MECHANISMS OF BIOLOGICAL CONTROL OF FUSARIUM ROOT AND STEM ROT OF GREENHOUSE CUCUMBER BY *GLIOCLADINUM CATENULATUM*

by

Syama Chatterton  
MSc, University of Guelph, 2002  
BSc, Simon Fraser University, 1999

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

In the  
Department of Biological Sciences

© Syama Chatterton 2010

SIMON FRASER UNIVERSITY  
Spring 2010

All rights reserved. However, in accordance with the Copyright Act of Canada, this work may be reproduced, without authorization, under the conditions for Fair Dealing. Therefore, limited reproduction of this work for the purposes of private study, research, criticism, review and news reporting is likely to be in accordance with the law, particularly if cited appropriately.
APPROVAL

Name: Syama Gauri Dasi Chatterton
Degree: Doctor of Philosophy

Title of Thesis:
Mechanisms of biological control of Fusarium root and stem rot of greenhouse cucumber by Gliocladium catenulatum

Examining Committee:
Chair: Dr. J. Reynolds, Professor

Dr. Z. Punja, Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. M. Moore, Professor
Department of Biological Sciences, S.F.U.

Dr. A. Plant, Associate Professor (Retired) and Adjunct Professor
Department of Biological Sciences, S.F.U.

Dr. R. Hamelin, Professor
Department of Forestry, The University of British Columbia
Public Examiner

Dr. T. Paulitz, Research Plant Pathologist
Department of Plant Pathology, Washington State University
External Examiner

11 January 2010
Date Approved
Declaration of Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the right to lend this thesis, project or extended essay to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or make a digital copy for use in its circulating collection (currently available to the public at the “Institutional Repository” link of the SFU Library website <www.lib.sfu.ca> at: <http://ir.lib.sfu.ca/handle/1892/112>) and, without changing the content, to translate the thesis/project or extended essays, if technically possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for scholarly purposes may be granted by either the author or the Dean of Graduate Studies.

It is understood that copying or publication of this work for financial gain shall not be allowed without the author’s written permission.

Permission for public performance, or limited permission for private scholarly use, of any multimedia materials forming part of this work, may have been granted by the author. This information may be found on the separately catalogued multimedia material and in the signed Partial Copyright Licence.

While licensing SFU to permit the above uses, the author retains copyright in the thesis, project or extended essays, including the right to change the work for subsequent purposes, including editing and publishing the work in whole or in part, and licensing other parties, as the author may desire.

The original Partial Copyright Licence attesting to these terms, and signed by this author, may be found in the original bound copy of this work, retained in the Simon Fraser University Archive.

Simon Fraser University Library
Burnaby, BC, Canada
ABSTRACT

_Gliocladium catenulatum_ strain J1446 (formulated as Prestop WP, Verdera Oy) is a biological control agent of Fusarium root and stem rot caused by _Fusarium oxysporum_ f. sp. _radicis-cucumerinum_ on greenhouse cucumber plants. The mechanisms involved in biocontrol efficacy are currently unknown. Following transformation of _G. catenulatum_ with the β-glucuronidase (uidA) gene, blue-stained mycelia could be seen growing on the surface and within epidermal and cortical cells of roots, stems and shoots 3 weeks after treatment. Application of _G. catenulatum_ preceding inoculation with _Fusarium_ significantly reduced pathogen populations on roots compared to plants inoculated with _Fusarium_ alone, while densities of the biocontrol agent increased in the presence of the pathogen. Factors influencing root population levels included nutrient solution pH, temperature and growing media type, while cucumber cultivar, root wounding and addition of nutrients did not appear to significantly affect colonization. In culture, _G. catenulatum_ produced chitinase and β-1,3-glucanase enzymes on chitin or laminarin as a sole carbon source, respectively, and caused localized degradation of _Fusarium_ hyphae. Cucumber root extracts from _G. catenulatum_-colonized plants had significantly higher levels of glucanase at 7 days post-application compared to untreated controls. Reverse-transcription polymerase chain reaction using primers designed to amplify a β-1,3-glucanase gene confirmed _G. catenulatum_ glucanase expression on roots. In a split-root system,
*G. catenulatum* applied to one-half of the roots prior to inoculation with *Fusarium* on the other half did not significantly reduce disease compared to plants treated with *Fusarium* only. There was no detectable increase in chitinase, peroxidase or polyphenol oxidase enzyme activity in roots and leaves following treatment with *G. catenulatum*. Competitive colonization of the rhizosphere by *G. catenulatum*, which is facilitated by its mycoparasitic ability, are the primary mechanisms by which pathogen development and disease incidence is reduced.

**Keywords:** *Clonostachys rosea f. catenulata*; biological control; greenhouse cucumbers; root colonization; mycoparasitism; induced systemic resistance; environmental factors
DEDICATION

I dedicate this thesis to my father, Peter Chatterton, and to my children Miriam and Elliot. Dad, you will always be remembered for your strength, for your songs that live on in your children and grandchildren, and for your unwavering belief in our abilities. To Miriam and Elliot, you are the joy in my life.
ACKNOWLEDGEMENTS

I would like to express my thanks to the individuals and organizations that supported me and offered their assistance during my degree program. I thank my advisor, Dr. Z. Punja for his encouragement, guidance and editing skills. I would like to thank Dr. M. Moore and Dr. A. Plant for serving on my advisory committee, and Dr. Hamelin and Dr. Paulitz for serving on my examination committee. Funding for this project was provided by the Biocontrol Network and the Natural Sciences and Engineering Research Council (NSERC) Discovery Grant. I would like to thank NSERC (post-graduate scholarship), the Biocontrol Network (post-graduate scholarship), SFU (graduate fellowship, PhD Research Stipend, and H.R. MacArthy Bursary), and the TSSU (childcare bursary) for financial assistance. I would like to thank Leslie Dodd for help with greenhouse operations and plant materials, Linda Pinto, Jayaraj Jayaraman and Jutta Buchhop for assistance with GUS transformations. Dr. Steve Marek (Oklahoma State University) and Dr. D. Guttmann (University of Toronto) kindly provided bacterial strains. Verdera Oy provided samples of Prestop, and RijkZwaan kindly donated cucumber seeds. Derrick Horne, UBC and Terry Holmes, PFC provided invaluable services with SEM processing and expertise, and light microscopy, respectively.

I am grateful for the friendship, assistance and interesting lunchtime conversations of the past and present members of the Punja lab. I would like to
thank my family, the Chatterton’s and Taylor’s, of whom there are now too many members to mention by name, for their encouragement, support, distractions and crowded Christmas dinners. To my extended family of friends, I thank you for nonstop silliness, laughter and zaniness. Most of all, I would like to express my appreciation, gratitude and love to my husband Warren for always believing in me, putting up with my never-ending education, and being my strength through everything.
TABLE OF CONTENTS

Approval ........................................................................................................................................ ii
Abstract ......................................................................................................................................... iii
Dedication ........................................................................................................................................ v
Acknowledgements ..................................................................................................................... vi
Table of Contents ........................................................................................................................ viii
List of Figures ............................................................................................................................... xi
List of Tables ................................................................................................................................. xiv
List of acronyms and abbreviations used ..................................................................................... xv

1: Introduction ................................................................................................................................. 1
  1.1 Pathogens in the greenhouse environment ................................................................. 1
  1.2 Biological control of pathogens in greenhouses ...................................................... 3
      1.2.1 Biological control of root pathogens using fungal biocontrol agents .......... 4
  1.3 Biocontrol by *Gliocladium catenulatum* .............................................................. 6
      1.3.1 Taxonomy of the genus *Gliocladium* ...................................................... 6
      1.3.2 Diseases suppressed by *Gliocladium catenulatum* ............................ 7
  1.4 Mechanisms of disease suppression by biological control agents ....................... 9
      1.4.1 Antibiosis .................................................................................................. 10
      1.4.2 Competition .............................................................................................. 12
      1.4.3 Induced systemic resistance .................................................................. 15
      1.4.4 Mycoparasitism ....................................................................................... 19
  1.5 Root colonization ............................................................................................................. 26
  1.6 Environmental factors determining the success of biocontrol agents .................. 29
  1.7 Research objectives ......................................................................................................... 32

2: Colonization of cucumber plants by the biocontrol fungus

*Gliocladium catenulatum* ............................................................................................................. 34
  2.1 Introduction .................................................................................................................... 34
  2.2 Materials and Methods ................................................................................................. 36
      2.2.1 Fungal strains and culture conditions ..................................................... 36
      2.2.2 Biological control activity of *G. catenulatum* .................................... 37
      2.2.3 Survival of *G. catenulatum* ................................................................. 38
      2.2.4 Scanning electron microscopic studies .................................................. 39
3: Chitinase and β-1,3-glucanase enzyme production by *Gliocladium catenulatum* against the fungal plant pathogens *Fusarium* and *Pythium* ................................................................. 66

3.1 Introduction ........................................................................................................ 66
3.2 Materials and Methods ...................................................................................... 68
   3.2.1 Production of antifungal metabolic compounds *G. catenulatum* that inhibit growth of *F. oxysporum* ................................................................. 68
   3.2.2 Chitinase and glucanase production in culture ............................................ 69
   3.2.3 Scanning electron microscopy (SEM) ......................................................... 71
   3.2.4 Effect of crude enzyme extracts on *Pythium* and *Fusarium* mycelial growth and conidial germination ............................................................ 72
   3.2.5 Release of glucose or NAGA from *Fusarium* or *Pythium* cell wall fragments ........................................................................................................... 73
   3.2.6 Measurement of chitinase and glucanase enzymes in cucumber plants ........................................................................................................ 73
   3.2.7 β-1,3-glucanase isoforms ............................................................................. 74
   3.2.8 Glucanase gene expression ......................................................................... 75
   3.2.9 Statistical analyses ...................................................................................... 76
3.3 Results ................................................................................................................ 77
   3.3.1 Production of antifungal metabolic compounds *G. catenulatum* that inhibit growth of *F. oxysporum* ................................................................. 77
   3.3.2 Chitinase and glucanase production in culture ............................................ 80
   3.3.3 Scanning electron microscopy .................................................................... 83
   3.3.4 Effect of crude enzyme extracts on *Pythium* and *Fusarium* growth and conidial germination ................................................................. 86
   3.3.5 Release of glucose or NAGA from *Fusarium* or *Pythium* cell walls ...... 86
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.6</td>
<td>Measurement of chitinase and glucanase in cucumber plants</td>
<td>89</td>
</tr>
<tr>
<td>3.3.7</td>
<td>β-1,3-glucanase isoforms</td>
<td>89</td>
</tr>
<tr>
<td>3.3.8</td>
<td>Glucanase gene expression</td>
<td>92</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>95</td>
</tr>
</tbody>
</table>

4: Factors influencing colonization of cucumber roots by *Gliocladium catenulatum* | 101 |
| 4.1     | Introduction | 101 |
| 4.2     | Materials and Methods | 103 |
| 4.2.1   | Fungal strains and plant growing conditions | 103 |
| 4.2.2   | Factors affecting population levels of *G. catenulatum* on cucumber roots | 105 |
| 4.2.3   | Statistical analysis | 107 |
| 4.3     | Results | 108 |
| 4.3.1   | Factors affecting population levels of *G. catenulatum* on cucumber roots | 108 |
| 4.4     | Discussion | 119 |

5: Evaluation of *Gliocladium catenulatum* to induce systemic resistance in cucumber | 126 |
| 5.1     | Introduction | 126 |
| 5.2     | Materials and Methods | 128 |
| 5.2.1   | Fungal strains and culture conditions | 128 |
| 5.2.2   | Disease development of Fusarium root and stem rot and biocontrol by *G. catenulatum* in hydroponic culture | 130 |
| 5.2.3   | Split-root assays | 131 |
| 5.2.4   | Measurement of defense-related enzymes in cucumber plants | 132 |
| 5.2.5   | Disease development of *P. syringae* pv. *lachrymans* (*Psl*) in cucumber leaves | 133 |
| 5.2.6   | Statistical analysis | 134 |
| 5.3     | Results | 134 |
| 5.3.1   | Disease development of Fusarium root and stem rot and biocontrol by *G. catenulatum* in hydroponic culture | 134 |
| 5.3.2   | Split-root assays | 135 |
| 5.3.3   | Measurement of defense-related enzymes in cucumber plants | 137 |
| 5.3.4   | Disease development of *P. syringae* pv. *lachrymans* in cucumber leaves | 142 |
| 5.4     | Discussion | 147 |

6: General discussion and conclusions | 152 |

7: References | 159 |
LIST OF FIGURES

Figure 2-1. Disease severity index (DSI) of cucumber plants and population densities of *G. catenulatum* on cucumber roots, tissues and growing medium. ................................................................. 47

Figure 2-2. Scanning electron micrographs of cucumber roots 7 days after inoculation with *G. catenulatum* (A-F), or *F. oxysporum* (G-I), or *G. catenulatum* followed 3 days later by *F. oxysporum* (J-L). .......... 48

Figure 2-3 Colonization of root, stem, cotyledon and leaf pieces by *G. catenulatum* after 0, 10, 30 or 60 s of surface sterilization, 3 weeks following application of Prestop WP to the seed............... 50

Figure 2-4 Colonization pattern of cucumber plants by *G. catenulatum* strain J1446 expressing the GUS gene. ................................................................. 53

Figure 2-5 Relationship between fungal biomass or protein content and GUS activity in a transformant of *G. catenulatum*................................. 55

Figure 2-6 Fungal biomass of *G. catenulatum* expressing the GUS gene in different tissues of cucumber plants following application to seed or rockwool block. ................................................................. 56

Figure 2-7 Population size of *Forc* in the roots (top) or crown area (bottom) of cucumber plants treated with *G. catenulatum* or Prestop WP ................................................................. 57

Figure 3-1 Dual culture plates of *G. catenulatum* (Gc) and *F. oxysporum* (Fo) or *P. aphanidermatum*. ........................................................................ 78

Figure 3-2 Colony diameter (top) and conidia production (bottom) of *F. oxysporum* that was grown alone or after 7 days exposure to cultures of *G. catenulatum* growing for 7 days or 14 days on a Petri dish attached by parafilm. ................................................................. 79

Figure 3-3 Time course of (A) chitinase and (B) β-1,3-glucanase production by *Gliocladium catenulatum* on MSM (no carbon source) and on medium containing chitin or laminarin as a carbon source, respectively. ....................................................... 81

Figure 3-4 Effect of carbon source on chitinase and β-1,3-glucanase production after 7 days of growth of *Gliocladium catenulatum*. .......... 82
Figure 3-5. Effect of initial medium pH on (A) chitinase and (B) β-1,3-glucanase production by *Gliocladium catenulatum* grown for 7 days on chitin or laminarin as the carbon source, respectively.  

Figure 3-6. Scanning electron micrographs of the interactions between *Gliocladium catenulatum* (c) and *Fusarium oxysporum* (f) in dual culture on water agar (A-D), or between *G. catenulatum* and *Pythium* (p) on excised cucumber roots (r) on water agar (E,F).  

Figure 3-7. Effect of *Gliocladium catenulatum* culture filtrates on (A) growth of *Fusarium oxysporum* or *Pythium aphanidermatum* mycelia or (B) germination of *Fusarium* conidia.  

Figure 3-8. Effect of *Gliocladium catenulatum* culture filtrates on the release of (A) NAGA from *Fusarium* cell wall fragments or (B) glucose from *Fusarium* or (C) *Pythium* cell wall fragments.  

Figure 3-9. Glucanase activity in roots of 10-day-old cucumber seedlings at 2, 3 and 7 days following inoculation with *Gliocladium catenulatum* (Gc), treatment with salicylic acid (SA) or water (Con).  

Figure 3-10. SDS-PAGE of β-1,3-glucanase isoforms from *G. catenulatum*.  

Figure 3-11. Detection of β-1,3-glucanase expression by *Gliocladium catenulatum* on colonized cucumber roots using reverse transcription – polymerase chain reaction (RT-PCR).  

Figure 3-12. Northern blot analysis of expression of a β-1,3-glucanase gene from *Gliocladium catenulatum*.  

Figure 4-1. Population levels of *G. catenulatum* on cucumber roots determined by colony plate counts (A) or GUS expression (B) as influenced by pH of the nutrient solution.  

Figure 4-2. Population levels of *G. catenulatum* on cucumber roots determined by colony plate counts (A) or GUS expression (B) as influenced by the temperature of the nutrient solution.  

Figure 4-3. Population levels of *G. catenulatum* on cucumber roots grown in different media types determined by colony plate counts (A) or GUS expression (B).  

Figure 4-4. Population levels of *G. catenulatum* on the roots of 5 cucumber cultivars (Averyl, Bodega, Ladner, Marcel or Sienna) determined by colony plate counts (A) or GUS expression (B).  

Figure 4-5. Population levels of *G. catenulatum* on cucumber roots as affected by addition of glucose or asparagine to the nutrient solution determined by colony plate counts (A) or GUS expression (B).
Figure 4-6  Population levels of \textit{G. catenulatum} on unwounded (control) cucumber roots or wounded roots determined by colony plate counts (A) or GUS expression (B). ......................................................... 118

Figure 5-1 Disease severity index (A) and root fresh weight (B) of cucumber plants inoculated with \textit{Fusarium} only or inoculated with \textit{Fusarium} three days after application of \textit{G. catenulatum} (1 x 10^6 conidia/ml NS) to the roots (Gc + Fus). ................................................................. 136

Figure 5-2 Disease severity index (A) and root fresh weight (B) of cucumber plants grown in hydroponic solution and either untreated (control) or treated with \textit{Gliocladium} on one half of the roots (Gc), treated with \textit{Forc} on one half of the roots only (Forc) or treated with \textit{Gliocladium} on one half of the roots 3 days before treatment with \textit{Forc} on the other half of the roots (Forc+Gc). .......... 138

Figure 5-3 Enzyme activities in roots or leaves of 10-day-old cucumber seedlings at 2, 3 and 7 days following application of INA (2 mg/L), \textit{G. catenulatum} (1x 10^6 cfu/ml) or SA (2 mM). .................. 141

Figure 5-4 Disease symptoms of \textit{Pseudomonas syringae pv. lachrymans} when applied as droplets on cotyledons of cucumber plants, 5 days after pathogen inoculation......................................................... 143

Figure 5-5 Disease severity of \textit{Pseudomonas syringae pv. lachrymans} when applied as droplets on cotyledons of cucumber plants measured by lesion area, mm^2 (A), % diseased leaf area (B), and population levels of \textit{Psl} on the surface of cotyledons (C). ............ 144

Figure 5-6 Disease symptoms of \textit{Pseudomonas syringae pv. lachrymans} when applied using a cheesecloth rub on cotyledons of cucumber plants, 5 days after pathogen inoculation.............................. 145

Figure 5-7 Disease severity of \textit{Pseudomonas syringae pv. lachrymans} when applied using a cheesecloth rub on cotyledons of cucumber plants measured by lesion area, mm^2 (A), % diseased leaf area (B), and population levels of \textit{Psl} on the surface of cotyledons (C) .............................................................. 146
LIST OF TABLES

Table 2-1  Slope values ($b$) from linear regression analysis of the population levels of *Forc* in the roots or crown area of cucumber plants treated with *G. catenulatum* ($10^7$ conidia/ml) versus log$_{10}$-transformed initial inoculum of *Forc*. ................................................................. 59

Table 2-2  Population levels of *G. catenulatum* in the roots or crown area of cucumber plants treated with *G. catenulatum* applied to the rockwool blocks ($10^7$ conidia/ml) prior to inoculation with *Forc* at an initial inoculum level of either $10^4$, $10^5$, or $10^6$ conidia/ml. ............... 60
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APDA</td>
<td>Acidified potato dextrose agar</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance.</td>
</tr>
<tr>
<td>AS</td>
<td>Acetosyringone</td>
</tr>
<tr>
<td>BCA</td>
<td>Biocontrol agent</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CHU</td>
<td>Chitinase units</td>
</tr>
<tr>
<td>DPA</td>
<td>Days post-application</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post-inoculation</td>
</tr>
<tr>
<td>DSI</td>
<td>Disease severity index</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Forc</td>
<td><em>Fusarium oxysporum</em> f. sp. <em>radicis-cucumerinum</em></td>
</tr>
<tr>
<td>gpd</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GU</td>
<td>Glucanase units</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>IM</td>
<td>Induction media</td>
</tr>
<tr>
<td>INA</td>
<td>2,6-dichloroisonicotinic acid</td>
</tr>
<tr>
<td>Log₁₀</td>
<td>Logarithm to base 10</td>
</tr>
</tbody>
</table>
M  Molarity
min  Minutes
MSM  Minimal synthetic (or salts) media
MU  Methylumbelliferone
MUG  Methylumbelliferyl β-D-glucuronide hydrate
NAGA  N-acetyl glucosamine
NS  Nutrient solution
PCA  Paraquat chloramphenicol agar
PDA  Potato dextrose agar
PDAtt  Potato dextrose agar + Triton + tetracycline
PDB  Potato dextrose broth
PsI  *Pseudomonas syringae pv lachrymans*
RFW  Root fresh weight
s  seconds
SA  Salicylic acid
SDW  Sterile distilled water
VOC  Volatile organic compound
1: INTRODUCTION

1.1 Pathogens in the greenhouse environment

One of the motivating factors in developing greenhouse cropping systems was the theoretical potential to eliminate or minimize plant diseases (Stanghellini and Rasmussen, 1994). Certainly, the diversity of pathogens is lower in the greenhouse, but most pathogens cannot be effectively excluded from the greenhouse environment (Paulitz and Belanger, 2001). Hence, several fungi have emerged as major root and foliar pathogens that can cause devastating crop losses when introduced into the enclosed system (Stanghellini and Rasmussen, 1994). Soilless substrates such as peat or rockwool lack the microbial diversity of natural soil. Thus, soilborne pathogens such as *Pythium aphanidermatum*, *Rhizoctonia solani* and *Fusarium oxysporum* can quickly grow and spread (Paulitz and Belanger, 2001). The warm temperatures and high humidity maintained in the greenhouse that ensure optimal plant growth also provide ideal conditions for infection by foliar pathogens such as *Botrytis cinerea* and powdery mildews. High density planting of greenhouse crops and the recirculation of nutrient solution can facilitate rapid spread of pathogens. Management practices, such as pruning and continuous harvesting, also provide sources of infection through wounds.

In British Columbia, two common root-infecting pathogens of greenhouse cucumbers are *P. aphanidermatum*, causing damping-off and root rot and
*Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Forc), causing Fusarium root and stem rot (Punja and Parker, 2000). *F. oxysporum* is an ascomycete fungus in the Order Hypocreales (Michielse and Rep, 2009). *P. aphanidermatum* is an oomycete, which are phylogenetically more closely related to algae than fungi, although its classification under a new Kingdom Stramenopiles or Chromista within the Domain Eukarya is under debate (Adl et al., 2005). Distinct characteristics of oomycetes are cell walls that lack chitin, and are instead composed primarily of β-1,3-glucan, and the production of motile flagellated aquatic zoospores (Viterbo et al., 2002b; Stanghellini and Rasmussen, 1994).

Symptoms of *P. aphanidermatum* infection include extensive browning and rotting of the root system and crown area (Wulff et al., 1998; Punja and Yip, 2003). Symptoms of *F. oxysporum* infection include a brown discoloration at the crown and vertical stem lesions accompanied by the presence of white mycelium and masses of orange conidia (Punja and Parker, 2000; Vakalounakis, 1996). In advanced stages of both diseases, plants begin to wilt and eventually die. While there are no cucumber cultivars resistant to *P. aphanidermatum* (Punja and Yip, 2003), cultivars of cucumber differ in their tolerance to *F. oxysporum*, with some cultivars showing high levels of resistance (Rose and Punja, 2004). Although seedlings appear to be most susceptible to infection by both *F. oxysporum* and *P. aphanidermatum*, disease symptoms often are most apparent in older plants (Punja and Parker, 2000; Favrin et al., 1988). Therefore, the pathogens may be present in the plant without causing disease until the plants become stressed by events such as fruiting, or by environmental factors such as high temperatures or
imbalances in the nutrient solution (Cherif et al., 1997; Favrin et al., 1988; Punja and Parker, 2000). Both pathogens are likely introduced into the greenhouse on infected plant material, contaminated growing substrates or irrigation water, with subsequent spread occurring throughout the greenhouse by water dispersal or contaminated equipment (Paulitz and Belanger, 2001; Stanghellini and Rasmussen, 1994).

1.2 Biological control of pathogens in greenhouses

Greenhouses and protected structures provide a unique niche for the implementation of biological control programs. Currently, over half of the commercially available biocontrol products are registered for use in greenhouses and nurseries, making the use of biocontrol agents in greenhouses more prevalent than in field crops (van Lenteren, 2000). Many of these biocontrol agents (BCAs) have been developed specifically against the major greenhouse pathogens, such as *Pythium*, *Rhizoctonia* and *Fusarium* (Paulitz and Belanger, 2001). Differences between field and greenhouse environments may help to explain the success of biocontrol agents in the greenhouse. Environmental parameters, such as temperature and relative humidity, can be adjusted to provide a consistency of performance of biocontrol agents that is not often observed under natural field conditions (Paulitz and Belanger, 2001). Greenhouses are relatively isolated units and therefore a limited number of pest species occur within them (van Lenteren, 2000). Introduced biological control agents are likely to establish and proliferate due to the low biological diversity in soilless substrates observed in the early stages of greenhouse production.
(Fravel, 2005). To aid in dispersal throughout the cropping system, biocontrol agents, if formulated correctly, can be easily added to the nutrient solution, thus facilitating uniform distribution (Paulitz, 1997).

Growers working in greenhouses often prefer biological control over chemical control agents for a number of reasons. Unlike their behaviour in soil, fungicides applied to the roots often cause toxicity to the crop plant under soilless conditions due to the lack of adsorption and dispersal (van Lenteren, 2000). In addition, there are relatively few fungicides registered for use in Canadian greenhouses. The use of biocontrol agents does not disrupt scheduled operations such as the prolonged re-entry times required after pesticide use (Harman, 2000). Consumer demands for pesticide-free foods also stimulate the use of biocontrol agents (van Lenteren, 2000). There is considerable precedence for the use of biological control against insect pests in greenhouses and this has become a well-established practice by growers. However, in spite of the great optimism and extensive research efforts, progress in achieving commercial, large-scale use of biological disease control has been slow and has yet to achieve the same successes realized for insect control (Spadaro and Gullino, 2005).

1.2.1 Biological control of root pathogens using fungal biocontrol agents

1.2.1.1 *Trichoderma* spp.

By far, the most studied biocontrol agents are *Trichoderma* spp. accounting for 90% of fungal strains that have been evaluated for use as biocontrol agents of plant diseases (Benitez et al., 2004). The most common
BCAs from the *Trichoderma* genus are strains of *T. virens, T. viride, T. asperellum* and *T. harzianum* (Punja and Utkhede, 2003). The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, their ability to survive under unfavourable environmental conditions, efficient utilization of nutrients, capability to colonize the plant rhizosphere, and strong aggressiveness against plant pathogenic fungi (Benitez et al., 2004). *T. harzianum* strain T-22 is marketed by Bioworks, Geneva, NY as a granular formulation (RootShield®) or as a wettable powder for soil drench (RootShield-Drench®) for suppression of root diseases in greenhouse crops, and specifically protects roots against *Pythium, Rhizoctonia* and *Fusarium*. Strain T-22 was produced using protoplast fusion between two parent strains in an attempt to obtain a biocontrol strain that was effective in competing with seed-borne bacteria, but still retained the ability to efficiently colonize developing roots (Ahmad and Baker, 1985; Harman, 2000). This biocontrol agent is registered in Canada for use against root pathogens in ornamental crops, tomato and cucumber.

