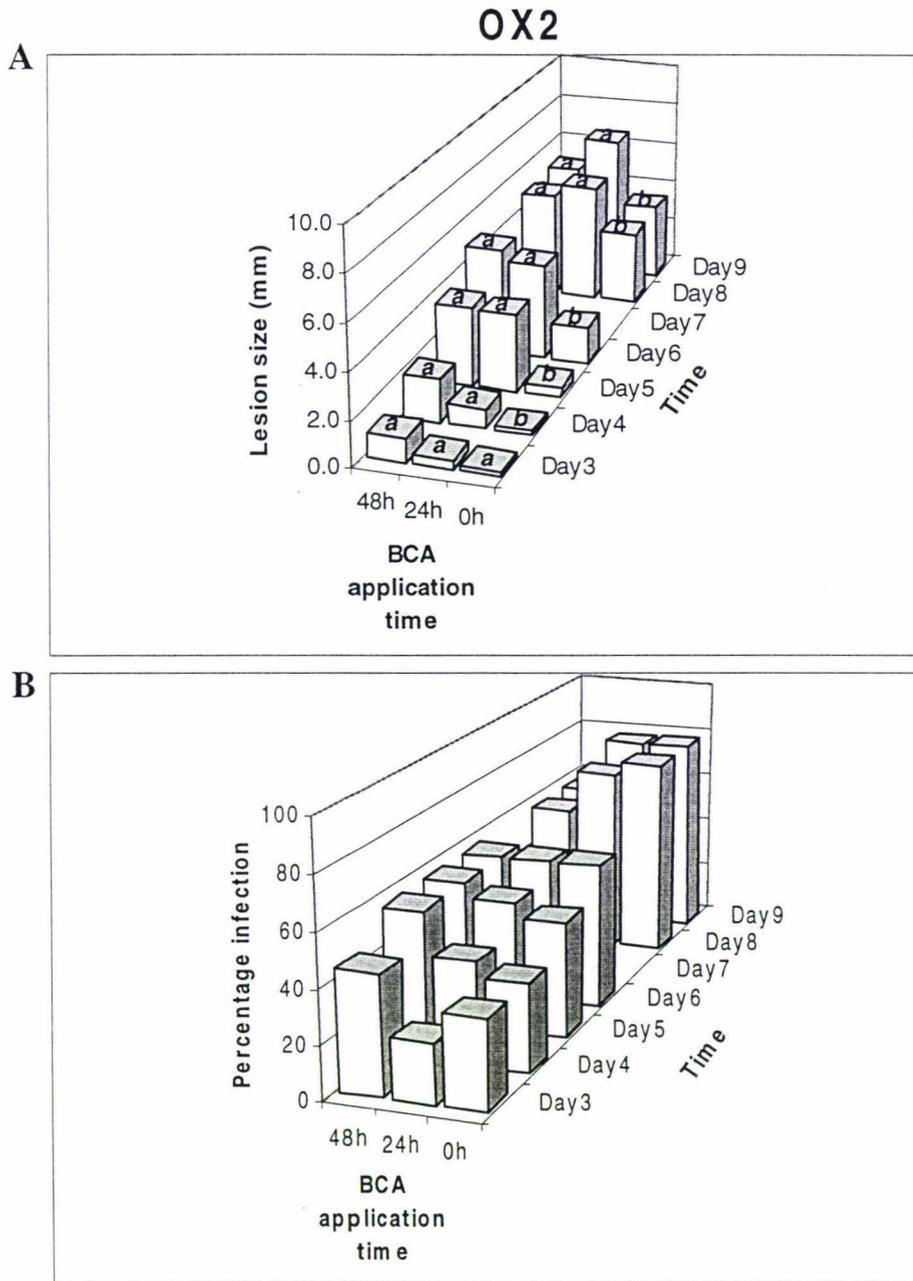


Fig 4-5 Effect on lesion size (A) and percentage infection (B) of *B. cinerea* by different time of application of bacterial isolate OX2 over time on bean leaf tissue. Bars with same letter in a day is not significantly different ( $P < 0.05$ ).

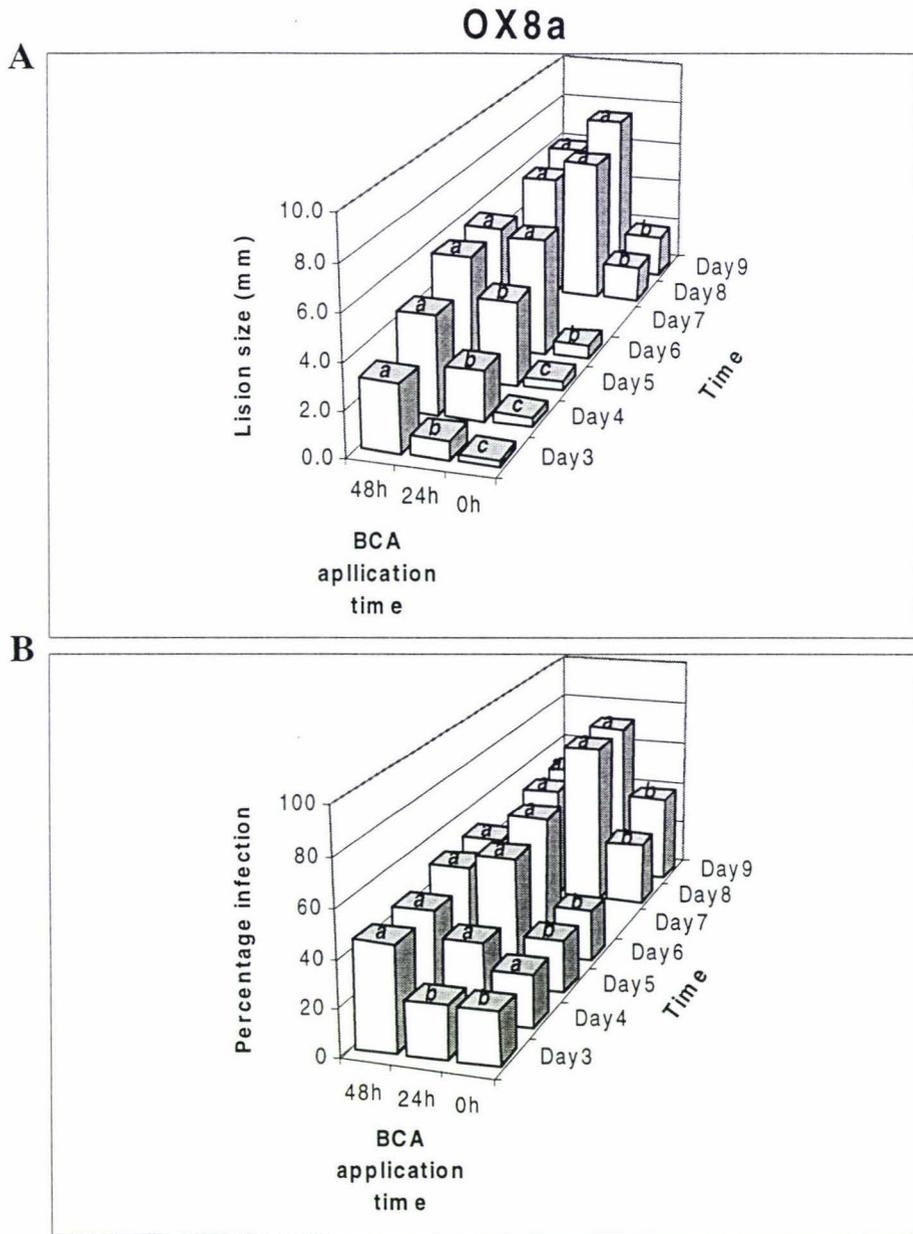


Fig 4-6 Effect on lesion size (A) and percentage infection (B) of *B. cinerea* by different time of application of bacterial isolate OX8a over time on bean leaf tissue. Bars with same letter in a day is not significantly different ( $P < 0.05$ ).

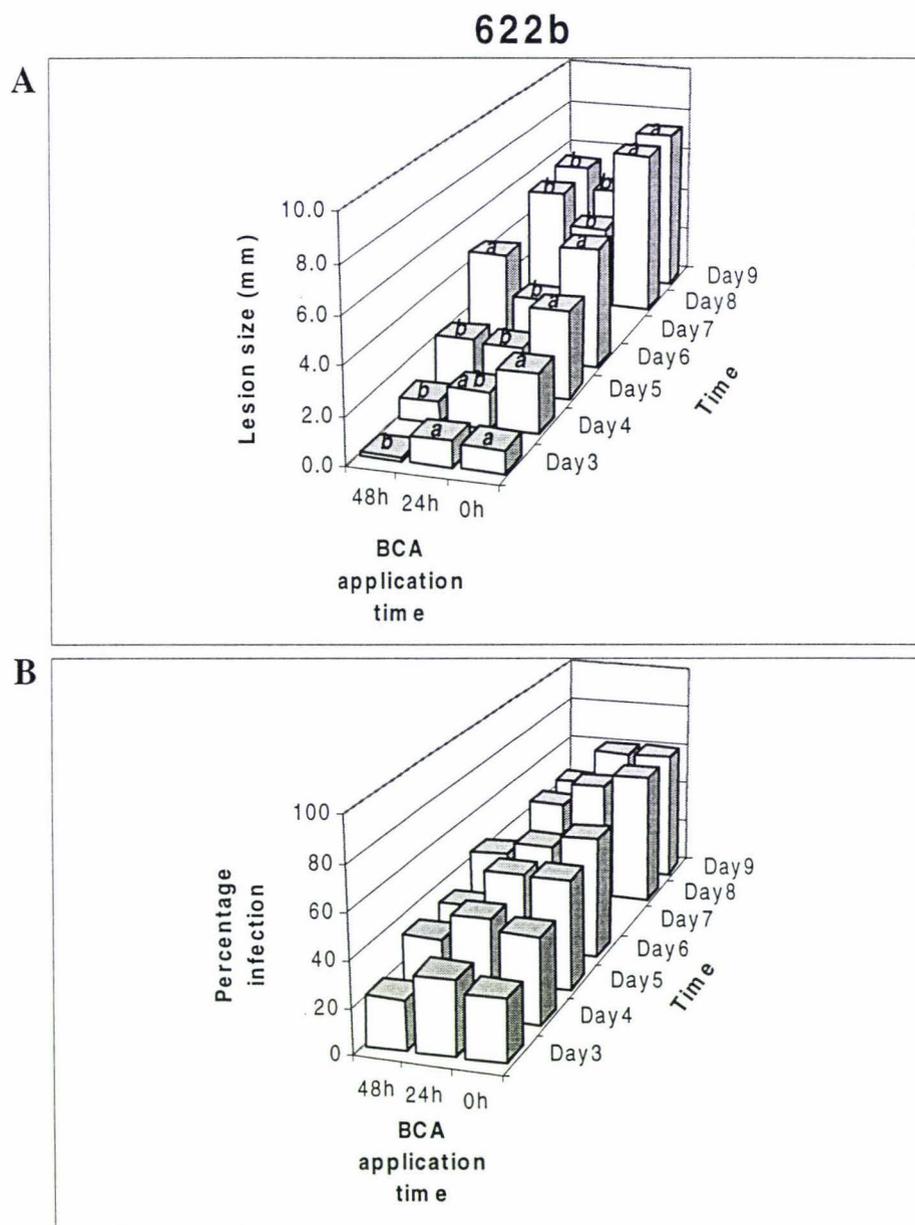


Fig 4-7 Effect on lesion size (A) and percentage infection (B) of *B. cinerea* by different time of application of bacterial isolate 622b over time on bean leaf tissue. Bars with same letter in a day is not significantly different ( $P < 0.05$ ).

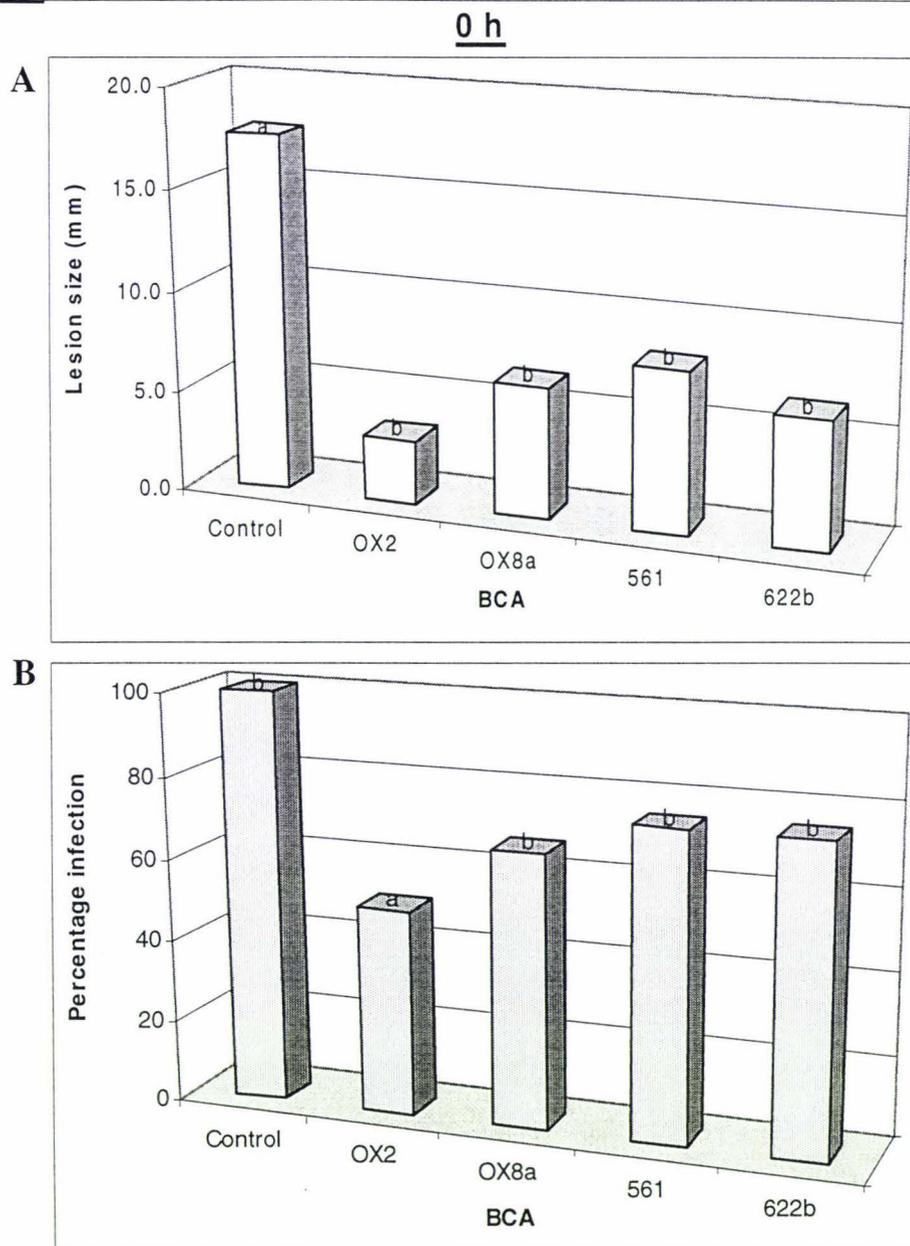


Fig 4-8 Lesion size and percentage infection measured at 5 days after inoculation on lettuce leaf tissue. BCAs added at 0 h after *B. cinerea* inoculation. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

