

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

A thesis presented in partial fulfilment of  
the requirements for the degree of  
Master of Applied Science  
at  
Massey University  
Palmerston North  
New Zealand

Vallipuram Vingnanasingam  
March 1998

## 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*.

### *Acknowledgements*

I wish to express my heartfelt gratitude to my supervisor Dr Peter Long for his invaluable help, encouragement and patience with the preparation of this thesis and his assistance throughout my investigation. I also wish to thank Mr Hugh Neilson for his constant companionship and appreciated assistance in the laboratory.

I am also greatly indebted to Ms Liz Nickless for her great assistance to work in Confocal Microscope. It was not possible to complete this study without her help with this wonderful machine. My sincere thanks also to Dr. Al Rowland for his permission to work with the confocal microscope in his laboratory. I am also very grateful to Dr. Duncan Hedderley from Statistics Research & Consulting Centre for his great support to statistical analysis.

Thanks are due all to other staff of the Dept. of Plant science and Plant Growth Unit for their help and use of their facilities.

I always be grateful to my friends Geogina Milne, Cristine Tayler, Song, Hyun Kim and my country mate Jegathees and Ruwan for their companionship and motivation during the period I spent for this study.

Finally, I acknowledge my deep appreciation to my special people, my wife Malliha, and my children Neelu and Keerth for their patience and encouragement during my study.

## Table of contents

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iii
TABLE OF CONTENTS.....	iv
TABLE OF FIGURES.....	ix
TABLE OF TABLES.....	xiii
<b>CHAPTER ONE</b>	
GENERAL INTRUCTION.....	1
1.1 <i>Botrytis cinerea</i> .....	1
1.1.1 Economic losses due to <i>B. cinerea</i> infection.....	1
1.1.2 Taxonomy and morphology.....	2
1.1.3 General characteristics of <i>B. cinerea</i> .....	2
1.1.4 Specific behaviour of <i>Botrytis cinerea</i> on plant surfaces.....	3
1.1.4.1. Survival of conidia.....	3
1.1.4.2 Germination of conidia.....	3
1.1.4.2.1 Spore concentration.....	3
1.1.4.2.2 Relative humidity and free water.....	4
1.1.4.2.3 Temperature.....	4
1.1.4.2.4 Other factors influencing spore germination.....	5
1.2 Control of <i>Botrytis cinerea</i> .....	6
1.2.1 Non-Chemical control.....	6
1.2.2 Chemical treatment.....	6
1.2.3 Biological control of <i>Botrytis cinerea</i> .....	7
1.2.3.1 General view on Biological control.....	7
1.2.3.2 Attachment biocontrol agents.....	9

1.2.3.3 Utilisation of attachment biocontrol agents in disease control.....	10
1.3 Confocal microscopy.....	11
1.3.1 Confocal microscope and computer image analysis.....	11
1.3.2 Staining for confocal microscopy.....	12
1.4. Objectives of this studies.....	12

## CHAPTER TWO

GENERAL MATERIALS AND METHODS.....	13
2.1 <i>Botrytis cinerea</i> isolates.....	13
2.1.1 culture preparation .....	13
2.1.1.1 Silica gel storage.....	13
2.1.2 Preparation of spore suspensions.....	14
2.2 Biological Control Agents (BCA); bacteria and yeast.....	14
2.2.1 Culture preparation .....	14
2.2.2 Preparation of cell suspension.....	15
2.2.3 Cells counts.....	15
2.3 Source of test materials.....	15
2.3.1 Bean ( <i>Phaseolus vulgaris</i> L.).....	15
2.3.2 Lettuce ( <i>Lactuca sativa</i> L.).....	15
2.3.3 Rose ( <i>Rosa hybrida</i> L.).....	16
2.4 Fluorescent treatment .....	16
2.4.1 KH4 antibody fluorescent dye.....	16
2.4.2 Trypan blue fluorescent dye.....	16
2.4.3 Glutaraldehyde fluorescent dye.....	17
2.4.3.1 Glutaraldehyde fluid (GA) preparation.....	17