### 1.2.1.2 Non-pathogenic *Fusarium oxysporum*

Non-pathogenic *Fusarium* species have been isolated from soils that naturally suppress Fusarium wilts, and as such are primarily effective against diseases caused by pathogenic *F. oxysporum* (Fravel et al., 2003). *Fusarium oxysporum* strain Fo47 has been extensively studied and is effective against Fusarium wilt diseases on carnation, tomato, cyclamen and Fusarium crown and root rot on tomato (Fravel et al., 2003). Fusaclean® is a biocontrol product
formulated from non-pathogenic *F. oxysporum* isolate Fo47 by Natural Plant Protection, Nogueres, France, but it is not yet registered for use in the US or Canada (Benhamou et al., 2002). This fungus has been extensively studied for its competitive ability and will be discussed further in section 1.4.2.

### 1.2.1.3 Gliocladium catenulatum

*G. catenulatum* strain J1466 was originally isolated from a Finnish field soil in 1989, in a screening for soil microbes that were effective in suppressing *Fusarium culmorum* on cereals (Teperi et al., 1998). The strain was then developed into a commercial biofungicide by Verdera Oy (Finland), and is marketed under the trade name Prestop®. The fungus is formulated as a soluble powder for spraying and drip irrigation (Prestop® WP). The final Prestop® product contains about $10^8$ colony-forming units per gram and has a shelf-life of one year when stored below $8^\circ$C. Prestop® Biofungicide WP has recently received registration for minor use in Canada against a variety of fungal diseases on greenhouse vegetables, herbs and ornamentals (Health Canada, 2008).

### 1.3 Biocontrol by Gliocladium catenulatum

#### 1.3.1 Taxonomy of the genus Gliocladium

The taxonomy of *Gliocladium* spp. is changing, and many species that were originally included in this genus have been moved to the genus *Clonostachys*. Schroers et al. (1999) proposed to reclassify *Gliocladium roseum*, a mycoparasite and known biocontrol agent, due to significant differences in morphology, ecology, and DNA sequence data from the type species of
Gliocladium, G. penicillioides. G. roseum (Clonostachys rosea), is distinguished by possession of two kinds of conidiophores: (i) early-formed Verticillium-like conidiophores with few long phialides; and (ii) later-formed penicillate conidiophores with small phialides and long conidial columns (Schroers et al., 1999). The teleomorph of G. roseum was first recognised as Nectria ochroleuca, and molecular data confirms that these two fungi are the same species. However, the genus N. ochroleuca is now classified in the Bionectria genus, and is distinct from the Nectria genus (Schroers, 2000). B. ochroleuca is found most commonly in subtropical and tropical regions, whereas its anamorph, C. rosea, is common in all areas. Shortly after this study was published, Schroers (2001) released a monograph of the genus Bionectria, proposing that G. catenulatum is a green-conidial variant of C. rosea, and thus should be renamed Clonostachys rosea f. catenulata (Schroers, 2001). The two variants, C. rosea and G. catenulatum, are readily distinguishable in culture. C. rosea produces a pale yellow to white mycelia with no discernible evidence of conidia on PDA, whereas G. catenulatum produces a pale peach to pink mycelia on the reverse with masses of green conidia on the surface. In this thesis, the name Gliocladium catenulatum will be used while recognizing the current taxonomical proposal to change it to C. rosea f. catenulata (Schroers, 2001).

1.3.2 Diseases suppressed by Gliocladium catenulatum

Efficacy trials with Prestop have shown that it is effective in reducing disease severity caused by a number of plant pathogens on a range of crops. In growth chamber trials, Prestop WP reduced the mortality of cucumber seedlings
due to damping-off caused by \textit{P. aphanidermatum} by 35\% under conditions of high disease pressure (Punja and Yip, 2003). In greenhouse experiments, \textit{G. catenulatum}, formulated as Prestop WP or Prestop Mix, was most effective in reducing plant mortality caused by \textit{P. aphanidermatum} (Punja and Yip, 2003). \textit{G. catenulatum} has also been shown to be effective in reducing pre- and post-emergence damping-off caused by \textit{P. ultimum} in pansy and snapdragon and post-emergence damping-off caused by \textit{R. solani} in salvia (McQuilken et al., 2001). In addition to \textit{Pythium}, \textit{G. catenulatum} was also successful in controlling Fusarium root and stem rot on cucumber plants, resulting in disease levels that were not significantly different from fungicide-treated plants (Rose et al., 2003). Damping-off on ginseng seedlings caused by a complex of soilborne pathogens was also reduced by \textit{G. catenulatum} (Rahman and Punja, 2007).

In addition to root-infecting pathogens, when applied to blossoms and developing fruit \textit{G. catenulatum} also had some efficacy against anthracnose on blueberry fruits caused by \textit{Colletotrichum acutatum} (Verma et al., 2006). \textit{Gliocladium} applications reduced the incidence of gummy stem blight on cucumbers, caused by \textit{Didymella bryoniae}, to approximately one-third of that observed in untreated plants (Utkhede and Koch, 2004). \textit{G. catenulatum} suppressed \textit{Botrytis cinerea} on tomato stems under semi-commercial conditions (Utkhede and Mathur, 2002), and was the most effective microbial agent against grey mould on lettuce seedlings (Lahdenpera and Korteniemi, 2005). It has also shown excellent control of \textit{Botrytis} on strawberries under field conditions in
Finland, and treatment with *G. catenulatum* increased the marketable yield (Lahdenpera and Korteniemi, 2005).

A number of diseases are also suppressed by isolates of *C. rosea* (*G. roseum*). The majority of research on this fungus has focused on its ability to suppress grey mould (*B. cinerea*) on a number of greenhouse crops including begonia, cyclamen, geranium, cucumber, pepper, and tomato and on field crops such as raspberry, strawberry and conifer seedlings (Sutton et al., 1997). *C. rosea* strain IK726 was recovered in the same screening project that resulted in the isolation of *G. catenulatum* J1446 (Jensen et al., 2007). This strain showed high efficacy in the biocontrol of both *F. culmorum* and *Bipolaris sorokiniana* on coated barley seeds (Jensen et al., 2000). By introducing strain IK726 at the start of carrot seed priming, *Alternaria* spp. were effectively controlled and field emergence was significantly improved (Jensen et al., 2004).

1.4 **Mechanisms of disease suppression by biological control agents**

Biocontrol agents are living organisms whose activities are dependent on the different physical and chemical environmental conditions to which they are subjected. For this reason, biocontrol efficacy can be unpredictable. Understanding the genetic diversity of strains and their mechanisms of biocontrol will lead to improved application of different strains of biocontrol agents. These mechanisms are often complex, but biocontrol results from the different mechanisms acting synergistically to achieve disease control. However, despite the assumed synergism between mechanisms, most mechanisms have been
studied in isolation in order to better decipher their role. Current research suggests there are four general mechanisms by which biocontrol is achieved: i) antibiosis; ii) mycoparasitism; iii) competition; or iv) induced systemic resistance. These are each discussed in more detail below.

1.4.1 Antibiosis

Antibiotic production by biocontrol fungi has most commonly been reported for isolates of *Trichoderma* and *Gliocladium* (Whipps, 2001). Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that inhibit growth of microorganisms, although antibiotic production does not always correlate with biocontrol ability (Benitez et al., 2004). Based on their antibiotic profiles, strains of *Trichoderma* (*Gliocladium*) *virens* can be separated into P and Q groups. Strains of the P group produce the antibiotic gliovirin, which has a very restricted activity spectrum, and is primarily effective against oomycetes such as *Pythium ultimum* (Howell, 2006). Strains of the Q group produce the antibiotic gliotoxin, which has broad spectrum activity against bacteria, actinomycetes and fungi. However, mutants of *T. virens* deficient for antibiotic production were as effective as parental strains in achieving biocontrol of cotton seedling diseases (Howell, 2006), indicating that antibiotics likely did not play a role in biocontrol by *T. virens*. On the other hand, biocontrol of *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all of wheat, by *T. harzianum* is related to the production of pyrone-like antibiotics (Vinale et al., 2008). Pyrones are low-molecular weight, non-polar, volatile compounds. High concentrations can build up in soil, resulting in long-range inhibition of microbial
communities. *T. harzianum* also produces peptaibols, linear peptides that have antimicrobial activity against bacteria and fungi (Lorito et al., 1996). Peptaibols inhibit β-glucan synthase in the target fungus, preventing the reconstruction of the pathogen cell wall, thus facilitating the disruptive action of β-glucanases (Lorito et al., 1996).

A novel fungal genus, *Muscodor albus*, an endophyte of *Cinnamomum zeylanicum*, was discovered to produce a broad range of volatile organic compounds (VOC) that have strong antimicrobial activity (Strobel, 2006). The mixture of VOCs consists primarily of various alcohols, acids, esters, ketones, and lipids. A number of plant pathogenic soilborne fungi, including *P. ultimum* and *R. solani*, are sensitive to these VOCs and exposure often results in death of the fungus. This fungus is now being explored for use as a mycofumigant and is being developed by AgraQuest, an agricultural biotech company in Davis, Calif., for use in agricultural settings to treat pathogen infested soils, plants and seeds (Strobel, 2006). An endophytic *Gliocladium* sp, isolated from the Gondwanaland tree in South America, has also been found to produce VOCs that are inhibitory against *P. ultimum* and other soilborne fungi, but was less effective against *F. oxysporum* (Stinson et al., 2003). The volatile organic compound [8]annulene was produced in the greatest amount by *Gliocladium* sp., and this was the first report of annulene production by a fungal species (Stinson et al., 2003). Very little is currently known about the mode of action of VOCs resulting in inhibition or death of the test microbes (Strobel, 2006). It is also not known if *G. catenulatum* produces antibiotics or volatile compounds as this has not been studied.
1.4.2 Competition

Competition can be divided into saprophytic competition for nutrients in the soil and rhizosphere, and competition for infection sites on the plant (Alabouvette et al., 2007). Plant roots are a major source of carbon and energy for microorganisms in the form of root exudates, and availability of these nutrients can often be the limiting factor in the germination of root-infecting plant pathogen propagules (Sivan and Chet, 1989). Starvation is the most common cause of death for microorganisms, so competition for limited nutrients can result in biocontrol if the introduced microorganisms can rapidly sequester or utilize existing nutrients (Vinale et al., 2008). F. oxysporum and P. aphanidermatum are opportunistic pathogens and thus are very susceptible to competition for nutrients (Paulitz and Belanger, 2001). The competitive ability of a biocontrol strain determines its capacity to establish in soil and in the plant rhizosphere, and is probably involved in its capability to colonize the root surface (Alabouvette et al., 2007). Different biocontrol strains have different abilities to colonize soil and the rhizosphere of different plants. The majority of studies addressing the role of competition in biocontrol efficacy against root-infecting pathogens have focused on non-pathogenic F. oxysporum and the biocontrol of vascular wilts. The growth kinetics of a collection of non-pathogenic F. oxysporum isolates introduced into sterilized soil amended with various amounts of glucose and a pathogenic strain of F. oxysporum f. sp. lini were used to develop a competitiveness index for each strain. These indices varied among strains, indicating that there is a large diversity among biocontrol strains in their competitive ability and capacity to utilize a carbon source (Couteaudier and
Alabouvette, 1990b). For example, isolate Fo47 could significantly inhibit chlamydospor germination of *F. oxysporum* f. sp. *lycopersici* in soil, but this repression decreased as glucose concentration in the soil was increased (Larkin and Fravel, 1999). Similarly, isolate Fo47 could not reduce the mycelial growth of pathogenic *F. oxysporum* when glucose levels in the growing media were high, but significant reductions in growth were observed under limiting glucose concentrations (Lemanceau et al., 1993).

Competition for infection sites has been considered an important mechanism as the root surface is thought to have a finite number of infection sites (Alabouvette et al., 2007). Many studies support this hypothesis of competition for infection sites. To demonstrate that pathogenic and non-pathogenic strains of *F. oxysporum* were competing for root colonization, a GUS-transformed strain of pathogenic *F. oxysporum* was introduced on the plant roots along with the biocontrol strain Fo47 (Eparvier and Alabouvette, 1994). Co-inoculation of the pathogen and biocontrol strain resulted in the same total fungal biomass as when either strain was inoculated alone, indicating that there was a maximum fungal population that could be supported on the plant roots. Furthermore, the biocontrol strain significantly reduced the pathogen population as measured by GUS activity. In a similar study, introduction of a non-pathogenic *F. oxysporum* strain reduced colonization intensity of the root tissues by *F. oxysporum* f.sp. *lycopersici* in tomato (Bao and Lazarovits, 2001). However, microscopic examination revealed that the pathogen strain was present inside the xylem vessels while the non-pathogenic strain was present at
the root surface and in the upper layers of cortical cells. This study demonstrated that the two strains could exclude each other from the same ecological niche, but since wilt pathogens preferentially colonize and damage the vascular system, pathogen development was not affected (Bao and Lazarovits, 2001). In another study, *F. oxysporum f. lycopersici* was transformed with DsRed2 while Fo47 was transformed with GFP (Olivain et al., 2006). When the two strains were coinoculated into soil, both strains were observed on the surface of tomato roots, often growing together at the same spot, indicating that the presence of one strain on the root did not prevent colonization by the other strain. Even when the protective strain was introduced at much higher inoculum levels than the pathogenic strain, it could never completely exclude the pathogen from reaching the root surface. However, the amount of root colonization by either strain was reduced relative to that colonized in a single inoculation with either strain alone at the same inoculum concentration. This reduced colonization is consistent with a reciprocal competitive interaction for nutrients. The authors concluded that competition for colonization of the root surface likely plays little role in the interaction between pathogenic and non-pathogenic *F. oxysporum*, and that competition relates to nutrients rather than to space (Olivain et al., 2006). It should be noted that there is no evidence that either mycoparasitism or antibiosis exists between strains of non-pathogenic *F. oxysporum* and pathogenic strains (Alabouvette et al., 2007).
1.4.3 Induced systemic resistance

A number of biocontrol fungi are reported to be efficient inducers of systemic and localized resistance in plants, i.e., activation of resistance mechanisms in the plant that combat subsequent pathogen attack. Resistance mechanisms of the plant include formation of thickened cell walls, deposition of phenolic compounds, phytoalexin synthesis and production of pathogenesis-related (PR) proteins, such as chitinases, β-1,3-glucanases, proteinase inhibitors and peroxidases (Harman and Shores, 2007; Conrath et al., 2006; Van Loon, 1997; Heil and Bostock, 2002). This mode of action has been implicated in the biocontrol efficacy of several Trichoderma spp. and non-pathogenic F. oxysporum isolates CS20 and Fo47. The induced resistance effect appears to be strongly dependent on the strain of the antagonist, the species/cultivar of the plant, the type of growing media and the pathogen (Hoitink et al., 2006; Woo and Lorito, 2007). Prerequisites for fungi to induce systemic resistance are the ability to colonize the root surface and penetrate the epidermis and the outer cell layers of the cortex. This root colonization behaviour has been demonstrated for strains of T. harzianum (T-203) and non-pathogenic F. oxysporum (Yedidia et al., 1999; Yedidia et al., 2000; Nahalkova et al., 2008; Olivain et al., 2006; Le Floch et al., 2005). Once the hyphae penetrate the roots, the fungus secretes metabolites that act as elicitors to signal plant resistance pathways resulting in plant cell-wall and biochemical changes that limit the growth of the fungus to a confined area (Harman, 2006; Harman et al., 2004). This interaction, which may also limit the
ingress of the biocontrol fungus, can result in both localized and systemic resistance to subsequent pathogen attack.

Many investigators have used a split-root method to study how biocontrol agents may induce plant resistance to root-infecting pathogens. In this system, since there is no direct interaction between the two microorganisms, disease reduction can be attributed to indirect effects resulting from increased plant defense responses due to root colonization by the biocontrol agent. *F. oxysporum* strain Fo47 protected tomatoes against Fusarium wilt in four different bioassay systems, in which the two fungi were physically separated from each other. In addition, colonization by Fo47 increased chitinase, $\beta$-1,3-glucanase, and $\beta$-1,4-glucosidase activity in plants (Fuchs et al., 1997). When induced resistance is the main mode of action, disease control can be achieved even when the pathogen population is much greater than that of the biocontrol fungus. Non-pathogenic *F. oxysporum* strain CS-20 could still reduce the incidence of vascular wilt in tomato when the pathogen population was up to 1000 times greater than that of the biocontrol agent (Larkin and Fravel, 1999).

Localized resistance has been implicated in the ability of *T. virens* to control cotton seedling diseases caused by *R. solani* through induction of terpenoid phytoalexins at the site of the infection (Howell, 2006). Resistance to Botrytis blight was observed in geranium cuttings that were transplanted into potting mix amended with binucleate *Rhizoctonia* isolates and *T. hamatum* isolate 382 (Olson and Benson, 2007). Treatment of tobacco roots with *G. roseum (C. rosea)* resulted in an increased activity of $\beta$-1,3-glucanases, $\beta$-1,4-
glycosidases and chitinases in leaf extracts, and leaves of these plants showed less severe symptoms of powdery mildew compared with the control (Lahoz et al., 2004). Pre-inoculation of tomato plants with the mycoparasite Pythium oligandrum triggered synthesis of several PR-proteins, which were increased further upon attack by B. cinerea (Le Floch et al., 2007).

Application of T. asperellum (T-203) to roots of cucumber plants induced resistance to Pseudomonas syringae pv. lachrymans (Psl) on the foliage and the physiological responses that were activated in the plant have been well characterized (Yedidia et al., 2003). Addition of the biocontrol agent to the roots resulted in a transient increase in defense-related proteins in both roots and shoots. When the leaves were subsequently inoculated with Psl, the expression of several genes encoding PR proteins, such as hydroperoxide lyase, chitinase, β-1,3-glucanase, and peroxidase, were increased compared to pathogen-only controls. Therefore, it appeared that the presence of T. asperellum potentiates resistance mechanisms in the plant to respond more rapidly to pathogen attack, implying that up-stream regulatory genes were activated by the biocontrol agent. This potentiation has been shown to be dependent on the jasmonate/ethylene pathways, but not the salicylate pathway (Shoresh et al., 2005a). Treatment of cucumber plants with jasmonate and ethylene inhibitors abolished the protective effect of T. asperellum, although colonization of the roots by the biocontrol agent was not affected. Furthermore, Lox1, a gene involved in jasmonate synthesis, and Pal1, an indicator of activation of the jasmonate pathway, were both upregulated following inoculation with T. asperellum. Therefore, there is strong
evidence to suggest that the application of *T. asperellum* to the roots potentiates the jasmonate/ethylene pathway, but this pathway is only fully activated when the pathogen is inoculated on the foliage.

During the interaction of a resistance-inducing biocontrol fungus with the plant, different classes of metabolites may elicit the induced response (elicitors). These molecules include: i) proteins with enzymatic activity, such as xylanase (Belien et al., 2006); ii) avirulence-like gene products (Harman and Shoresh, 2007); or iii) low-molecular-weight compounds released from fungal or plant cell walls (Vinale et al., 2008). Penetration of the epidermis and subsequent ingress into the outer cortex of cucumber roots by fungal biocontrol agents likely requires secretion of cell wall lytic enzymes (Yedidia et al., 1999). Xylan containing $\alpha$-L-arabinofuranoside side chains is a major component of plant cell walls. A xylanase isolated from the filtrate of *T. atroviride* induced ethylene biosynthesis genes and PR protein accumulation when applied to tobacco leaves, but this response was cultivar-specific (Avni et al., 1994). Two fungal $\alpha$-L-arabinofuranosidases and two aspartyl proteases were differentially expressed when *T. asperellum* was cultivated in the presence of cucumber roots, and were upregulated during the first 24 hours of plant interaction (Viterbo et al., 2004). These findings indicate that plant cell-wall degrading enzymes are actively secreted by *Trichoderma* to penetrate the root epidermis, and that these enzymes may also function as elicitors. In *T. virens*, a protein with elicitor activity homologous to a serine protease was isolated (Hanson and Howell, 2004), providing further evidence for the role of proteases in induced systemic
resistance. Recently, a small protein (Sm1) isolated from *T. virens* was shown to induce the expression of defense-related genes in cotton (Djonovic et al., 2007b). Conversely, expression of Sm1 by *T. virens* was also significantly enhanced in the presence of the host plant (Djonovic et al., 2006). Interestingly, this protein belongs to a family of phytotoxic proteins, common among fungal pathogens, but Sm1 lacks toxicity against plants and microbes. A number of proteins, such as TasHyd1 (hydrophobin), swollenin, and peptaibols, from *T. asperellum* T-203 are involved in both plant root colonization and induction of systemic resistance, and their genes have been characterized (Brotman et al., 2008; Viterbo et al., 2007; Viterbo and Chet, 2006). Despite these advances, a thorough understanding of the molecular communication processes that occurs during fungal-plant recognition is lacking, and the majority of research has focussed on *Trichoderma*-plant interactions (Djonovic et al., 2006).

### 1.4.4 Mycoparasitism

Mycoparasitism is the direct attack of one fungus on another for utilization of the host fungus as a nutrient source. It generally involves four sequential steps: chemotropism, recognition, attachment and coiling, and cell wall penetration followed by digestion of the host cell (Benitez et al., 2004; Harman and Shoshresh, 2007). An antagonist will direct its growth towards the host, usually along a chemical gradient of amino acids or sugars, which is likely not host-specific (Markovich and Kononova, 2003). Once an antagonist encounters a potential host, the antagonist must recognize the host as a specific target. The exact mechanism of recognition is not fully understood but it can be mediated by
lectin-carbohydrate binding in some cases or by sensing of a diffusible factor from the host fungus (Inbar and Chet, 1997). This interaction is necessary to activate the genes involved in the mycoparasitic process (Viterbo et al., 2002a). Immediately following recognition, the antagonist hyphae attach and coil around the host hyphae, forming appressorium-like structures to penetrate the host (Steyaert et al., 2003). Host penetration and dissolution of the cell wall for release of nutrients is achieved through the concerted action of a variety of cell-wall degrading enzymes (Viterbo et al., 2002a; Cohen-Kupiec et al., 1999). Because this final step is crucial to the success of a mycoparasitic interaction, the majority of research on mycoparasitism has focussed on the role of cell-wall degrading enzymes.

Most fungi have cell walls that contain chitin as a structural backbone and laminarin (β-1,3-glucan) as a filling material (Martin et al., 2007). Oomycetes are unique in that their cell walls lack chitin, and are comprised solely of glucan. Laminarin is a polymer of D-glucose in a β-1,3 configuration, arranged as helical coils, from which polymers of β-1,6-glucose branch (Martin et al., 2007). Chitin is a β-(1,4)-linked polymer of N-acetyl D-glucosamine (GlcNAc), arranged in parallel (β) or antiparallel (α) sheets (Duo-Chuan, 2006). Fungal cell walls contain more than 60% laminarin, chemically bonded to chitin layers, forming a complex net of glucan and N-acetylglucosamine oligomers (Cohen-Kupiec et al., 1999). Laminarin is hydrolyzed mainly by β-1,3-glucanases, which can be further classified as exo- or endo-β-glucanases (Pitson et al., 1993; Viterbo et al., 2004; Markovich and Kononova, 2003). Exo-β-1,3-glucanases hydrolyze laminarin by
sequentially cleaving glucose residues, and the sole hydrolysis products are glucose monomers (Martin et al., 2007; Pitson et al., 1993). Endo-β-glucanases cleave β-1,3-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides (Martin et al., 2007; Pitson et al., 1993). Both enzyme types are required for the full digestion of laminarin. Chitin is hydrolyzed by chitinases, which catalyze the cleavage between the C1 and C4 bonds of two consecutive GlcNAc units (Viterbo et al., 2002b; Duo-Chuan, 2006; Dahiya et al., 2006). Chitinases are divided into three principal classes: 1) 1,4-β-N-acetylglucosaminidases, which cut the chitin polymer in an exo-type manner to release single GlcNAc monomers; 2) endochitinases, which cleave randomly at internal sites along a chitin fibril; and 3) exochitinases or chitobiosidases, which release only diacetylchitobiose units (Adams, 2004; Cohen-Kupiec and Chet, 1998; Duo-Chuan, 2006; Viterbo et al., 2002a; Viterbo et al., 2002b). The role of each enzyme in the complex appears to be different, and enzymes with different or complementary modes of action are required for maximal antifungal effects on different pathogens (Viterbo et al., 2002b).

An understanding of the genetic control of mycoparasitism has improved significantly in recent years (Harman, 2006), and a number of chitinase and glucanase genes have been identified, particularly from different Trichoderma isolates. Different strains of a specific fungal species appear to produce a variety of types and numbers of cell-wall degrading enzymes. For example, the chitinolytic system of the well-characterized fungus, T. harzianum, contains two β-(1,4)-N-acetylglucosaminidases (102 and 73 kDa), four endochitinases (52, 42,
33, and 31 kDa), and one exochitinase (40 kDa) (Markovich and Kononova, 2003). *T. harzianum* CECT 2413 has been shown to produce at least three extracellular β-1,3-glucanases, while strain IMI206040 produces seven extracellular glucanases in the presence of laminarin as an inducer, of which five have been partially characterized (Markovich and Kononova, 2003).

Direct evidence for the involvement of glucanases in mycoparasitism was first demonstrated by Lorito et al. (1994a) using *T. harzianum*. A purified 78kDa endo-β-1,3-glucanase inhibited *B. cinerea* spore germination, and when chitinase was present, the two enzymes showed synergistic cooperation in inhibiting the pathogenic fungus (Lorito et al., 1994b). Transformation studies have also demonstrated the link between cell-wall degrading enzymes and biocontrol efficacy. Transformants of *T. longibrachiatum* overexpressing the β-1,4-endoglucanase gene, *egl1*, were generally more suppressive to *P. ultimum* on cucumber plants compared to the wild-type (Migheli et al., 1998). A gene encoding one of the three endochitinases from *T. virens* was overexpressed or disrupted, resulting in enhanced or reduced biocontrol against *R. solani*, respectively (Baek et al., 1999). However, mutants of *T. harzianum* that were either deficient or enhanced in *chit42* expression displayed the same biocontrol efficacy as the wild-type against *R. solani* or *Sclerotium rolfsii*, illustrating the complexity of the mycoparasitic response and the redundancy of multiple chitinase genes (Carsolio et al., 1999). However, in another study, a mutant of *T. harzianum* P1, also disrupted in the *chit42* gene, exhibited the same biocontrol effect against *Pythium ultimum* as strain P1, showed reduced antagonism
against *B. cinerea* on bean leaves compared with strain P1, but performed better than the wild type against the soilborne fungus *R. solani* (Woo et al., 1999).

These results indicate that the antagonistic interactions between fungal mycoparasitic strains and various fungal hosts is likely based on different mechanisms, and the mycoparasitic response can vary greatly within a fungal species. In another study, *T. virens* transformants constitutively overexpressing β-1,3- and β-1,6-glucanase genes provided enhanced protection of cotton seedlings against *P. ultimum, R. solani* and *Rhizopus oryzae* (Djonovic et al., 2007a). Furthermore, the biocontrol activity was greatest when pathogen pressure was the highest.