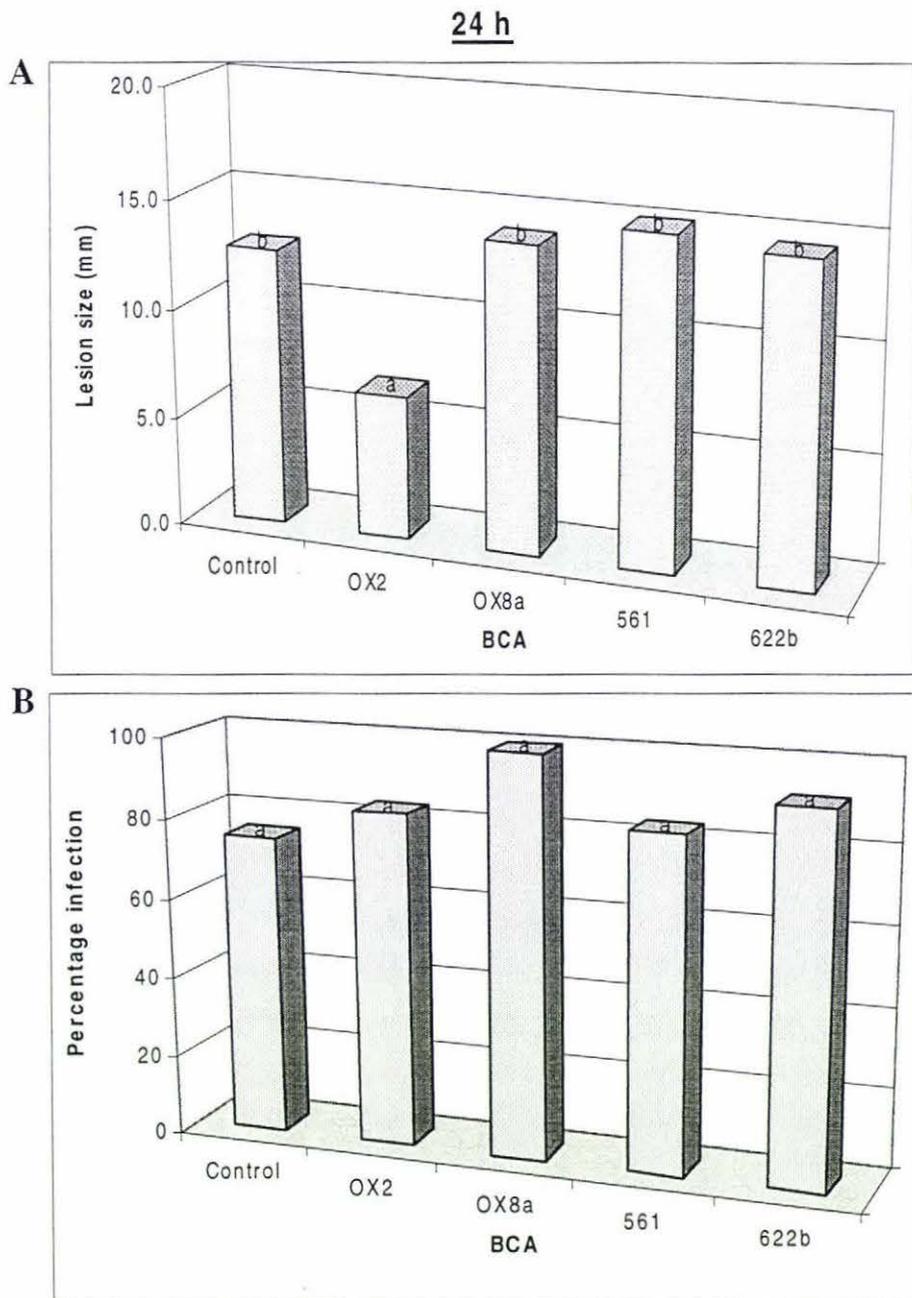


Fig 4-9 Lesion size and percentage infection measured at 5 days after inoculation on lettuce leaf tissue. BCAs added at 24 h after *B. cinerea* inoculation. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

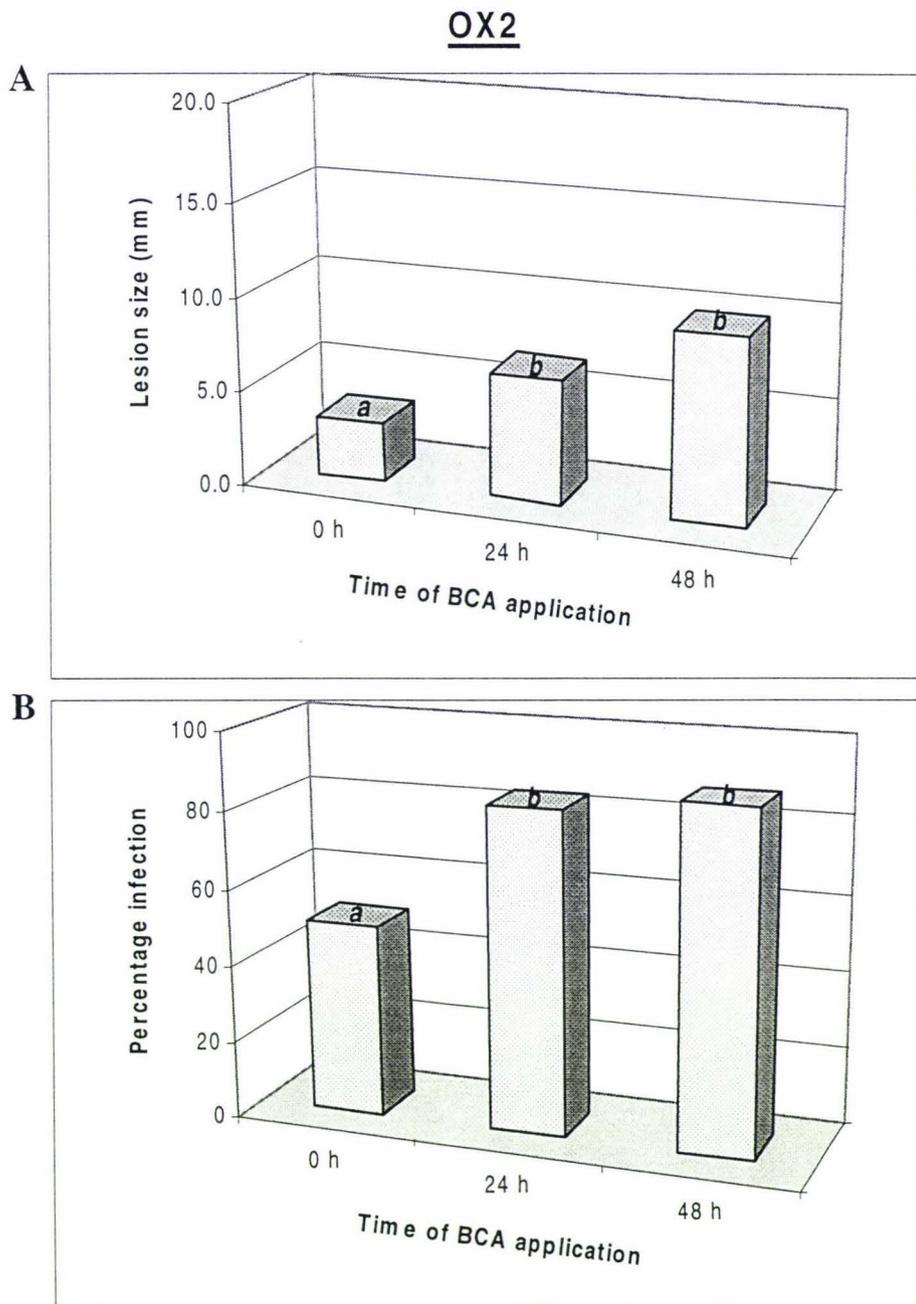


Fig 4-10 Lesion size (A) and percentage infection (B) measured at 5 days after inoculation on lettuce leaf tissue. BCAs added at 0h, 24h and 48 h after *B. cinerea* inoculation. Means accompanied by same letter are not significantly different at  $P < 0.05$ .

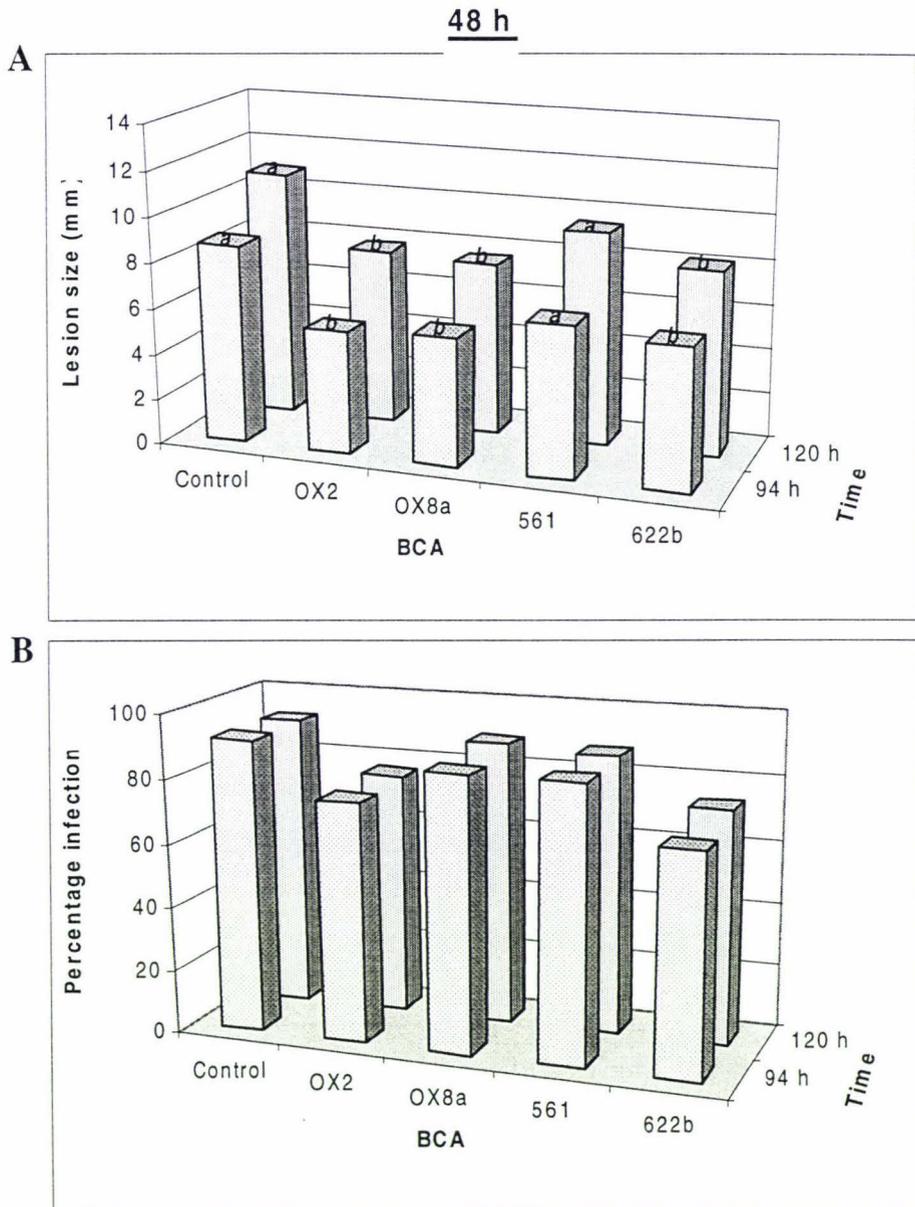


Fig 4-11 Lesion size (A) and percentage infection (B) measured at 4<sup>th</sup> and 5<sup>th</sup> days after inoculation on rose petal tissue. BCAs added at 48 h after *B. cinerea* inoculation. Bars with same letter in an inoculation time is not significantly different ( $P < 0.05$ ).