---

2.4.3.2 Buffered sucrose solution preparation.....	17
2.4.3.3 Procedure of glutaraldehyde fixation.....	17
2.5 Confocal microscopy .....	18
2.6 Computer Image Analysis.....	18
2.7 Assessment of <i>Botrytis cinerea</i> infection.....	19
2.7.1 Data collection.....	19
2.7.2 Observation and recording.....	19
2.8 Experimental designs and statistical analysis.....	19

### CHAPTER THREE

INFECTION OF DETACHED PLANT MATERIALS BY <i>BOTRYTIS CINEREA</i> .....	20
3.1 Introduction.....	20
3.2 Objective.....	21
3.3 Materials and Methods.....	22
3.3.1 <i>B. cinerea</i> infection on bean and lettuce leaves and rose petals.....	22
3.4 Results.....	25
3.5 Discussion.....	37

### CHAPTER FOUR

BIOLOGICAL CONTROL OF INFECTION OF ROSE, BEAN AND LETTUCE BY <i>BOTRYTIS CINEREA</i> .....	39
4.1 Introduction.....	39
4.2 Objective.....	40
4.3 Materials and Methods.....	41

---

4.4 Results.....	45
4.5 Discussion.....	57

## CHAPTER FIVE

ATTACHMENT OF BACTERIA AND YEAST TO <i>B. CINEREA</i> HYPHAE AS A BIOCONTROL MECHANISM.....	60
5.1 Introduction.....	60
5.2 Objectives.....	61
5.3 Materials and Methods.....	61
5.3.1 In vitro bioassay for attachment BCAs .....	61
5.3.1.1 <i>B. cinerea</i> and BCA preparation.....	61
5.3.1.2 Media preparation.....	62
5.3.1.3 Inoculation and incubation.....	62
5.3.1.4 Dark field microscopy.....	62
5.3.2 In vivo bioassay for attachment BCAs.....	63
5.4 Results.....	63
5.4.1 Biocontrol and BCAs attachment assay – in vitro.....	63
5.4.1.1 Biocontrol of <i>B. cinerea</i> .....	63
5.4.1.2 Dark field microscopy on attachment assay.....	64
5.4.2 Biocontrol and BCAs attachment assay – in vivo.....	64
5.5 Discussion.....	70

## CHAPTER SIX

BIOMASS MEASUREMENT OF <i>BOTRYTIS CINEREA</i> USING LASER SCANNING CONFOCAL MICROSCOPE.....	72
6.1 Introduction.....	72

---

6.2 Objective.....	74
6.3 Materials and Methods.....	75
6.3.1 <i>B. cinerea</i> inoculation and BCAs application.....	75
6.3.2 Fluorescent treatment.....	75
6.3.3 Confocal microscopic examination.....	75
6.3.4 Image analysis.....	76
6.4 Results.....	76
6.4.1 Fluorescent treatment.....	76
6.4.2 Image analysis.....	77
6.5 Discussion.....	84

## CHAPTER SEVEN

GENERAL DISCUSSION.....	86
Introduction.....	86
<i>B. cinerea</i> infection on plant tissue.....	86
Application of BCAs.....	87
BCA's attachment and biocontrol.....	88
Potential biocontrol agents.....	88
Biomass measurement.....	90