Although not as well studied as *Trichoderma* spp., strains of *C. rosea* (*G. roseum*) and *G. catenulatum* (*C. rosea* f. *catenulata*) have shown mycoparasitic activity against several plant pathogenic fungi. Parasitism of several fungal plant pathogens by *G. catenulatum in vitro*, including *R. solani, P. ultimum, Botrytis cinerea* Pers.: Fr., and *Sclerotinia sclerotiorum* (Lib.) de Bary has been reported (Huang, 1978; McQuilken et al., 2001; Simay, 1988; Turhan, 1993). Microscopic observations showed that the biocontrol agent destroyed hyphal cells of *S. sclerotiorum* and *Fusarium* spp. through direct contact, resulting in collapse and disintegration of the host cells without visible penetration (Huang, 1978). Hyphae of *G. catenulatum* were observed to coil loosely around hyphae of *P. ultimum* and *R. solani*, causing partial destruction (McQuilken et al., 2001). Penetration of hyphae of *Alternaria alternata* by *G. catenulatum* was achieved without the formation of appressorium-like structures, and parasitized conidia were distorted
and eventually collapsed (Turhan, 1993). *G. roseum* parasitized *B. cinerea* by direct penetration of hyphal tips, host cell walls were ruptured at penetration sites, and conidia and germ tubes of *B. cinerea* displayed signs of cytoplasmic disintegration (Li et al., 2002). An isolate of *C. rosea*, isolated from senescent chickpea stems in Washington State, inhibited growth of *Didymella rabiei* on chickpea debris and formed appressoria on pathogen hyphae (Dugan et al., 2005). Enzymatic hydrolysis is believed to be involved in the penetration and dissolution of pathogen cell walls by *G. catenulatum* (Lahdenpera and Korteniemi, 2005). Although there are no published reports detailing the production or regulation of cell-wall degrading enzymes by this fungal biocontrol agent, several recent studies have described the enzyme production by strains of *C. rosea*.

An isolate of *G. roseum* was reported to produce β-1,3-glucanases and chitinases in culture that were effective in degrading cell walls of *Fusarium equiseti* but not *P. ultimum* (Inglis and Kawchuk, 2002). An endochitinase gene, *Crchi1*, was cloned from *Clonostachys rosea* (syn. *Gliocladium roseum* Bainier) and its expression was found to be induced by cell walls of *R. solani* and repressed by glucose (Gan et al., 2007). In a series of recent papers, the cell-wall degrading complex of *C. rosea* strain IK726 was characterized using gene expression analyses (Mamarabadi et al., 2009; Mamarabadi et al., 2008b; Mamarabadi et al., 2008a). Three endochitinase genes (*cr-ech58, cr-ech42* and *cr-ech37*), were identified and gene expression was characterized. Expression of *cr-ech42* and *cr-ech37* was found to be triggered by cell walls of *F. culmorum*.
and chitin, while cr-ech58 was not induced by these carbon sources. Mutants that were disrupted in these endochitinase genes showed no significant difference in biocontrol efficacy against F. culmorum on barley or A. radicina on carrot compared to the wildtype (Mamarabadi et al., 2008b). In a separate study, expression patterns of four chitinases and two endoglucanase genes from C. rosea IK726 were analyzed using real-time RT-PCR in vitro and in strawberry leaves during interaction with B. cinerea (Mamarabadi et al., 2008a). One exochitinase gene (cr-nag1), one endochitinase gene (cr-ech42), and two endoglucanase genes (cel12B and cel12D), were upregulated in the in vitro interaction with B. cinerea. When B. cinerea was pre-inoculated on detached strawberry leaves, expression of cr-nag1, cr-ech37, and cr-ech42 was upregulated compared to leaves inoculated with C. rosea only, while there was no change in expression levels of cr-ech58 and the two endoglucanase genes. An enhanced expression level of cr-nag1 was found in interactions between C. rosea and F. culmorum, but enhanced expression was not observed in interactions between C. rosea and P. ultimum (Mamarabadi et al., 2009). Furthermore, expression of cr-nag1 was specifically repressed in medium containing a high glucose content, but was induced by chitin or F. culmorum cell walls as sole carbon sources.

Production of cell-wall degrading enzymes has been demonstrated in response to a range of polysaccharides, including cell-wall preparations from different pathogens. High levels of glucose appear to inhibit the expression of glucanases and chitinases, although basal levels of many cell-wall degrading
enzymes have been detected under non-inducing conditions (Steyaert et al., 2003). This basal level may be necessary to release low levels of inducing polysaccharides from the host cell, which then activate the mycoparasitic gene expression cascade (Vinale et al., 2008). The exact nature of the diffusible molecule that induces the complex of degrading enzymes has not yet been determined (Cortes et al., 1998). The factors activating the biocontrol gene cascade in T. atroviride strain P1 containing the green fluorescent protein (gfp) gene reporter system controlled by different inducible promoters from the nag1 or ech42 genes have been evaluated (Lu et al., 2004). Microscopic analysis of gfp expression revealed that induction of both chitinase genes is an early event during the interaction with R. solani on cucumber seeds, and that the genes were induced by the presence of the host, chitin and chitoligomers. This study also demonstrated that direct mycoparasitism between an antagonist and its fungal host can occur on the plant surface, but early colonization of the plant surface by the biocontrol agent was required.

1.5 Root colonization

To control root diseases, an antagonist should ideally possess the capability to colonize the root surface despite competition from other microorganisms (Green et al., 2001). A biocontrol isolate is determined to be rhizosphere competent when it can colonize the rhizosphere of developing roots when initially applied to seeds (Ahmad and Baker, 1987). The effectiveness of an introduced biocontrol agent in controlling disease is influenced by its root colonization ability, since this will determine the population size of the agent on
the roots at the time of pathogen infection, and the proportion of the pathogen population potentially affected by the biocontrol agent (Paulitz, 2000; Larkin and Fravel, 1999). Therefore, inconsistent root colonization has often been attributed to the variable success of biocontrol of root diseases in soil (Whipps, 2001). As such, a threshold rhizosphere population density of the introduced biocontrol agent is often critical for disease suppression.

The ability to preferentially colonize the rhizosphere is a prerequisite for successful expression of all biocontrol mechanisms. Competition relies on the ability of the biocontrol agent to rapidly utilize scarce nutrients, occupy niches in the root zone before the pathogen can, and establish and maintain population densities that are high enough to exclude the pathogen. Effective competition is highly dependent on the population ratio between the biocontrol agent and the pathogen. Typically, the population of the biocontrol fungus must be larger than that of the pathogen population to achieve control (Larkin and Fravel, 1999). Mycoparasitism is also contingent on rhizosphere colonization since the agent must be ideally situated in the root zone to interact with the host fungi to succeed in reducing pathogen numbers or prevent infection. Induced systemic resistance is dependent on the ability of the biocontrol agent to penetrate and colonize the outermost root tissues of the host without causing disease.

The detection and quantification of microorganisms is usually performed by dilution-plating of plant parts, soil or soil extracts onto selective media. The use of dilution-plating does not differentiate among different propagules (hyphal fragments, conidia and chlamydospores) all of which may generate colonies
when plated on agar, and thus is not a true estimate of fungal biomass (Bae and Knudsen, 2000). Furthermore, the majority of colonies arise from conidia rather than hyphae, which makes a correlation to fungal activity difficult (Green and Jensen, 1995). These methods also rely on the ability to easily distinguish the fungus of interest from each other and from other species. Genetic engineering of biocontrol agents with marker genes provides a useful tool to detect and monitor introduced biocontrol agents. Two reporter genes have primarily been used in ecological studies of introduced fungi; β-glucuronidase (GUS), originally used to assess gene activities in transgenic plants (Jefferson et al., 1986) and the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Chalfie et al., 1994). GUS expression in fungi is easily detectable by conventional enzymatic methods, and by histochemical procedures that allow observation of the fungus within plant tissues (Bae and Knudsen, 2000). When production of GUS is under the control of a constitutive promoter, the production of the enzyme should correlate with the general physiological condition of the organism, and thus varies with phases of the life cycle and varying environmental conditions. Therefore, the level of enzyme production, measured by a standard fluorometric assay, qualifies as an expression of the metabolic activity of the fungus *in planta* (Green and Jensen, 1995). GUS activity can also be related to fungal biomass, and thus can be used to quantify colonization levels in plant tissues (Bao et al., 2000; Thrane et al., 1995). GFP requires UV or blue light to induce green fluorescence, and unlike GUS, does not require an exogenous substrate for detection (Bae and Knudsen, 2000). However, although the GFP method is
useful to directly observe individual spores and mycelia in planta, it is not suitable for enumeration of fungal biomass (VandenWymelenberg et al., 1997).

The use of a GUS transformant of T. harzianum strain T3a showed that this strain could not colonize healthy roots of various plants in non-sterile soil; growth depended on access to dead organic substrates such as seed coats, decaying roots and wounds (Green et al., 2001). C. rosea strain IK726 transformed with GUS could grow and sporulate on intact healthy carrot root tissue and on newly-developed leaves of barley, indicating its ability to thrive in very different niches (Lubeck et al., 2002). In the soil environment, the biomass of C. rosea was present mainly in the form of conidia unless an exogenous nutrient source, such as root exudates or host hyphae, was present (Lubeck et al., 2002). Bao and Lazarovits (2001) demonstrated that the GUS-transformed non-pathogenic F. oxysporum strain 70T01 colonized the epidermal and cortical cell layers of tomato roots in a discontinuous and uneven pattern, but newly elongating roots were not colonized.

1.6 Environmental factors determining the success of biocontrol agents

Basic environmental conditions, such as temperature, moisture, and soil physical and chemical characteristics, not only affect the physiology of the host plant, but will also alter the interactions among the plant, pathogen and biocontrol agent (Larkin and Fravel, 2002). Environmental conditions will affect the survival of BCAs, but also their efficacy against pathogens, thus changing environmental conditions can be a major impediment to successful biocontrol (Fravel, 2005).
BCAs that have the ability to survive under a wide range of environmental conditions can be more easily developed into commercial products since disease control will be less variable (Longa et al., 2008). The identification and quantification of variables that affect the efficacy of an antagonist should make biocontrol more predictable and effective. Variability in disease resistance and susceptibility among host cultivars can also affect the disease response and may influence the degree of biological control (Hoitink and Boehm, 1999).

Biological control of Fusarium wilt of tomato by non-pathogenic *Fusarium* isolate CS-20 was not affected by greenhouse temperatures, whereas isolates CS-1 and CS-24 were less effective at 27°C, the optimum temperature for disease development (Larkin and Fravel, 2002). Similarly, isolate CS-20 was effective in reducing disease development in four different kinds of soil varying in texture and organic matter, whereas biocontrol efficacy by isolate CS-1 was dependent on soil type. All three isolates displayed the same biocontrol efficacy on eight different tomato cultivars with varying levels of inherent resistance to Fusarium wilt (Larkin and Fravel, 2002). *T. harzianum* strain T-95 was rhizosphere competent on cucumber grown in soil at pH 5.0, 6.0, and 7.0 and temperatures of 19, 26, and 33°C, although population densities associated with the roots were highest at 26°C and pH 7.0 (Ahmad and Baker, 1987).

The pH of the growing media can affect mycelia growth of the biocontrol agent and can play a role in the regulation of extracellular enzyme production and mycoparasitic ability (Kredics et al., 2003). The ability to thrive over a wide range of external pH conditions is an important component of the complex set of
characteristics that a biocontrol agent encounters during its interaction with the host plant (Vinale et al., 2008). External pH is also a determining factor in the virulence of many pathogens, as pathogenicity factors are often produced within a very narrow range of pH (Caracuel et al., 2003). Therefore, biocontrol strains that can survive under a range of pH conditions by adapting their own metabolism, especially those functions related to biocontrol activity, could consequently reduce the virulence of phytopathogens, which are unable to withstand changes in pH.

The biotic and abiotic components of the soil or plant matrix may affect the biocontrol activity of fungal strains, especially in relation to the receptivity of the growing media to the introduced strain (Simon and Sivasithamparam, 1989). For example, there was a greater density of fungi associated with cucumber roots when plants were grown in soil, followed by rockwool and sawdust, with the lowest number occurring in nutrient solution (Menzies et al., 2005). Throughout the experimental period, the density of the natural, endemic fungal community in the rockwool substrate remained low, supporting the hypothesis that an introduced biocontrol agent may flourish in this system due to low microbial competition. Higher levels of microbial soil biomass induced a shift from hyphal growth to sporulation in *T. harzianum*, resulting in reduced biocontrol efficacy (Bae and Knudsen, 2005). To minimize the potential of suppression of introduced biocontrol agents in natural environments, a detailed study of the metabolites produced by microorganisms present in the soil environment should be performed (Vinale et al., 2008). Induction of resistance to Phytophthora leaf
blight in cucumber by *T. hamatum* was more effective in plants grown in compost-amended media than sphagnum peat media, illustrating that organic matter quality influences biocontrol efficacy (Khan et al., 2004).

### 1.7 Research objectives

To maximize consistency and efficacy of disease control afforded by *G. catenulatum*, a thorough understanding of the mechanisms of action through which disease suppression occurs is necessary. Biocontrol often results from a concurrent or synergistic action of several of these mechanisms, and is dependent on factors such as environmental conditions and pathogen inoculum level. Understanding the ecology of a microbial antagonist is critical for continued success of biocontrol and its practical implementation. The overall objective of this thesis was to elucidate the mode(s) of action of *G. catenulatum* against *Forc* on cucumber. Several research objectives were proposed that were aimed at understanding the biology and ecology of this biocontrol fungus.

1. Transform *G. catenulatum* with a reporter gene to characterize colonization of the fungus in the rhizosphere and phyllosphere of cucumber.
2. Determine the mycoparasitic ability of *G. catenulatum* by evaluating cell-wall degrading enzyme profiles and interactions with *F. oxysporum* f. sp. *radicis-cucumerinum* and *P. aphanidermatum*.
3. Determine the ability of *G. catenulatum* to produce antifungal metabolic compounds that inhibit growth of *F. oxysporum* f. sp. *radicis-cucumerinum* and *P. aphanidermatum*. 
4) Evaluate the ability of *G. catenulatum* to induce systemic resistance in cucumber plants.

5) Determine the environmental factors that affect colonization of cucumber roots by *G. catenulatum*.
2: COLONIZATION OF CUCUMBER PLANTS BY THE BIOCONTROL FUNGUS GLIOCLADIUM CATENULATUM


2.1 Introduction

The biocontrol fungus Gliocladium catenulatum Gilman & Abbott strain J1446 (syn. Clonostachys rosea f. catenulata (Gilman & Abbott) Schroers; teleomorph Bionectria ochroleuca (Schw.) Schroers & Samuels (Schroers, 2001; Schroers et al., 1999), commercially available as Prestop WP and Prestop Mix (Verdera Oy, Finland), shows antagonistic properties against a number of phytopathogenic fungi (McQuilken et al., 2001). For example, studies have shown that G. catenulatum can reduce root and stem rot caused by Fusarium oxysporum Schlechtend.:Fr. f. sp. radicis-cucumerinum D.J. Vakalounakis (Forc) on greenhouse cucumbers (Cucumis sativus L.) in growth room trials and under semicommercial growing conditions (Rose et al., 2003). This biocontrol agent was also reported to reduce damping-off caused by Pythium ultimum and Rhizoctonia solani on ornamental bedding plants (McQuilken et al., 2001) and by P. aphanidermatum on cucumber (Punja and Yip, 2003). In addition to root-infecting pathogens, G. catenulatum also had some efficacy against anthracnose on blueberry fruits caused by Colletotrichum acutatum when applied to blossoms
and developing fruit (Verma et al., 2006). Applications of *G. catenulatum* also suppressed sporulation of *Botrytis* spp. on dead onion and lily leaves (Kohl et al., 1995). These results indicate that *G. catenulatum* has broad-spectrum activity against many fungi.

The mechanisms of action of *G. catenulatum* that result in disease suppression are not well understood. While the fungus has been reported to parasitize several fungal plant pathogens including *Rhizoctonia solani*, *Pythium ultimum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* (McQuilken et al., 2001; Huang, 1978; Simay, 1988; Turhan, 1993), these studies were all conducted in culture with only the 2 interacting fungal species present. *G. catenulatum* survived in peat-based growing media for up to 28 days after application and was observed colonizing cucumber roots 5 weeks after application suggesting it has the ability to grow in the rhizosphere (McQuilken et al., 2001). It has been postulated that the rhizosphere colonization ability of this fungus coupled with its mycoparasitic ability may contribute to biocontrol efficacy (Punja and Utkhede, 2003; Rose et al., 2003). Root colonization is an important attribute of many soilborne antagonists, such as *Trichoderma* spp. (Whipps, 2001). However, the extent of colonization of plant roots by *G. catenulatum*, both externally and internally, and its effect on pathogen populations and disease development has not been previously investigated.

To facilitate research to determine root colonization potential of biocontrol fungi, reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP) have been used. In particular, the GUS gene has been used to
study the ecology, distribution and rhizosphere competence of biocontrol agents such as *Trichoderma harzianum* (Lo et al., 1998), non-pathogenic *F. oxysporum* (Bao and Lazarovits, 2001) and *F. moniliforme* (Yates et al., 1999), as well as for ecological studies of *C. rosea* (Lubeck et al., 2002). In this research, we developed a GUS-marked strain of *G. catenulatum* and used light microscopy, coupled with scanning electron microscopic observations, to study colonization of cucumber roots and spread to other parts of the plant. The objectives were to determine the population densities and survival of the biocontrol agent over time, the potential for endophytic colonization, and the effect of *G. catenulatum* on development of *F. oxysporum* on cucumber roots.

### 2.2 Materials and Methods

#### 2.2.1 Fungal strains and culture conditions

An isolate of *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (*Forc*) was obtained from a commercial greenhouse containing cucumber plants displaying symptoms of Fusarium root and stem rot (Punja and Parker, 2000), and was maintained on potato dextrose agar (Difco) amended with 2 ml/liter of lactic acid (APDA). To ensure the isolate retained its virulence, reisolations were made from artificially inoculated plants by surface-sterilizing diseased tissues in 20% bleach (Javex) for 30 s, followed by 70% ethanol for 60 s, and then rinsing in sterile distilled water. The tissues were plated onto Komada’s medium (Komada, 1975), followed by transfer to APDA after 10-14 days of growth. Conidia were obtained by flooding 14-day-old cultures with sterile distilled water (SDW), followed by scraping the surface with a glass rod. The resulting suspension was
filtered through a double layer of cheesecloth to remove mycelia. Strain J1446 of G. catenulatum was derived from a commercial formulation (Prestop WP provided by Verdera Oy, Finland) by plating samples onto PDA amended with tetracycline (20 mg/liter) and Triton X-100 (2 ml/liter) (PDAtt) (McQuilken et al., 2001). The fungus was then cultured onto PDA and grown under laboratory conditions (21-24°C) under ambient fluorescent lights.

2.2.2 Biological control activity of G. catenulatum

Seeds of cucumber cv. Mystica (Rijk Zwaan, Netherlands), highly susceptible to Forc (Rose and Punja, 2004), were planted into the seeding cavities of 10-cm² rockwool blocks (Westgro, Delta, BC) and covered with 50 ml of vermiculite. A suspension (10 g/liter) of Prestop WP (formulated to contain 1 x 10⁷ spores/ml of G. catenulatum) was applied as a drench (50 ml) to each rockwool block, which was contained inside a plastic bag. Plants were fertilized with a nutrient solution consisting of N-P-K (7-11-27) soluble fertilizer (0.73 g/liter) (Plant Products Ltd., Bramalea, ON) with CaNO₃ (0.48 g/liter) as needed. Thirty days later, one set of 10 plants was inoculated with Forc by pouring 50 ml of conidial suspension (5 x 10⁴ conidia/ml) to the base of the plant. Treatments consisted of Prestop WP with Forc, Prestop WP alone, Forc alone and a water control. The plants were arranged in a randomized complete block design, with 10 replicates per treatment. Mortality of plants and height of surviving plants were recorded 15 and 30 days after the Forc treatment. The mortality and height data were used to calculate a disease severity index as follows: DSI = (no. of dead plants at 15 days/15) + (no. of dead plants at 30 days/30) + [1-(height of
surviving plants/height of controls)]. Experiments were conducted in a growth room under ambient temperature (21-24°C) with a 16 h photoperiod provided by sodium vapour lights (light intensity of 100 μmoles/m²/s). Growth room trials were conducted three times.

2.2.3 Survival of G. catenulatum

To monitor changes in population density of G. catenulatum on cucumber roots over time, plants treated with Prestop WP at seeding were grown as described above. After 30 days, and at 10-day intervals thereafter, 1 g samples of roots, which had emerged below the rockwool blocks, were collected from each of 10 replicate plants. They were rinsed under running tap water and vigorously shaken in 10 ml of sterile distilled water (SDW). Suspensions were serially diluted and plated onto PDAtt, with four replicate dishes per sample. After 60 days of plant growth, tissue samples were obtained from the crown region of each plant and five 1 cm³ samples were arbitrarily removed from the rockwool block. Samples were vigorously shaken in SDW and suspensions were serially diluted as before. One ml samples of nutrient solution that had filtered through the rockwool blocks were also collected at 60 days and serially diluted as above. The number of colonies of G. catenulatum from each sample was determined after 72 h of incubation under ambient laboratory conditions. The experiment was conducted three times.
2.2.4 Scanning electron microscopic studies

To microscopically assess growth and colonization of root surfaces by *G. catenulatum*, surface-sterilized cucumber seeds were placed on filter paper strips (3 mm thick) on a wire mesh suspended 1 cm above nutrient solution (120 ml) inside autoclaved Magenta boxes (Sigma). The containers were incubated on a bench under ambient conditions (21-24°C) with a 16 h photoperiod. After 10 days, when the seeds had germinated and roots reached the nutrient solution, *G. catenulatum* was added at a final concentration of $10^5$ spores/ml of the nutrient solution. Three days later, one set of plants was inoculated with *Forc* by adding conidia to the nutrient solution at a final concentration of $10^4$ spores/ml.

Treatments consisted of *G. catenulatum* only, *Forc* only, *G. catenulatum* with *Forc*, and a non-treated control. Root segments (5 mm long) were excised from the main root at 3 and 7 days after inoculation with *Forc*. Samples were fixed in 0.1M cacodylate buffer containing 2% glutaraldehyde, 4% formaldehyde and 4% sucrose using standard microwave processing (Gerrity and Forbes, 2003). Samples were post-fixed in 2% (w/v) tannic acid, followed by fixation in 1% osmium tetroxide in the above buffer for 1 h at room temperature, and 2% aqueous uranyl acetate for 1 h at room temperature in the dark. The samples were then dehydrated in a graded ethanol series and were critical point-dried using CO$_2$. Samples were mounted on stubs using colloidal silver, sputter-coated with gold-palladium in a Nanotech SEMPrep II Sputter Coater and examined in a scanning electron microscope (Hitachi S4700).
2.2.5 Extent of internal colonization

To determine if *G. catenulatum* could colonize cucumber roots, stems and shoots internally, seeds were placed in 10-cm$^2$ rockwool blocks as described previously and Prestop WP (10g/liter, 50 ml/plant) was applied at seeding. After 3 weeks, 5 mm long root and stem pieces and 6 mm-diameter disks from the cotyledons and true leaves were collected. Tissues were immersed in 70% ethanol for 0, 10, 30 or 60 s each followed by 2% sodium hypochlorite (40% bleach) for 0, 10, 30 or 60 s each, respectively, and then rinsed three times in sterile distilled water. They were plated onto PCA (0.1 ml paraquat, 200 mg chloramphenicol and 12 g agar per L) (Peng and Sutton, 1991). The percentage of tissue pieces yielding colonies of *G. catenulatum* was recorded after 14 days of incubation at ambient temperature. Each treatment was replicated three times, with five tissue pieces per replicate for each sterilization treatment. The experiment was conducted three times.

2.2.6 GUS-transformation

A 4.12 kb *HindIII-EcoRI* fragment from plasmid pAN7-2 (provided by Dr. Y. Wei, University of Saskatchewan) containing the promoter and *E. coli* hygromycin B phosphotransferase (*hph*) genes (Punt and Vandenhondel, 1992) was isolated and ligated to the 4.95 kb *HindIII-EcoRI* fragment from pNOM1 (provided by Dr. Y. Wei, University of Saskatchewan) containing the promoter and *E. coli* β-glucuronidase (*uidA*) genes (Roberts et al., 1989) in the *EcoRI* site of the pCambia0380 vector. Both genes were driven by the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*).
promoter. *Agrobacterium tumefaciens* strain AGL-1 (provided by Dr. S. Marek, Oklahoma State University) was transformed with the binary vector (An et al., 1988). Positive transformants were verified using PCR, stored in -80°C glycerol stocks and were used to transform *G. catenulatum* according to the following procedure (Dr. S. Marek, personal communication). Cells from glycerol stock were plated onto *Agrobacterium* minimal medium containing kanamycin (100μg/ml) and carbenicillin (100μg/ml) and incubated at 28°C for 2-3 days. Single colonies were used to inoculate liquid minimal medium and incubated at 28°C with shaking at 250 rpm for 2 days. Cultures were then diluted in induction medium (IM) containing 40 mM MES, 0.5% glycerol (w/v) and 200 μM acetylsyringone (AS) (Mozo and Hooykaas, 1991) to an optical density of 0.2 absorbance units at 600 nm and then incubated overnight at 28°C with shaking at 250 rpm. The induced suspension was then diluted again to an optical density of 0.2 absorbance units at 600 nm in IM for use in transformations. Conidia of *G. catenulatum* were diluted in IM to a concentration of 2x10^6, 2x10^4 or 2x10^3 conidia/ml and 500 μl of each conidia suspension was mixed with 500 μl of the induced *Agrobacterium* cells. Aliquots (200μl) were plated onto Hybond membranes (Amersham) placed on IM plates containing 200 μM AS and incubated at room temperature for 72 h. The membranes were transferred to YPS plates containing 300 μg/ml timentin to inhibit *Agrobacterium* growth and 200 μg/ml hygromycin to select the *Gliocladium* transformants and incubated at room temperature for 7-10 days. Colonies growing on the selective medium were tested for GUS activity by placing mycelia from each colony in the well of a
microtiter plate containing 200 µl of 10 mM sodium phosphate buffer (pH 7.0) and 4 µl of X-Gluc substrate (12 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid substrate, Sigma). For each positive isolate, single-conidial isolations were carried out to ensure mitotic stability. The transformed isolate displaying the highest level of GUS expression as determined by staining intensity was chosen for further studies. To test for genetic stability, this isolate was cultivated under non-selective conditions for 2 months, after which time colonies were tested for hygromycin B resistance and GUS activity. Integration of the uidA gene was verified by Southern blot hybridization following EcoRI digestion of the genomic DNA (Wally et al., 2008).

2.2.7 Growth of GUS-transformed G. catenulatum on cucumber tissues

Cucumber plants were grown aseptically in Magenta boxes or in rockwool blocks as described previously. Seeds were treated with GUS-transformed G. catenulatum prior to planting by soaking in 10^7 conidia/ml for 10 minutes, then air-drying for 30 minutes and immediately planted, or G. catenulatum was applied to the nutrient solution in Magenta boxes when plants were 10 days old or to rockwool blocks at seeding as described above. Tissues were harvested at intervals between 2 and 10 days and histochemical staining of germinating seeds, root, stem, apical meristem and leaf pieces was performed according to Jefferson et al. (1986) with modifications according to Wally et al. (2008). Tissue segments that displayed characteristic blue staining were mounted in resin, sectioned (4 µm thickness) and viewed under 40 X magnification using bright-field microscopy. To verify the results of GUS staining, root, stem and leaf
pieces were simultaneously plated onto PDA+hygromycin to confirm presence of the transformed fungus.