## 4.5 Discussion

Some yeast and bacteria species have potential as biological control agents for *B. cinerea* on postharvest products, and control of such diseases of kiwifruit and tomato by these agents have been demonstrated successfully by Cook et al. (1997a and 1997b). Some of the same biocontrol agents (*Enterobacter agglomerans*, *Enterobacter aerogenes*, *Candida sake* and *Trichosporon pullulans*) have been used in these experiments and gave significant biocontrol on bean and lettuce leaves, and on rose petals when applied at the same time and/or with a delay after application of *B. cinerea* inoculation. Isolates OX2 (*Enterobacter agglomerans*) and OX8a (*Enterobacter aerogenes*) were effective for reduction of lesion size and percentage infection when applied at the same time or when delayed after application of *B. cinerea* on bean leaf tissue. On the other hand, these isolates were not effective when applied at the same time as *B. cinerea* on rose petal tissues although they were effective when applied after a 48 h delay. Similar results were also obtained by Cook et al. (1997b) on tomato stem tissues when they found that four of the six isolates tested maintained strong biocontrol when applied 48 h after *B. cinerea*. This result is contrary to the observations of Redmond et al. (1987) who demonstrated biological control of *B. cinerea* on rose flowers at 20°C only when the antagonists were applied before the pathogen arrival, and also to reports that biocontrol acting by the inhibition of infection by *B. cinerea* is seldom able to confer protection should the pathogen arrival precede BCA inoculation (Hammer & Marois, 1989; Malathrakis & Kritsotaki, 1992).

Further, isolates OX2 and OX8a proved their ability to reduce infection as measured by disease severity and disease incidence for comparatively long periods if they were present at the time of pathogen arrival on the bean leaf tissue while isolate 622b (*Trichosporon pullulans*) was effective for a long period of time when applied after pathogen arrival. These observation indicated that each biocontrol

agent is specific in its ability to control the pathogen in or on a host even when selected for a particular characteristic (in this case attachment to the pathogen) to suppress the pathogen.

The long period of effectiveness on control of pathogen development by isolates OX2 and OX8a at the time of arrival of pathogen could be due to colonisation of the spores. On the other hand, the late application of isolate 622b must have given control of further development of hyphae by colonisation of the germ tubes of the pathogen rather than by colonisation of the spores.

In lettuce tissue, these four isolates were generally effective only when applied at same time as *B. cinerea* although isolate OX2 was still effective with up to a 24 h delayed application. These observations indicated that the effectiveness of the biocontrol agents were dependent on the crops as well as on time of application relative to pathogen establishment. In addition there was a differential effect of biocontrol isolates on the different host plant. Isolate OX2, for example, effectively controlled infection when applied together with *B. cinerea* or with a delayed application on bean but on lettuce it was only effective with a delayed application.

Although a significant reduction of lesion size was obtained when *B. cinerea* was co-inoculated with OX2, OX8a and 622b on rose petal tissue, the magnitude of the reduction was minimal. In bean, the magnitude of lesion reduction over time was higher when these isolates were applied at the same time as *B. cinerea* than with a delayed application. The results of Hammer and Marois (1989) on magnitude of lesion reduction by application of biocontrol agents on rose petals previously inoculated with *B. cinerea* agreed with the results presented here for rose petals

but were contrary to these on bean leaves when BCAs were applied at same time as *B. cinerea*.

These results indicate that the BCAs of Cook et al. (1997b), successfully used in kiwifruit and tomato, have potential for use in other crops against the same pathogen. The time of application with the different BCAs is an important factor, which can determine the magnitude of disease reduction over time, and it needs to be assessed when considering commercial application of these antagonists to the other crops. Biocontrol agents used in this study have the ability to attach to spores or to mycelia as Cook (1997) demonstrated for their intensive colonisation on spores, germ tubes, and elongated hyphae of *B. cinerea*. Therefore, the action of BCAs applied at the same time (or after) pathogen inoculation can probably be attributed to a mode of antagonism centred on microbial adhesion (Cook et al. 1997a).

The question of a differential reaction of an isolate in different crops towards the same pathogen requires further investigation of host (crop) effects on the biocontrol agents.

## *Chapter Five*

### **Attachment of bacteria and yeast to *B. cinerea* hyphae as a biocontrol mechanism**

#### **5.1 Introduction**

Understanding the mode of action of antagonists is important because it will allow the development of more reliable procedures for the effective application of known antagonists and it should provide a rationale for selecting more effective antagonists (Wilson and Wisniewski 1989). Droby and Chalutz (1991) stated that an effective biological control agent may inhibit plant pathogens by producing antibiotics, by successfully competing with pathogens for nutrients and space, by inducing host resistance, or by interacting directly with the pathogen. Antibiotic production appears to be a major mode of action of many of the antagonists but Baker (1987) has described the other modes of action in detail.

Competition for nutrients is a widespread phenomenon in the interaction between microorganisms on the phylloplane (Droby et al. 1990). The mechanism of antagonism due to nutrient competition can be demonstrated when the pathogen requires an external source of nutrients in order to penetrate the living host. Fokkema (1976) stated that when the pathogen infects the host via dead plant material or wounds, competition for nutrients with other saprophytic fungi could occur during the pathogen's saprophytic growth on these substrates.

Another mode of action, induced host resistance, involves wound healing processes and other defence reaction of the host tissue that may be induced by an antagonist (Droby and Chalutz 1991). Yeast cells applied to a grapefruit peel wound stimulated ethylene production by the tissue (Droby et al. 1989) and phenylalanine ammonia-lyase (PAL) and peroxidase activity (Chalutz et al. 1988b).

Direct parasitism or interaction with the pathogen is another type of mode of action described by Baker (1987) and by Droby and Chlutz (1991). For example, antagonist yeast cells may affect fungal pathogens directly, thereby decreasing infectivity. The antagonist cells tend to attach to mycelium of the fungus, may deplete it of nutrients or may otherwise adversely affect the vital processes of the fungus (Wisniewski et al. 1988b). They may also interfere with the recognition process between the fungus and the host cell wall, thereby interfering with infection (Droby and Chlutz 1991). Using scanning electron microscopy (SEM), other work has shown attachment to fungi by bacteria (Nelson et al. 1986; Wisniewski et al. 1989; Toyota and Kimura 1993; Yang et al. 1994) or yeast (Wisniewski et al. 1988a, 1991). Some yeast and bacteria have been identified and tested for their biocontrol efficacy recently in kiwifruits and tomato by Cook (1997). These antagonists have no production of antibiotics but effectively attached to the *Botrytis* hyphae and reduced its development in the tissue. Some of these bacteria and yeast were used in this study.

## 5.2 Objectives

To observe and evaluate BCA attachment on *B. cinerea* hyphae in vitro and in vivo.

## 5.3 Materials and Methods

### 5.3.1 In vitro bioassay for attachment BCAs

#### 5.3.1.1 *B. cinerea* and BCA preparation

*B. cinerea* isolate Bc-C1 spore suspension was prepared as described in chapter 2 (section 2.1.2) to use in this study. The spore concentration was adjusted to  $1 \times 10^3$  spores per ml.

Bacterial and yeast cell suspensions were prepared as described in Chapter 2. Bacteria cell concentration was measured using a slide counter chamber and yeast cell concentration was determined using a haemocytometer as  $1.5 \times 10^6$  cells per ml of bacterial isolate OX2,  $1.6 \times 10^5$  cells per ml of bacterial isolate OX8a,  $2.0 \times 10^5$  cells per ml of yeast isolate 561, and  $6.5 \times 10^6$  cells per ml of yeast isolate 622b.

#### **5.3.1.2 Media preparation**

Nutrient broth medium was used in this experiment. 3.2 g. of nutrient broth powder (GIBCOBRL) dissolved in 400 ml. of water and 10 ml. of this solution were poured in to forty 25 ml. conical flasks, and autoclaved for 15 minutes at 15 psi and  $121^{\circ}\text{C}$ .

#### **5.3.1.3 Inoculation and incubation**

Ten  $\mu\text{l}$ . of *B. cinerea* inoculum, was pipetted in to nutrient broth medium in 25 ml. conical flasks and each BCA was applied separately at the same time, and a 24 h delayed after *B. cinerea* inoculation as a 10  $\mu\text{l}$  drop of cell suspension. BCA cultures were incubated at  $20^{\circ}\text{C}$  on a continuous shaker at 150 rpm for 3-5 days after *B. cinerea* inoculation. After incubation, flasks were observed for the change of colour of media, mycelial growth and BCA attachment to mycelium.

#### **5.3.1.4 Dark field microscopy**

A small piece of mycelium was taken from each flask, placed in a watch glass and two or three times washed with SDW to remove unattached BCAs and medium. The washed mycelium was mounted on a slide in SDW and observed under a transmission microscope ("Reichert" GEO. W. Wiltion & Co Ltd) set for dark field background and examples of BCA attachment to *B. cineria* hyphae were photographed.

### 5.3.2 In vivo bioassay for attachment BCAs

Aliquots of the *B. cinerea* spore suspension ( $1 \times 10^6$  spores per ml) and BCA cells suspensions prepared above were used in this assay.

Bean and lettuce leaves, and rose petals were prepared for inoculation as described in Chapter 2 (section 2.3) and Chapter 3 (Section 3.3.1).

Detached leaves and petals were placed in a tissue culture plate cover (see Chapter 4, Section 4.3) and inoculated with 5  $\mu$ l of *B. cinerea* spore suspension after making a slight wound in each site. Bean and lettuce leaves were inoculated in six sites per leaf and rose petals in two sites per petal then incubated at 20<sup>0</sup>C for 24 h. After incubation 5  $\mu$ l of BCAs was applied to each *B. cinerea* inoculated site and were again incubated for another 24 h in the same condition. Two leaves or petals were used for each BCA candidate.

Incubated tissues were cut in 1 mm<sup>2</sup> pieces which covered wounded, inoculated areas and were placed in a universal bottle to fix with glutaraldehyde (see Chapter 2 Section 2.4.3) which induces fluorescence of *B. cinerea* hyphae and BCAs.

## 5.4 Results

### 5.4.1 Biocontrol and BCAs attachment assay – in vitro

#### 5.4.1.1 Biocontrol of *B. cinerea*

Media inoculated with *B. cinerea* spores and BCAs at same time become a pale white colour and no spore germination had occurred after 48 h incubation.

Medium inoculated with *B. cinerea* only (control), became a yellowish colour and spores were well germinated and produced mycelia. BCAs applied at 24 h after *B. cinerea* inoculation, differentially affected colour of the media and of mycelial

growth. Spore germination and a little mycelial growth of *B. cinerea* was observed in all media to which these isolates were applied but, that with isolate 561 produced abundant mycelial growth and that with isolate 622b became yellow. Isolates OX2 and OX8a changed the medium colour to pale white (Table 5-1).

#### **5.4.1.2 Dark field microscopy on attachment assay**

BCA attachment to *B. cinerea* hyphae was observed by dark field microscopy. All four BCAs applied 24 h after *B. cinerea* inoculation showed some attachment to hyphae of *B. cinerea*. Yeast isolate 561 had abundant attachment to the hyphae (Fig 5-2A) while isolate 622b showed sparse attachment only (Fig 5-2B).

Of the bacterial BCAs, isolate OX8a showed more intensive attachment (Fig 5-1B) than isolate OX2 which appeared to cause amorphous extracellular materials to be released from the hyphae (Fig 5-1A).

#### **5.4.2 Biocontrol and BCAs attachment assay – in vivo**

Yeast isolates 561 and 622b added on lettuce and bean leaf tissue respectively, and Bacterial isolate OX8a was added to lettuce leaf tissue and attached to *B. cinerea* hyphae as observed by confocal microscopy (Fig 5-3). However, attachment by OX8a was laser than that observed *in vitro* [Fig 5-1 (B) Fig 5-3 (B)]. Fig 5-4 (A) shows the poor attachment by yeast isolate 561 which showed aggressive colonisation around hyphae *in vitro* [Fig 5-2 (A)]. Isolate 622b showed the same trend in attachment (sparse adhesion) to the hyphae both *in vitro and in vivo* [Figs 5-2 (B) and 5-3 (B)].

Table 5-1 Observation of BCA attachment to hyphae of *B. cinerea* and other interactions at 0 h and 24 h after application of BCAs in vitro.

BCA	Application time	Colour of media	Spore germination and mycelial growth	BCA attachment
Control (Media only)	0 h	Very light yellow and translucent	-	-
Control ( <i>B. cinerea</i> only)	0 h	Yellow and slight translucent	Spore germinated and abundant mycelial production	-
OX2	0 h	Pale white and no translucent	No spore germination and spores were coagulated in the media suspension	Very few attachment to spores
OX8a	0 h	Pale white and no translucent	No spore germination and spores were coagulated in the media suspension	Bacterial attachment to spores
561	0 h	Pale white but translucent	No spore germination and spores were coagulated in the media suspension	No attachment
622b	0h	No colour change	No spore germination	No attachment
OX2	24 h	Pale white and no translucent	Spores were germinated and a small amount of mycelial production.	Very few bacterial attachment to mycelia.
OX8a	24 h	Pale white and no translucent	Spores were germinated and a small amount of mycelial production.	Considerable amount of bacterial attachment to hyphae and spores.
561	24 h	Pale white but translucent	Spores were germinated and abundant mycelial production.	Aggressive yeast attachment to mycelia.
622b	24 h	Yellow but translucent	Spores were germinated and fair amount of mycelial production.	Sparse yeast attachment to mycelia.