## CHAPTER EIGHT

CONCLUSION AND RECOMMENDATION FOR FUTURE STUDIES.....	91
REFERENCES.....	93

## Table of figures

<b>Fig 3-1.</b> 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 $P < 0.05$ .....	27
<b>Fig 3-2.</b> Lesion size and percentage infection by dry and wet ( $1 \times 10^6$ and $1 \times 10^8$ spores per ml.) spore inoculation on bean, lettuce and rose tissue. Means accompanied by same letter are not significantly different at $P < 0.05$ .....	28
<b>Fig 3-3.</b> Lesion size and percentage infection by <i>B. cinerea</i> 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 $P < 0.05$ .....	29
<b>Fig 3-4.</b> Lesion size and percentage infection by <i>B. cinerea</i> 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 $P < 0.05$ .....	30
<b>Fig 3-5.</b> Lesion size and percentage infection by <i>B. cinerea</i> 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 $P < 0.05$ .....	31
<b>Fig 3-6.</b> Differences of lesion size and percentage infection between wounded and unwounded tissues of bean, lettuce and rose.....	32
<b>Fig 3-7.</b> Lesion size measured 48 h and 96 h after inoculation of <i>B. cinerea</i> on bean leaves. Dark = leaves left 24 h in dark before inoculation; W= Spores in water suspension; T= Infected tissue with spores.....	33
<b>Fig 3.8.</b> Lesion size measured at 48 h and 96 h after <i>B. cinerea</i> inoculation on different age and position bean leaves. Coty = cotyledon.....	34
<b>Fig 3-9.</b> Percentage infection measured after different <i>B. cinerea</i> isolates were inoculated on bean and lettuce leaves and rose petals.....	35

<b>Fig 3-10.</b> Lesion development over time. A) Rose petals inoculated only with <i>B. cinerea</i> . 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.....	36
<b>Fig 4-1.</b> BCAs added at 0h (A), 24h (B) and 48 h (C) after inoculation of rose petals by <i>B. cinerea</i> . 1) Control 1: no BCAs or <i>B. cinerea</i> inoculated 2) Control2: only <i>B. cinerea</i> was inoculated 3) OX2 4) OX8a 5) 561 6) 622b.....	44
<b>Fig 4-2.</b> 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 <i>B. cinerea</i> inoculation. Bars with same letter in a day is not significantly different ( $P<0.05$ ).....	47
<b>Fig 4-3.</b> 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 <i>B. cinerea</i> inoculation. Bars with same letter in a day is not significantly different ( $P<0.05$ ).....	48
<b>Fig 4-4.</b> 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 <i>B. cinerea</i> inoculation. Bars with same letter in a day is not significantly different ( $P<0.05$ ).....	49
<b>Fig 4-5.</b> Effect on lesion size (A) and percentage infection (B) of <i>B. cinerea</i> 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 ( $P<0.05$ ).....	50
<b>Fig 4-6.</b> Effect on lesion size (A) and percentage infection (B) of <i>B. cinerea</i> 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 ( $P<0.05$ ).....	51
<b>Fig 4-7.</b> Effect on lesion size (A) and percentage infection (B) of <i>B. cinerea</i> 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 ( $P<0.05$ ).....	52

- 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  $P < 0.05$ .....53
- 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  $P < 0.05$ .....54
- 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  $P < 0.05$ .....55
- 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 ( $P < 0.05$ ).....56
- 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<sup>o</sup>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  $\mu$ m)....66
- 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>o</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).....67
- 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. ....68

- 
- 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.....69
- 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.....80
- 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.....81
- 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.....82
- Fig 6-4.** Confocal micrograph of *B. cinerea* hyphae (A) before and (B) after removal of fluorescing background plant cells.....83

---

## Table of tables

- Table 5-1.** Observation of BCA attachment to hyphae and other interaction at 0 h and 24 h application of BCAs after *B. cinerea* inoculation in vitro.....65
- 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 or 24 h after inoculation. All tissues were fixed With glutaraldehyde 24-36 h after *B. cinerea* inoculation.....79
- Table 6-2.** Hyphal volume of *B. cinerea* per unit area measured by LSCM and image analysis. Yeast BCAs 561 and 662b applied 12 or 24 h after inoculation. All tissues were treated with glutaraldehyde 24-36 h after *B. cinerea* inoculation.....79