2.2.8 Estimation of fungal biomass

Fungal biomass of GUS-transformed *G. catenulatum* on cucumber tissues was estimated from GUS activity using a modification of the fluorometric method (Jefferson et al., 1986). A standard curve correlating fungal biomass to 4-methylumbelliferyl (MU) concentration was first developed by measuring GUS activity in protein extracts from fungal mycelia as described by Bao et al. (2000). Briefly, five-day-old mycelia, grown in PDB, was harvested by vacuum filtration through Whatman No. 1 filter paper, and washed twice with SDW. 50 mg aliquots were ground in 1 ml ice-cold GUS buffer in 2-ml polypropylene tubes containing one chrome steel bead (6.35 mm) and glass beads (0.5 mm). The tissue was homogenized for 1 min using a MiniBeadBeater-8 (BioSpec Products, Bartlesville, OK) and the resulting homogenate was then serially diluted. The dry weight of tissue in each serial dilution was determined by transferring 200 μl aliquots to weigh boats, which were then dried for 24 h and reweighed. Homogenates were centrifuged twice at 10 000 x g for 15 min at 4°C, and the supernatants were measured for protein concentration using the Bradford assay and were also used for GUS activity assays. 20 μl of extract was mixed with 200 μL 1mM MUG in GUS buffer and incubated for 1 h at 37°C. The reaction was terminated by adding 780 μl stop buffer (0.2 M Na₂CO₃) and the MU concentration was measured using a fluorometer (Hoefer DyNA Quant 200, Amersham Biosciences), with an excitation wavelength of 365 nm and emission
wavelength of 455 nm. GUS activity was calculated as the amount (nmoles) of MU produced per hour per milligram of mycelium.

2.2.9 Survival of *F. oxysporum* on cucumber roots in the presence of *G. catenulatum*

To determine the effect of *G. catenulatum* on survival of *F. oxysporum* on cucumber roots, cucumber plants were grown in rockwool blocks as described previously. Rockwool blocks were treated with Prestop WP as described above or 50 ml of 1x10^7 conidia/ml of transformed *G. catenulatum* (GcA1) was applied to the rockwool blocks at the time of seeding. Three-week-old plants were then inoculated with a 50 ml suspension of either 10^4, 10^5 or 10^6 conidia/ml of *Forc*. Treatments consisted of *Forc* alone, Prestop WP alone, the transformant alone, *Forc*+Prestop WP and *Forc*+transformant. There were 10 replicate plants per treatment, and the experiment was conducted twice. Two weeks post-inoculation, samples of roots and crown tissues were collected. Root samples were divided into 3 portions: main taproot within the rockwool blocks, lateral roots that were growing outside the rockwool blocks, and root tips excised from the lateral roots. All root and crown tissues were rinsed thrice before grinding for 30s in 1 ml SDW using one glass bead (6.35 mm) in a Mini Beadbeater. The suspensions were diluted and plated onto *Fusarium*-selective medium (Komada, 1975) and onto PDAtt. Suspensions from treatments with GUS-transformed *G. catenulatum* were plated onto PDA+hygromycin. Root samples from these plants were also stained with X-gluc to visualize colonization.
2.2.10 Statistical analyses

For all experiments, analysis of variance was performed using the Proc GLM method in Statistical Analysis System, version 8.0 (SAS Institute, 1999). Means of treatments were compared to the appropriate control treatment using Dunnett’s test or contrast partitioning. Density estimates of *G. catenulatum* associated with roots were log transformed (\(y+1\)) prior to analysis. Density estimates of *Forc* associated with roots or crown relative to *Forc* inoculum levels were subjected to regression analysis, and slopes of curves from the various treatments were compared using contrast partitioning. Normality of all dependent variables was tested by means of the univariate procedure, and homogeneity of experimental errors was examined by plotting residuals versus predicted values using the mixed model and plot procedure. Outlier detection was performed using studentized residuals and Cook’s distance measure. Data for repetitions of the experiments were pooled for analysis when \(F\)-tests indicated that variances of the data did not differ significantly. The Type 1 error rate (\(\alpha\)) was set at 0.05 for all statistical tests.

2.3 Results

2.3.1 Biological control activity and survival of *G. catenulatum*.

Cucumber seeds treated with Prestop WP followed by treatment with *Forc* 30 days later resulted in a significantly lower DSI compared to cucumber plants treated with *Forc* alone in growth room trials (Fig. 2-1A). When applied at seeding, *G. catenulatum* persisted on cucumber roots for at least 50 days at levels above \(1 \times 10^5\) CFU/g root fresh weight (RFW) (Fig. 2-1B). By 60 days post-
application (DPA), population levels were slightly below $1 \times 10^5$ CFU/g RFW. The biocontrol agent was also recovered from the crown region of cucumber plants at densities of $5 \times 10^4$ CFU/ g stem tissue at 60 DPA (Fig. 2-1C). Rockwool blocks also had population levels at 60 DPA of $1 \times 10^5$ CFU/ cm$^3$ of rockwool. Lower levels of *G. catenulatum* were recovered from the nutrient solution inside the plastic bags.

### 2.3.2 Scanning electron microscopic observations.

Cucumber roots were extensively colonized by *G. catenulatum* hyphae within 7 days after application and formed a dense network over the root surface. Hyphae were found intertwined near root hairs and were closely associated with the junction of epidermal cells (Fig. 2-2A-C). Sporulation was observed, with production of characteristic verticilliate conidiophores and conidia on the root surface (Fig. 2-2D-F). A dense network of hyphae was also visible on the surface of cucumber roots treated with *Forc* alone 7 days after inoculation (Fig. 2-2G-I) but no penetration or collapse of epidermal cells was observed. On roots treated with *G. catenulatum* followed by *Forc*, hyphae of *G. catenulatum* and *Forc* were distinguishable on the basis of their size (Fig. 2-2K). Colonization by *G. catenulatum* was clearly visible and conidiophores and conidia were present on the root surface (Fig. 2-2J); there was less evidence of the presence of *Forc* hyphae. There was no visible hyphal interaction between the two fungi even when they were observed growing in close proximity to one another (Fig. 2-2J-L).
Figure 2-1. Disease severity index (DSI) of cucumber plants and population densities of *G. catenulatum* on cucumber roots, tissues and growing medium.

(A) DSI of cucumber plants treated with Prestop WP (containing *G. catenulatum*) 30 days before inoculation with *F. oxysporum* f. sp. *radicis-cucumerinum*. (B) Population densities, expressed as log(10) colony forming units/ g root fresh weight, of *G. catenulatum* associated with cucumber roots and (C) cucumber crown region and growing medium over a 60 day period. (C) Crown = log(10) CFU/ g tissue; substrate = log(10) CFU/cm$^3$; nutrient solution = log(10) CFU/ml
Figure 2-2. Scanning electron micrographs of cucumber roots 7 days after inoculation with *G. catenulatum* (A-F), or *F. oxysporum* (G-I), or *G. catenulatum* followed 3 days later by *F. oxysporum* (J-L).

(A) Colonization of root hair zone; scale bar = 100 μm; (B, C) Extensive colonization of the root surface; scale bar = 100 μm; (D, E, F) Sporulation of *G. catenulatum* on the cucumber root surface; scale bar (D,E) = 100 μm, scale bar (F) = 30 μm; (G, H, I) Extensive colonization of the root surface by *F. oxysporum*; scale bar (G) = 500 μm, scale bar (H,I) = 100 μm; (J, K) Hyphal contact between *G. catenulatum* (g) and *F. oxysporum* (f) on the root surface; scale bar = 100 μm; (L) Hyphae and conidia of *G. catenulatum* (g) and *F. oxysporum* (f) in close proximity to each other; scale bar = 100 μm.
2.3.3 Extent of internal tissue colonization.

When root, stem and leaf tissues were not surface-sterilized, *G. catenulatum* was recovered at frequencies of almost 100%. By comparison, the biocontrol agent was not recovered from leaf tissues after surface-sterilization for 10, 30 or 60 s (Fig. 2-3). It was recovered from 50 % of root and stem pieces after sterilization for 10 s, but recovery from both tissues was reduced to 25% after 30 s of sterilization. Recovery was 20% from root pieces after 60 s of sterilization and 0% from stem pieces (Fig. 2-3). There was no recovery of any other microbes at any of the sterilization times from the tissues plated.

2.3.4 GUS transformation

Approximately 25 colonies per plate grew on the selection medium when *Agrobacterium* was incubated with *G. catenulatum* at a concentration of $10^3$ conidia/ml; at higher conidial densities, the putative transformant colonies were too numerous and were not discernible. All 25 putative transformants were screened for GUS activity by histochemical staining on microtiter plates and for hygromycin resistance. Approximately 20% of colonies displayed spontaneous resistance to hygromycin and were not transformed. In total, 12 colonies displayed the GUS phenotype, yielding a transformation frequency of 3%. Southern hybridization analysis showed that the transformants contained a single copy of the *uidA* gene while the wild-type strain did not contain sequences similar to this gene (data not shown). Transformants were grown for three successive transfers on non-selective agar medium. Suspensions of conidia were then plated onto PDA and randomly selected colonies were tested for GUS
Colonization of root, stem, cotyledon and leaf pieces by *G. catenulatum* after 0, 10, 30 or 60 s of surface sterilization, 3 weeks following application of Prestop WP to the seed.
expression. All the single-spore colonies were positive for hygromycin resistance and GUS activity, indicating that the genes were stably maintained. The colony morphology of transformant GcA1 and its ability to colonize cucumber roots were not significantly different from the parent strain GcJ1446. The average recovery from cucumber roots was $5 \times 10^6$ CFU/g RFW 5 weeks post-inoculation, which is similar to the wild-type strain. When stem and root pieces were plated onto PDA+ hygromycin, 100% of tissue pieces were colonized by the transformed strain. Randomly selected colonies arising from the tissues all stained positively for GUS, indicating the transformant was stable.

2.3.5 Growth of GUS transformed G. catenulatum on cucumber tissues

Following seed treatment with conidia of GUS-transformed G. catenulatum, colonization of the seed coat was visible within 24 hr and the endosperm and emerging radicle were also colonized (Fig. 2-4A). On seeds in Magenta boxes, colonization of the developing cotyledons and the main root occurred along the margin and predominantly near the root tips, respectively, within 3 days after seed germination (Fig. 2-4B). After 14 days, roots were primarily colonized at the root tip and at the junctions of lateral root emergence (Fig. 2-4C, D), although discontinuous colonization along the surface of the mature root zone was also observed. The crown area, shoot meristem and emerging true leaves were also colonized by G. catenulatum (Fig. 2-4E-G). G. catenulatum was found associated externally with trichomes on the stem and appeared to form a network of hyphae over the epidermis (Fig. 2-4E). When the tissues were sectioned, hyphae could be seen growing internally in epidermal
Colonization pattern of cucumber plants by *G. catenulatum* strain J1446 expressing the GUS gene.

(A) Seed coat and endosperm and (B) developing cotyledons colonized by *G. catenulatum* 24 h and 72 h after seed application, respectively. (C, D) Colonization of roots, (E, F) stem, (G) meristem and true leaves (F) by *G. catenulatum* 14 days after seed treatment in Magenta boxes. Colonization of roots was visible at junctions of lateral roots and at root tips (D), while hyphae were found associated with (E) trichomes on the stem surface. (H-M) Light micrographs showing internal colonization by *G. catenulatum*. (H) Colonization of the epidermal and cortical cells in roots of plants grown in Magenta boxes and (I) 3-week-old plants grown in rockwool blocks. (J) Hyphae on the surface of the stem with ingress into trichomes and cortical cells and (K) xylem vascular elements of plants grown in Magenta boxes. (L) Hyphae in cortical cells of stems of plants grown in rockwool blocks. (M) Hyphae in the epidermal layer of young true leaves of plants grown in Magenta boxes.
and cortical cells of roots (Fig 2-4H, I). On stems, blue-stained hyphae were observed on the surface of trichomes (Fig. 2-4J), and internal colonization of the epidermal, cortical and vascular regions was evident (Fig. 2-4J-L). Colonization of the epidermal layer of young true leaves was observed on plants grown both in Magenta boxes and rockwool blocks (Fig. 2-4M).

2.3.6 Estimation of fungal biomass

There was a linear relationship between GUS activity and mycelial dry weight and between GUS activity and protein content (Fig. 2-5). Using GUS activity, as little as 1 ng of mycelia could be detected (Fig. 2-5). Application of G. catenulatum to the rockwool blocks resulted in significantly higher root colonization compared to the lower stem and other plant tissues (Fig. 2-6). Seed treatment resulted in colonization of the true leaves, lower stem and roots at low levels that were not significantly different from each other (Fig. 2-6).

2.3.7 Survival of F. oxysporum on cucumber roots in the presence of G. catenulatum

There was no significant difference in the levels of Forc or G. catenulatum on the three different root portions; therefore, data were combined to give overall CFU levels per mg of root. In addition, there was no significant difference in the CFU levels of G. catenulatum applied as Prestop WP or GcA1, so the data were combined for these treatments. Application of G. catenulatum to the rockwool blocks before inoculation with Forc resulted in a significant decrease in Forc levels on the roots and crown when compared to plants inoculated with Forc only, regardless of the initial Forc inoculum concentration (Fig. 2-7). At a Forc
Figure 2-5 Relationship between fungal biomass or protein content and GUS activity in a transformant of *G. catenulatum*.

GUS activity was measured in dilutions of mycelial extracts from 3-day-old cultures. The lines were generated by simple regression analysis.
Figure 2-6 Fungal biomass of *G. catenulatum* expressing the GUS gene in different tissues of cucumber plants following application to seed or rockwool block.

Fungal biomass was determined indirectly from GUS activity. Means represent the combined values from two independent trials (n=10). Vertical bars indicate standard error of the mean.
Figure 2-7 Population size of *Forc* in the roots (top) or crown area (bottom) of cucumber plants treated with *G. catenulatum* or Prestop WP

Prestop was applied at the recommended rate or conidia of GcA1 (10^7 cfu/ml) was applied to the rockwool blocks prior to inoculation with *Forc* at an initial inoculum level of either 10^4, 10^5, or 10^6 conidia/ml. Plants were sampled 2 weeks after inoculation with *Forc*. Population levels were determined by plating supernatant from ground tissues onto selective media for *Forc*. The means and standard error were obtained from 10 replicates per treatment.
concentration of $10^4$ conidia/ml, treatment with *G. catenulatum* reduced pathogen levels on the roots to undetectable levels. Linear regression coefficients for slopes were significantly lower in the roots and crown of plants treated with the biocontrol agent followed by Forc inoculum compared to plants treated with Forc only (Table 2-1), indicating that *Forc* survival was reduced in the presence of *G. catenulatum*.

Population levels of *G. catenulatum* on the roots were significantly higher at 4000, 6700 or 5200 CFU/ mg fresh weight ($P=0.0469, 0.0001,$ and $0.0072,$ respectively, Table 2-2) when *Forc* was added at inoculum levels of $10^4$, $10^5$ or $10^6$ conidia/ml compared to *G. catenulatum* only treatments (1200 CFU/ mg). On the crown, population densities of *G. catenulatum* on plants receiving *Forc* at all inoculum levels were not significantly different from plants treated with *G. catenulatum* only (913 CFU/ mg) ($P=0.4695$) (Table 2-2).

### 2.4 Discussion

The results from this study showed that *G. catenulatum* provided protection to cucumber against Fusarium root and stem rot for a period of up to 60 days following a single application to the rockwool growing medium at seeding. In previous studies, pathogen challenge occurred within 24 h to 3 days after *G. catenulatum* was applied (McQuilken et al., 2001; Punja and Yip, 2003; Rose et al., 2003). We observed that disease suppression occurred even when pathogen challenge occurred 30 days following application of the biocontrol agent. Root colonization plating data revealed that the density of *G. catenulatum* was about $5 \times 10^5$ CFU/g
Table 2-1 Slope values (b) from linear regression analysis of the population levels of *Forc* in the roots or crown area of cucumber plants treated with *G. catenulatum* (10⁷ conidia/ml) versus log₁₀-transformed initial inoculum of *Forc*.

<table>
<thead>
<tr>
<th>Treatmentᵃ</th>
<th>Rootsᵇᶜ</th>
<th>Crown</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Forc</em> only</td>
<td>389.7 (25.9)</td>
<td>15.4 (2.1)</td>
</tr>
<tr>
<td><em>Forc</em> + Prestop WP</td>
<td>49.8 (28.7)*</td>
<td>1.2 (2.1)*</td>
</tr>
<tr>
<td><em>Forc</em> + GcA1</td>
<td>75.9 (25.9)*</td>
<td>1.6 (2.1)*</td>
</tr>
</tbody>
</table>

ᵃPlants were sampled 2 weeks after inoculation with *Forc*. *Forc* was applied to rockwool blocks at inoculum levels of 10⁴, 10⁵ or 10⁶ conidia/ml, 3 weeks following application of *G. catenulatum* (10⁷ conidia/ml) at seeding. The means and standard errors were obtained from 10 replicates per treatment. Population levels were determined by plating tissues onto selective media for *Forc*.

ᵇValues in parentheses indicate standard error of the mean

ᶜValues within a column followed by an asterisk are significantly different from the pathogen control (contrast partitioning, α < 0.05). Graphical depiction of regression lines are shown in Figure 2-7.
Table 2-2  Population levels of *G. catenulatum* in the roots or crown area of cucumber plants treated with *G. catenulatum* applied to the rockwool blocks (10⁷ conidia/ml) prior to inoculation with *F orc* at an initial inoculum level of either 10⁴, 10⁵, or 10⁶ conidia/ml.

<table>
<thead>
<tr>
<th><em>F orc</em> levels</th>
<th><em>Gc</em> CFU/ mg fresh weight³</th>
<th>Roots²</th>
<th>Crown²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1199 (867)</td>
<td>913 (141)</td>
<td></td>
</tr>
<tr>
<td>10⁴</td>
<td>4141 (862)*</td>
<td>1019 (199)</td>
<td></td>
</tr>
<tr>
<td>10⁵</td>
<td>6707 (862)*</td>
<td>1056 (199)</td>
<td></td>
</tr>
<tr>
<td>10⁶</td>
<td>5172 (944)*</td>
<td>750 (115)</td>
<td></td>
</tr>
</tbody>
</table>

³Plants were sampled 5 weeks and 2 weeks after inoculation with *G. catenulatum* and *F orc*, respectively. The means and standard errors were obtained from 10 replicates per treatment. Population levels were determined by plating tissues onto selective media for *G. catenulatum*.

²Values in parentheses indicate standard error of the mean

³Values in a column followed by an asterisk differed significantly from the *G. catenulatum* only control (contrast partitioning, α < 0.05)
root fresh weight at the time of pathogen challenge, and populations remained near this level over the duration of the experiment. These high densities of the biocontrol agent likely play an important role in biocontrol activity since the proportion of the root surface colonized and the efficacy of biocontrol is dependent on a high ratio of the biocontrol strain to the pathogen (Larkin and Fravel, 1999; Whipps, 2001). Plating of root segments also indicated that almost 100% of root sections were colonized by G. catenulatum even after 60 days. Scanning electron microscopic observations confirmed the ability of G. catenulatum to sporulate abundantly on the root surface, and colonization was evident in the root hair zones and at the origins of lateral roots. As well, G. catenulatum sporulated extensively on the surface of the rockwool, with some spread occurring to leaves and stems in growth room trials. Since the rockwool medium was initially sterile with no competing microorganisms, this may have allowed G. catenulatum to establish high densities. The competitive saprophytic ability of G. catenulatum in nonsterile soil is not known.

In this study, G. catenulatum was transformed with the GUS marker gene using Agrobacterium to facilitate further study of its colonization behaviour and distribution on greenhouse cucumber plants. The use of Agrobacterium-mediated transformation of fungi is increasing due to the reported high transformation efficiencies, ease and reliability of the procedure (de Groot et al., 1998; Duarte et al., 2007). However, G. catenulatum was resistant to hygromycin at 200 mg/L, a characteristic that has also been observed in closely related fungi (Lubeck et al., 2002). This made selection of transformants very
difficult as spontaneous resistance to hygromycin occurred at a fairly high rate (20%), even on nutrient-poor media. Nonetheless, 12 positive transformants were identified and found to be mitotically stable after single spore transfers. Transformation did not change the growth or antagonistic properties of the transformant compared to the wildtype.

The ability of *G. catenulatum* to colonize cucumber plants internally was initially suggested by its recovery from root and stem pieces even after 60 s of sterilization. Recovery of a microbe after such a rigorous sterilization procedure has been suggested to be indirect evidence of its ability to be an endophyte (Schulz and Boyle, 2005). Direct evidence was obtained by the visualization of blue-stained hyphae in sections of the epidermis and cortical regions of roots and stems using the GUS-marked strain. Hyphae were also observed in the xylem vessels of cucumber stems in plants grown in nutrient solution in enclosed Magenta boxes. Endophytic growth of fungi within roots can be inter- or intracellular and become systemic (Schulz and Boyle, 2005). Histochemical observations showed that *G. catenulatum* also colonized the epidermal layer of true leaves. Colonization of above-ground tissues by fungal endophytes is usually intracellular and results in localized growth (Schulz and Boyle, 2005). Our observations suggest that *G. catenulatum* meets the criteria of an opportunist, since it has the capability to grow both endophytically and saprophytically (Schulz and Boyle, 2005). Many endophytes can induce plant defence reactions that limit their growth inside the plant (Peters et al., 1998). For example, *T. harzianum* was shown to colonize the epidermis of cucumber roots
and induce systemic resistance during the early stages of colonization (Yedidia et al., 2000). Whether or not *G. catenulatum* can also induce defense responses in cucumber plants remains to be determined.

We observed *G. catenulatum* to actively colonize the seed coat and developing roots following seed treatment. This rhizosphere competence can protect the germinating seed and emerging radicle against damping-off pathogens, such as *Pythium* spp. and *Rhizoctonia solani* (McQuilken et al., 2001; Punja and Yip, 2003). Furthermore, seed treatment or application to rockwool blocks resulted in colonization of above-ground parts, including the apical shoot meristem. *G. catenulatum* has been shown to reduce the incidence of anthracnose caused by *C. acutatum* when applied to blueberry flowers, suggesting that colonization must have occurred to preclude pathogen invasion (Verma et al., 2006). Strains of *G. catenulatum* which colonize roots or leaves have also been shown to be effective biocontrol agents against seed-borne diseases of cereals (Lubeck et al., 2002) and against *Botrytis cinerea* on strawberry and raspberry leaves (Peng and Sutton, 1991; Sutton et al., 1997).

Population levels of *Forc* on roots and crown tissue were significantly lower in the presence of *G. catenulatum* compared to plants inoculated with *Forc* alone, suggesting that this fungus can exclude *Forc* from colonizing the roots. Using scanning electron microscopy, mycelia of *Forc* were rarely observed at sites colonized by the biocontrol agent and infrequently found on the roots compared to roots from treatments with *Forc* alone. Infection by *F. oxysporum f. sp. radicis-lycopersici* on tomato was shown to occur near root hairs and through cellular
junctions along the main root (Lagopodi, 2002; Bolwerk et al., 2005). *G. catenulatum* was often observed associated with root hairs and formed a dense network over the cucumber root epidermis, with hyphae closely associated with junctions of epidermal cells. Therefore, colonization of these niches by *G. catenulatum* prior to *Forc* infection likely reduced the infection sites available for *Forc*. Interestingly, levels of *G. catenulatum* on roots were higher in the presence of *Forc* compared to plants inoculated with *G. catenulatum* alone. Nutrients released from infection sites could have favoured growth of the biocontrol agent. Other studies have observed a similar phenomenon: *T. harzianum* levels were higher on roots that were more diseased and had higher populations of *P. ultimum* (Green et al., 2001). Similarly, densities of non-pathogenic *F. oxysporum* were higher in roots infected with pathogenic *F. oxysporum* f. sp. *radicis-lycopersici* (Bao and Lazarovits, 2001). A higher density of the biocontrol agent relative to the pathogen is usually required for biocontrol in which competition for nutrients or infection sites is the primary mode of action (Larkin and Fravel, 1999; Bolwerk et al., 2005). Therefore, in all of the biocontrol and population density experiments, *G. catenulatum* was introduced at densities much higher than the pathogen, usually at $1 \times 10^6$ cfu/ g roots. Since *G. catenulatum* was also applied preceding the pathogen, it is probable that roots were colonized at the time of pathogen introduction, thus effectively excluding *Forc* infection. This strategy is an important factor to ensure the success of *G. catenulatum* in biocontrol approaches in commercial greenhouse settings. Since Fusarium stem and root rot develops when primary infections occur early in the
growing season (Punja and Parker, 2000), protection through pre-emptive colonization by *G. catenulatum* can result in long-term protection against this disease. *G. catenulatum* is also reported to be a mycoparasite of several fungal pathogens (Huang, 1978; Simay, 1988; Turhan, 1993; McQuilken et al., 2001) and produces cell-wall degrading enzymes in culture that inhibit *Forc* spore germination and growth. The role of mycoparasitism *in situ* and its impact on *Forc* population densities remains undetermined.
3: CHITINASE AND β-1,3-GLUCANASE ENZYME PRODUCTION BY GLIOCLADIUM CATENULATUM AGAINST THE FUNGAL PLANT PATHOGENS FUSARIUM AND PYTHIUM


3.1 Introduction

The fungus Gliocladium catenulatum Gilman & Abbott strain J1446 [syn. Clonostachys rosea f. catenulata (Gilman & Abbott) Schroers; teleomorph Bionectria ochroleuca (Schw.) Schroers & Samuels (Schroers, 2001; Schroers et al., 1999)], is a commercially formulated biocontrol agent (Prestop WP and Prestop Mix, Verdera Oy, Finland) with broad-spectrum activity against plant pathogens. Application of Prestop was shown to reduce root diseases caused by Fusarium oxysporum Schlechtend.:Fr. f. sp. radicis-cucumerinum D.J. Vakalounakis and Pythium aphanidermatum Edson (Fitz.) on greenhouse cucumber (Cucumis sativus L.) (Punja and Yip, 2003; Rose et al., 2003). In addition, diseases caused by Pythium ultimum Trow and Rhizoctonia solani Kühn on ornamental bedding plants (McQuilken et al., 2001) and damping-off on ginseng seedlings caused by a complex of soilborne pathogens (Rahman and
Punja, 2007) were reduced by *G. catenulatum*. The biocontrol agent has also shown efficacy in reducing anthracnose development caused by *Colletotrichum acutatum* Simmonds when applied to blueberry blossoms and developing fruit (Verma et al., 2006) and suppressed sporulation of *Botrytis* spp. on dead onion leaves (Kohl et al., 1995). These studies indicate that *G. catenulatum* has activity against both rhizosphere- and phyllosphere-infecting fungi of plants.

The mechanisms of action of *G. catenulatum* involved in disease suppression are unknown. As described in the previous chapter, this biocontrol agent was shown to be rhizosphere competent and can endophytically colonize roots as well as stems of cucumber plants. Parasitism of several fungal plant pathogens *in vitro*, including *R. solani*, *P. ultimum*, *Botrytis cinerea* Pers.: Fr., and *Sclerotinia sclerotiorum* (Lib.) de Bary has been reported (Huang, 1978; McQuilken et al., 2001; Simay, 1988; Turhan, 1993). Microscopic observations showed that the biocontrol agent destroys hyphal cells of *S. sclerotiorum* and *Fusarium* spp. through direct contact, resulting in collapse and disintegration of the host cells without penetration (Huang, 1978). Hyphae of *G. catenulatum* were observed to coil loosely around hyphae of *P. ultimum* and *R. solani*, causing partial destruction (McQuilken et al., 2001). Penetration of hyphae of *Alternaria alternata* by *G. catenulatum* was achieved without the formation of appressorium like structures, and parasitized conidia showed distortion and eventual collapse (Turhan, 1993). Enzymatic hydrolysis is most likely involved in the penetration and dissolution of pathogen cell walls by *G. catenulatum* (Lahdenpera and Korteniemi, 2005). However, there are no published reports
detailing the production or regulation of cell-wall degrading enzymes by this fungus. The ability to produce these enzymes, in particular chitinases and \( \beta-1,3 \)-glucanases, is a widely distributed property of rhizosphere competent biocontrol fungi, and is critical for the mycoparasitic process (Viterbo et al., 2002b). An endochitinase gene, \( Crchi1 \), has been cloned from \( Clonostachys rosea \) (syn. \( Gliocladium roseum \) Bainier) and its expression was found to be induced by cell walls of \( R. solani \) and repressed by glucose (Gan et al., 2007).