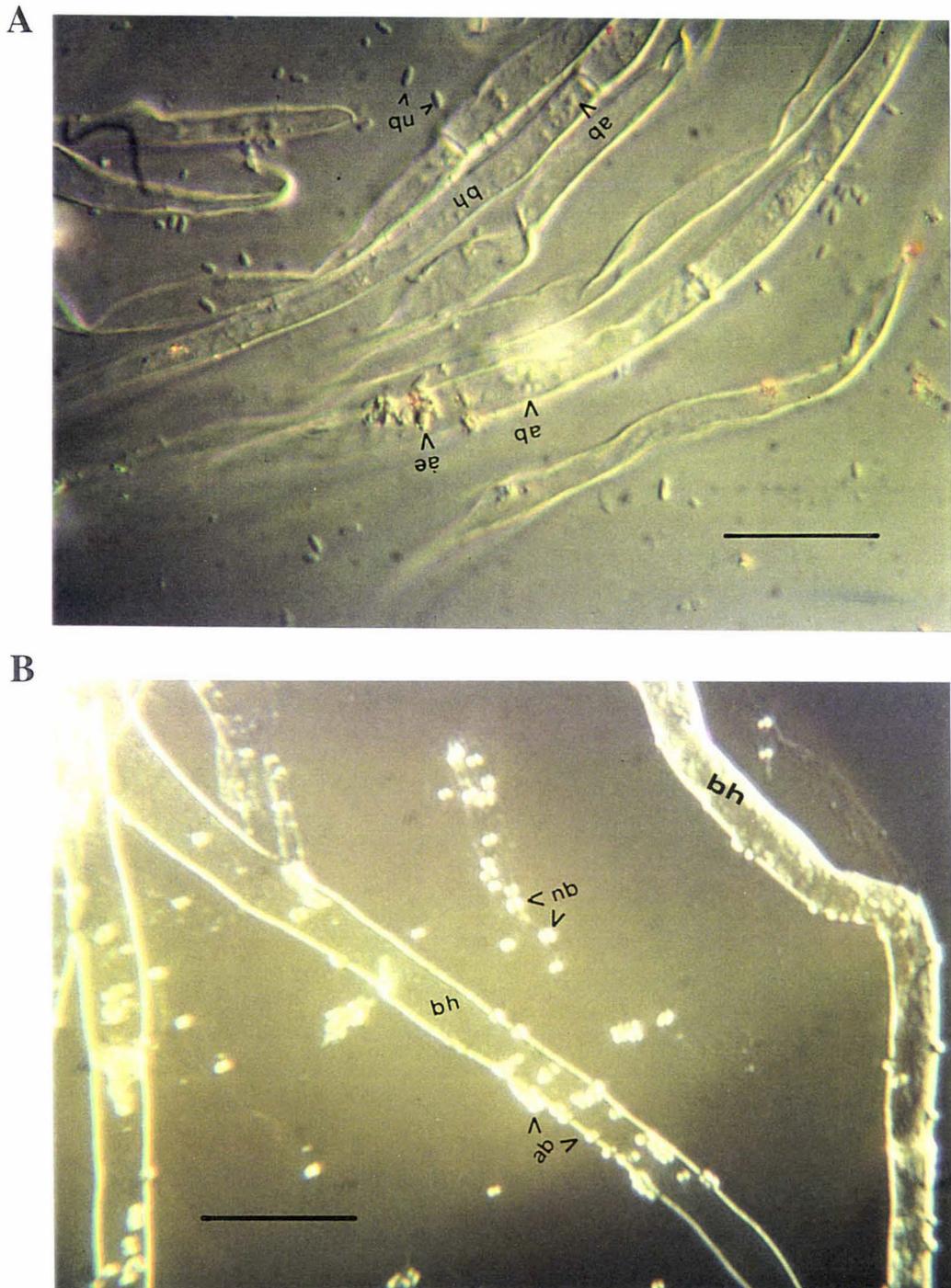


Fig 5-1. Bacterial isolates OX 8a (A) and OX2 (B) attached to *B. cinerea* hyphae after 72 h incubation in NB medium at 20°C and observed under dark field microscopy. Bacteria added to the medium 24 h after *B. cinerea* spores. ab: Bacteria attached to the mycelial cell wall, nb: Non-attached bacteria scattered in the medium, bh: *B. cinerea* hyphae washed with SDW before mounted on slide, ae: amorphous extra-cellular material. (bar 20 µm).



Fig 5-2. Yeast isolate 561 (A) and 622b (B) attached to *B. cinerea* hyphae after 72 h incubation in NB medium at 20<sup>0</sup>C and observed under dark field microscopy. Yeast added to the medium 24 h after *B. cinerea* spores. ay: Yeast attached with the mycelial cell wall, bh: *B. cinerea* hyphae washed with SDW before mounted on slide. (bar 20  $\mu$ m).

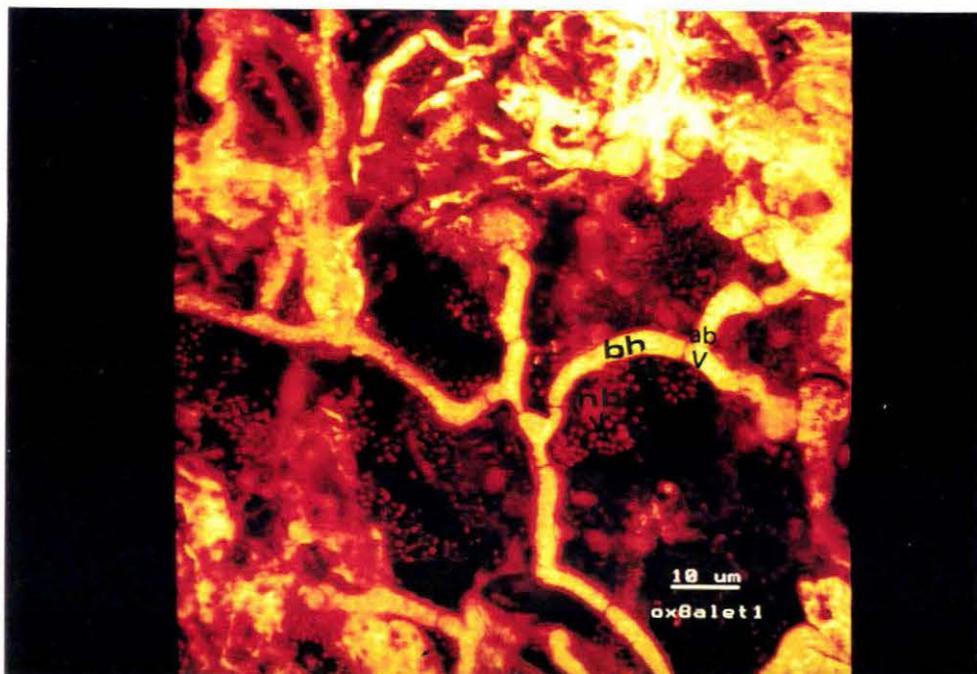


Fig 5-3 Confocal micrograph of bacteria isolate OX8a attached to hyphae of *B. cinerea* on lettuce leaf tissue treated with Glutaraldehyde. Bacteria applied 24 h after the tissue was inoculated with *B. cinerea*. ab: Bacteria attached with the mycelial cell wall, nb: Non-attached bacteria scattered on the surface of the leaf tissue, bh: Glutaraldehyde treated *B. cinerea* hyphae.

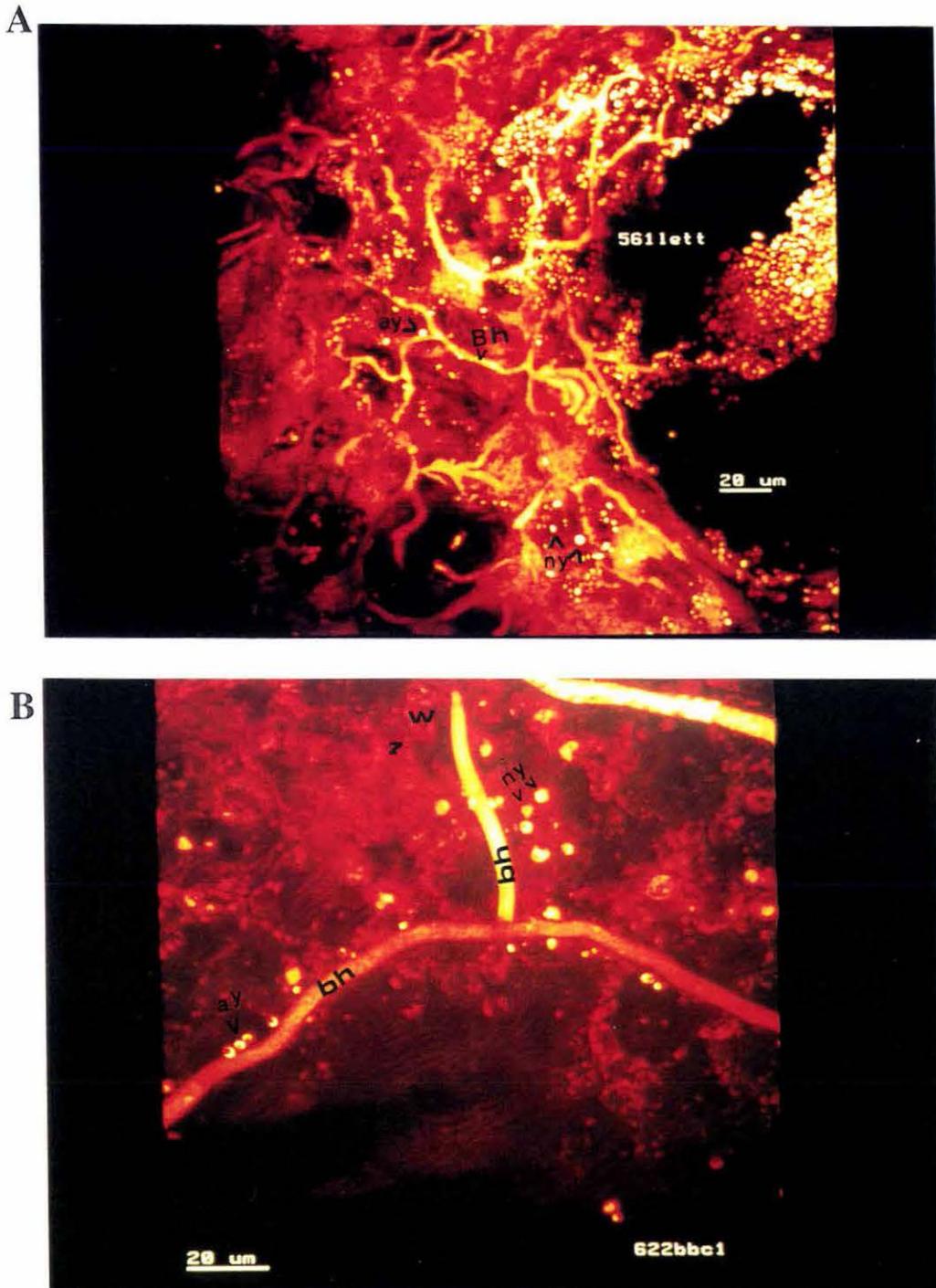


Fig 5-4 Confocal micrograph of Yeast isolate 561 (A) and 622b (B) attached to hyphae of *B. cinerea* on bean (B) and lettuce (A) leaf tissue treated with Glutaraldehyde. Yeast applied 24 h after the tissue was inoculated with *B. cinerea*. ay: Yeast attached with the mycelial cell wall, ny: Non-attached yeast scattered on the surface of the leaf tissue, bh: Glutaraldehyde treated *B. cinerea* hyphae.

## 5.5 Discussion

The isolates of bacteria and yeast used in this study, had already been examined with several other bacteria and yeast isolates for their ability to adhere to *B. cinerea* hyphae *in vitro* and *in vivo* on autoclaved bean leaves and on tomato stem pieces by Cook (1997). Electron micrographs showed aggressive colonisation of bacterial isolate OX 8a, end-on adhesion of isolate OX2 and tentative adhesion of yeast isolate 622b of the hyphal surface of *B. cinerea* on tomato stem tissue (Cook et al. 1997b and Cook 1997). Malajczuk et al. (1977), Nesbitt et al. (1978) and Homma (1984) observed cell crowding in other fungal-bacterial interactions in light microscopic studies *in vitro*. Attachment bacteria have also been observed by Korsten et al. (1995) on avocado post harvest pathogens.

Isolates 561 and OX8a were particularly good colonisers along the pathogen hyphae *in vitro*. Isolates OX2 and 622b showed little attachment *in vitro* or *in vivo*, but they did control mycelial growth. Similar results were obtained by Cook (1997) and he pointed out that attachment alone should not be viewed as the sole mechanism, through which biocontrol is expressed. Indeed, the isolates tested in this study have given biocontrol whether they had few or many attachment either *in vitro* or *in vivo*. *In vivo* study (see Chapter 4) showed no significant biocontrol by isolate 561 but significant biocontrol was observed by isolate OX8a. Both isolates are good hyphal colonisers, thus that good colonisation does not necessarily indicate good biocontrol result. On the other hand, isolates OX2 and 622b are poor colonisers but gave effective biocontrol. These observations indicate that increasing attachment ability could increase the biocontrol mechanism functioning through attachment.

Mycelial morphological changes were reported in previous studies when bacteria or yeast – fungal interaction occurred. Pitting and holes in the *B. cinerea* hyphal

surface was observed when *P. guilliermondii* interact with hyphae (Wisniewski et al. 1991) and growth destruction of germ tubes from *C. sativus* (Fradkin and Patrick 1985). The same kinds of morphological changes of mycelia were observed in vitro in this study in the presence of isolate OX2. *B. cinerea* hyphae were pitted, distorted and activated to segregate some extra-cellular compounds. In some cases, bacterial cells were observed inside the mycelial cells under the light microscope.

## Chapter Six

### Biomass measurement of *Botrytis cinerea* using Laser Scanning Confocal Microscope

#### 6.1 Introduction

An alternative biocontrol agent (BCAs) selection criterion was developed by Cook et al. (1997a) and the function of the BCAs was based on the ability to adhere to conidia or hyphae of *B. cinerea*. A test for biocontrol ability against the pathogen, which can be done within a short period, would be extremely useful.

Conventional testing procedure by measuring disease severity and incidence can be a time consuming process. For example, assessment of biocontrol effectiveness in kiwifruit can take up to three months to reach a final conclusion (P.G. Long, Personal communication).

