The infection process of

*Fusarium oxysporum* f. sp. *vasinfectum* in Australian cotton

and associated cotton defence mechanisms

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Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

2007

School of Botany

The University of Melbourne
ABSTRACT

*Fusarium oxysporum* f. sp. *vasinfectum* (Fov) was first identified in Australia in 1993, and has since become one of the most significant threats to the country's thriving cotton industry. The interaction between a unique Australian biotype of Fov and cotton hosts with varying susceptibilities to Fusarium wilt was studied. This research described the infection process and associated host defence mechanisms of two commercial cotton varieties after inoculation with Fov, and quantified their subsequent accumulation of antimicrobial terpenoids.

A rapid, reliable glasshouse bioassay that correlated with field resistance was developed for the study of Fusarium wilt of cotton. Detailed observations of the infection process obtained through light microscopy were used to formulate the disease cycle of Australian Fusarium wilt of cotton. Using pathogen growth assays, varietal differences in root exudates and vascular tissues in the cotton hosts were documented. Root diffusate from the most susceptible cotton variety to Fusarium wilt, Siokra 1-4, contained a lipophilic compound that promoted the germination of Fov microconidia. On the other hand, a lipophilic compound present in diffusate from the least susceptible variety, Sicot 189, inhibited the growth of Fov germ tubes.

A bioassay using inoculated whole plants showed that Fov colonisation of the vascular tissues of Sicot 189 was restricted after 3 days. The basis for this inhibition was investigated further using light and transmission electron microscopy. Infection induced the reorganisation of contact cells in host vascular tissue, including an increase in cytoplasmic content and the partitioning of vacuoles, which was concurrent with the accumulation of materials in adjacent vessel lumens, via pits. Histochemical analysis indicated these globular materials secreted into the vessels were terpenoids. These structural and terpenoid responses in Siokra 1-4 and Sicot 189 were similar, however, they were more intense and rapid in the latter, less susceptible variety. The responses in Sicot 189 also corresponded to the time period that pathogen inhibition was observed. Thus, a correlation was demonstrated between the rapid and intense induction of both structural and biochemical responses with decreased susceptibility to Fusarium wilt. Detailed HPLC analysis of vascular tissues confirmed that terpenoids accumulated more rapidly and at higher concentrations in the less susceptible cotton variety. These findings provided strong evidence for the involvement of antimicrobial terpenoids in the determination of Fusarium wilt susceptibility of Australian cotton varieties.

This work represents the most complete survey to date of the interaction of Australian biotypes of Fov with cotton. These insights can contribute to future cotton breeding efforts and cultural management of Fusarium wilt in the field. Thus, each part of this study has advanced complementary facets of our understanding of Fov, and has provided a framework from which future studies on phytoalexins and other putative cotton defences can be studied.
DECLARATION

This is to certify that

(i) this thesis comprises only my original work towards the PhD except where indicated,
(ii) due acknowledgement has been made in the text to all other material used,
(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

[Signature]

27/07/2009
ACKNOWLEDGEMENTS

The generous provision of knowledge and guidance by my supervisors, Professor David Guest and Dr Robyn Heath, was invaluable throughout this work. David’s pursuit of the big picture, “attitude,” patience and trust in my abilities is warmly acknowledged, as is Robyn’s honesty, insight and enthusiasm.

I am thankful for the support of the cotton pathology community, especially David Nehl, Stephen Allen, and Joe Kochman. Their welcoming encouragement, perspective and guidance were integral to this project. There have been many other cotton friends made along the way.

I have very fond memories of my old Mycology Lab: Kerrie Pellen, Emer O’Gara, Kaija Jordon, Tonia Griffith-Jones, Laura Fitzgerald, Ken Brown, Gabrielle Smetham and Rose Daniel amongst others. They contributed advice, knowledge, skills, tears, and laughter over those very enjoyable years. Thanks to Rose for her continued support with limitless hospitality at both her new lab and home. It was also a relief to be welcomed into my friendly foster-lab, The Enterprise Lab, after Mycology closed.

Many people have contributed practical and technical skills to this project. In particular I have benefited from the years of experience of Bruce McGuinness, Steve ELEFTERIADIS and Simon Crawford. The inspirational Gretta Weste will be remembered for her input and mycology parties over the years.