The objectives of this study were to: (i) investigate the ability of \( G. catenulatum \) to parasitize hyphae of \( F. oxysporum \) f. sp. \( radicis-cucumerinum \) (hereafter referred to as \( Fusarium \)) and \( P. aphanidermatum \) (hereafter referred to as \( Pythium \)) \textit{in vitro} and \textit{in situ}; (ii) to determine the conditions that promote the production of lytic enzymes \textit{in vitro} and (iii) to study the effects of these hydrolytic enzymes on growth of \( Fusarium \) and \( Pythium \).

### 3.2 Materials and Methods

#### 3.2.1 Production of antifungal metabolic compounds \( G. catenulatum \) that inhibit growth of \( F. oxysporum \).

To determine whether production of non-volatile antifungal compounds by \( G. catenulatum \) were directly active against \( F. oxysporum \) or \( P. aphanidermatum \), mycelial discs of \( G. catenulatum \) (5 mm) from a 14-day-old culture were placed on one side of PDA or water agar in 9-cm Petri dishes. The plates were incubated for 7 days to allow time for the production and diffusion of metabolites into the agar. An agar disc with actively growing \( Forc \) or \( P. aphanidermatum \) mycelium (7-day-old culture) was then placed mycelial side down, onto the
opposite side of the inoculated plates, and the plates were incubated for a further 5 days. Inhibition of *Forc* growth was determined by measuring mycelial growth in the direction of the *G. catenulatum* colony in comparison with control plates of *Forc*.

To determine whether the production of volatile antifungal compounds by *G. catenulatum* were inhibitory against *F. oxysporum*, PDA plates were inoculated with a disk of *G. catenulatum*, and grown for 7-14 days. Fresh PDA plates were inoculated with a disc of *Forc*. The lids were removed; and the plates containing *Forc* were inverted over the *G. catenulatum* plate (Fig 3-1). The two plates were sealed with Parafilm. After 7 days incubation, the colony diameter and sporulation of *Forc* growing in the presence of *G. catenulatum* was measured and compared to the control.

### 3.2.2 Chitinase and glucanase production in culture

*G. catenulatum* strain J1446 was grown on minimal salts medium (MSM) supplemented with the appropriate carbon source at 1 mg/ml and NaNO$_3$ as the nitrogen source at 1 mg/ml (Tweddell et al., 1994). For chitinase induction, MSM was supplemented with colloidal chitin purified from crab shells (Roberts and Selitrennikoff, 1988). For β-1,3-glucanase induction, MSM was supplemented with laminarin from *Laminaria digitata* (Sigma). Flasks were inoculated with a mycelial plug (6-mm-diameter) of *G. catenulatum* from a 10-day-old potato dextrose agar (PDA; EMD Chemicals Inc.) culture. To determine the time course of enzyme production on chitin and laminarin, triplicate flasks were harvested after 3, 7, 10, 14 and 21 days of growth and assayed for enzyme activity.
Additional carbon sources also tested (at 1 mg/ml dry weight) for enzyme induction included glucose, sucrose, N-acetyl-D-glucosamine, *Fusarium* and *Pythium* cell walls and cucumber roots. Control flasks contained no carbon source. Cell walls of *Fusarium* and *Pythium* were prepared according to the method of Tweddell et al. (1994). Cucumber roots from 14-day-old seedlings grown aseptically in nutrient solution were homogenized in a Waring blender at an initial concentration of 30 mg root fresh weight/ml of MSM. To determine the effect of initial pH on enzyme production, media containing chitin and laminarin (1 mg/ml) were adjusted with 1 M KOH or HCl to pH 4, 5, 6, 7, or 9. Enzyme activity was assayed after 7 days of growth for all carbon sources and initial pHs. For each carbon source and initial pH, culture filtrates from 3 replicate flasks were filtered through Whatman No. 1 filter paper, passed through a 0.22 μm sterile filter, and then freeze-dried prior to conducting enzyme activity assays. For chitinase assays, culture filtrates were rehydrated in 0.1M phosphate buffer, pH 6.8, whereas for glucanase assays, culture filtrates were rehydrated in 0.1M citrate buffer, pH 4.7. Each experiment was conducted twice.

Chitinase activity was assayed by monitoring the release of N-acetylglucosamine (NAGA) from colloidal chitin according to the method of Reissig et al. (1955). The reaction mixture contained 0.5 ml of supernatant and 0.5 ml colloidal chitin (2%, w/v) in 0.1 M phosphate buffer, pH 6.8. Samples were incubated for 2 h at 37°C and the reaction was stopped by boiling for 15 min. Controls were boiled for 15 min before incubation to determine background levels of N-acetylglucosamine. Residual chitin in the samples was removed by
centrifugation at 3000 g. Chitinase specific activity (CHU) was expressed as micromoles of N-acetylglucosamine per mg protein per h at 37°C.

β-1,3-glucanase activity was measured using the glucose oxidase reagent (Sigma) according to the manufacturer’s recommendations. The reaction mixture contained 25 μl supernatant, 25 μl laminarin (10 mg/ml) and 100 μl sodium acetate buffer (0.2 M, pH 5.0). Samples were incubated at 40°C for 2 h and the reaction was terminated by boiling for 4 min. Controls were boiled for 15 min prior to incubation to determine background levels of free glucose. Specific activity of β-1,3-glucanase (GU) was expressed as micromoles of glucose released from laminarin per mg of protein per h at 40°C. The protein concentration of the culture filtrates was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin (Sigma) as the standard.

3.2.3 Scanning electron microscopy (SEM)

To visualize hyphal interactions between G. catenulatum and Fusarium or Pythium in dual culture, the fungi were grown on an autoclaved cellophane sheet placed on PDA or water agar in 9-cm diameter Petri dishes. The cellophane sheet was inoculated with a 5-mm plug of the antagonist placed 3 cm away from a 5-mm diameter plug of the pathogen. The cultures were grown at 20-22°C in the dark. When the colonies came into contact with each other (7 days), pieces of cellophane from the contact zone were cut and prepared for SEM. In a similar experiment, 3 cucumber root segments (5 cm in length) were placed midway between the two fungal colonies and dishes were inoculated as above. Samples
were fixed in 0.1M cacodylate buffer containing 2% glutaraldehyde and 4% formaldehyde at room temperature for 1.5 h. Samples were post-fixed in 2% (w/v) tannic acid, followed by fixation in 1% osmium tetroxide in the above buffer for 1 h at room temperature, and 2% aqueous uranyl acetate for 1 h at room temperature in the dark. The samples were then dehydrated in a graded ethanol series and were dried in 100% hexamethyldisilizane. Samples were mounted on stubs using colloidal silver, sputter-coated with gold-palladium in a Nanotech SEMPrep II Sputter Coater and examined in a scanning electron microscope (Hitachi S4700).

3.2.4 Effect of crude enzyme extracts on *Pythium* and *Fusarium* mycelial growth and conidial germination

Crude enzyme extracts in filtrates following growth of *G. catenulatum* on chitin or laminarin as a carbon source were used to determine their effect on hyphal growth of *Pythium* and on hyphal growth and germination of conidia of *Fusarium*. The cell-free filtrates were mixed with cool, molten PDA in a 1:2 ratio (v/v) and poured into 3 cm-diameter Petri dishes and each dish was inoculated with a 3-mm-diameter plug of *Fusarium* or *Pythium*. Boiled culture filtrates and phosphate buffer were used as controls. Colony growth of *Fusarium* or *Pythium* was measured after 5 days of growth in two perpendicular directions. Each treatment was replicated 4 times. To determine the effect of filtrates on germination of conidia of *Fusarium*, a conidial suspension (2 x 10^5 spores/ml) of 50 μl was mixed with 50 μl of the culture filtrate in wells of double concave microscope slides placed inside Petri dishes lined with moistened filter paper.
Slides were incubated in the dark for 18 h under ambient laboratory temperatures (21-24°C). The frequency of germination of 100 conidia was counted in a haemocytometer and compared to controls receiving MSM alone. Each treatment was replicated three times and the experiment was conducted twice.

3.2.5 Release of glucose or NAGA from *Fusarium* or *Pythium* cell wall fragments

To determine the ability of crude enzyme extracts of *G. catenulatum* to degrade *Fusarium* or *Pythium* cell walls, culture filtrates following growth of *G. catenulatum* on MSM, chitin, laminarin, *Fusarium* or *Pythium* cell walls were collected after 7 days, passed through a 0.22 µm filter, freeze-dried and rehydrated in phosphate buffer (0.1M, pH 6.8). One ml of each filtrate was incubated with 5 mg of *Fusarium* or *Pythium* cell wall fragments at 37°C for 12 h. Glucose and NAGA concentrations were determined as described previously. Boiled culture filtrates and phosphate buffer were used as controls. Each treatment was replicated 3 times and the experiment was repeated twice.

3.2.6 Measurement of chitinase and glucanase enzymes in cucumber plants

Cucumber seedlings were grown aseptically inside Magenta boxes with nutrient solution as described above. After 10 days, the following treatments were initiated: i) conidia of *G. catenulatum* were added to the nutrient solution (5x10^5 conidia/ml nutrient solution) ; ii) 2,6-dichloroisonicotinic acid (INA) (2 µg/ml) was added to the nutrient solution; iii) salicylic acid (5 mM) was applied to the foliage until run-off; iv) control plants. At 2, 3 and 7 days after treatment, the
roots and shoots were excised, weighed, and ground separately in a mortar and pestle under liquid nitrogen. The material was suspended in cold phosphate buffer (10 mM, pH 6.0) and ground again with silica sand in microcentrifuge tubes using a polypropylene pestle (Sigma). The resulting suspension was centrifuged twice at 10,000 x g at 4°C and the supernatant was stored at -20°C and used for enzyme assays. Chitinase and \( \beta \)-1,3-glucanase activities in the root and shoot samples were assayed as described above. The protein concentration was determined as previously described. There were 3 replicate plant samples for each treatment. The experiment was conducted three times.

3.2.7 \( \beta \)-1,3-glucanase isoforms

To detect isoforms of \( \beta \)-1,3-glucanase in culture filtrates of \( G. \) catenulatum and in plant tissues inoculated with \( G. \) catenulatum, polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed according to the Davis system, using 15% (w/v) polyacrylamide resolving gels and 5% (w/v) polyacrylamide stacking gels containing 0.1% (w/v) SDS. The resolving gel contained 0.6 mg/ml of alkali-soluble \( S. \) cerevisiae \( \beta \)-1,3-glucan per ml and 0.1% (w/v) SDS (Grenier and Asselin, 1993). Gels were run with a voltage of 27 mAmp at room temperature. After electrophoresis, the enzymes were renatured by incubating the gels for 2 h at 37°C in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100. Gels were then stained in 0.025% (w/v) aniline blue with 150 mM \( K_2HPO_4 \) for 15 min at room temperature, followed by destaining in distilled water. Lytic zones were revealed by fluorescence using a long wave UV transilluminator. Purified \( \beta \)-1,3-glucanase
from *Trichoderma harzianum* (InterSpex Products, Inc.) was used as a positive control. Low-range Rainbow© molecular weight marker (RPN755, Amersham BioSciences) was used to determine the approximate size of polypeptides with glucanase activity bands.

### 3.2.8 Glucanase gene expression

#### 3.2.8.1 RT-PCR

To confirm expression of β-1,3-glucanase by *G. catenulatum* on colonized cucumber roots, plants were grown in Magenta boxes and inoculated with the biocontrol agent as described above. Roots were harvested 7 days after application of *G. catenulatum* and immediately frozen in liquid nitrogen. Total RNA was extracted from freeze-dried root tissue as described by Chomczynski and Sacchi (1987). First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase according to manufacturer’s directions (Invitrogen) using 10 μg of total RNA as the template. First-strand cDNA was used as the template in PCR amplification using Glu1F (AAGGGAGACGGTATTGCAAGAT) and Glu1R (TTCCGTCTGGATGAATGTCA) primers that were designed to amplify a 700 bp amplicon from the partial Glu1 mRNA sequence for *G. catenulatum* in the GenBank database (accession number DQ975304). The PCR product from the above reaction was used as the template in a second PCR amplification using primers Glu1npF (CCCAAACCTATGCTTCGTGT) and Glu1npR (CGTGGGAACATTTGGAGTCT) designed to amplify a 220 bp band from the internal sequence of the Glu1 fragment. The identity of the amplicon was confirmed by sequencing (Macrogen, Korea).
3.2.8.2 Northern analysis

For expression studies of the β-1,3-glucanase gene, *G. catenulatum* was grown on MSM supplemented with glucose (1.0 or 20.0 g/liter), ground cell walls of *Pythium* or *Fusarium* (2.0 g/liter) or homogenized cucumber roots (2.0 g/liter). Liquid cultures were grown as described above, except conidia of *G. catenulatum* from 14-day-old PDA plates were used to inoculate the medium at a final concentration of $10^6$ spores/ml. Mycelium was collected after 3 or 7 days of growth and immediately frozen in liquid nitrogen. Total RNA was extracted from freeze-dried mycelium as described by Chomczynski and Sacchi (1987), separated electrophoretically on a 1.2 % formaldehyde-agarose gel and transferred onto Hybond XL (Amersham, Uppsala, Sweden) nylon membranes. Random primers were used to label the 750 bp Glu1 mRNA fragment using $[\alpha-^{32}\text{P}]$ dCTP and Prime-A-gene labelling kit (Promega, Madison, WI, USA) following manufacturers protocols, and used as a radioactive RNA probe. Hybridization procedures were carried out as described by Wally et al. (2008).

3.2.9 Statistical analyses

For all data collected, variance analysis was performed using the `proc glm` method using Statistical Analysis System, version 8.0 (SAS Institute, 1999). Means of treatments were compared using Tukey’s HSD test. Normality of all dependent variables was tested by means of the univariate procedure, and homogeneity of experimental errors was examined by plotting residuals versus predicted values using the mixed model and plot procedure. Outlier detection was performed using studentized residuals and Cook’s distance measure. Data
for repetitions of the experiments were pooled for analysis when $F$-tests indicated that variances of the data did not differ significantly. The Type 1 error rate ($\alpha$) was set at 0.05 for all statistical tests.

### 3.3 Results

#### 3.3.1 Production of antifungal metabolic compounds *G. catenulatum* that inhibit growth of *F. oxysporum*.

*G. catenulatum* did not inhibit the growth of *Forc*, when the two fungi were co-inoculated on PDA (Fig. 3-1A). In contrast, on WA, hyphae of *G. catenulatum* overgrew and sporulated on *Fusarium* hyphae (Fig 3-1B). On PDA, *G. catenulatum* overgrew colonies of *P. aphanidermatum* (Fig 3-1C). In the experiments to detect volatile antibiotic production by *G. catenulatum* (Fig 3-1D, E), radial growth of *Forc* was not significantly inhibited by the presence of *G. catenulatum* at any of the treatment combinations (Fig 3-2). Sporulation of *Forc* was significantly decreased in all treatments in the presence of *G. catenulatum*, but only by an average 5-fold decrease from approximately $1 \times 10^7$ conidia per ml in control treatments to $2 \times 10^6$ conidia per ml in *Forc* + *G. catenulatum* treatments (Fig 3-2).
Figure 3-1  Dual culture plates of *G. catenulatum* (Gc) and *F. oxysporum* (Fo) or *P. aphanidermatum*.

Dual culture plates of *G. catenulatum* (Gc) and *F. oxysporum* (Fo) on (A) PDA and (B) water agar, and (C) *G. catenulatum* and *P. aphanidermatum* (Pa) on PDA, 7 days after co-inoculation. Samples for SEM processing were taken from the interaction zones (circles).  (D) Method to test for production of volatile organic compounds (VOCs) by *G. catenulatum*.  (E) Control culture of *F. oxysporum* (Fo), and culture of *F. oxysporum* (Fo) exposed to a 14-day-old culture of *G. catenulatum* (Gc) for 7 days.
Figure 3-2 Colony diameter (top) and conidia production (bottom) of *F. oxysporum* that was grown alone or after 7 days exposure to cultures of *G. catenulatum* growing for 7 days or 14 days on a Petri dish attached by parafilm.

Means are the combined values from two independent trials. Vertical bars indicate standard error of the mean (n=6).
3.3.2 Chitinase and glucanase production in culture

*G. catenulatum* produced extracellular chitinase and β-1,3-glucanase on chitin or laminarin as the sole carbon source, respectively. In chitin-amended medium, production of chitinase increased steadily and peaked at 14 days (30 CHU) after which time the levels remained constant (Fig. 3-3A). When grown on MSM only, chitinase activity was not detected. Production of β-1,3-glucanase peaked at 3 days when grown on laminarin and was highest at 150 glucanase units (GU) than at any other time period (Fig. 3-3B). Glucanase levels were lower after 7 days in culture and remained steady at around 40 GU for the duration of the experiment. When grown on MSM only, *G. catenulatum* produced a constant low level of glucanase over the time period assayed.

An incubation period of 7 days was chosen to test the effect of different carbon sources on production of chitinase and β-1,3-glucanase by *G. catenulatum*. Chitinase production was significantly higher in medium containing *Fusarium* cell walls (28 CHU), with activity almost 3.5-fold higher than on chitin-containing medium (8 CHU) (*P* < 0.0001) (Fig. 3-4). Chitinase activity was not detected in extracts from cultures grown on laminarin, glucose, sucrose, and N-acetylglucosamine (data not shown). The production of β-1,3-glucanase by *G. catenulatum* was not significantly different on laminarin (32 GU) compared to when *Fusarium* or *Pythium* cell walls were used as the sole carbon source (24 and 31 GU, respectively). *G. catenulatum* also produced extracellular β-1,3-
Figure 3-3 Time course of (A) chitinase and (B) β-1,3-glucanase production by *Gliocladium catenulatum* on MSM (no carbon source) and on medium containing chitin or laminarin as a carbon source, respectively.

Chitinase and β-1,3-glucanase activities are expressed as micromoles of N-acetylglucosamine (CHU) or as micromoles of glucose (GU) per milligram of protein per hour, respectively. Means are the combined values from two independent trials. Vertical bars indicate standard error of the mean (n=6).
Figure 3-4 Effect of carbon source on chitinase and β-1,3-glucanase production after 7 days of growth of *Gliocladium catenulatum*.

Chitinase and β-1,3-glucanase activities are expressed as micromoles of N-acetylglucosamine (CHU) or as micromoles of glucose (GU) per milligram of protein per hour, respectively. Values are the combined means from two independent trials. Carbon sources were as follows (1mg/ml): minimal synthetic medium with no carbon source (MSM), chitin, laminarin (Lam) *Fusarium* cell wall (FCW), *Pythium* cell walls (PCW), and homogenized cucumber roots (cuc). Values for columns in each group followed by the same letter did not differ significantly (Tukey’s HSD, *P* < 0.05). Vertical bars indicate standard error of the mean. Means represent the combined values from two independent trials (n=6).
glucanase in liquid culture when chitin or autoclaved and homogenized cucumber roots were used as the sole carbon source (Fig. 3-4). Chitinase production was not induced by cucumber roots. Similar to chitinase, glucanase was not detected in extracts of cultures grown on sucrose or glucose. The background levels of glucose in boiled culture filtrates from these treatments were an average of 1.6 μmol glucose/ml.

Maximum production of chitinase was observed at pH 6 (15 CHU) and there was no significant difference in enzyme activity at pH 4, 5, 7 and 9 (7-10 CHU) (Fig. 3-5A). Glucanase activity was significantly repressed by acidic pH, and was maximum at pH > 6 (Fig. 3-5B).

3.3.3 Scanning electron microscopy

During growth in culture, the hyphal diameters of Fusarium and Pythium were both approximately 30 μm whereas the hyphal diameter of G. catenulatum was approximately 10 μm, thereby allowing the fungi to be distinguished from one another (Fig. 3-6A). In dual culture, hyphae of G. catenulatum and Fusarium grew in close proximity to each other, and there was evidence of contact of G. catenulatum hyphae with Fusarium (Fig. 3-6B, C), with likely penetration points observed along the pathogen’s hyphae (Fig.3- 6D inset). Hyphal strands of G. catenulatum often grew in parallel along the hyphae of Fusarium, but lysis of Fusarium hyphae was not observed. When excised cucumber roots were placed on water agar between G. catenulatum and Fusarium cultures, the hyphae of the two fungi were rarely seen growing together. In areas of the root segments
Figure 3-5. Effect of initial medium pH on (A) chitinase and (B) β-1,3-glucanase production by *Gliocladium catenulatum* grown for 7 days on chitin or laminarin as the carbon source, respectively.

Chitinase and β-1,3-glucanase activities are expressed as micromoles of N-acetylglucosamine (CHU) or as micromoles of glucose (GU) per milligram of protein per hour, respectively. Values followed by the same letter did not differ significantly (Tukey’s HSD, *P* < 0.05). Vertical bars indicate standard error of the mean. Means are the combined values from two independent trials. (*n*=6).
Figure 3-6 Scanning electron micrographs of the interactions between *Gliocladium catenulatum* (c) and *Fusarium oxysporum* (f) in dual culture on water agar (A-D), or between *G. catenulatum* and *Pythium* (p) on excised cucumber roots (r) on water agar (E,F).

(A) Hyphae of *G. catenulatum* and *Fusarium oxysporum* growing in close proximity. The hyphae of *Fusarium* are 3X greater in diameter compared to *G. catenulatum*; scale bar = 30 μm; (B, C) Contact of *G. catenulatum* hyphae with *Fusarium* scale bar = 30 μm; (D) Penetration of *Fusarium* hyphae by *G. catenulatum*; scale bar = 30 μm; inset = 10 μm; (E) Hyphae of *G. catenulatum* branching and coiling around *P. aphanidermatum* on a cucumber root; scale bar = 50 μm; (F) Appressorium-like structures (arrows) of *G. catenulatum* attached to a hyphae of *Pythium*; scale bar = 30 μm.
colonized by both fungi, there was evidence of contact of *Fusarium* hyphae by *G. catenulatum* similar to that observed in dual cultures, but penetration was not observed (data not shown). In contrast, on excised cucumber roots, *G. catenulatum* hyphae were observed to coil around the hyphae of *Pythium*, producing short branches that surrounded the host hyphae (Fig. 3-6E). Spherical, appressorium-like structures were produced when attachment of the host hyphae to the pathogen’s hyphae was observed (Fig. 3-6F).

### 3.3.4 Effect of crude enzyme extracts on *Pythium* and *Fusarium* growth and conidial germination

Colony growth of *Fusarium* was significantly reduced (*P* < 0.05) on PDA containing crude extracts of filtrates from *G. catenulatum* with glucanase or chitinase activity when compared to boiled extracts or PDA alone (Figure 3-7A). Chitinase and glucanase levels in these filtrates ranged from 15 to 30 CHU or 40 to 50 GU, respectively (data not shown). Germination of *Fusarium* conidia was also significantly reduced (by up to 50 %) in the presence of these extracts (Figure 3-7B). Colony growth of *Pythium* was significantly reduced (*P* < 0.05) on PDA containing crude extracts of filtrates with glucanase, but not chitinase, activity compared to boiled extracts or PDA alone (Figure 3-7A).

### 3.3.5 Release of glucose or NAGA from *Fusarium* or *Pythium* cell walls

When *Fusarium* or *Pythium* cell walls were incubated with culture filtrates of *G. catenulatum* containing chitinase or β-1,3-glucanase activity, NAGA or glucose was released, respectively (Fig. 3-8). All filtrates containing glucanase activity were capable of degrading cell walls of *Fusarium* and *Pythium* to release
Figure 3-7  Effect of *Gliocladium catenulatum* culture filtrates on (A) growth of *Fusarium oxysporum* or *Pythium aphanidermatum* mycelia or (B) germination of *Fusarium* conidia.

Filtrates were obtained from extracts when *G. catenulatum* was grown on: no carbon source (MSM), laminarin medium (Lam) or chitin medium (Chit). Boiled filtrates were used as controls. Means represent the combined values from two independent trials. Values for columns in each group followed by the same letter did not differ significantly (Tukey’s HSD, *P* < 0.05) Vertical bars indicate standard error of the mean (n=6).
Figure 3-8  Effect of Gliocladium catenulatum culture filtrates on the release of (A) NAGA from Fusarium cell wall fragments or (B) glucose from Fusarium or (C) Pythium cell wall fragments.

Filtrates were obtained from extracts when G. catenulatum was grown on: no carbon source (MSM), chitin medium (Ch), laminarin medium (Lam), or Fusarium or Pythium cell walls. Boiled filtrates or phosphate buffer was used as a control for background levels of glucose or NAGA. Means represent the combined values from two independent trials. Values in a column followed by the same letter did not differ significantly (Tukey's HSD, $P < 0.05$). Vertical bars indicate standard error of the mean ($n=6$).
glucose. Filtrates containing chitinase activity released NAGA from *Fusarium* cell walls only. Filtrates obtained from chitin or *Fusarium* cell wall-containing media possessed chitinase and glucanase activity that released both NAGA and glucose from *Fusarium* cell wall fragments (Fig. 3-8A, B). Filtrates obtained from laminarin-containing medium possessed glucanase activity that was capable of releasing glucose, but not significant amounts of NAGA, from *Fusarium* or *Pythium* cell wall fragments (Fig. 3-8A, B). All activity levels were significantly higher than those in boiled filtrates and phosphate buffer.

### 3.3.6 Measurement of chitinase and glucanase in cucumber plants

Chitinase and glucanase activities were measured in the leaves and roots 2, 3 and 7 days after treatment with *G. catenulatum*. There was no detectable increase in chitinase activity in the roots or leaves after treatment with the biocontrol agent at most of the sampling times (see Chapter 5). However, there was a significant increase in glucanase activity in the roots (Fig. 3-9), but not leaves (see Chapter 5) 7 days after treatment with *G. catenulatum*.

### 3.3.7 β-1,3-glucanase isoforms

When *G. catenulatum* was grown on laminarin, three bands corresponding to β-1,3-glucanase activity were resolved on SDS-PAGE gels (Fig. 3-10), two of which were approximately 20 kDa and one was approximately 45kDa in size. Only one band, corresponding to 45 kDa, was detected in culture filtrates when *G. catenulatum* was grown on MSM. Two 20 kDa bands were visible in extracts originating from roots that had been inoculated with *G. catenulatum* 7 days prior.
Figure 3-9  Glucanase activity in roots of 10-day-old cucumber seedlings at 2, 3 and 7 days following inoculation with *Gliocladium catenulatum* (*Gc*), treatment with salicylic acid (SA) or water (Con).

Enzyme activity is expressed as micromoles of glucose (GU) per milligram of protein per hour. Values for columns in each sampling time followed by the same letter did not differ significantly (Tukey's HSD, *P* < 0.05). Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean (n=9).
Figure 3-10 SDS-PAGE of β-1,3-glucanase isoforms from *G. catenulatum*.

*G. catenulatum* was grown on media containing no carbon source (MSM) (lane 1) or laminarin (lane 2), or in extracts from cucumber roots treated with *G. catenulatum* for 7 days (lane 3), control roots (lane 4) and plants treated with INA (2 μg/ml) (lane 5). Samples (60 μl) of culture filtrates or plant extracts were loaded on a gel containing β-1,3-glucan. Bands with glucanase activity (size = 45 or 20 kDa) appeared as dark zones under UV illumination after staining with aniline blue.
to extraction. No bands were visible in root extracts from control plants or those treated with INA.