It has been often assumed that biocontrol of a pathogen is closely related to reductions in its biomass. A measure of the biomass of a pathogen in its target substrate (plant materials) could be a very useful tool for the quick estimation of biocontrol efficiency. The biomass of a pathogen within the biocontrol interaction has been measured using both direct and indirect methods by a number of workers in various substrates. Food-borne fungi were quantified by counting cfu and measuring mycelial length through microscopic observation (Jarvis et al. 1977; Schnürer 1993). Bakken and Olsen (1983) estimated biomass of several isolates of bacteria and fungi with respect of buoyant density and dry weight. Fungal biomass has been measured indirectly by measuring differences on chemical composition between fungi and their substrate. One such method is the quantification of ergosterol (Seitz 1977, 1979), a fungus specific membrane lipid (Weete 1980). The enzyme-Linked Immunosorbent Assay (ELISA) technique was used to quantitatively detect *Penicillium* spp. in apple (Notermans et al. 1986), *Verticillium dahliae* in potato (Plasencia et al. 1996), and *Humicola lanuginosa* in rice grains (Dewey et al. 1989, 1992). However, a more absolute measure of

fungal biomass, such as fungal dry weight or fungal volume through microscopical determination of hyphal volume in a substrate could give direct estimation without further complication of chemical measurement of biomass.

Although, estimation of fungal dry weight is a more absolute measure of fungal biomass, it is not possible when fungi are growing in a solid substrate, except agar (Notermans et al. 1986). Hence, the method is not applicable when fungi are grown in plant materials. The only other alternative is to measure fungal biomass through microscopical determination of hyphal volume using advanced computerised microscopic technique. Kessel et al. (1996) used image analysis of tissues labelled with the MAB BC-KH4 and immunofluorescence for spatial analysis and quantification of *B. cinerea* biomass after interacting with BCA (*Ulocladium atrum*) on necrotic lily leaves.

The Confocal microscope and associated computer packages could be used to estimate fungal biomass within plant tissue. This is because the images produced by the confocal microscope cover the area that detracts from the focal plane in normal microscopy with optimal contrast and resolution (Kwon et al. 1993). In addition to a clear image from a focal plane, it has the ability to yield rapid and accurate three-dimensional perspectives. Further, confocal microscopy provides an opportunity to quantitatively measure organelle distributions (Kwon et al. 1993). The three dimensional image is transferred to an image analysing computer which can estimate fungal hyphal volume automatically.

Successful image production in confocal microscopy depends on stains that cause fungal hyphae within the plant tissue to fluoresce. Different stains or fixatives have been used by various workers to fluoresce fungal hyphae and thus obtain a clear image from the confocal microscope. Monoclonal antibody-FITC conjugate (Cole et al. 1996), 0.05% trypan blue in lactophenol (Wei et al. 1997) and

glutaraldehyde, a fixative (Singh et al. 1997) were used with different fungi in different plant materials and clear images from confocal microscopy have been reported.

## **6.2 Objective**

To determine whether the biomass of *B. cinerea* can be estimated before and after application of BCAs using confocal microscopy after glutaraldehyde fixation to stimulate fluorescence of fungal hyphae in plant tissue.

## 6.3 Materials and Methods

### 6.3.1 *B. cinerea* inoculation and BCAs application

Fully developed bean leaves and lettuce leaves were taken from plants grown in the plant growth unit as described in Chapter 2, Section 2.3. A single leaf was placed in a culture plate cover (see Chapter 3 for details) and inoculated with 2 $\mu$ l of *B. cinerea* spore suspension containing approximately 100 spores per site using a micro pipette. Likewise, 6-8 sites per bean leaf and 4-6 sites per a lettuce leaf were inoculated. All inoculated sites were wounded by pressing gently on the tissue surface with the end of a glass rod. Inoculated leaves were then, incubated for 12-24 h (see Chapter 3 for details). After incubation, 5 $\mu$ l suspension of each of isolates OX2 ( $1.5-2.4 \times 10^9$  cells per ml), OX8a ( $1.8-2.3 \times 10^9$  cells per ml), 561 ( $1.0-5.0 \times 10^8$  cells per ml) and 622b ( $96.2 \times 10^7 - 1.4 \times 10^8$  cells per ml) was applied to each inoculated site. BCAs were not applied to the control treatment. After application of BCAs, leaves were incubated for another 12-24 h under the same conditions as previously.

### 6.3.2 Fluorescent treatment

After incubation, the tissue was treated with BC-KH4 monoclonal antibody, trypan blue and glutaraldehyde separately as described in Chapter 2, Section 2.4. The treated specimens were immersed in phosphate buffer, trypan blue lactophenol and buffered sucrose solution on a glass slide respectively and were then placed in desiccator vacuum chamber for 6 to 12 h to remove the air bubbles from the tissues. The specimens then mounted on the glass slides with CITI fluor mounting medium.

### 6.3.3 Confocal microscopic examination

The specimens were examined in a laser scanning confocal microscope (Model Leica TCS/NT) using a different wavelength for each treatment as described in

Chapter 2, Section 2.5. Each specimen was observed under 1x40 (oil) or 1x100 (oil) objectives lenses and the images were created from 15 to 30 optical sections taken 5 $\mu$ m apart in depth and then recombined to form the final three dimensional image. Some images were photographed by a built in camera in the confocal microscopic system. All of those images obtained in good condition were saved on a hard or a floppy diskette to transfer the image to the analysing computer when required.

#### 6.3.4 Image analysis

Images with Multiple optical sections were transferred to an image analysis computer (Indy Silicon Grafics Computer System) to be analysed for hyphal volume. Image Space Ver. 3.10 programme was used for the analyses. Each optical section of the image with fungal hyphae and plant tissue was obtained on the screen and the threshold manipulated to remove the marginal difference between fungal hyphae and some fluorescing plant tissue cells in order to get an accurate measurement of hyphal volume (Fig 6-4). Fluorescing plant tissue cells that are closely connected to the fungal hyphae were removed manually using the paintbrush programme. This manipulation was done on each optical section of the image and reduced the background effect due to fluorescing plant tissue around fungal hyphae. Then, the total volume of fungal hyphae was measured automatically in pixel or cubic micrometer by giving the appropriate commands to the computer.

### 6.4 Results

#### 6.4.1 Fluorescent treatment

*B. cinerea* hyphae treated with monoclonal antibody BC-KH4 in the bean tissue fluoresced poorly on the section of hyphae close to the conidia and other parts of the hyphae either did not fluoresce or fluoresced poorly. Trypan blue (0.05%) in

lactophenol treated specimens did not produce a clear image of fungal hyphae due to lack of fluorescence under confocal microscopy examination. The fungal hyphae in the specimen treated with Trypan blue (0.05%) fluoresced better than the specimen treated with BC-KH4 antibody. Glutaraldehyde treated specimens produced a very clear image of fungal hyphae (Fig 6-1 to 6-3).

Glutaraldehyde rendered the hyphae strongly auto-fluorescent and the background plant cells slightly auto-fluorescent.

Confocal micrographs were obtained from glutaraldehyde treated bean and lettuce leaf specimens inoculated with *B. cinerea* alone (fig 6-1) and co-inoculated with *B. cinerea* and BCAs OX2 {Fig 6-2 (A)}, OX8a {Fig 6-2 (B)}, 561 {Fig 6-3 (A)} and 622b {Fig6-3 (B)}.

Healthy and well developed hyphae were observed in *B. cinerea*-only inoculated bean and lettuce tissue (Fig 6-1) while those in inoculated tissues to which BCAs were applied 12-24 h later showed unhealthy and distorted hyphae, thin in size compared with healthy hyphae (Fig 6-2 and 6-3).

#### 6.4.2 Image analysis

The control treatment (inoculated with *B. cinerea* only) and those co-inoculated with BCAs OX2, OX8a and 561 on bean leaves and BCAs OX8a, 561 and 622b on lettuce leaves were analysed and the hyphal volume of *B. cinerea* measured. In both types of leaf, *B. cinerea*-only inoculated tissues had a large biomass of *B. cinerea* per unit area compared with BCA treated tissues (Table 6-1 and 6-2). Among BCA treated tissues, OX8a reduced fungal biomass more than OX2 in bean leaves and in lettuce leaves, and 622b reduced fungal biomass more than 561 (Table 6-1 and 6-2). Since only a limited number of samples could be measured

due to time constraints, the results were not statistically analysed. However, the difference between control and treatments were very large (10-40 fold on bean leaves and 2-3 fold on lettuce leaves).

Table 6-1. Hyphal volume of *B. cinerea* per unit area of inoculated bean leaf tissue measured by LSCM and image analysis. Bacterial BCAs OX2 and OX8a were applied 12-24 h after inoculation. All tissues were fixed with glutaraldehyde 24-36 h after *B. cinerea* inoculation.

BCAs / <i>B. cinerea</i>	Biomass of <i>B. cinerea</i>	
	Hyphal volume (Pixel)	Hyphal volume ( $\mu\text{m}^3$ )
<i>B. cinerea</i> alone	1121844	430967.59
<i>B. cinerea</i> with OX2	59185	48315.09
<i>B. cinerea</i> with OX8a	21774	15691.01

Table 6-2. Hyphal volume of *B. cinerea* per unit area of inoculated lettuce leaf tissue measured by LSCM and image analysis. Yeast BCAs 561 and 662b applied 12-24 h after inoculation. All tissues were treated with glutaraldehyde 24-36 h after *B. cinerea* inoculation.

BCAs / <i>B. cinerea</i>	Biomass of <i>B. cinerea</i>	
	Hyphal volume (Pixel)	Hyphal volume in $\mu\text{m}^3$
<i>B. cinerea</i> alone	428710	85479.06
<i>B. cinerea</i> with 561	108291	33490.07
<i>B. cinerea</i> with 622b	118923	23989.35

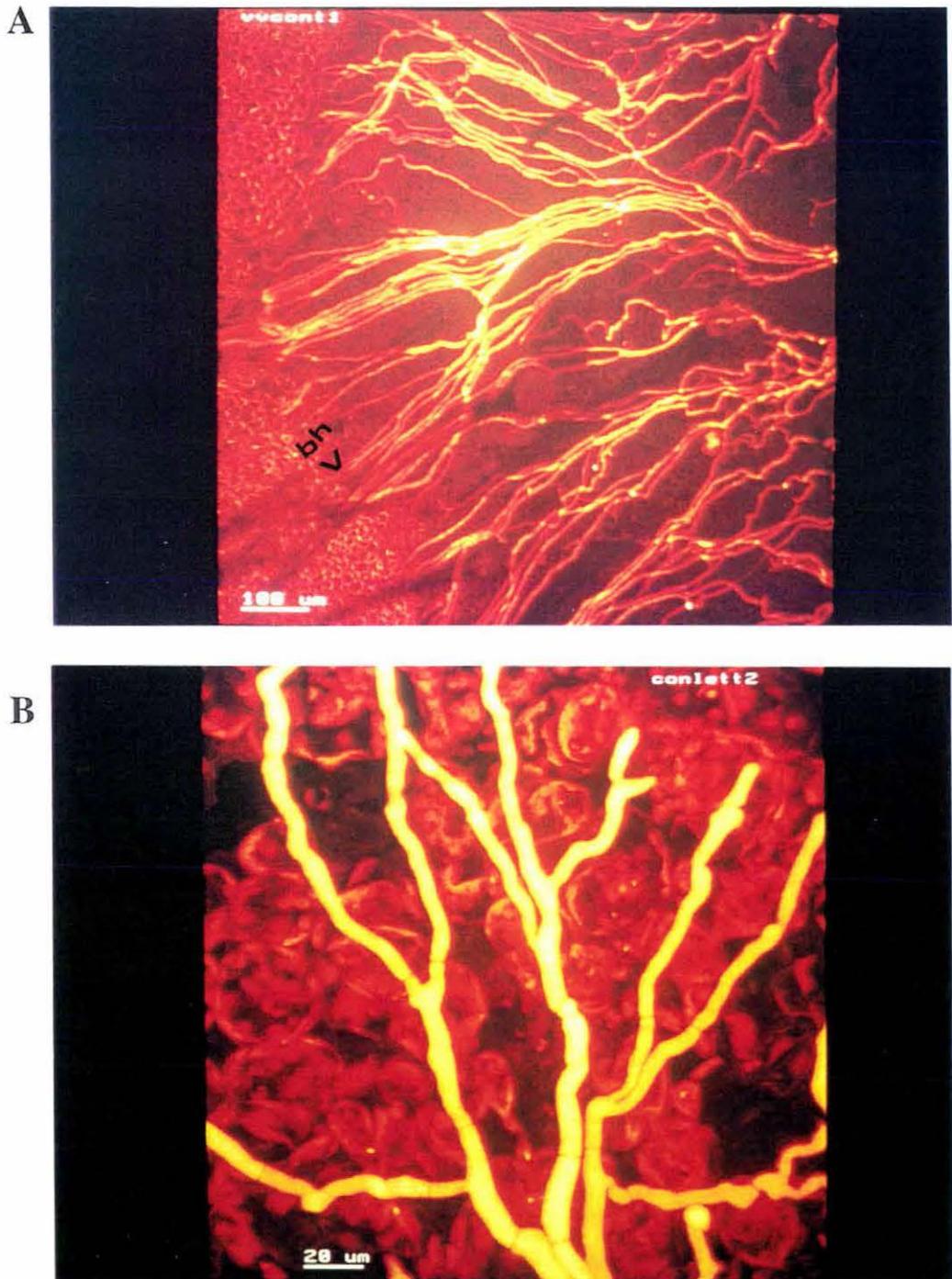


Fig 6-1 Confocal micrograph of *B. cinerea* inoculated onto a wounded lettuce leaf surface and incubated for 72 h. Specimen treated with glutaraldehyde. A) Hyphal development and penetration into the tissue (bh). B) A portion of the mycelium from the advance margin of the infection.