I am indebted to Robert Stipanovic of the Southern Plains Agricultural Research Center Cotton Pathology Research Unit, USDA, whose generous gift of terpenoid standards contributed a significant component of this work. The International Cotton Advisory Committee graciously assisted this project with the provision of gratis access to their statistical resources.

I gratefully acknowledge the Cotton Research and Development Corporation for their provision of my stipend and generous financial support to travel far and wide to attend meetings, conferences and workshops. The School of Botany has continually provided me with exceptional facilities. I acknowledge the receipt of University of Melbourne and School of Botany Overseas Travel Scholarships. Thanks also to Happy Horticulture, University of Sydney, who made a significant contribution by allowing me to battle with their new HPLC.

My family’s encouragement and devotion made this PhD possible. My friends, particularly Gina, have kept me sane throughout the project. Its completion is testament to Tom’s patience and unflinching support.
# TABLE OF CONTENTS

Abstract .................................................................................................................. i

Declaration ............................................................................................................. ii

Acknowledgements ............................................................................................... iii

Table of Contents ................................................................................................. iv

Table of Figures ...................................................................................................... ix

Table of Tables ....................................................................................................... xii

Chapter 1  Cotton: production, diseases and defence responses.............. 1

1.1  Cotton production ........................................................................................... 2

1.2  The Australian cotton industry ......................................................................... 2

1.3  Diseases of cotton ............................................................................................ 4
   1.3.1  Diseases of Australian cotton ..................................................................... 9

1.4  Fusarium wilt of cotton .................................................................................. 10
   1.4.1  Causal organism ...................................................................................... 11
   1.4.2  Management strategies for Fusarium wilt of cotton ............................... 15
       1.4.2.1  Cultural management ....................................................................... 15
       1.4.2.2  Resistant cotton cultivars ................................................................. 17
           1.4.2.2.1  Conventional .............................................................................. 17
           1.4.2.2.2  Transgenic .................................................................................. 18

1.5  Disease resistance ........................................................................................... 19
   1.5.1  The infection process of Fusarium oxysporum ...................................... 22
   1.5.2  Phenolic and terpenoid defence responses of cotton ............................ 24
       1.5.2.1  Phenolics ......................................................................................... 26
       1.5.2.2  Terpenoids ...................................................................................... 27
           1.5.2.2.1  Biosynthetic pathway of cotton terpenoids ................................. 27
           1.5.2.2.2  Cotton terpenoids related to insect attack ................................. 29
       1.5.2.3  Cotton terpenoids related to pathogen challenge .............................. 30
           1.5.2.3.1  Roots ......................................................................................... 31
           1.5.2.3.2  Stele .......................................................................................... 31
       1.5.2.4  Effects of terpenoids ........................................................................ 33
           1.5.2.4.1  Gossypol .................................................................................... 33
           1.5.2.4.2  Gossypol-related phytoalexins ..................................................... 33
           1.5.2.4.3  Toxicity ....................................................................................... 34
   1.5.3  The vascular wilt disease model ............................................................... 35
       1.5.3.1  Usefulness of the vascular wilt model .............................................. 36

1.6  Aim and scope of this study ......................................................................... 36
Chapter 2  General materials and methods .................................................. 39

2.1  Host plants ...................................................................................... 39
  2.1.1  Source and cultivar ................................................................. 39
  2.1.2  Potting mix ............................................................................. 39
  2.1.3  Glasshouse conditions ............................................................ 39
  2.1.4  Seed Germination ................................................................... 39

2.2  Pathogen ....................................................................................... 40
  2.2.1  Source and strain .................................................................... 40
  2.2.2  Storage ................................................................................... 40
  2.2.3  Growth Media ......................................................................... 41
         2.2.3.1  Fusarium-selective (Peptone PCNB) agar ....................... 41
         2.2.3.2  Potato dextrose broth / agar .......................................... 41
         2.2.3.3  Carnation leaf agar ......................................................... 41
  2.2.4  Inoculum Production ............................................................... 42
         2.2.4.1  Solid inoculum ................................................................. 42
         2.2.4.2  Inoculum suspension ....................................................... 42