3.3.8 Glucanase gene expression

3.3.8.1 PCR

PCR with first-strand cDNA synthesized from total RNA extracted from cucumber plants colonized by *G. catenulatum* using the primer pair designed to amplify a 750 bp fragment did not consistently produce amplicons that were visible on an agarose gel (Fig. 3-11A). Therefore, to detect glucanase expression, the PCR products were used in a subsequent nested PCR protocol using a primer pair designed to amplify an internal fragment of a β-1,3-glucanase gene from *G. catenulatum* (GenBank accession DQ975304). PCR from cDNA synthesized from total RNA from cucumber plants colonized by *G. catenulatum* produced an amplicon of approximately 250 bp, which was absent in control plants not treated with *G. catenulatum* (Fig. 3-11B). The identity of the 750 and 250 bp amplicons, as determined by sequencing, showed 100% similarity with the sequence of the glucanase gene fragment from *G. catenulatum*.

3.3.8.1 Northern analysis

Glucanase mRNA was detected when *G. catenulatum* was grown on *Fusarium* or *Pythium* cell walls (0.2% w/v), homogenized cucumber roots (0.2% w/v), or in low glucose levels (0.1% w/v) representing starvation conditions (Fig. 3-12). Lower intensity of probe hybridization was observed when the initial
Figure 3-11 Detection of β-1,3-glucanase expression by *Gliocladium catenulatum* on colonized cucumber roots using reverse transcription – polymerase chain reaction (RT-PCR).

(A) Glu1F and Glu1R primer set and (B) nested PCR with PCR products from above using Glu1npF and Glu1npR primer set. Lane 1, water control; Lanes 3, 4, uninoculated cucumber roots; Lanes 2, 5, 6, cucumber roots colonized by *G. catenulatum*; Lane 7, *G. catenulatum* mycelium grown on laminarin; Lane M, DNA ladder. Amplicons of (A) 750 bp or (B) 220 bp are indicated by an arrow.
Figure 3-12 Northern blot analysis of expression of a β-1,3-glucanase gene from
*Gliocladium catenulatum*.

The fungus was grown as a shake culture in MSM supplemented with 0.2% cell wall fragments of *Fusarium* (Fo) or *Pythium* (Pa), homogenized cucumber roots (R), 2% glucose (G2) or 0.1% glucose (G0.1). Approximately five micrograms of total RNA, extracted after 3 days or 7 days of growth, was electrophoresed on a formaldehyde gel, blotted, and hybridized to a radiolabelled probe designed from a 750 bp fragment of a β-1,3-glucanase gene isolated from *G. catenulatum* (top panel). The bottom panel shows ethidium bromide-stained rRNA.
glucose concentration was increased to 2%, both 3 and 7 days after incubation (Fig. 3-12). Background glucose levels in boiled culture filtrates from 2% glucose treatments were 42 and 4.8 µmol glucose/ml at day 3 and 7, respectively. In contrast, glucose level was 0.6 µmol/ml in the 0.1% glucose treatment at day 3, and by day 7, glucose was not detected in the culture filtrates. Growth of G. catenulatum on Fusarium or Pythium cell walls enhanced glucanase expression compared to cucumber roots and low glucose treatments. Detection of glucanase mRNA was higher after 7 days than 3 days of incubation.

3.4 Discussion

Secretion of extracellular enzymes capable of lysing cell walls of pathogenic fungi is important in the mycoparasitic process and these enzymes are well characterized in many biocontrol agents, especially T. harzianum (Viterbo et al., 2002b; Whipps, 2001). Purified endo-β-1,3-glucanase and endochitinases from T. harzianum inhibited spore germination and had lytic activity against the cell walls of a number of plant pathogenic fungi, including Botrytis cinerea, Rhizoctonia solani, Sclerotium rolfsii and F. oxysporum f. sp. melonis (Lorito et al., 1994a; Viterbo et al., 2001), providing evidence for their involvement in mycoparasitism. Hydrolytic enzyme activity has not been previously characterized in G. catenulatum. We observed that enzymes capable of degrading chitin and β-1,3-glucan, both major cell wall components in Fusarium (Schoffelmeer et al., 1999), were produced in culture by G. catenulatum. Both enzymes inhibited the growth and germination of conidia of Fusarium, while only glucanase inhibited the growth of Pythium. The lack of
effect of chitinase on *Pythium* was not unexpected because the cell walls of plant-pathogenic oomycetes, such as *P. aphanidermatum*, are composed mainly of β-1,3- and β-1,6-glucans and cellulose instead of chitin (Viterbo et al., 2002b). Interestingly, even though glucanase activity was detected in culture filtrates of *G. catenulatum* grown on chitin, these filtrates did not inhibit growth of *P. aphanidermatum* in culture. Glucanase activity in filtrates from chitin media was approximately 1.5-fold lower than that from laminarin media. Therefore, it is likely that the glucanase levels from chitin-amended media were too low to inhibit *Pythium* growth, since growth inhibition by cell-wall degrading enzymes is often concentration dependent. For example, growth inhibition of *B. cinerea* by chitinases and glucanases produced by *T. harzianum* followed a dose-dependent response (Lorito et al., 1994a).

Activities of both chitinase and glucanase from *G. catenulatum* were repressed by glucose and were induced by fungal cell wall extracts, or by polymers such as laminarin and chitin. Northern analysis of glucanase mRNA generally revealed the same trends as enzyme activity data, demonstrating that expression of glucanase mRNA is induced by fungal cell walls. However, in enzyme activity assays, glucanase was not detected in low percent glucose-amended media, even though glucanase mRNA was detected in these glucose concentrations (0.1% w/v). To ensure that adequate levels of growth would be obtained for RNA extractions, the initial inoculum of *G. catenulatum* in the MS medium (1 x 10⁶ conidia/ml) was much higher in the experiments designed for Northern analysis. Therefore, it may be that carbon starvation was attained
much more rapidly in these cultures than those grown for enzyme activity assays, where a single mycelial plug was used as the initial inoculum source. The enhanced expression of genes encoding hydrolytic enzymes under conditions of physiological stress or carbon starvation has been reported for mycoparasitic fungi such as *T. harzianum* and *Coniothyrium minitans* (Donzelli et al., 2005; Giczey et al., 2001). Interactions between a mycoparasite and its host can also induce expression of chitinase and glucanase genes due to the action of small diffusible molecules derived from host cell walls (Lu et al., 2004; Viterbo et al., 2002a; Cohen-Kupiec et al., 1999).

The initial pH of the growth medium affected the activity of both glucanase and chitinase. In *T. harzianum*, pH has also been found to be an important factor regulating the production of hydrolytic enzymes (Donzelli et al., 2005). The pH of homogenized cucumber root extracts grown in rockwool cubes and watered with nutrient solution ranged from 5.8 to 6.3. Since the optimal pH range for glucanase and chitinase production in *G. catenulatum* (pH 6.0-7.0) coincides with the pH of cucumber roots, enzyme production *in situ* is likely to occur. The high initial peak of glucanase activity after 3 days in culture, along with its detection on cucumber roots *in vivo*, suggest that this enzyme may be important in the biocontrol activity of *G. catenulatum* against root pathogens. In contrast, fungal chitinase activity was not enhanced on colonized cucumber roots, and activity was not induced by cucumber roots, suggesting chitinase may be less important than glucanase.
Although attachment of *G. catenulatum* to *Fusarium* hyphae was observed in dual culture, this mycoparasitic behaviour was rarely detected on the root surface. It may be that direct interactions between an antagonist and a pathogen in the rhizosphere were rare, or just not observed (Whipps, 2001). In contrast, on excised cucumber roots, coiling and formation of appressorium-like structures by *G. catenulatum* on *Pythium* were frequently seen. This mycoparasitic behaviour by *G. catenulatum* was not observed on colonized cucumber roots in the absence of the pathogen (described in the previous chapter), indicating that hyphal branching and appressoria-like structures were induced by the presence of fungal host hyphae. In *T. atroviride*, hyphal branching and formation of specialized structures such as appressoria and papillae on colonized cucumber roots appeared to be an active response to the presence of a fungal host (Lu et al., 2004).

Despite the absence of direct penetration of *Fusarium* hyphae by *G. catenulatum* on cucumber roots, it is conceivable that glucanase levels could inhibit growth of *Fusarium in situ* when the two fungi are in close proximity to each other. This mechanism, termed hyphal interference, can occur through the action of diffusible metabolites, despite a physical separation between the interacting organisms (Thrane et al., 1997). Under conditions of carbon starvation and reduced growth, many fungi can actively secrete high levels of hydrolytic enzymes (Tweddell et al., 1994; Ramot et al., 2000; Viterbo et al., 2002a). For example, *T. harzianum* retained its ability to produce glucanase in
the presence of easily fermented carbon components such as those found in plant exudates (Thrane et al., 2000).

There were no detectable changes in enzyme activity in cucumber roots or leaves 2-3 days following treatment with G. catenulatum or salicylic acid. Salicylic acid and INA treatments were included to compare the effects of G. catenulatum to known plant chemical defense inducers, since SA and INA have been reported to induce resistance to fungal pathogens by enhancing expression of defense-related proteins (Walters et al., 2005; Jayaraj et al., 2008). In our experimental system, however, neither SA nor G. catenulatum appeared to induce the production of plant β-1,3-glucanase or chitinase. However, a significant elevation in β-1,3-glucanase or chitinase levels may not be revealed with the application of a biocontrol agent alone, but may also require pathogen challenge. For example, inoculation with T. asperellum resulted in a significant increase in mRNA levels of both chitinase and glucanase genes in cucumber leaves, but only when challenge-inoculated with a pathogen (Shoresh et al., 2005b). Therefore, whether or not G. catenulatum can induce defense responses in cucumber plants remains to be determined. In our study, glucanase activity was significantly higher in roots 7 days after application of G. catenulatum when compared to the control and SA-treated plants, and the glucanase isoform patterns indicated that the glucanase activity was of fungal origin. This was further supported by detection of fungal glucanase mRNA expression on colonized cucumber roots using reverse-transcription PCR. An amplicon corresponding to a 250 bp fragment of a G. catenulatum β-1,3-
glucanase was detected in nested PCR samples from colonized cucumber roots which was absent in roots not treated with *G. catenulatum*. This provides strong evidence for the *in situ* expression of an important fungal enzyme known to be involved in biological control activity.

Rhizosphere competence is also strongly related to biocontrol efficacy in mycoparasitic isolates of *Trichoderma* spp. (Thrane et al., 1997; Whipps, 2001). Therefore, production of antifungal β-1,3-glucanases by *G. catenulatum* in the rhizosphere could create an environment that is inhibitory to growth and colonization by *Fusarium* or *Pythium*, prior to the introduction of pathogen inoculum (pre-emptive colonization). This is supported by the significant biocontrol efficacy of *G. catenulatum* when applied 24 h to 3 days prior to pathogen inoculation (McQuilken et al., 2001; Punja and Yip, 2003; Rose et al., 2003) and by findings, described in Chapter 2, that the density of *Fusarium* propagules on cucumber root and crown tissues was significantly reduced in the presence of *G. catenulatum*. Preliminary investigations into the role of volatile and nonvolatile antibiotics by *G. catenulatum* indicated that these were absent in culture. The extensive root colonization ability of this fungus, coupled with its ability to rapidly produce glucanase *in situ* as reported here, are likely major contributors to its efficacy as a biocontrol agent against *Fusarium* and *Pythium*. 
4: FACTORS INFLUENCING COLONIZATION OF CUCUMBER ROOTS BY *GLIOCLADIUM CATENULATUM*


4.1 Introduction

The fungus *Gliocladium catenulatum* Gilman & Abbott strain J1446 [syn. *Clonostachys rosea* f. *catenulata* (Gilman & Abbott) Schroers; teleomorph *Bionectria ochroleuca* (Schw.) Schroers & Samuels (Schroers, 2001; Schroers et al., 1999)] is a commercially formulated biocontrol agent (Prestop WP, Verdera Oy, Finland) with broad-spectrum activity against plant pathogens (Lahdenpera and Korteniemi, 2005; McQuilken et al., 2001). A comparative study on biological control strategies to reduce Fusarium root and stem rot of cucumbers demonstrated that this biocontrol agent was effective in reducing seedling mortality and was the best of three commercially available fungal biocontrol agents in reducing disease severity in growth room trials (Rose et al., 2003). Under semicommercial growing conditions, *G. catenulatum* also reduced root and stem rot when applied prior to pathogen inoculation (Rose et al., 2003). When applied to rockwool blocks at seeding, *G. catenulatum* persisted on cucumber roots for at least 50 days at levels above 1 x 10⁵ CFU/g root fresh
weight (Chapter 2). Hyphae of *G. catenulatum* were found to extensively colonize cucumber roots, forming a dense network over the root surface within 7 days after application, and hyphae were often observed growing internally in root epidermal cells. Application of *G. catenulatum* to rockwool blocks before inoculation with *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*) resulted in a significant decrease in pathogen levels on the roots and crown when compared to plants inoculated with *Forc* only. The biocontrol agent also secretes cell-wall degrading enzymes that can degrade hyphae of *Forc* and β-1,3-glucanase activity was detected on colonized cucumber roots (Chapter 3). Therefore, the root colonization ability of *G. catenulatum* coupled with mycoparasitism appear to be important for its efficacy.

A major challenge in biocontrol programs to manage root diseases is ensuring that stable populations of the introduced agents are maintained in the rhizosphere so that beneficial effects are sustained throughout the growing season (Leandro et al., 2007). The survival of biocontrol agents and their efficacy against pathogens are known to be influenced by environmental conditions. Inconsistent environmental conditions in the field have often been cited as a reason for failure or variable performance of biocontrol agents (Fravel, 2005). Therefore, identification of factors that can affect the efficacy of an antagonist should make biocontrol a more predictable and successful strategy. Since most biocontrol agents have an optimum environmental range for best efficacy, these conditions should be defined from laboratory studies (Paulitz, 2000). Environmental conditions including temperature, moisture, and soil
physical and chemical characteristics can affect the physiology of the host plant and can also alter the interactions among the plant, pathogen and biocontrol agent (Larkin and Fravel, 2002). Understanding the parameters that favour establishment of *G. catenulatum* on cucumber roots may help identify strategies for use under greenhouse conditions that will maximize survival of the biocontrol agent, potentially resulting in more consistent disease reduction. The objectives of this study, therefore, were to determine the influence of root zone temperature, nutrient solution pH, cucumber cultivar, root wounding and growing media on root colonization and population density of *G. catenulatum* on cucumber plants.

### 4.2 Materials and Methods

#### 4.2.1 Fungal strains and plant growing conditions

A GUS-modified, hygromycin-resistant strain of *G. catenulatum* (Chapter 2) was maintained on PDA amended with hygromycin (50 mg/liter). To produce inoculum, the fungus was cultured onto half-strength PDA amended with tetracycline (20 mg/liter) and Triton X-100 (2 ml/liter) (PDAtt, McQuilken et al. 2001) and grown under laboratory conditions (21-24°C) for 10-14 days with ambient fluorescent lights. Conidia were obtained by flooding colonies with sterile distilled water, followed by scraping the surface with a glass rod. The resulting suspension was filtered through a double layer of cheesecloth to remove mycelia. The total number of spores was determined using a haemocytometer and adjusted to the desired concentration.

Surface-sterilized cucumber seeds cv. Averyl (Rijk Zwaan, De Lier, the Netherlands) were placed on filter paper strips (3 mm thick) suspended on a wire
mesh 1 cm above nutrient solution (120 ml) inside autoclaved Magenta boxes (Sigma). The containers were incubated on a bench under ambient conditions (21-24°C) with a 16 h photoperiod. Nutrient solution was prepared with 0.73 g of soluble fertilizer (N:P:K, 7:11:27: Plant Products, Brampton, ON) and 0.48 g Ca(NO₃)₂ per liter of water, and adjusted to pH 5.8 using 1M KOH. After 10 days, when the seeds had germinated and roots reached the nutrient solution, seedlings were transferred to individual hydroponic containers (800 ml, Fisher Scientific) containing nutrient solution. Five seedlings were placed in each container by inserting roots through 1-cm holes cut in Styrofoam lids. A piece of foam was wrapped around the crown region to prevent the seedling from falling into the nutrient solution. For aeration, compressed air was bubbled continuously into the solution in each container via plastic tubing. Nutrient solution levels were replenished with distilled water as needed, and the nutrient solution in the container was replaced weekly with fresh solution. Plants were grown in a growth room under ambient temperature (21-24 °C, except for temperature trials) with a 16 h photoperiod provided by sodium vapour lights (light intensity of 100 μmoles/m²/s). One week after transplant, conidia of *G. catenulatum* were applied directly to the nutrient solution at a final concentration of 5 x 10⁵ conidia/ml of solution.

To determine population levels, roots were harvested 7, 14 and 21 days after application of *G. catenulatum*, by removing the entire root system from each plant (3 plants/sampling time). Whole roots were washed briefly with distilled water, blotted dry and weighed, and random segments of roots were excised to
obtain samples that did not exceed 500 mg. Tissues were homogenized for 30s in 1 ml ice-cold GUS extraction buffer (Bao et al., 2000) using one glass and one chrome steel bead (6.35 mm each) in a Mini Beadbeater-8 (BioSpec Products, Bartlesville, OK). A 100 µl aliquot of the homogenate was diluted and plated onto PDA to determine colony-forming units (CFU) of *G. catenulatum* per mg of root fresh weight. Dishes were then incubated at room temperature for 5 days before colonies were counted. To determine GUS activity levels in roots, the remaining homogenate was ground for an additional 60 s, proteins were extracted, and the resulting supernatant was used to determine GUS activity expressed as nM MU/mg root fresh weight as described in Chapter 2.

4.2.2 Factors affecting population levels of *G. catenulatum* on cucumber roots

4.2.2.1 Nutrient solution pH

To determine the effect of pH on colonization by *G. catenulatum*, the pH of the nutrient solution was modified by adding 1N HCl or 1M KOH to adjust the pH to 4.0, 5.0, 6.0, 7.0 or 8.0. The pH was monitored daily and adjusted as needed.

4.2.2.2 Temperature of the nutrient solution

The effect of temperature on colonization was determined by varying the temperature of the nutrient solution to 16, 18, 22, 26 or 30 °C. The desired temperatures were obtained by growing plants in a heated water bath (26 and 30 °C treatments), at room temperature (22 °C) or in cooling growth chambers (16 and 18 °C). The temperature of the nutrient solution was monitored daily, and on average, temperature fluctuated by +/- 1 °C.
4.2.2.3 Growing media

The effect of growing media on colonization was determined by growing cucumber plants in one of the following substrates: hydroponic containers, rockwool blocks, sawdust, soilless potting mix (Sunshine Mix #4, Sun Gro Horticulture Canada Ltd., Vancouver, BC) and field soil collected from a local organic garden. Cucumber plants were grown in hydroponic containers as described above. For rockwool blocks, cucumber seeds were placed in the seeding cavities of 10-cm² rockwool blocks (Westgro, Delta, BC) and covered with 50 ml of vermiculite. For all other media types, cucumber seeds were planted directly into the growing media which was contained in 8-cm plastic pots (500 cm³ volume). Conidia of G. catenulatum were applied to the growing media when plants were approximately 17 days old to give a final concentration of 5 x 10⁵ conidia per cm³ of growing media. Roots were sampled at 14 and 21 days after application of the biocontrol agent, to ensure enough tissue was available.

4.2.2.4 Cucumber cultivar

To determine whether the cultivar influenced population levels, five cucumber cultivars, Averyl (Rijk Zwaan), Bodega (Rijk Zwaan), Ladner (Rijk Zwaan), Marcel (Nunhems) and Sienna (Nunhems) were grown under hydroponic conditions as previously described. Roots were excised and population levels determined as described previously.
4.2.2.5 Addition of nutrients to the hydroponic solution

To determine whether addition of nutrients to the nutrient solution affected colonization by *G. catenulatum*, plants were grown in hydroponic solution as described above. Glucose (0.01% w/v), asparagine (0.005% w/v) or glucose and asparagine (0.01% and 0.005% w/v, respectively) were added to the nutrient solution just prior to application of *G. catenulatum* to the roots. The nutrient solution containing the additives was replaced with regular nutrient solution after 3 days. Plants grown in nutrient solution without any additives served as the control.

4.2.2.6 Wounding

To determine the effect of wounding on colonization by *G. catenulatum*, roots were either not wounded (control), wounded by cutting off the distal 2 cm of the roots at the time of application (cut), wounded by piercing the main root 10 times with a sterilized fine needle (an insect mounting pin) at the time of application (stab), or wounded by piercing the main root 10 times with a fine needle daily after application of the biocontrol agent (daily).

4.2.3 Statistical analysis

For all experiments conducted using the hydroponic system, there were 3 replicate containers per treatment, and 3 plants were harvested on each sampling day. Each experiment was conducted three times, except for the growing media experiment, which was conducted two times. For the growing media experiment, there were 10 replicate pots, and 5 plants were sampled on
each harvesting day. Data from repeated experiments were combined for analysis when F-tests indicated that variances of the data did not differ significantly. Density estimates of _G. catenulatum_ associated with cucumber roots were log transformed (y+1) prior to analysis. Analysis of variance was performed using the _proc mixed_ statement to determine significance of main treatment effects and the interaction of treatment with other experimental effects (day, variety) using the Statistical Analysis System, version 9.1 (SAS Institute, 2008). Data sets from each sampling day were analyzed separately when analysis of variance indicated that day was a significant source of variation. For all experiments, significant differences between treatment means were separated using Fisher`s Protected LSD. The Type 1 error rate (α) was set at 0.05 for all statistical tests.

4.3 Results

4.3.1 Factors affecting population levels of _G. catenulatum_ on cucumber roots

4.3.1.1 Nutrient solution pH

The pH of the nutrient solution had a significant effect on the population levels of _G. catenulatum_ on cucumber roots at all sampling days. Seven days after application of _G. catenulatum_, population levels were highest on roots that were grown at pH 5, 6, or 7 with average populations of 3.7 - 3.8 log_{10} CFU per mg root fresh weight at all 3 pH’s (Figure 4-1). Populations on roots grown at pH 4 or 8 were significantly lower, at 3.5 and 3.1 log_{10} CFU per mg root fresh weight (\(P = 0.018\) and 0.0001, respectively). GUS activity was highest in roots grown at
pH 5 (31.2 nM MU/mg root fresh weight/hr), but there was no significant difference in GUS activity from pH 4, 6 and 7 treatments, which ranged in value from 25 to 27 nM MU per mg root fresh weight (Figure 4-1). GUS activity was lowest on roots grown at pH 8, with a value of 20 nM MU. Fourteen days after application, population levels of *G. catenulatum* were highest on roots from the pH 5 treatment (3.4 log\(_{10}\) CFU) but these levels were not significantly different from the pH 6 treatment (3.2 log\(_{10}\) CFU, \(P = 0.0862\)). The lowest level was observed on roots from the pH 8 treatment (2.5 log\(_{10}\) CFU) which was significantly lower compared to the other pH treatments (\(P < 0.0001\)). GUS activity levels from pH 5, 6 and 7 treatments were not significantly different from each other (24.2, 24.4 and 26.4 nM MU/mg root fresh weight, respectively), while activity was significantly lower at pH 8 (14.4, \(P = 0.0001\)). By day 21, population levels on roots had decreased in all treatments, with the highest level occurring at pH 5 (2.9 log\(_{10}\) CFU) and the lowest level at pH 8 (1.9 log\(_{10}\) CFU). GUS activity levels from pH 4, 5, 6 and 7 treatments were not significantly different from each other, while a significantly lower value was observed in the pH 8 treatment (\(P < 0.0003\)). There was no GUS activity detected in control roots not receiving *G. catenulatum* treatment.

4.3.1.2 Temperature of the nutrient solution

The temperature of the root zone had a significant effect on colonization by *G. catenulatum* on all sampling days. Seven days after application of the biocontrol agent, population levels were highest at 22 °C (4.0 log\(_{10}\) CFU/mg root
Figure 4-1 Population levels of *G. catenulatum* on cucumber roots determined by colony plate counts (A) or GUS expression (B) as influenced by pH of the nutrient solution.

Plants were sampled 7, 14 and 21 days weeks after application of *G. catenulatum* at $5 \times 10^5$ CFU/ml. Colony counts were determined by plating supernatant from ground tissues onto selective media for *G. catenulatum*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 9 replicates per treatment. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
fresh weight). However, this level was not significantly different from that observed at 16 °C (3.7 log_{10} CFU, \( P = 0.0743 \)) and 18 °C (3.8 log_{10} CFU, \( P = 0.3486 \)), but was significantly higher than levels at 26 °C (3.6 log_{10} CFU, \( P = 0.0284 \)) and 30 °C (3.3 log_{10} CFU, \( P = 0.0032 \)) (Figure 4-2). GUS activity was highest at 18 °C (53.5 nM MU), but this value was not significantly different than at 22 °C (45.69 nM MU, \( P = 0.2650 \)). GUS activity in roots that were grown at 16, 26 and 30 °C were not significantly different from each other, with mean levels close to 25 nM MU per mg root fresh weight in all three treatments. By day 14, population levels were highest in roots grown at 18 °C (3.6 log_{10} CFU) and this number was significantly higher than levels observed on roots from 16 °C (2.8 log_{10} CFU, \( P = 0.0043 \)), 22 °C (3.2 log_{10} CFU, \( P = 0.0471 \)), 26 °C (3.1 log_{10} CFU, \( P = 0.0383 \)) and 30 °C (2.2 log_{10} CFU, \( P = 0.0002 \)). Similar trends were observed by day 21, with the highest levels of colonization occurring on roots that were grown at 18 °C (3.2 log_{10} CFU). These levels were significantly higher than those observed in all other treatments (\( P < 0.0001 \)). However, there was no significant difference in GUS activity levels between any of the treatments (\( P = 0.2851 \)) at day 14 or 21.

### 4.3.1.3 Growing media

Colonization of cucumber roots by *G. catenulatum* was significantly affected by the medium in which the cucumbers were grown. Plants grown in nutrient solution in hydroponic containers supported the highest population levels of *G. catenulatum* at both 14 and 21 days after application of the biocontrol agent (Figure 4-3). Colony forming units of *G. catenulatum* on these roots on day 14
Figure 4-2 Population levels of *G. catenulatum* on cucumber roots determined by colony plate counts (A) or GUS expression (B) as influenced by the temperature of the nutrient solution.

Nutrient solution of the plants was maintained at 16, 18, 22, 26 or 30°C. Plants were sampled 7, 14 and 21 days weeks after application of *G. catenulatum* at $5 \times 10^5$ CFU/ml. Colony counts were determined by plating supernatant from ground tissues onto selective media for *C. rosea*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 9 replicates per treatment. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
Figure 4-3 Population levels of *G. catenulatum* on cucumber roots grown in different media types determined by colony plate counts (A) or GUS expression (B).

Cucumber plants were grown in nutrient solution (Hydro), rockwool blocks (rock), sawdust (saw), soilless potting mix (peat) or field soil (soil). Plants were sampled 14 and 21 days weeks after application of *G. catenulatum*. Colony counts were determined by plating supernatant from ground tissues onto selective media for *G. catenulatum*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 10 replicates per treatment. Means represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.
were significantly higher than levels on roots grown in rockwool or sawdust ($P = 0.0222$ and $0.0001$, respectively), which in turn were significantly higher than populations found in peat and soil ($P < 0.0001$). By day 21, however, colony forming units on roots grown in nutrient solution or in rockwool were not significantly different from each other ($P = 0.1222$), whereas these levels were significantly higher than those found in all of the other growing media. GUS activity was also highest on roots grown in nutrient solution at 14 days (58.9 nM MU) and was significantly higher than activities from all of the other media types (Figure 4-3). By 21 days after application, GUS activities on roots grown in nutrient solution, rockwool or sawdust were not significantly different from each other. Lowest colonization levels, as determined by both colony counts and GUS activity, were found on cucumber roots grown in the potting mix and field soil at both sampling times.