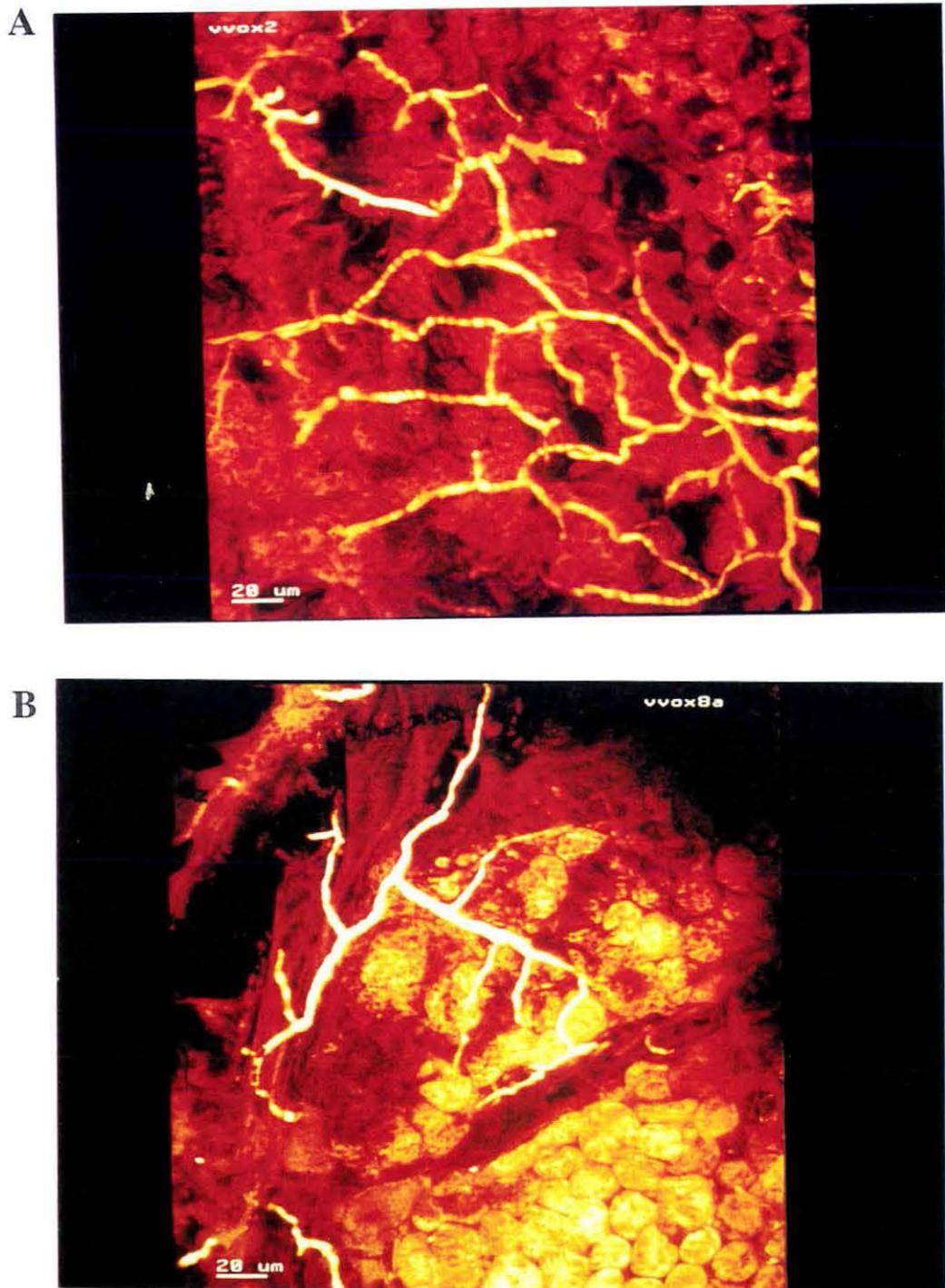


Fig 6-2 Confocal micrograph of *B. cinerea* hyphal development following addition of bacterial isolates OX2 (A) and OX8a (B) application to the surface of bean leaf tissue. BCAs applied 24 h after *B. cinerea* inoculation. Specimen treated with glutaraldehyde.

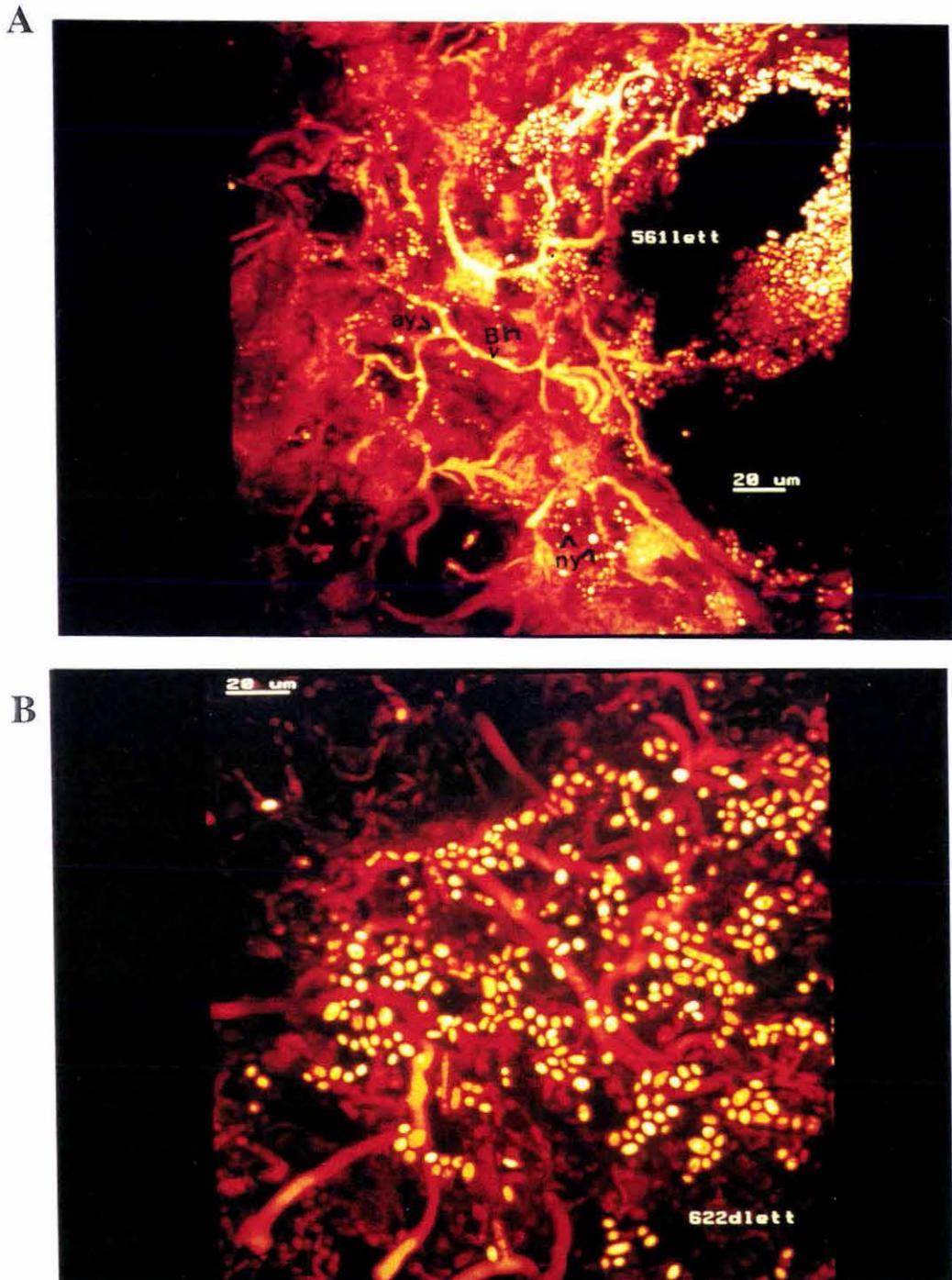


Fig 6-3 Confocal micrograph of *B. cinerea* hyphal development following addition of yeast isolate 561 (A) and 622b (B) to the surface of lettuce leaf tissue. BCAs applied 24 h after *B. cinerea* inoculation. Specimen treated with glutaraldehyde.

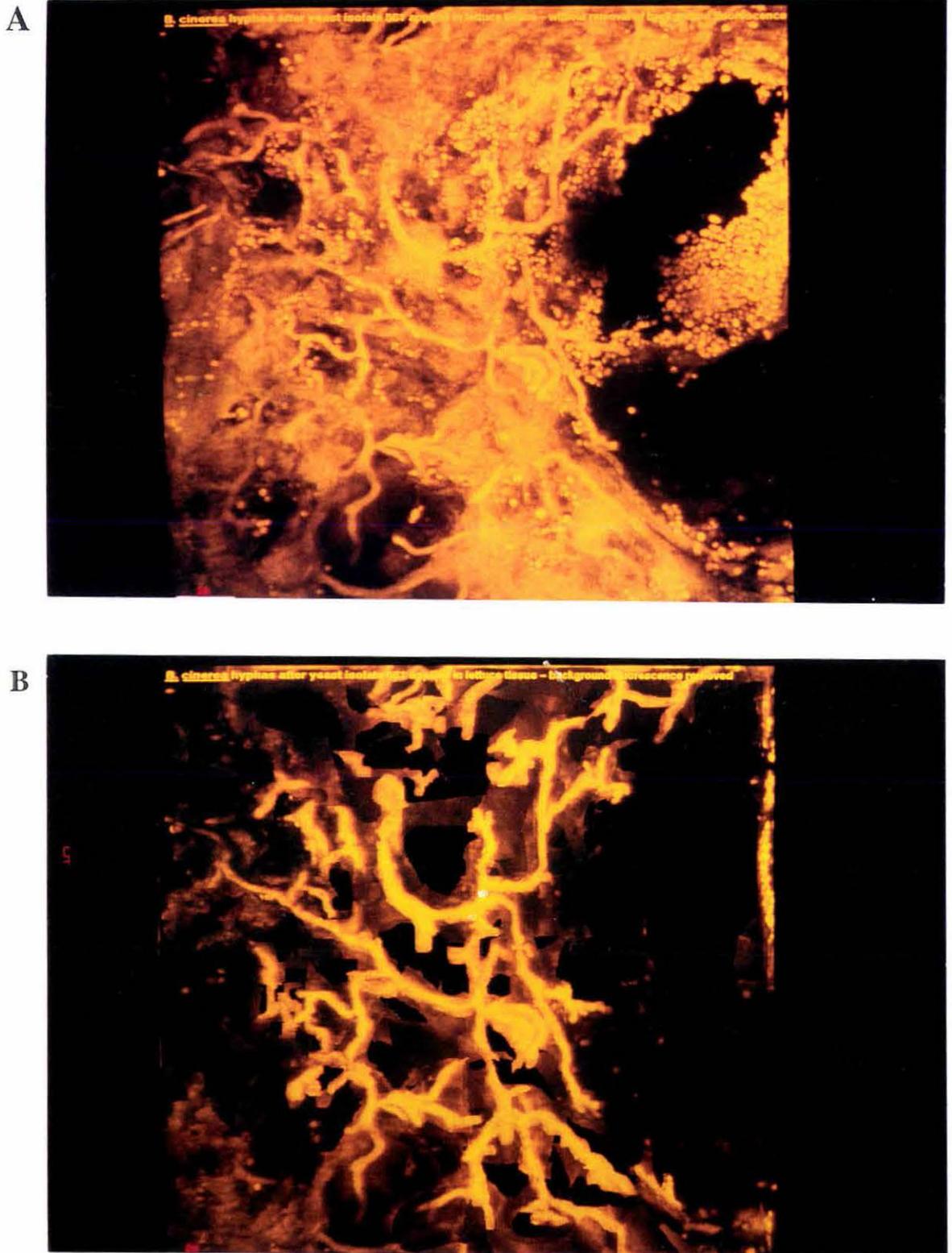


Fig 6-4 Confocal micrograph of *B. cinerea* hyphae (A) before and (B) after removal of fluorescing background plant cells (bar 20 μm).

## 6.5 Discussion

Confocal laser scanning microscopy is a relatively new technique for determining the 3-dimensional structure of transparent or opaque specimens using either fluorescence or reflectance imaging (Donaldson 1998). Fluorescence is probably the most important optical readout mode in biological confocal microscopy because it works so well with epi-illumination but this advantage is critically dependent on the availability of suitable fluorophores that can either be tagged onto biological macromolecules to show their location, or whose optical properties are sensitive to the local environment (Tsien and Waggoner 1995). Botrytis-specific monoclonal antibody, BC-KH4 was used as an immunofluorescence label for *B. fabae* in bean leaf tissue and apparently labelled the surface of conidia and germ tubes although short germtubes of dry inoculated conidia were not immunolabelled (Cole et al. 1996). In this study, BC- KH4 antibody treated *B. cinerea* hyphae in the bean tissue fluoresced poorly on a portion of the hyphae close to the conidia and the other parts of the hyphae did not fluoresce or did so only slightly. Previous work by Cook (1997) showed good labelling of *B. cinerea* with the BC-KH4 antibody followed by the Sigma anti-mouse FITC conjugated antibody so it is possible that one or both batches of antibody have deteriorated in storage.

Shelly et al. (1998) obtained images of fungal hyphae that were well differentiated from the substrate of biodegraded wood samples treated with glutaraldehyde. The same result was obtained from this study with *B. cinerea* hyphae in leaves or petals after glutaraldehyde fixation. The hyphae fluoresce brightly and are usually differentiated from background plant cells. However, some plant cells do fluoresce brightly (not more than the fungal hyphae) because of glutaraldehyde induced fluorescence. Such plant cells interfere with the image analysis measurement of the volume of neighbouring fluorescing hyphae. These cells were

eliminated before analysis by manually using the paintbrush module in the system. Although simple to perform, this method was laborious and while removing unwanted objects, there were some instances where the distinction between such cells and portions of fungal hyphae were not clear. This process took considerable time [eg: 2-3 h to convert the images that make up Fig 6-4(A) to those that make the image in Fig 6-4 (B)]. This is the reason for lack of replication at this stage. It would be better to develop a method to suppress induced fluorescence of plant cells and eliminate this problem thereby speeding up the process considerably.