2.3  Statistical analyses ......................................................................... 42

Chapter 3  Glasshouse bioassay ............................................................. 43

3.1  Introduction .................................................................................... 43
  3.1.1  Past bioassay protocols ............................................................ 44

3.2  Methods ......................................................................................... 45
  3.2.1  Fov isolate selection ............................................................... 45
         3.2.1.1  In vitro ........................................................................... 45
         3.2.1.2  In vivo ............................................................................. 45
         3.2.1.2.1  Cut taproot ................................................................. 45
         3.2.1.2.2  Taproot puncture ......................................................... 45
  3.2.2  Glasshouse bioassays ............................................................... 46
         3.2.2.1  Infested growth substrate assay ....................................... 46
         3.2.2.1.1  Fresh solid inoculum ................................................... 46
         3.2.2.1.2  Powdered solid inoculum .......................................... 47
         3.2.2.2  Root dip assay ................................................................. 48
         3.2.2.3  Taproot puncture assay ................................................... 48
  3.2.3  Statistical analysis .................................................................... 49

3.3  Results .......................................................................................... 50
  3.3.1  Fov isolate selection ............................................................... 50
         3.3.1.1  In vitro ........................................................................... 50
         3.3.1.2  In vivo ............................................................................. 50
         3.3.1.2.1  Cut taproot inoculation ................................................ 50
         3.3.1.2.2  Taproot puncture inoculation ....................................... 50
  3.3.2  Glasshouse bioassays ............................................................... 50
         3.3.2.1  Infested growth substrate assay ....................................... 50
         3.3.2.1.1  Fresh solid inoculum ................................................... 50
         3.3.2.1.2  Powdered solid inoculum .......................................... 51
         3.3.2.2  Root dip assay ................................................................. 52
3.3.2.3 Taproot puncture assay .......................................................... 52

3.4 Discussion ...................................................................................... 53
3.4.1 Fov isolate selection ................................................................. 53
3.4.2 Glasshouse bioassays ................................................................. 53
3.4.2.1 Infested growth substrate assay ........................................... 53
3.4.2.2 Root dip assay .................................................................. 55
3.4.2.3 Taproot puncture assay ......................................................... 56
3.4.3 Conclusions .............................................................................. 57

Chapter 4 Infection process ................................................................. 79

4.1 Introduction .................................................................................... 79

4.2 Materials and Methods ................................................................. 81
4.2.1 Histopathology of Fov-infected cotton ...................................... 81
4.2.1.1 Plant and pathogen .......................................................... 81
4.2.1.2 Histochemical techniques ................................................. 81
4.2.1.2.1 Cleared tissue / lactophenol cotton blue stain ........... 81
4.2.1.2.2 Embedded tissue / toluidine blue stain ..................... 81
4.2.1.2.3 Fresh tissue / DioC6(3) stain .................................... 82
4.2.1.2.4 Scanning electron microscopy .................................. 82
4.2.2 Fov growth assays .................................................................. 83
4.2.2.1 Growth in root diffusate ................................................. 83
4.2.2.1.1 Treatment of diffusate ............................................. 83
4.2.2.2 Growth in stele tissue .................................................... 84
4.2.2.2.1 Root boxes ............................................................... 84
4.2.2.2.2 Taproot puncture in root boxes .............................. 84

4.3 Results .......................................................................................... 86
4.3.1 Histopathology ........................................................................ 86
4.3.1.1 Root surface ................................................................... 86
4.3.1.2 Penetration of root epidermis ......................................... 86
4.3.1.3 Colonisation of root epidermis and cortex .................... 86
4.3.1.4 Colonisation of vascular tissue ..................................... 86
4.3.1.5 Heavily infested tissue ............................................... 87
4.3.2 Fov growth assays .................................................................. 87
4.3.2.1 Growth in root diffusate ................................................. 87
4.3.2.2 Growth in stele tissue .................................................... 88

4.4 Discussion .................................................................................... 89
4.4.1 Histopathological insights ....................................................... 89
4.4.2 Fov growth assays .................................................................. 92
4.4.2.1 Growth in root diffusate ................................................. 92
4.4.2.2 Growth in stele .............................................................. 95
4.4.3 Conclusions .............................................................................. 96