4.3.1.4 Cucumber cultivar

Analysis of variance indicated that cultivar type was not a significant source of variation contributing to colony forming unit values at day 7, 14, or 21 ($P = 0.1323$, 0.3523, or 0.0626, respectively) or to GUS activity values at day 7, 14 or 21 ($P = 0.2162$, 0.6881, or 0.0602) (Figure 4-4).

4.3.1.5 Addition of nutrients to the hydroponic solution

Analysis of variance indicated that the addition of glucose, asparagine or glucose + asparagine to the nutrient solution was not a significant source of variation contributing to colony forming unit values at day 7, 14, or 21 ($P =$
Figure 4-4 Population levels of *G. catenulatum* on the roots of 5 cucumber cultivars (Averyl, Bodega, Ladner, Marcel or Sienna) determined by colony plate counts (A) or GUS expression (B).

Plants were sampled 7, 14 and 21 days weeks after application of *G. catenulatum* at $5 \times 10^5$ CFU/ml. Colony counts were determined by plating supernatant from ground tissues onto selective media for *G. catenulatum*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 9 replicates per treatment. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
0.3870, 0.0940, or 0.0960, respectively) or to GUS activity values at day 7, 14 or 21 \((P = 0.8703, 0.1153, \text{ or } 0.2061)\) (Figure 4-5).

### 4.3.1.6 Wounding

Wounding of the roots had a significant effect on the population levels of *G. catenulatum*, but only at 14 and 21 days after application of the biocontrol agent. Seven days after application, there was no significant difference between the treatments and colonization levels in all treatments ranged from 3.6 to 3.9 \(\log_{10}\) CFU/mg root fresh weight \((P = 0.1160)\) and GUS activity levels ranged from 26.6 to 38.2 nM MU/mg root fresh weight \((P = 0.2649)\) (Figure 4-6). By day 14, population levels on the roots that were wounded daily with a fine needle were significantly lower than those on unwounded roots \((P = 0.0002)\) or on roots that were wounded at the time of inoculation only \((P = 0.0001)\). Roots that were wounded by cutting had population levels that were significantly higher than all other treatments \((P = 0.0001)\). GUS activity levels were significantly higher on roots that were wounded by cutting (27.7 nM MU) compared to all other treatments, which showed similar GUS activity levels ranging from 16.7 to 19.7 nM MU. By day 21, population levels on roots that were wounded daily fell to 0.6 \(\log_{10}\) CFU/ mg root fresh weight, which was significantly lower than all other treatments \((P < 0.0001)\). Population levels on the roots that were wounded by cutting were higher than on non-wounded roots \((P = 0.0052)\) and on roots that were wounded once by stabbing \((P = 0.0052)\). Only roots that were wounded daily with a needle displayed GUS activities that were significantly lower than all other treatments.
Figure 4-5 Population levels of *G. catenulatum* on cucumber roots as affected by addition of glucose or asparagine to the nutrient solution determined by colony plate counts (A) or GUS expression (B).

Nutrient solution of the plants was either left untreated or glucose (0.01%w/v), asparagine (0.001% w/v) or glucose + asparagine were added to the nutrient solution at the time of *G. catenulatum* application. Plants were sampled 7, 14 and 21 days weeks after application of *G. catenulatum* at 5 x 10⁵ CFU/ml. Colony counts were determined by plating supernatant from ground tissues onto selective media for *G. catenulatum*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 9 replicates per treatment. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
Figure 4-6  Population levels of *G. catenulatum* on unwounded (control) cucumber roots or wounded roots determined by colony plate counts (A) or GUS expression (B).

Roots were wounded by stabbing with a fine tip needle daily (daily) or once at the time of biocontrol application (stab) or by cutting the distal 2 cm of roots at the time of application (cut). Plants were sampled 7, 14 and 21 days weeks after application of *G. catenulatum* at 5 x 10⁵ CFU/ml. Colony counts were determined by plating supernatant from ground tissues onto selective media for *G. catenulatum*. Fungal biomass was determined indirectly from GUS activity and is expressed as nM MU per mg root fresh weight. The means and standard error were obtained from 9 replicates per treatment. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
4.4 Discussion

Inconsistencies in achieving biocontrol of root pathogens due to varying environmental conditions have often been cited as a limiting factor in using biocontrol agents for disease management strategies (Fravel, 2005). Currently, over half of the commercially available biocontrol products are registered for use in greenhouses, making the use of biocontrol agents in this environment more prevalent than in field crops (van Lenteren, 2000). In greenhouses, environmental conditions are relatively uniform, and can be adjusted to provide optimal conditions for growth of a biocontrol agent, provided that these conditions coincide with conditions required for optimal plant health (Paulitz and Belanger, 2001). The success of an introduced biocontrol agent in suppressing disease can be influenced by the conduciveness of the environment to the disease and to the establishment and proliferation of the antagonist in the root zone (Landa, 2004). In this study, a number of abiotic factors were evaluated for their effects on colonization of cucumber roots by the biocontrol fungus *G. catenulatum* since there have been no previous studies to determine this.

There were some discrepancies in data values between population levels expressed as CFU/mg root fresh weight or nM MU/mg root fresh weight. However, the general data trends and the conclusions that could be drawn from the results did not differ greatly between the two enumeration methods. The largest difference in results between CFU and GUS activity was for the temperature experiments, and especially at 7 days after application. The biomass of *G. catenulatum* on the roots was much higher at 18 and 22°C
compared to the other temperatures, as measured by GUS activity levels, whereas this difference was not observed in the results for CFU/ mg root fresh weight. The dilution-plating technique does not differentiate among different propagules (hyphal fragments, conidia and chlamydospores), all of which may generate colonies when plated on agar, and thus is not a true estimate of fungal biomass (Park et al., 1992; Bae and Knudsen, 2000). When expression of GUS is under the control of a constitutive promoter, the production of the enzyme correlates with the general physiological condition of the organism, and thus qualifies as an expression of the metabolic activity of the fungus in planta (Green and Jensen, 1995; Bao et al., 2000). Therefore, in this case, GUS activity measurements were likely correlated to the fungal biomass of G. catenulatum on the roots, whereas CFU/mg would more closely describe sporulation occurring on the roots, thus accounting for any inconsistency between the two results.

Population levels of G. catenulatum associated with cucumber roots were significantly affected by pH of the nutrient solution, temperature, and growing media, while root wounding, cucumber cultivar and nutrient additives did not have a significant effect. The ability to thrive over a wide range of external pH values is an important component of the complex set of variables that a biocontrol agent encounters during its interaction with the host plant (Benitez et al., 2004). Biocontrol agents that can survive over a range of pH conditions by adapting their own metabolism, especially those functions related to biocontrol activity, could consequently reduce the activity of phytopathogens which are unable to withstand changes in pH (Benitez et al., 2004). In this study, G.
*catenulatum* survived over the range of pH levels tested, but population levels were highest on roots that were grown at pH 5 - 7. The target pH of the nutrient solution supplied to cucumber plants is between 5.5 and 6.0 (OMAFRA, 2003). The pH optimum levels of *G. catenulatum* fall within the common range for other biocontrol fungi such as *T. atroviride* and *T. harzianum* (Longa et al., 2008; Ahmad and Baker, 1987). Ambient pH regulates gene expression involved in the mycoparasitic response and antagonistic abilities have been shown to function at an optimal pH in filamentous fungi such as *T. harzianum* strain CECT 2413 (Moreno-Mateos et al., 2007) and *T. atroviride* strain P1 (Donzelli et al., 2005). Production of chitinase and β-1,3-glucanase by *G. catenulatum* was influenced by pH, with highest activities of both enzymes occurring at pH 6 (Chapter 3).

*G. catenulatum* was able to grow and sporulate on healthy cucumber roots, and wounding of cucumber roots at the time of *G. catenulatum* application did not significantly affect population levels compared to healthy roots. This single wounding event likely induced transient changes in root exudates, and their effect on population levels would not be detected at the first sampling time which occurred 7 days after application of the antagonist. However, when cucumber roots were wounded daily, population levels of *G. catenulatum* significantly declined over time, and staining with X-Gluc revealed that *G. catenulatum* was not present at the wound sites (data not shown). Wounding of plants can induce responses that strongly resemble those induced by pathogen attack, resulting in elevated levels of defense-related proteins and phenolic compounds that function in preventing fungal growth (Baron and Zambryski, 2003).
Therefore, it is likely that daily wounding resulted in reduced *G. catenulatum* colonization due to an enhanced tissue defense response. In contrast, hyphae of *T. harzianum* (T3) were found especially in association with small wounds on cucumber roots, and the presence of organic substrates such as seed coats, decaying roots and wounds enhanced root colonization (Thrane et al., 1995; Green et al., 2001).

Temperature is a key factor influencing colonization by biocontrol agents and expression of biocontrol mechanisms (Landa, 2004). The optimum temperature range for colonization of cucumber roots by *G. catenulatum* was 18-22 °C, although at 18 °C, populations were maintained at higher levels on the roots over time. In culture, *G. catenulatum* grew best at 25 °C, but growth also occurred at temperatures ranging from 15 to 30°C. The target root temperature for cucumber plants in commercial greenhouses is 18-22°C (OMAFRA, 2003). Pre-emptive colonization of the cucumber root zone by *G. catenulatum* prevented infection by *Forc* and significantly reduced pathogen populations on the roots when plants were grown at 22°C (Chapter 2). Severity of Fusarium root and stem rot on cucumber seedlings is highest at 17-24°C (Vakalounakis, 1996; Punja and Parker, 2000). This would indicate that even under disease-conducive temperatures, *G. catenulatum* is effective at suppressing *Fusarium* infections, and these two fungi have similar temperature requirements for root establishment.

The type of growing medium in which cucumbers were grown appeared to have the greatest influence on colonization of roots by *G. catenulatum*. Plants
grown under conditions where roots were suspended in a nutrient solution supported the highest levels of root colonization. When roots were grown in a rockwool or sawdust substrate, populations of *G. catenulatum* were similar to those observed in nutrient solution alone. In the greenhouse, biological control agents introduced to the root zone in soilless substrates are expected to establish and proliferate due to the low microbial diversity, especially during the early stages of greenhouse production (Fravel, 2005; Menzies et al., 2005). For example, tomatoes grown in a hydroponic system supported more abundant growth of non-pathogenic *F. oxysporum* Fo47 on the root surface, allowing the fungus to colonize the root elongation zone and apex. This colonization pattern was rarely observed in soil environments (Nahalkova et al. 2008; Olivain et al. 2006). A greater density of fungi was found associated with cucumber roots grown in soil, followed by rockwool and sawdust, with the least number occurring in nutrient solution (Menzies et al., 2005). Higher levels of microbial soil biomass induced a shift from hyphal growth to sporulation in *T. harzianum*, resulting in reduced biocontrol efficacy (Bae and Knudsen, 2005). These types of effects may be due to soil fungistasis, which is largely dependent on the soil microbial community composition (de Boer et al., 2003). This could explain the very low population levels of *G. catenulatum* associated with cucumber roots in field soil.

In two types of peat-based potting mixes, Rhizoctonia damping off of poinsettia was not controlled effectively by *T. hamatum* (T-382), due to a rapid decline in the population levels of the biocontrol agent (Krause et al., 2001). Peat-based potting mixes often have a lower microbial carrying capacity (Hoitink et al., 2006),
and this could explain the low colonization levels by *G. catenulatum* in the potting mix used in this study. Most biocontrol agents have a threshold level required for biocontrol activity (Paulitz, 2000). Biocontrol activity by *Trichoderma* strains has been reported at populations of $10^5$–$10^7$ CFU/g of growing medium, and when *Trichoderma* strains are present at lower levels, biocontrol can be ineffective (Leandro et al., 2007). A similar population level of *G. catenulatum* was observed on roots of cucumber plants grown in nutrient solution, rockwool and sawdust throughout the duration of the experiment, but levels in potting mix and field soil were below $10^5$ CFU/g root fresh weight 21 days after application.

There was no influence of cucumber cultivar on the ability of *G. catenulatum* to colonize cucumber roots. The cultivars used in this study represent host genotypes with different levels of resistance to Fusarium root and stem rot (Rose and Punja, 2004), suggesting host genotype did not influence the antagonist-plant association. Of the cultivars tested, Sienna is highly susceptible, Ladner and Bodega display moderate susceptibility and Averyl and Marcel are resistant to infection by *Forc* (Rose and Punja, 2004). In studies on biocontrol of tomato wilt caused by *F. oxysporum* f.sp. *lycopersici*, similar disease control was achieved by non-pathogenic isolates of *F. oxysporum* regardless of the tomato cultivar used (Larkin and Fravel, 2002). However, on chickpea, different cultivars significantly affected the extent and consistency of suppression of Fusarium wilt of chickpea using non-pathogenic *F. oxysporum* (Hervas et al., 1998).
Addition of exogenous nutrient sources can be used to enhance population levels of an introduced biocontrol agent to the maximum carrying capacity of the growth medium (Weaver and Kenerley, 2005; Schlatter et al., 2009). However, application of glucose or asparagine to the nutrient solution did not significantly increase the population density of *G. catenulatum* in the present study. This could indicate that the maximum carrying capacity of the roots in the nutrient solution had already been reached. In addition, addition of glucose and asparagine to the nutrient solution caused a significant increase in the number of bacteria (data not shown), which may have reduced proliferation of *G. catenulatum*, through competition.
5: EVALUATION OF GLIOCLADIUM CATENULATUM TO INDUCE SYSTEMIC RESISTANCE IN CUCUMBER

5.1 Introduction

The fungus *Gliocladium catenulatum* Gilman & Abbott strain J1446 [syn. *Clonostachys rosea* f. *catenulata* (Gilman & Abbott) Schroers; teleomorph *Bionectria ochroleuca* (Schw.) Schroers & Samuels (Schroers, 2001; Schroers et al. 1999)], is a commercially formulated biocontrol agent (Prestop WP, Verdera Oy, Finland) with broad-spectrum activity against plant pathogens (Lahdenpera and Korteniemi, 2005; McQuilken et al., 2001). Recent studies on the use of biological control strategies to reduce root diseases of cucumbers have shown that Prestop WP and Prestop Mix (Verdera Oy, Finland) were effective in reducing disease severity of Fusarium root and stem rot and Pythium rot in growth room trials (Rose et al., 2003; Punja and Yip, 2003). The biocontrol agent has also shown efficacy in reducing damping-off on ginseng seedlings caused by a complex of soilborne pathogens (Rahman and Punja, 2007), reducing anthracnose development caused by *Colletotrichum acutatum* Simmonds in blueberries (Verma et al., 2006), and suppressed sporulation of *Botrytis* spp. on dead onion leaves (Kohl et al., 1995). These studies indicate that *G. catenulatum* has activity against both rhizosphere- and phyllosphere-infesting fungi of plants.
In general, methods by which a microbial antagonist can achieve disease suppression include competition for nutrients or space in the rhizosphere or on the roots, mycoparasitism, antibiosis, and induced systemic resistance (ISR) (Whipps, 2001). Biocontrol often results from a concurrent or synergistic action of several of these mechanisms, and is dependent on factors such as environmental conditions and pathogen inoculum level (Green et al., 2001). Induced systemic resistance, as a mode of disease suppression, has been implicated in the biocontrol efficacy of several *Trichoderma* spp. and non-pathogenic isolates of *F. oxysporum*. Non-pathogenic *F. oxysporum* strain Fo47 protected tomatoes against Fusarium wilt in a split-root assay in which the biocontrol agent and the pathogen were physically separated from each other (Larkin and Fravel, 1999). Application of *T. asperellum* T-203 to roots of cucumber significantly reduced lesions caused by *Pseudomonas syringae* pv. *lachrymans* (Psl) on foliage (Yedidia et al., 2003; Shoresh et al., 2005a) and enhanced enzyme levels of chitinase, glucanase and peroxidase in the absence of pathogen inoculation (Yedidia et al., 1999; Yedidia et al., 2000). Treatment of tobacco roots with *G. roseum* resulted in increased activity of glucanases and chitinases in leaf extracts, and leaves of these plants showed less severe symptoms of powdery mildew compared with the control (Lahoz et al., 2004).

There are generally four characteristics of a biocontrol agent that support ISR as a likely mechanism of biocontrol: i) nonspecificity of control of diseases caused by unrelated pathogens; ii) requirement of host colonization prior to challenge by the pathogen; iii) dose-independence in application and iv) the
ability to reduce disease when spatially separated from the pathogen (Olson and Benson, 2007). Results reported previously in this thesis (Chapter 2) show that G. catenulatum exhibits dose-independence in its ability to reduce pathogen propagules of Forc, and that host colonization is an essential aspect of biocontrol by G. catenulatum. However, the ability of G. catenulatum to reduce Fusarium root and stem root when spatially separated from the pathogen or to protect against foliar pathogens when applied to the roots has not yet been assessed. Pseudomonas syringae pv lachrymans, the causal agent of angular leaf spot of cucumber, has been utilized for demonstration of nonspecificity and spatial separation in short-term cucumber experiments (Yedidia et al., 2003; Shoresh et al., 2005b; Shoresh, 2006; Viterbo et al., 2007). Control of a foliar pathogen by G. catenulatum would support induced systemic resistance as a mechanism of biological control. Therefore, the objectives of this study were to determine the ability of G. catenulatum to a) reduce Fusarium root and stem rot in a split-root system, b) induce defense enzymes in cucumber plants in the absence of a pathogen, and c) reduce foliar infection by P. syringae pv lachrymans.

5.2 Materials and Methods

5.2.1 Fungal strains and culture conditions

An isolate of Fusarium oxysporum f.sp. radicis-cucumerinum (Forc) was obtained from cucumber plants displaying symptoms of Fusarium root and stem rot in a commercial greenhouse. The isolate was maintained on potato dextrose agar (Difco) amended with 2 ml/liter of lactic acid (APDA). To ensure the isolate retained its virulence, reisolations were made at regular intervals from artificially
inoculated plants by surface-sterilizing diseased tissues in 20% bleach (Javex) for 30 s, followed by 70% ethanol for 60 s, and then rinsing in sterile distilled water. The tissues were plated onto Komada’s medium (Komada, 1975), followed by transfer to APDA after 10-14 days of growth. For inoculum production, conidia were obtained by flooding 14-day-old cultures with sterile distilled water, followed by scraping the surface with a glass rod. The resulting suspension was filtered through a double layer of cheesecloth to remove mycelia. The total number of both macro- and micro-conidia was determined using a haemacytometer and adjusted to the desired concentration.

Strain J1446 of *G. catenulatum* was recovered from a commercial formulation (Prestop Mix, Verdera Oy, Finland) by plating samples onto PDA amended with tetracycline (20 mg/liter) and Triton X-100 (2 ml/liter) (McQuilken et al., 2001). The fungus was then cultured onto PDA and grown under laboratory conditions (21-24°C) under ambient fluorescent lights.

*P. syringae* pv. *lachrymans* (*Psl*, obtained from Dr. D. Guttmann, University of Toronto) was grown in tryptic soy broth (TSB) overnight at 25°C. Bacterial cells were centrifuged at 5,000 rpm. The pellet was resuspended in sterile saline-phosphate buffer, and the concentration of the suspension was determined spectrophotometrically. The bacterial density of the suspension was adjusted to $OD_{600}=0.3$ in 0.5 X PBS + 0.01% Tween 20 for plant inoculation.
5.2.2 Disease development of Fusarium root and stem rot and biocontrol by *G. catenulatum* in hydroponic culture

Cucumber seeds were planted into cavities made in rockwool blocks (3.5 x 3.5 cm, Westgro, Delta, BC) and maintained in a growth room as described previously (Chapter 4). After 10 days, seedlings were placed into individual hydroponic units which consisted of 800 ml plastic containers (Fisher Scientific) filled with nutrient solution (NS), as described in previously (Chapters 2 and 4). Seven days after transplant, conidia of *G. catenulatum* were applied to the nutrient solution at a final concentration of 1 x 10^6 cfu/mL NS. Three days later, plants were inoculated with *F. oxysporum* by adding a conidial suspension to the NS at a final concentration of 5 x 10^2, 5 x 10^3 or 5 x 10^4 cfu/mL NS. Treatment combinations consisted of i) *Gc* + *Forc* at each of the 3 inoculum doses, ii) *Forc* only at each of the 3 inoculum doses, and iii) a control treatment which received *Gc* only. Percent plant mortality was assessed at 15 and 30 days, while shoot height and root fresh weight was determined at 30 days. The mortality and height data were used to calculate a disease severity index as follows: DSI = (no. of dead plants at 15 days/15) + (no. of dead plants at 30 days/30) + [1-(height of surviving plants/height of controls)]. Experiments were conducted in a growth room under ambient temperature (21-24°C) with a 16 h photoperiod provided by sodium vapour lights (light intensity of 100 μmoles/m²/s). There were three replicate plants per treatment and the experiment was repeated twice.
5.2.3 Split-root assays

Cucumber seeds were planted into cavities made in rockwool blocks (3.5 x 3.5 cm, Westgro, Delta, BC) and maintained in a growth room as described in Chapter 2. After 3 weeks, the lower portion of the stem was split in half from the hypocotyl down to the main root system using a sterile scalpel. Each half of the root system was placed into an individual hydroponic unit which consisted of a 400 ml plastic container (Fisher Scientific) filled with NS. Seven days later, the following treatments were applied: i) conidia of *G. catenulatum* were added at a final concentration of $10^6$ per ml NS to one-half of the roots while the other half received no treatment; ii) *G. catenulatum* was added to one-half of the roots, followed 3 days later by *Fusarium* on the other half at a final concentration of $5 \times 10^4$ conidia per ml NS; iii) *Fusarium* was applied to one-half of the roots only; iv) control.

To ensure that there was no contamination between *Gliocladium* and *Fusarium* on the two sides of the root system, root pieces from each side were plated, at various time intervals, onto Komada’s medium and onto PDAAtt for detecting the presence of *G. catenulatum*. The number of dead plants in all of the treatments was recorded 14 and 28 days after treatment. At the end of the experiment, the root fresh weight of each half of the root system was measured, along with plant height and shoot fresh weight. The mortality and height data were used to calculate a disease severity index as follows: $\text{DSI} = \frac{\text{no. of dead plants at 14 days}}{14} + \frac{\text{no. of dead plants at 28 days}}{28} + \left[1 - \frac{\text{height of surviving plants}}{\text{height of controls}}\right]$. The experiment was conducted six times.
5.2.4 Measurement of defense-related enzymes in cucumber plants

Cucumber seedlings were grown aseptically inside Magenta boxes with NS as described previously (Chapters 2 and 3). After 10 days, the following treatments were applied: i) conidia of *G. catenulatum* were added to the NS; ii) 2,6-dichloroisonicotinic acid (INA) (2 μg/ml) was added to the NS; iii) salicylic acid (5 mM) was applied to the foliage; iv) control plants. At 2, 3 and 7 days after treatment, the roots and shoots were excised, weighed, and ground separately in a mortar and pestle under liquid nitrogen. The material was suspended in cold phosphate buffer (10 mM, pH 6.0) and ground again with silica sand in microcentrifuge tubes using a polypropylene pestle (Sigma). The resulting suspension was centrifuged twice at 10,000 x g at 4°C and the supernatant was stored at -20°C and used in enzyme assays. The protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin (Sigma) as the standard. There were 3 replicate plant samples for each treatment. The experiment was conducted three times.

Chitinase and β-1,3-glucanase activity in root and shoot samples was assayed as described in Chapter 3. Peroxidase activity was determined according to the method described by Chen et al. (2000). The reaction mixture contained 100 μl of root or shoot extract, 792 μl of phosphate buffer (5 mM, pH 6.5) and 7.5 μl pyrogallol (60 mM). The reaction was started by adding 100 μl of 0.6M H₂O₂ and the increase in absorbance at 420 nm was measured at 30 s intervals for 3 min. Enzyme activity was expressed as the change in absorbance/min/mg protein. Polyphenol oxidase activity was determined
according to the procedure of Mayer et al. (1966). Briefly, 200 \( \mu l \) of the enzyme extract from roots or shoots was mixed with 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 \( \mu l \) of 0.01 M catechol was added to the mixture and activity was expressed as the change in absorbance at 495 nm/min/mg protein.

### 5.2.5 Disease development of *P. syringae pv. lachrymans (Psl)* in cucumber leaves

Cucumber seeds cv. Sienna were planted in small rockwool blocks (3.5 x 3.5 cm, Westgro, Delta, BC), groups of 2 blocks each were placed in individual Petri dish lids, and watered daily with NS. When seedlings were 10 days-old, 3 ml of each of following treatments were applied to the roots: i) SDW as a control treatment, ii) a spore suspension of *G. catenulatum* (5 x 10^6 cfu/ml), iii) an autoclaved spore suspension of *G. catenulatum*, iv) an autoclaved suspension of *Forc* cell walls (1mg/10 ml), prepared as described previously (Chapter 3), and v) 0.02% (w/v) chitosan. Three days later, plants were inoculated with *Psl* in one of two ways i) 3 X 10 \( \mu l \) drops of *Psl* suspension was applied to each cotyledon or ii) 500 \( \mu l \) of *Psl* suspension was applied to a sterile cheesecloth pad (3 cm x 3 cm, 4 layers), which was then rubbed on each cotyledon twice. Cotyledons were also mock inoculated with 0.5 X PBS + 0.01% Tween 20. For each of the two inoculation methods, there were six treatments in total: i) control, ii) *Psl* only, iii) Gc + *Psl*, iv) autoclaved Gc + *Psl*, v) *Forc* cell walls + *Psl*, and vi) chitosan + *Psl*. Plants were kept in a humidex chamber for the duration of the experiment. Five days after *Psl* inoculations, the cotyledons were harvested, photographed and
weighed. Cotyledons were then immediately washed 2 times in SDW, and suspended in 10 ml sterile PBS. To determine colony-forming units of \( Psl \), leaves were ground using a mortar and pestle, and dilutions plated onto King’s B media. The number of lesions, lesion size and percent diseased leaf area was calculated from photographs using Quantity One 1-D Analysis Software (BioRad, Hercules, California). There were 3 replicate plants per treatment, and the experiment was repeated twice.

5.2.6 Statistical analysis

Data from individual trials were combined for analysis when \( F \)-tests indicated that variances of the data did not differ significantly. Density estimates of \( Psl \) associated with cucumber were log transformed \((y+1)\) prior to analysis. Analysis of variance was performed using the \textit{proc glm} statement to determine significance of treatment effects using the Statistical Analysis System, version 9.1 (SAS Institute, 2008). For all experiments, except those with \( Psl \), significant differences between treatment means were separated using Tukey’s HSD test. For the \( Psl \) experiments, treatment means were compared to the \( Psl \) only control using Dunnet’s Method. The Type 1 error rate (\( \alpha \)) was set at 0.05 for all statistical tests.