This work has shown that the volume of *B. cinerea* hyphae in plant tissue can be measured by a confocal image analysis system and that it detected differences in *B. cinerea* biomass between BCA treated tissue and control tissue (only *B. cinerea* inoculated) at a stage when symptom development alone would have been an unreliable guide. Thus, in bean leaf tissue there was less fungal biomass obtained from treatments that included biocontrol agents OX2 and OX8a. This result supported those obtained in Chapter 4 where a significantly smaller lesion size was measured from OX2 and OX8a treatments. Further experiments are required to statistically analyse differences of fungal biomass between specimens treated with different BCAs against *B. cinerea*. However, the results from this study indicate that the measurements of fungal biomass in the plant tissue using confocal microscopy and glutaraldehyde fixation, as a fluorescence treatment are possible and that it should be possible to detect differences in *B. cinerea* biomass at an early stage of infection before symptom development is a reliable guide.

## *Chapter Seven*

### **General Discussion**

#### **Introduction**

Biological control offers an alternative to fungicide use for the control of plant diseases and it is possible to control plant pathogens biologically by manipulating the epiphytic microflora on plant surfaces (Droby and Chlutz 1991). Numerous biocontrol agents have been isolated and experimentally tested for their efficacy against their respective plant pathogens under laboratory condition. For such tests it is important to get consistent infection of the pathogen on a targeted substrate. Since, *B. cinerea* is considered a weak postharvest pathogen but, is capable of attacking crops at almost any stage in their growth and in storage (Maude 1980), one must consider the factors that affect the infection process and that could result in inconsistency in infection. In this study, experiments in chapter 3 provide information on condition for consistent infection of *B. cinerea* in different crop tissues. Procedures for subsequent experiments were based on these results. Chapter 5 and 6 dealt with the attachment ability of biocontrol agents by testing them with *in vitro* and *in vivo* techniques.

Biocontrol agents were evaluated by the conventional method of measuring percentage infection and disease severity as a comparison with new technology (Chapter 4). A Computerised Confocal Laser Scanning Microscope with a suitable fluorescing stain was used to measure biomass of *B. cinerea* in different plant tissues (Chapter 6) as a test of the assumption that pathogen biomass reduction is related to the biocontrol efficiency,

#### ***B. cinerea* infection on plant tissue**

There are number of factors which determine the success of infection of a pathogen in a host tissue. For *B. cinerea* infection, particularly on postharvest product, relative humidity and free water on the tissue surface play an important

role in the infection process (Rijkenberg et al. 1980; Salinas et al. 1989; Pie and De Leeuw 1991). Harrison et al. (1994) stated that humidity has a profound effect on fungal diseases of plants, because it determines the duration of surface water on the aerial plant parts. In contrast, infection of *B. cinerea* conidia in the absence of free water at high humidity has been observed in table grapes (Nelson 1951) and rose (Williamson et al. 1995). Some other fungal spores, eg; of the powdery mildews are adapted to germinate and infect the host with out free water (Butt 1978). In this study, consistent infection of *B. cinerea* was observed on lettuce leaves and on rose petals when they were inoculated with dry spores or a low concentration spore suspension providing that a high relative humidity and free water were maintained on the tissue surface. Purkayastha and Deverall (1965a), Jarvis (1962) and Carre (1984) have also demonstrated the importance of free water for *B. cinerea* spore germination. On bean leaves, consistent infection was only obtained with high relative humidity, free water and a high spore concentration. The bean plant has been well studied for antifungal substances (Purkayastha and Deverall 1965b; Hargreaves et al. 1977; Deverall and Vessey 1969) known as phytoalexins that influence the germination and infection processes of pathogens. These inhibitors may be host specific, and may be produced by epidermal cells in response to substances diffusing from ungerminated conidia or from epiphytic microflora (Mansfield and Deverall 1974). However, the observation in this study on bean suggests that the inhibition of spore germination and infection by these antifungal substances can be over come by increasing the inoculum concentration of *B. cinerea*.

### **Application of BCAs**

The results from Chapter four showed that biocontrol efficiency varied with time of BCA application and with different host. For example, bacterial isolate OX2 was effective with simultaneous or delayed (by 48 h) application on inoculated

bean leaf tissue but, it was only effective with a simultaneous application in lettuce tissue, or with delayed (by 48 h) application in rose tissue. Therefore, it seems that the activity of the BCA is influenced by tissue that is specific to each host and by time of arrival at infection court relative to that of the pathogen. Those results indicate that application procedures for these BCAs cannot be generalised but must be tailored to each host.

### **BCA's attachment and biocontrol**

El-Ghaouth et al. (1998), observed attachment of a biocontrol agent, *Candida saitoana*, to *B. cinerea* hyphal walls that restricted the proliferation of *B. cinerea* and suppressed disease in apple. Biocontrol agents involved in this study showed both sparse and aggressive attachment to *B. cinerea* hyphae (see chapter 3 and 6). Isolate 561 did not control lesion development satisfactorily even though it has the ability to aggressively attach to the pathogen but the other aggressive attacher OX8a did control in all three crops. Wisniewski, et al (1990,1991) observed attachment of two yeast species: *P. guilliermondii* and *Debaryomyces hansenii* on *B. cinerea* hyphae but *P. guilliermondii* attached fastidiously and effectively controlled hyphal development whereas *D. hansenii* was ineffective. On the other hand sparse attachers, OX2 and 622b did control lesion development in all three crops at any one stage of application time. Such observations indicate that all attachers are not necessarily good biocontrol agents but it can be assumed that these biocontrol agents need to be attached to the pathogen aggressively to some degree to give biocontrol effect.

### **Potential biocontrol agents**

Yeast and bacteria have been used as biocontrol agents against *B. cinerea* in kiwifruit (Cheah and Hunt 1994; Cook 1997), rose (Redmond et al. 1987; Hammer and Marois 1989), apple (Wisniwski et al. 1988b; Janisiewicz 1987; 1988), citrus

fruit (Chalutz et al. 1988b; Chalutz and Willson 1989; Singh and Deverall 1984), bean (Elad et al. 1994a, 1994b) and tomato (Cook et al. 1997b; Elad et al. 1994a, 1994b). Bacteria and yeast with the ability to attach to the hyphae have been used by Cook (1997) as biocontrol agents against *B. cinerea* in kiwifruit and tomato. Cook (1997) used this character as the main criterion for the isolation of biocontrol agents and he established a relationship between attachment and biocontrol ability of these isolates against *B. cinerea* on kiwifruit and tomato. However, one of his yeast (isolate 561) aggressively attached to the *B. cinerea* hyphae and effectively reduced infection in kiwifruit and tomato (Cook et al. 1997b) but it did not control lesion development in bean and lettuce (Chapter 4). However, a reduction in lesion size was observed at an early stage of infection only. Further more, a high amount of biomass reduction was found when the biomass was measured at an early stage of pathogen development in lettuce leaves (Chapter 6). On the other hand, bacterial isolate OX8a which aggressively colonised hyphae did control lesion development from an early stage of infection in all three crops (Chapter 4) and a high reduction of pathogen biomass was also observed at an early infection stage in bean leaves (Chapter 6). These observations indicated that the biomass reduction occurred due to colonisation of BCA and that could be the reason for the control of lesion development at an early stage. However, factors such as temperature and BCA concentration may affect the biocontrol efficiency differentially to different BCA as observed by Cook et al. (1997b) when they applied BCAs at different concentrations and in different temperatures to tomato stems. Only one temperature and a single concentration of BCAs were used in this study and it would be worth repeating this work at different temperature and BCA concentration to determine whether the biocontrol effects by the various isolates on different hosts are consistent.

**Biomass measurement**

Several methods have been used directly (Jarvis et al. 1983; Schnürer 1993) or indirectly (Seitz 1977, 1989; Weete 1980) to measure fungal biomass in an artificial substrate. However, it has not been possible to measure directly or indirectly absolute fungal biomass when fungi are growing in plant tissue (Notermans et al. 1986). However, in this study, a direct method has been developed to measure fungal biomass in plant tissue. A Computerised Confocal Laser Scanning Microscope has been used to produce a clear image of the fungal hyphae in plant tissue with the help of suitable fluorescent staining.

Glutaraldehyde fluoresces fungal hyphae brightly and facilitates production of a clearer image in the confocal microscope than the other two stains used in this study. However, in some cases adjoining plant cells also fluoresced and caused a mis-reading of the computerised measurement of volume of fungal biomass. The problem can be overcome by carefully removing the fluorescing cells from the image using a paintbrush programme but this takes considerable time. A technique must be developed either in the computer or in the staining process to avoid confusion of plant cell fluorescence with that of hyphae.

## *Chapter Eight*

### **Conclusion and recommendation for future studies**

For experimental purposes, consistent infection of *B. cinerea* on a host plant is important. Various factors and conditions were identified as important for consistent infection on bean, lettuce and rose host tissues. In general, a medium temperature (about 21<sup>0</sup>C) and high humidity with a film of free water on the tissue surface are necessary for infection of all host tissues. In addition, a high inoculum concentration of *B. cinerea* was required for consistent infection of bean leaf tissue. Lettuce and rose tissues became infect with dry spore inoculation of *B. cinerea* under high humidity conditions. This inoculation technique was much easier than the wet technique due to the formation of a film of free water on the tissue surface. However, the number of spores inoculated on each site was not known. To solve this problem, a technique must be developed to estimate the dry spore concentration on inoculated sites.

Both the bacterial isolates and one yeast isolate show promise for biocontrol of *B. cinerea* infection in terms of reduction of either lesion size or percentage infection on all three host plants. However, biocontrol efficiency depends upon time of application and varied with biocontrol agents as well as host plants. Since, one temperature and one BCA concentration were used in this study it is important to investigate, in future, the effect of temperature and the concentration of BCA for biocontrol efficiency on these crops.

All four isolates tested in this study showed sparse or aggressive attachment to *B. cinerea* hyphae and showed efficiency in biocontrol in terms of reduction of lesion size or percentage infection on at least one crop or in one application time. Nevertheless, futher work is required to the relationship between aggressiveness of attachment and biocontrol efficiency in order to increase biocontrol efficiency by increasing the attachment ability of BCAs.

The feasibility of a quick investigation of biocontrol efficiency was established by measuring biomass of *B. cinerea* in the host tissue at early stage of infection using Laser Scanning Confocal Microscope with the use of glutaraldehyde as a fluorescing agent for *B. cinerea* hyphae. All four BCA isolates tested in this technique caused a reduction of biomass of *B. cinerea*.

The constraint in this technique was the need to differentiate or eliminate fluorescing host cells adjacent to the pathogen to facilitate the correct measurement of volume of hyphae. Due to this constraint, repeatable measurements could not be taken in this study for use in statistical evaluation of the results. Further study is required to eliminate this constraint perhaps by a differential fluorescent staining technique or by an easily manipulated computer program to eliminate the background fluorescent effect.

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### **Estimating Biomass of *Botrytis cinerea***

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#### **Abstract**

The estimation of biomass of a pathogen at an early stage of disease development could be useful for infection studies and could save considerable time when assessing the efficacy of biocontrol agents (e.g for control of *Botrytis cinerea* infections of kiwifruit). Glutaraldehyde has been shown to give useful autofluorescence of fungal hyphae in wood. We used this technique to study the effect of biological control agents on *Botrytis cinerea* infection of bean and lettuce leaves by Lesser Scanning Confocal Microscopy. Bacterial isolates of *Enterobacter agglomerans* and *Enterobacter aerogenes*, and yeast isolates of *Candida sake* and *Trichosporon pullulans* which were already used as biocontrol agents in kiwifruit and tomato were used in this study. Images of the Glutaraldehyde fixed specimens produced by the confocal microscopy clearly facilitate to measure the volume of fungal biomass. There was less hyphal development in leaf tissue when bacterial and yeast biocontrol agents were applied with the pathogen than in pathogen only treatments.

#### **Introduction**

Efficacy of biocontrol agents is often judged by symptom development but this can take some time as with *Botrytis* infection of kiwifruit and an alternative, quicker approach would be useful. Pathogen biomass would be limited by a successful biocontrol agent (BCA) hence a measure of pathogen biomass in plant material could be an alternative.

The laser scanning confocal microscope (LSCM) provides a clear fluorescent image with accurate three-dimensional perspectives from which fungal hyphal volumes can be measured. Successful image production in LSCM depends on fungal hyphae fluorescing more brightly than plant tissue. Glutaraldehyde has been used with wood rotting fungi (Singh et al. 1997) and was assessed against *B. cinerea* in bean and lettuce leaf tissue in this study.

#### **Objective**

To determine whether the biomass of *B. cinerea* in plant tissue can be estimated by confocal microscopy of glutaraldehyde fixed tissue

#### **Materials & Methods**

Bean and lettuce leaves wounded with a glass rod tip and inoculated with 2ml of *B. cinerea* spore suspension (100 spores) were incubated at 21°C for 12-24 h. Bacterial (OX2 and OX8a) and yeast (561 and 622b) isolates ( $1 \times 10^6$  -  $1 \times 10^9$  cells/ml) were then applied as 2ml droplets to inoculation sites and the plant tissues were incubated for a further 12-24 h. Pieces (1-2 mm) of inoculated tissues were incubated in glutaraldehyde and mounted on a slide in CITI fluor before observation with a Leica LSCM using 490 and 568 nm exciter

wavelengths and, 530 and 590 nm imaging wavelengths. Each image was created from 15-30 optical sections (5mm apart).

Background fluorescence was removed and fungal biomass measured with Image Space ver. 3.10 on an "Indy Silicon Graphics Computer". Fungal volume was measured as pixel number and/or cubic mm.

## Results

Gluteraldehyde treated hyphae of *Botrytis fluoresce* brightly and the images can be formatted into a 3-D image (Fig. 1) or can be used for estimating hyphal volume. Background fluorescence can be a problem (Fig. 2B and 3B) but removal of the background gives a clear image (Fig. 2c and 3c) from which hyphal volumes can be measured (Tables 1 and 2).

**Table 1.** Hyphal volume of *B. cinerea* measured by LSCM in inoculated bean leaf tissue with and without Bacterial BCAs OX2 and OX8a.