Chapter 5 Histological responses to infection ........................................ 122

5.1 Introduction .................................................................................... 122

vi
5.2 Materials and Methods ................................................................. 124
  5.2.1 Plant and pathogen ................................................................. 124
  5.2.1.1 Inoculation .................................................................. 124
  5.2.2 Histochemical techniques ..................................................... 124
      5.2.2.1 Embedded tissue / toluidine blue stain ......................... 124
      5.2.2.2 Transmission electron microscopy .................................. 124
      5.2.2.3 Fresh tissue / toluidine blue or SbCl$_3$ – HClO$_4$ reagent ..... 125

5.3 Results ....................................................................................... 126
  5.3.1 Embedded tissue / toluidine blue ........................................... 126
  5.3.2 Transmission electron microscopy ........................................... 127
  5.3.3 Fresh tissue stained with toluidine blue ................................. 127
  5.3.4 Fresh tissue stained with SbCl$_3$-HClO$_4$ ............................... 128

5.4 Discussion .................................................................................. 129
  5.4.1 Structural changes ................................................................. 129
  5.4.2 Chemical defences ................................................................. 130
      5.4.2.1 Phenolics .................................................................. 130
      5.4.2.2 Terpenoids ................................................................ 131
  5.4.3 Conclusion ............................................................................ 132

Chapter 6 Antimicrobial terpenoids ................................................. 159

6.1 Introduction ............................................................................... 159

6.2 Materials and Methods ............................................................... 162
  6.2.1 Pathogen and host ................................................................. 162
  6.2.2 Extraction of antimicrobial terpenoids ................................. 162
  6.2.3 HPLC analysis .................................................................... 163
  6.2.4 Fov growth in stele ............................................................... 163
  6.2.5 Statistical analysis ................................................................. 163

6.3 Results ....................................................................................... 164
  6.3.1 Identification ....................................................................... 164
  6.3.2 Quantification ...................................................................... 164
      6.3.2.1 Timing and magnitude of antimicrobial terpenoids ........ 164
      6.3.2.2 Total antimicrobial terpenoid production relative to Fov spread ... 165
      6.3.2.3 Location of antimicrobial terpenoids ............................. 166
      6.3.2.4 Desoxyhemigossypol content of stele extract ................ 167

6.4 Discussion .................................................................................. 168
  6.4.1 Qualitative analysis ............................................................... 168
  6.4.2 Role in defence ...................................................................... 169
  6.4.3 Conclusions ......................................................................... 172

Chapter 7 General discussion ......................................................... 196

7.1 Bioassay .................................................................................... 197
  7.1.1 Future applications and further questions ............................. 199

7.2 Infection process ....................................................................... 200
TABLE OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Map of cotton growing regions in Australia (provided by Cotton Research and Development Corporation)</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Cotton plants with advanced Fusarium wilt symptoms</td>
<td>11</td>
</tr>
<tr>
<td>1.3</td>
<td>Fov spores on potato dextrose agar plates: (A) microconidia (m) and macroconidia (M) on the surface of the agar; (B) microconidia formed in false heads on hyphae; and (C) macroconidia formed on branched conidiophores</td>
<td>13</td>
</tr>
<tr>
<td>1.4</td>
<td>Proposed biosynthetic pathway for cotton terpenoids within the acetate-mevalonate pathway. Adapted from Liu et al. (1999) and Benedict et al. (2004)</td>
<td>27</td>
</tr>
<tr>
<td>3.1</td>
<td>Isolates of Fov on agar plates</td>
<td>59</td>
</tr>
<tr>
<td>3.2</td>
<td>Distance travelled by Fov in cotton stele after cut taproot inoculation</td>
<td>61</td>
</tr>
<tr>
<td>3.3</td>
<td>Distance travelled by Fov in cotton stele after taproot puncture inoculation</td>
<td>63</td>
</tr>
<tr>
<td>3.4</td>
<td>Emergence of seedlings in potting medium inoculated with different concentrations of solid (fresh) inoculum</td>
<td>65</td>
</tr>
<tr>
<td>3.5</td>
<td>Emergence and survival of seedlings in potting medium inoculated with solid (fresh) inoculum</td>
<td>67</td>
</tr>
<tr>
<td>3.6</td>
<td>Emergence of seedlings in potting medium inoculated with different concentrations of solid (powdered) inoculum</td>
<td>69</td>
</tr>
<tr>
<td>3.7</td>
<td>Emergence and survival of seedlings in potting medium inoculated with solid (powdered) inoculum</td>
<td>71</td>
</tr>
<tr>
<td>3.8</td>
<td>Survival of cotton plants after root-dip inoculation</td>
<td>73</td>
</tr>
<tr>
<td>3.9</td>
<td>Survival of cotton plants after inoculation by taproot puncture</td>
<td>75</td>
</tr>
<tr>
<td>3.10</td>
<td>Survival of cotton plants after inoculation by taproot puncture with the provision of a 12 hour photoperiod</td>
<td>77</td>
</tr>
<tr>
<td>4.1</td>
<td>Method used for quantification of in vivo pathogen growth</td>
<td>98</td>
</tr>
<tr>
<td>4.2</td>
<td>Colonisation of the root surface</td>
<td>100</td>
</tr>
<tr>
<td>4.3</td>
<td>Penetration of the root epidermis</td>
<td>102</td>
</tr>
<tr>
<td>4.4</td>
<td>Penetration of root hairs</td>
<td>104</td>
</tr>
<tr>
<td>4.5</td>
<td>Colonisation of host cells</td>
<td>106</td>
</tr>
</tbody>
</table>
Figure 6.1 Graph showing mobile phase gradient used for HPLC analysis. .............. 174