5.3 Results

5.3.1 Disease development of Fusarium root and stem rot and biocontrol by \textit{G. catenulatum} in hydroponic culture

Development of Fusarium root and stem rot in hydroponic culture was dependent on the initial inoculum dose of \textit{Forc}. Cucumber plants only displayed
mortality at an initial Forc dosage of $1 \times 10^5$ cfu/ml NS (~ 60%), and the resulting DSI was close to 6 (Fig. 5-1). The percent mortality in treatments that received $1 \times 10^3$ or $1 \times 10^4$ cfu/ml suspension of Forc was very low (< 20%), and the corresponding DSI levels were close to 1. Application of G. catenulatum conidia to the NS before inoculation with Forc resulted in low percent mortality (< 20%) in all treatments, regardless of the Forc dose, with corresponding DSI levels ranging from 0.5 to 1.0. Although inoculation with Forc did not always result in mortality of cucumber plants, the roots in all treatments were brown and decayed, with a significant reduction in root mass compared to the control (5.8 g). There was no significant difference in the fresh weight of roots in any of the Forc-treated plants, regardless of the initial inoculum dosage. Furthermore, treatment with G. catenulatum prior to Forc inoculation did not reduce the severity of decayed roots as measured by root fresh weight.

5.3.2 Split-root assays.

The split-root assay provided a physical separation of the antagonist and pathogen on either side and permitted direct observation of development of disease symptoms and root growth. However, only three of the six replicate experiments could be combined for analysis, owing to the variability in disease development of Fusarium root and stem rot. Treatment with G. catenulatum on one-half of the roots did not significantly reduce the DSI on plants treated with Forc on the other half of the roots (DSI = 0.73), compared to treatments with Forc only on one half (DSI = 1.46) (Fig. 5-2). However, the disease levels in the hydroponic system were low (mortality < 15%), and there was significant
Figure 5-1 Disease severity index (A) and root fresh weight (B) of cucumber plants inoculated with *Fusarium* only or inoculated with *Fusarium* three days after application of *G. catenulatum* (1 x 10⁶ conidia/ml NS) to the roots (Gc + Fus).

*Fusarium* was applied at an initial inoculum dose of 5 x 10², 5 x 10³ or 5 x 10⁴ conidia/ml NS. Root weight was measured 28 days after treatment with Forc. Means represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.
variation in disease levels between repeated trials. A better assessment of disease severity in the split-root system was obtained from root weight, which was proportional to the level of root decay observed (Fig. 5-2). Fresh weight of cucumber roots treated with Forc on one half of the roots and G. catenulatum on the other half was not significantly different from that of plants treated with Forc only on one half of the roots, and was significantly lower than the root weight of the uninoculated control (Fig. 5-2). All roots that were inoculated with Forc were extensively colonized and were decayed. The split-root system was effective in spatially separating the two fungi as G. catenulatum colonies were not detected growing on roots treated with Forc. Conversely, Fusarium colonies were not present on roots treated with G. catenulatum (data not shown).

5.3.3 Measurement of defense-related enzymes in cucumber plants.

Chitinase, peroxidase, polyphenol oxidase and glucanase activities were measured in the leaves and roots 2, 3 and 7 days after treatment with G. catenulatum. Although the experiment was repeated three times, data could not be combined for analysis due to the variability in enzyme levels among trials. Therefore, representative results from the best trial are shown. Polyphenol oxidase (PPO) was not detected in the roots of any of the treatments, at any of the sampling days (Fig. 5-3). In leaves, however, PPO activity was measurable in all the treatment at all sampling days. There was no difference in PPO activity on day 2 between any of the treatments. At day 3, leaves from plants treated with G. catenulatum to the roots showed significantly higher levels of PPO than any other treatment, but this was not observed by day 7. In salicylic acid treated-
Figure 5-2 Disease severity index (A) and root fresh weight (B) of cucumber plants grown in hydroponic solution and either untreated (control) or treated with *Gliocladium* on one half of the roots (Gc), treated with *Forc* on one half of the roots only (Forc) or treated with *Gliocladium* on one half of the roots 3 days before treatment with *Forc* on the other half of the roots (Forc+Gc).

Root weight was measured 28 days after treatment with *Forc*. Means represent the combined values from three independent trials. Vertical bars indicate standard error of the mean.
plants, leaves showed significantly higher levels of PPO at day 7 only.

There was no significant difference in peroxidase units in the roots between any of the treatments on 2 or 3 days after application (Fig. 5-3). By day 7, peroxidase levels in the roots of INA, SA and *G. catenulatum*-treated plants were significantly higher compared to control plants. In leaves, peroxidase units were significantly higher in SA-treated plants at 2, 3 and 7 days compared to the corresponding sampling days in both control and *G. catenulatum*-treated plants. There was a significant increase in peroxidase units in leaves of INA-treated plants, but only at day 7.

Measurable units of chitinase were only detected in the roots of control and treated plants at day 7, and there was no significant difference in these levels between any of the treatment (Fig. 5-3). In the leaves, there was no detectable increase in the levels of chitinase after treatment with INA, SA or *G. catenulatum* at any of the sampling times.

Glucanase activity in the roots was not significantly different between any of the treatments at day 2 (Fig. 5-3). However, application of INA, SA and *G. catenulatum* caused an increase in glucanase activity in the roots at day 3 compared to the control, but differences from the control were not significant. There was a marked increase in glucanase activity in the roots 7 days after treatment with *G. catenulatum*, at levels that were significantly higher than in any of the other treatments. Glucanase activity was not detected in the leaves from any of the treatments at day 2. Glucanase activity in the leaves was not significantly different between any of the treatments at day 3 or day 7.
Figure 5-3 Enzyme activities in roots or leaves of 10-day-old cucumber seedlings at 2, 3 and 7 days following application of INA (2 mg/L), *G. catenulatum* (1x 10^6 cfu/ml) or SA (2 mM).

Enzyme activities are expressed as follows: Polyphenol oxidase (PPO) and peroxidase units = \( \Delta \) absorbance/min/mg protein; chitinase units = \( \mu \)moles NAGA/mg protein/h; and glucanase units = \( \mu \)moles glucose/mg protein/h respectively. Values represent the means from one representative trial, with three replicate samples per treatment. Vertical bars indicate standard error of the mean.
5.3.4 Disease development of *P. syringae pv. lachrymans* in cucumber leaves

When *Psl* was applied to cotyledons as 10-μl droplets, disease symptoms were not very severe as visible lesions did not spread beyond the initial drop size (Fig. 5-4). Nevertheless, when compared to the *Psl* control, percent diseased leaf area was significantly reduced by application of *G. catenulatum* and chitosan (*P* = 0.0045 and 0.0304, respectively), whereas application of autoclaved *G. catenulatum* or *Forc* cell walls did not significantly reduce disease severity (*P* = 0.6648 and 0.0893, respectively) (Fig. 5-5). Although lesion area (mm²) was not significantly reduced by any of the treatments, the density of bacteria associated with the cotyledons was significantly reduced by all root treatments compared to the *Psl* control (Fig. 5-5). Application of *P. syringae* inoculum on a cheesecloth pad introduced sufficient wounding to the surface of the cotyledon to allow for development of spreading lesions and severe disease symptoms (Fig. 5-6).

When compared to the *Psl* control, application of *G. catenulatum* or chitosan to the roots significantly reduced both lesion area (*P* = 0.0099 and 0.0002, respectively) and percent diseased leaf area (*P* = 0.0084 and 0.0009, respectively) (Fig. 5-7). In contrast, treatment with autoclaved *G. catenulatum* and *Forc* cell walls did not significantly reduce lesion area (*P* = 0.75 and 0.1192, respectively) and percent diseased area (*P* = 0.9997 and 0.1287, respectively).

Similarly, treatment with *G. catenulatum* and chitosan significantly reduced the population density of *Psl* on the cotyledons (*P* = 0.0024 and 0.0001, respectively), whereas treatment with autoclaved *G. catenulatum* and *Forc* cell walls did not (*P* = 0.284 and 0.7135, respectively).
Figure 5-4  Disease symptoms of *Pseudomonas syringae* pv. *lachrymans* when applied as droplets on cotyledons of cucumber plants, 5 days after pathogen inoculation.

The following treatments were applied to the roots: SDW (control) (A), SDW (*Psl* only control) (B), *G. catenulatum* (C), Forc cell walls (D), chitosan (E), or autoclaved *G. catenulatum* (F). 24 h after root treatments, *Psl* was applied to the cotyledons in 10 μl droplets, except for the control.
Figure 5-5  Disease severity of *Pseudomonas syringae* pv. *lachrymans* when applied as droplets on cotyledons of cucumber plants measured by lesion area, mm$^2$ (A), % diseased leaf area (B), and population levels of *Psl* on the surface of cotyledons (C).

The following treatments were applied to the roots: SDW (control) (con), SDW (*Psl* only control) (Ps), autoclaved *G. catenulatum* (auto), Forc cell walls (CW), chitosan (Chit), or *G. catenulatum* (Gc). *Psl* was applied to the cotyledons 24 h after root treatments in 10 μl droplets in all treatments, except the control. Means represent the combined values from two independent trials. Vertical bars indicate standard error of the mean. Bars with an asterisk were significantly different from the *Psl* control (Dunnett's Control test, $\alpha < 0.05$)
Figure 5-6  Disease symptoms of *Pseudomonas syringae* pv. *lachrymans* when applied using a cheesecloth rub on cotyledons of cucumber plants, 5 days after pathogen inoculation.

A cheesecloth pad soaked in inoculum of *Psl* was rubbed on the surface of the cotyledons 24 h after root treatment. The following treatments were applied to the roots: SDW (control) (A), SDW (*Psl* only control) (B), *G. catenulatum* (C), Forc cell walls (D), chitosan (E), or autoclaved *G. catenulatum* (F).
Figure 5-7  Disease severity of *Pseudomonas syringae* pv. *lachrymans* when applied using a cheesecloth rub on cotyledons of cucumber plants measured by lesion area, mm² (A), % diseased leaf area (B), and population levels of *Psl* on the surface of cotyledons (C).

The following treatments were applied to the roots: SDW (con), SDW (*Psl* only control) (Ps), autoclaved *G. catenulatum* (auto), Forc cell walls (CW), chitosan (Chit), or *G. catenulatum* (Gc). 24 h after root treatment, a cheesecloth pad soaked in a *Psl* suspension was rubbed on the leaf surface in all treatments, except the control. Means represent the combined values from two independent trials. Vertical bars indicate standard error of the mean. Bars with an asterisk were significantly different from the *Psl* only control (Dunnett’s Control test, α < 0.05).
5.4 Discussion

An *F. oxysporum* inoculum dosage of $5 \times 10^4$ was chosen for the split root assays based on the results obtained from disease development in hydroponics. At this inoculum level, *Forc* was capable of infecting the plant, resulting in symptom development in the roots, but did not always cause mortality. The lower inoculum dosage more accurately reflects inoculum levels that are likely to be present in a commercial greenhouse. However, unlike assays performed with whole root systems, in the split-root system, infection by *Forc* on one-half of the roots rarely resulted in visible lesions on the crown and stem area even though inoculated roots displayed symptoms of *Fusarium* infection. Thus, it was difficult to assess the effect of the biocontrol agent on disease suppression as measured by a disease severity index. However, in both the whole root and split-root systems, pre-treatment with *G. catenulatum* did not reduce root browning or root biomass loss caused by *F. oxysporum*. Interestingly, in the whole root assays, *G. catenulatum* was successful in reducing mortality caused by *Forc*, despite root infection, likely by preventing the spread of pathogen hyphae to the crown and stem regions. *F. oxysporum* f. sp. *lycopersici* was better and faster at colonizing tomato roots growing in hydroponic culture than in soil (Nahalkova et al., 2008), indicating that the outcome of biocontrol studies can be influenced by the culture conditions. Therefore, although the hydroponic system was invaluable for visualization and quantification of root health, it did not provide the ideal conditions for measuring disease suppression using a split-root assay.
Another method of evaluating the ability of a biocontrol agent to induce resistance is to determine whether application of the fungus to the plant will induce changes in defense-related enzymes relative to the control. A number of biocontrol fungi have been shown to initiate increased levels of defense-related enzymes, such as peroxidases, β-1,3-glucanase, chitinase, cellulase, polyphenol oxidase and phenylalanine ammonia lyase, after their application (Yedidia et al., 1999; Yedidia et al., 2000; Xue et al., 1998; Duijff et al., 1998; Fuchs et al., 1997). Therefore, cucumber roots treated with *G. catenulatum* were assayed for enhanced levels of defense enzymes relative to control plants. Although detectable changes in enzyme activity in roots or leaves following treatment with *G. catenulatum* were observed for some enzymes at different time points, there was no consistent trend evident from the data that allowed conclusions to be drawn regarding induced systemic resistance by *G. catenulatum*. Only glucanase levels were significantly higher in roots compared to SA, INA and control-treated plants at 7 days post-application of *G. catenulatum*. But as discussed in Chapter 3, this enhanced glucanase activity was due to fungal, not plant, activity. These results are in contrast to findings published for other biocontrol fungi. However, results presented in the literature on induction of defense related enzymes are sometimes contradictory (Alabouvette et al., 2007). Nonpathogenic strain Fo47 was ineffective in inducing systemic resistance in tomato, yet its application enhanced levels of PR proteins (Duijff et al., 1998). In the absence of pathogen challenge, glucanase, chitinase and peroxidase levels in cucumber plants increased at 48 and 72 h after application of *T. harzianum*
(T-203) compared to untreated controls (Yedidia et al., 1999; Yedidia et al., 2000). However, there was no change in chitinase, glucanase and peroxidase gene expression levels, measured using quantitative PCR, after application of *T. asperellum* (T-203) only (Shoresh et al., 2005a). An increase in transcript levels, after application of T-203, relative to non-inoculated plants was not detected until the plants were challenge inoculated with *Psl* on the leaves. Similarly, non-pathogenic binucleate *Rhizoctonia* spp. caused substantial increases in activities of peroxidases, glucanases and chitinases in bean seedlings in the absence or presence of plant pathogens (Xue et al., 1998). However, gene expression studies showed that levels of glucanase, phenylalanine ammonia lyase, and chalcone synthase were significantly lower in plants treated with non-pathogenic *Rhizoctonia* spp. in either the absence or presence of pathogenic *R. solani* (Wen et al., 2005). Therefore, the technique that is used to measure host-mediated changes resulting from biocontrol application, the timing of the plant sampling after application, and the presence or absence of pathogen challenge can greatly affect the outcome and conclusions of the experiment.

The induced resistance effect also appears to be strongly dependent on the strain of the antagonist, the species/cultivar of the plant, the type of growing media and the pathogen (Hoitink et al., 2006; Woo and Lorito, 2007). It is possible that although *G. catenulatum* does not appear to induce systemic resistance in cucumber against *F. oxysporum*, this mechanism can be effective against other pathogens. Therefore, the ability of *G. catenulatum*, when applied to the roots, to reduce disease severity of the foliar pathogen *P. syringae pv.*
lachrymans was also tested. When Psl was applied as 10 μl droplets to cucumber cotyledons, spreading lesions did not develop much past the border of the inoculating drop, and measurements of disease severity were low. Inoculating the pathogen on a cheesecloth pad resulted in much higher disease levels, most likely due to wounding of the leaf surface, allowing entry and colonization of the bacteria. However, in both systems, application of G. catenulatum to the roots resulted in a 4-fold reduction in the percentage of diseased leaf area. A similar reduction in the pathogen population on the leaf surface was also observed. Application of autoclaved G. catenulatum mycelia or Forc cell walls to the roots did not appear to have any effect on disease reduction, indicating that only live G. catenulatum exerted a protective effect.

Application of chitosan to the roots resulted in the same disease suppression levels provided by G. catenulatum. Chitosan is a deacetylated derivative of chitin that can elicit a resistance response in plant tissues against pathogens by induction of structural barriers and defense-related enzymes (chitinase and β-1,3-glucanases) (Bautista-Baos et al., 2006; Amborabe et al., 2008; Benhamou and Theriault, 1992; ElGhaouth et al., 1994). Similar results for suppression of Psl were found for T. asperellum T-203, where application of T-203 to the roots of cucumber plants resulted in significant reduction of necrotic lesion area and bacteria populations on cucumber leaves (Shoresh et al., 2005b; Yedidia et al., 2003). This reduction in disease severity was correlated with an increase in the plant mRNA of defense-related genes such as PAL and hydroxyperoxide lyase (HPL), along with an accumulation of phenolic products (Yedidia et al., 2003).
Preliminary evidence from this study indicates that *G. catenulatum* does have the capability of inducing a defense response in cucumber, as indicated by its ability to reduce disease symptoms caused by a foliar bacterial pathogen. However, application of *G. catenulatum* to the roots, in the absence of pathogen challenge, does not appear to stimulate the production of defense enzymes in the leaves. In Chapter 2, it was shown that *G. catenulatum* can colonize cells in the epidermal layer of the roots, and that under axenic hydroponic growing conditions, can penetrate even further into the root zones. Similar patterns of colonization have been found for *Trichoderma* spp. (Yedidia et al., 1999; Yedidia et al., 2000), and invasion of the outer root cells by *Trichoderma* strains likely results in systemic induced resistance (Harman et al., 2004). Therefore, to ensure that the biocontrol fungus is contained within the first few cell layers of the roots, the same type of defense reactions should be exhibited against colonization by *G. catenulatum*. Using the techniques in this study, we were unable to find evidence of such a defense response. In order to conclude that *G. catenulatum* can induce a biochemical response in the host plant, future studies should focus on quantifying mRNA levels of defense-related genes in treated plants with and without pathogen challenge at specific time points. Compared to quantification of enzyme activities, measurement of mRNA levels using qPCR appears to be a more robust and consistent method for determining changes that occur in the plant after application of a biocontrol agent (Wen et al., 2005; Shoresh et al., 2005a).
6: GENERAL DISCUSSION AND CONCLUSIONS

The mechanisms of action of the biocontrol agent Gliocladium catenulatum in suppressing disease severity of Fusarium root and stem rot of cucumbers were explored in detail. Methods of disease suppression by a biocontrol agent include four recognized principles: a) competition for nutrients or space in the rhizosphere, b) mycoparasitism, c) antibiosis, and d) induced systemic resistance. Data gathered in this thesis supports evidence for competition in conjunction with mycoparasitism. The success of an introduced biocontrol agent in suppressing disease can be influenced by its root colonization ability and the population size on the roots at the time of pathogen infection. Therefore, the root colonizing ability of G. catenulatum was evaluated in detail.

Carbon is most likely the primary growth-limiting factor in agricultural systems; therefore, effective use of carbon resources may be of greatest importance in governing interactions between fungal species in the rhizosphere (Sivan and Chet, 1989; Couteaudier and Alabouvette, 1990a). The rhizosphere competence of G. catenulatum demonstrated in this thesis indicates that this strain is able to successfully outcompete other rhizosphere microflora for the limited carbon resources available on a plant root. This implied rhizosphere competence is challenged by the observation that population density of G. catenulatum on roots was highest in hydroponic culture, a growing medium that is initially low in indigenous rhizosphere. In field soil and soil-less potting mix,
which have their own populations of indigenous microflora, colonization of cucumber by *G. catenulatum* was low. Similarly, when exogenous nutrients were introduced to the nutrient solution, allowing bacterial species to flourish, the population density of *G. catenulatum* was reduced. These findings could indicate that the competitive ability of *G. catenulatum* in microbial-rich environments may be fairly poor. However, McQuilken et al. (2001) found that *G. catenulatum* survived in pathogen-containing peat-based growing media at levels over $10^6$ CFU/cm$^3$ for up to 28 days after application. Similarly, *G. catenulatum* survived in rockwool blocks containing *F. oxysporum* for up to 60 days at levels close to $10^5$ CFU/ g root fresh weight. Therefore, it is likely that the presence of pathogen propagules, especially when artificially inoculated at high dosages, can provide an alternate carbon source for this mycoparasitic fungi, thus enhancing population densities. Furthermore, under greenhouse conditions, where cucumbers are planted into new sterile rockwool blocks, the initial microbial population present in the rhizosphere is low. Therefore, for adequate establishment of propagules of *G. catenulatum*, the biocontrol product should be applied early in the growing season.

Although *G. catenulatum* has long been considered a mycoparasite of plant pathogenic fungi, this thesis provided evidence for the first time for the production of cell-wall degrading enzymes, a characteristic that is required for mycoparasitism. Results from this study showed that β-1,3-glucanase was produced on the roots *in vivo* by *G. catenulatum* at detectable levels. An interesting area for further research would be to evaluate the synergistic effect
between mycoparasitism, root colonization and competition. An increase in $\beta$-1,3-glucanase activity was not observed on the roots until 7 days after application of *G. catenulatum*. This could indicate that either the population density of *G. catenulatum* was not sufficiently high enough on the roots until this time, or that population levels of *G. catenulatum* were sufficiently high enough to have utilized all the carbon exudates from the roots, and thus a switch to nutrition through mycoparasitism would be warranted. Cell-wall degrading enzymes are often regulated through catabolite repression, and the cellular processes required for a mycoparasitic mode of nutrition are not activated until glucose levels are low (Viterbo et al., 2002a; Martin et al., 2007). This form of regulation was also observed for *G. catenulatum*. Thus, it is evident that the mycoparasitic behaviour of *G. catenulatum* could also function to enhance its competitive ability to utilize the scarce carbon resources present in the rhizosphere environment. By producing cell-wall degrading enzymes on the roots, an inhibitory environment is created that would succeed in limiting growth of other rhizosphere microflora. Furthermore, these cell-wall degrading enzymes are also induced by the cell walls of other fungi, thus providing a positive feedback loop for enhanced production of these enzymes. Therefore, the population dynamics of *G. catenulatum* in relation to enzyme production on the plant root, combined with the effect of pathogen presence, warrants further investigation, to conclusively prove that these three activities acting in concert with each other are responsible for the biocontrol effect of this fungus. The efficacy of introduced BCAs in suppressing disease can also be influenced by the environmental conditions and
the host plant susceptibility (Larkin et al., 2002). Temperature, pH, wounding and plant cultivar are critical factors for disease development of *Fusarium* diseases. Severity of Fusarium root and stem rot on cucumber seedlings is highest at 17-24°C (Punja and Parker, 2000; Vakalounakis, 1996). External pH is a determining factor in the virulence of many pathogenic *F. oxysporum* strains, as pathogenicity factors are often produced within a very narrow range of pHs (Caracuel et al., 2003; Penalva and Arst, 2002). Cucumber cultivars display a range of different resistance levels to Fusarium root and stem rot, with some cultivars showing complete resistance to infection by *Forc* (Rose and Punja, 2004). *F. oxysporum* infects roots through wound sites. Therefore, the effect of these environmental and host factors on the association of *G. catenulatum* with the roots was studied in depth. Results showed that environmental factors such as temperature and pH appeared to have a larger influence on colonization by *G. catenulatum* than did host factors such as cultivar and wounding. A further step in this research would be to determine how varying these environmental factors affects disease suppression provided by *G. catenulatum* against *Forc*. However, considering that the majority of the biocontrol studies were performed under the optimal conditions for *Forc* development (17-24°C, pH of 6), this would suggest that biocontrol is effective even under the most conducive conditions for Fusarium root and stem rot development.

Another factor often cited as the cause for inconsistent performance of biological control agents is the variability in root colonization by the introduced BCA (Weller, 1988; Whipps, 2001; Fravel, 2005). Colonization of the roots by *G.*
*catenulatum* was fairly consistent among experimental repetitions and different experiments, with levels remaining above $1 \times 10^5$ cfu/g root fresh weight in rockwool and hydroponic trials. Furthermore, it was found that *G. catenulatum* appears to persist on cucumber roots for at least up to 60 days. Of equal importance to biocontrol efficacy is the pattern of distribution of the biocontrol agent on the roots relative to pathogen distribution. To visualize the distribution patterns of *G. catenulatum* on the roots, a GUS-marked strain was developed. This strain showed that colonization by *G. catenulatum* often occurred at the root hair zone, forming a dense network over the cucumber root epidermis. However, colonization of the entire root zone was discontinuous, with some root areas not supporting any growth of *G. catenulatum*. Nonetheless, the presence of *G. catenulatum* was sufficient to significantly reduce pathogen propagules levels detected on the roots. As discussed earlier, efficient root colonization ability driven by competition for the scarce nutrient resources in the rhizosphere, coupled with the mycoparasitic ability of *G. catenulatum*, may act synergistically to produce an inhibitory environment against fungal root-infecting pathogens.

The ability to induce systemic resistance in the host has been shown be an important characteristic of fungal biocontrol agents (Harman et al., 2004). However, it was difficult to determine whether part of the biocontrol efficacy of *G. catenulatum* against *Forc* resulted from induced resistance in the host. This is likely due to the complexity of mechanisms that are involved in host resistance to *F. oxysporum* pathogens. Major resistance (R) genes have been found against specific *F. oxysporum* races in several crops, including cultivars of tomato,
melon, cucumber, and avirulence genes have been identified in *F. oxysporum* f.sp. *lycopersici* (Michielse and Rep, 2009). Alternately, some cultivars display resistance to infection by *F. oxysporum* through polygenic resistance mechanisms. Using *Arabidopsis thaliana* as a model host plant for determining resistance mechanisms to *F. oxysporum* infection, it appears that the salicylic acid, jasmonate and ethylene pathways are all involved in limiting disease development. However, some studies suggest that the jasmonate pathway is not involved in resistance to *F. oxysporum*, and that PR proteins do not contribute to resistance (Diener and Ausubel, 2005). Given the complexity of the nature of a plant’s response to infection by *F. oxysporum*, it is not surprising that unravelling the potential for induced resistance against this pathogen by a biocontrol agent is equally complex. This could be one potential reason why we failed to prove that induced systemic resistance occurred against *F. oxysporum*. Nevertheless, induced resistance by *G. catenulatum* did reduce disease development by the foliar-infecting pathogen, *P. syringae* pv. *lachrymans*. Furthermore, endophytic colonization of the epidermis and outer cortex region of the cucumber roots suggests that a plant response to *G. catenulatum* invasion should occur. To provide incontrovertible evidence for an induced systemic resistance response that is effective in preventing *F. oxysporum* infection, it may first be necessary to determine which resistance pathway is elicited by *G. catenulatum* colonization. In addition, the choice of cultivar used for ISR experiments might affect the outcome of the experiment. Therefore, further research into the ability of *G. catenulatum* to induce resistance in cucumbers against root-infecting pathogens
is justified, but caution must be used when selecting the appropriate cultivar and resistance indicators for the experiment. The mechanism of resistance governing the interaction between cucumber cultivars and *Forc* is poorly characterized, and needs to be better understood first to fully evaluate ISR by *G. catenulatum* against this pathogen.

The development of a biocontrol strategy to control both Pythium root rot and Fusarium root and stem rot of greenhouse cucumbers based on application of the formulated product, Prestop®, which contains *G. catenulatum* as the active ingredient is needed. This product can be effective as a broad-spectrum antifungal agent, as the fungus appears to be active against many different fungal pathogens (Lahdenperä and Korteniemi, 2005; McQuilken et al., 2001). A drawback of biocontrol programs to manage diseases is often the narrow target range of the antagonist. Furthermore, the finding that *G. catenulatum* was also present on the leaves after application to the roots, indicates that this fungus may have usefulness against foliar-infecting pathogens. Ultimately, the goal of this study was to determine the primary mechanisms of action responsible for the efficacy of this biocontrol agent, with the aim to improve understanding of its implementation in a biocontrol program. Results indicate that the biocontrol agent will be most effective when applied early in the growing season, will be more advantageous in greenhouse crops grown in a soilless system, and that populations will persist for some time in the roots. Growers that employ this biocontrol agent may also have the added benefit of some foliar protection against pathogens, but that aspect warrants further investigation.
7: REFERENCES


Fusarium strain during biocontrol of tomato foot and root rot. *Molecular Plant-Microbe Interactions, 18*, 710-721.


fungus Clonostachys rosea (syn. Gliocladium roseum). Journal of Microbiology, 45, 422-430.


OMAFRA staff. (2005), Growing greenhouse vegetables. Publication order #371, Agdex #290.