BCAs / <i>B. cinerea</i>	Biomass of <i>B. cinerea</i> Hyphal volume (Pixel)	Biomass of <i>B. cinerea</i> hyphal volume ( $\mu\text{m}^3$ )
<i>B. cinerea</i> alone	1121844	430967.59
<i>B. cinerea</i> with OX2	59185	48315.09
<i>B. cinerea</i> with OX8a	21774	15691.01

**Table 2.** Hyphal volume of *B. cinerea* measured by LSCM in lettuce leaf tissue with and without yeast BCAs 561 and 662b.

BCAs / <i>B. cinerea</i>	Biomass of <i>B. cinerea</i> Hyphal volume (Pixel)	Biomass of <i>B. cinerea</i> hyphal volume in $\mu\text{m}^3$
<i>B. cinerea</i> alone	428710	85479.06
<i>B. cinerea</i> with 561	108291	33490.07
<i>B. cinerea</i> with 622b	118923	23989.35

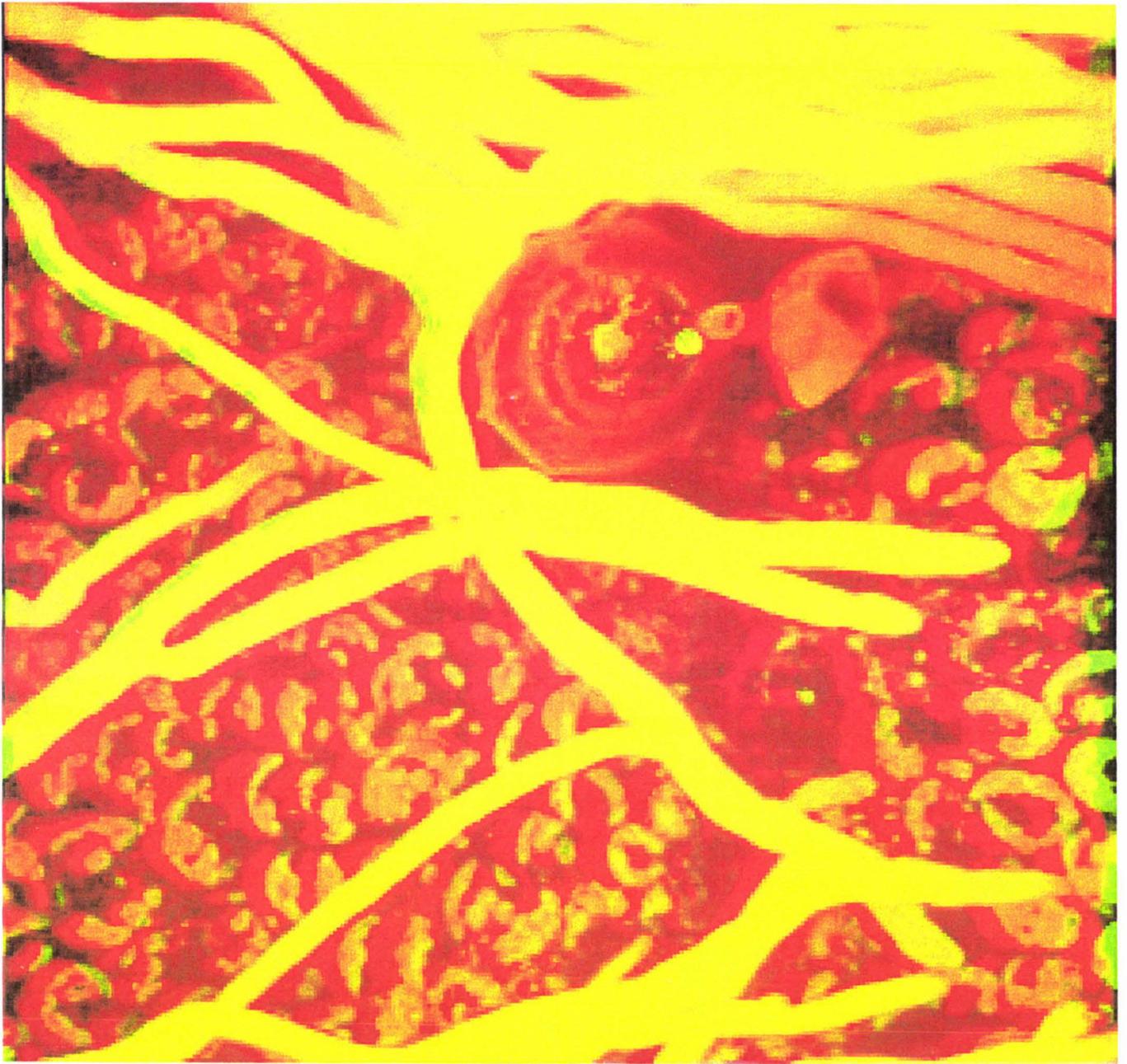
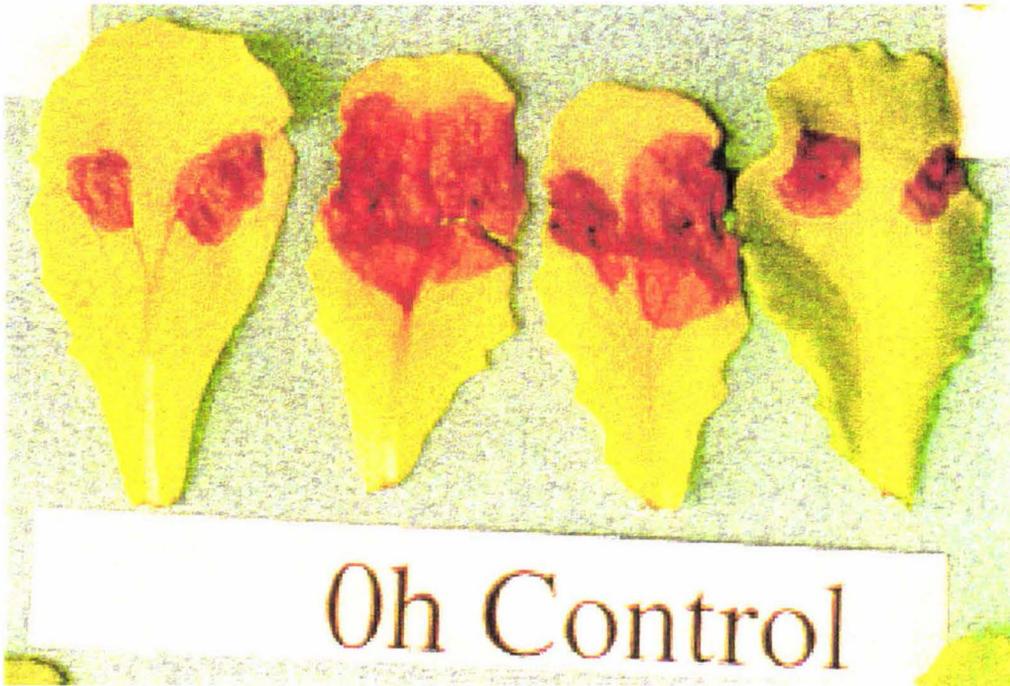


Fig 1. Image of *Botrytis cinerea* hyphae in a 3 D format

A



B

C

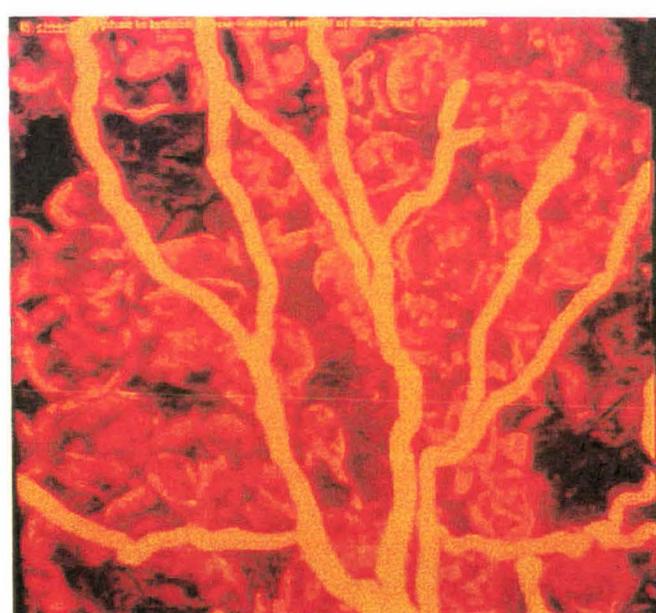
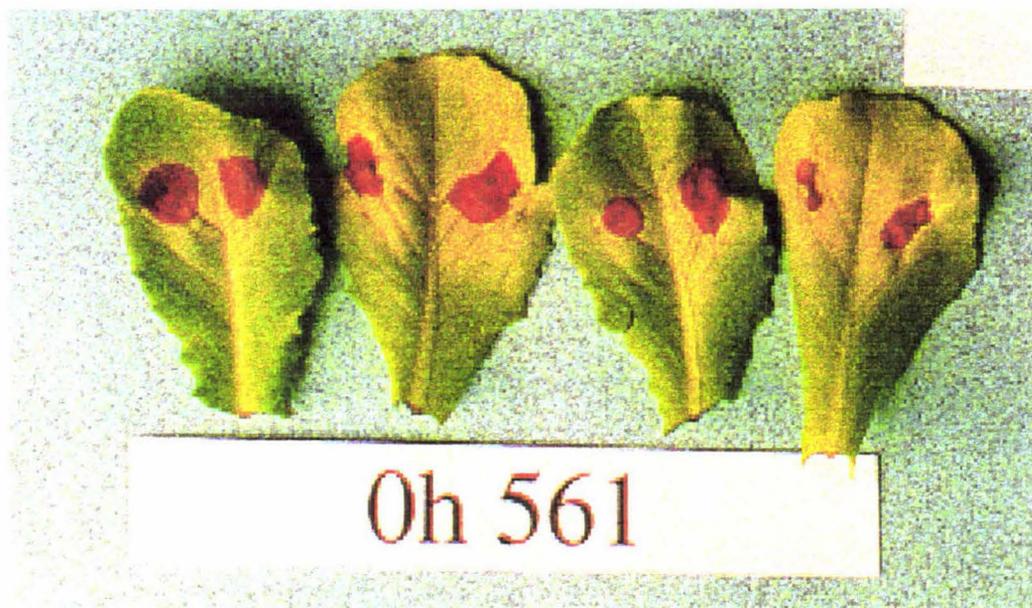
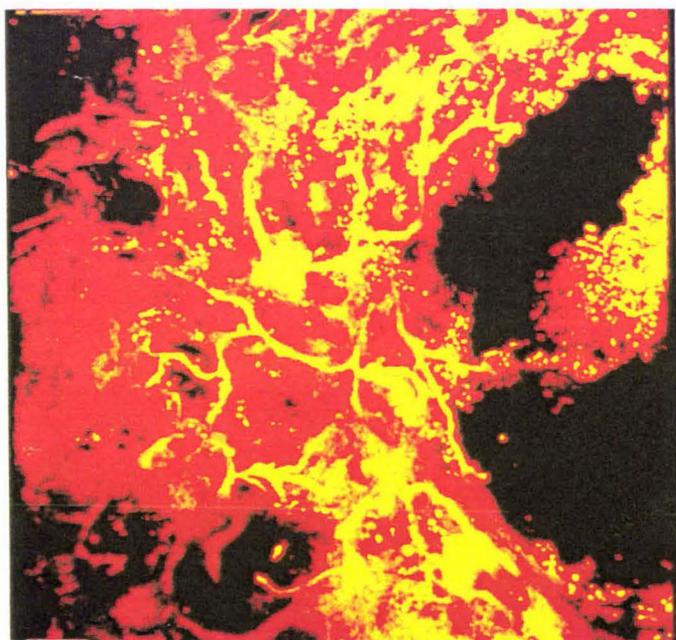


Fig 2. Lettuce leaves inoculated with *Botrytis cinerea* A) Lesion on the leaves, B) and C) LSCM image before and after background removal.

A



B



C

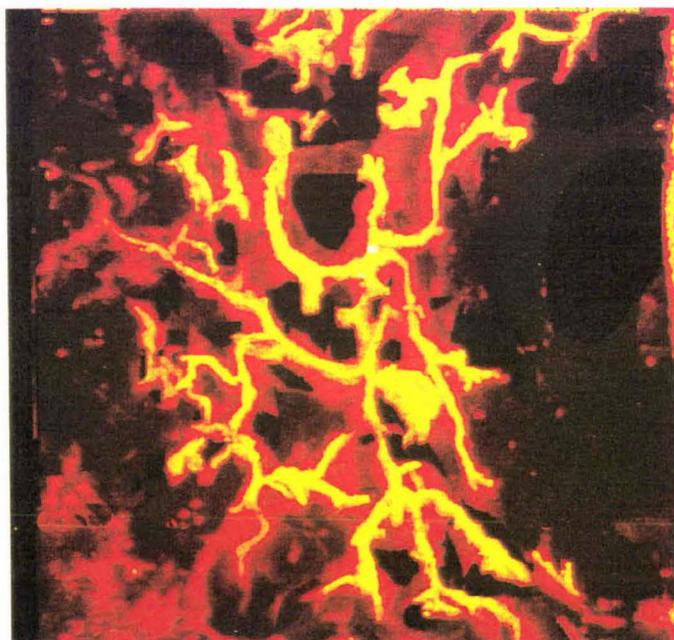


Fig 3. Lettuce leaves inoculated with *Botrytis cinerea* followed by BCA 561 a) Lesion on the leaves, b) and c) LSCM image before and after background removal.

## Conclusion

1. *B. cinerea* hyphae fluoresce brightly after glutaraldehyde fixation when illuminated with blue light (490 nm wave length)
2. It is necessary to remove some background host fluorescence before measuring hyphal volumes
3. Direct measurement of the biomass of a fungal pathogen in plant tissue is possible using LSCM
4. It should be possible to assess the efficacy of biocontrol agents by measuring fungal biomass in plant tissue using this technology.