Figure 6.2 Chromatograms of cotton stele extracts taken prior to inoculation .......... 176

Figure 6.3 Representative chromatograms of cotton stele extracts 5 days after mock- and Fov-inoculation ......................................................................................... 178

Figure 6.4 Antimicrobial terpenoid contents of cotton stele extracts: dHG, and HG ... 180

Figure 6.5 Antimicrobial terpenoid contents of cotton stele extracts: dMHG and G ... 182

Figure 6.6 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 1 .................................................................................................................. 184

Figure 6.7 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 3 .................................................................................................................. 186

Figure 6.8 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 5 .................................................................................................................. 188

Figure 6.9 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 3 .................................................................................................................. 190

Figure 6.10 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 5 .................................................................................................................. 192

Figure 6.11 Desoxyhemigossypol content of stele extracts on Days 1(A), 3(B) and 5(C). ...................................................................................................................... 194
TABLE OF TABLES

Table 1.1 Worldwide cotton production estimates for 2005..............................................2
Table 1.2 World chemical usage for plant protection.........................................................4
Table 6.1 Synonyms for cotton terpenoids ........................................................................161
CHAPTER 1  COTTON: PRODUCTION, DISEASES AND DEFENCE RESPONSES

Humankind has used cotton for centuries. The genus, *Gossypium*, owes its utility to the length and structural morphology of its seed hairs that can be processed into yarn. This lint was so valued that cotton was domesticated independently in four different regions of the world: Meso-America; South America; West Africa; and India (Diamond, 1997). The oldest archaeological record of cotton usage dates back to a Neolithic burial site (6th millennium BC) in Pakistan (Moulherat *et al*., 2002). The acquisition of the crop and techniques for producing fabric have historically been so prized that cotton has been described as “a world power” of “revolutionary capacity” (Farnie and Jeremy, 2004).

Cotton lint is valued primarily for its strong, fine, yet durable fibres. Clothing is the primary good produced with cotton (Australian Bureau of Agricultural and Resource Economics, 2006) but the fibre is used to manufacture a wide variety of textiles and industrial products. The seed of the cotton plant provides high quality vegetable oil widely consumed by humans, and is a protein-rich stockfeed (Alford *et al*., 1996; Mujahid *et al*., 2000).

Cottonseed oil is used for food products ranging from margarine to salad dressing. Whole cottonseed and cottonseed meal are used in animal feed. Cottonseed contains high quality protein, however it also contains the toxic glandular pigment gossypol (C_{30}H_{30}O_{8}) (Mujahid *et al*., 2000; Robinson *et al*., 2001), that damages the reproductive system, heart, liver and membranes of grazing animals (Kovacic, 2003). Death by heart failure may follow ingestion due to gossypol’s reduction of the oxygen-carrying capacity of the blood (Arieli, 1998). It is less toxic to ruminants due to the binding of free gossypol to soluble proteins in the rumen (Reiser and Fu, 1962). After oil extraction, the meal is stripped of gossypol and fed to poultry and swine (Murray *et al*., 1993).

The term “cotton” usually refers to the four species of the genus *Gossypium* that produce useful lint: *G. arboretum*, *G. herbaceum*, *G. barbadense*, and *G. hirsutum*. The diploid cottons *G. arboretum* L. and *G. herbaceum* L. produce low yields and variable fibre qualities, which results in their accounting for little of the world’s cotton production. The allotetraploid species *G. hirsutum* L. (Upland cotton) and *G. barbadense* L. (Egyptian, Pima, or extra-long-staple cotton) originated from South and
Meso-America, respectively, but have been dispersed widely throughout the world. *Gossypium hirsutum* accounts for the vast majority of production, and in some areas farmers have been legally bound to cultivate this species alone (Wilhem *et al.*, 1974a). This species represents an average of 97% of the total US production in the last five years, with *G. barbadense* accounting for the remainder (International Cotton Advisory Committee, 2006a). Extensive variation of cultivars within these species exists after much intercrossing with morphotypes, wildtypes and ancestral types, and more recently with genetic engineering.

### 1.1 Cotton production

The value of world cotton production in 2004/05 was estimated at US$30 billion (International Cotton Advisory Committee, 2005b) and demand for the crop is increasing rapidly. Cotton consumption rose 5.5% in 2004, representing the sixth consecutive year of expansion, and the largest increase since 1986 (International Cotton Advisory Committee, 2005a). Continued growth to record levels of world raw cotton consumption is forecast over the next five years (Australian Bureau of Agricultural and Resource Economics, 2006)

China and the USA represented roughly 23 and 21% of global cotton production in 2004/2005, respectively, and with India, Pakistan and Brazil account for nearly three quarters of world production (International Cotton Advisory Committee, 2006a).

**Table 1.1 Worldwide cotton production estimates for 2005 (International Cotton Advisory Committee, 2006a)**

<table>
<thead>
<tr>
<th>Country</th>
<th>Production (million tons)</th>
<th>Consumption (million tons)</th>
<th>Average Yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>5.77</td>
<td>9.80</td>
<td>1.14</td>
</tr>
<tr>
<td>USA</td>
<td>5.20</td>
<td>1.31</td>
<td>0.93</td>
</tr>
<tr>
<td>India</td>
<td>4.12</td>
<td>3.60</td>
<td>0.47</td>
</tr>
<tr>
<td>Pakistan</td>
<td>2.09</td>
<td>2.39</td>
<td>0.65</td>
</tr>
<tr>
<td>Brazil</td>
<td>1.07</td>
<td>0.87</td>
<td>1.26</td>
</tr>
<tr>
<td>Australia</td>
<td>0.58</td>
<td>0.01</td>
<td>1.81</td>
</tr>
<tr>
<td><strong>TOTAL</strong>*</td>
<td><strong>24.76</strong></td>
<td><strong>24.93</strong></td>
<td><strong>0.74</strong></td>
</tr>
</tbody>
</table>

*Totals include countries not shown.

### 1.2 The Australian cotton industry

While cotton seeds arrived in Australia with the first European settlement in 1788, cotton was grown as an ornamental plant for years, and struggled as a crop until the
industry was all but non-existent in the mid-20th century (Harding, 1924; Hearn and Fitt, 1992). Two American cotton growers, Paul Kahl and Frank Hadley, were so enthused by the potential for cotton production in Australia that they bought land and planted their first crop in the Namoi Valley, New South Wales, in 1961. This crop, with the support of an encouraging government and a new irrigation system, was the forerunner of the Australian cotton industry.

There are currently approximately 1200 Australian growers (www.crdc.com.au, July 2006). Although grower numbers are declining due to limited water availability and amalgamations of farms, the vast majority of Australian cotton is still produced by family run businesses on plots of an average size of 400 hectares. All of these farms grow additional crops, and many graze sheep and cattle. Approximately 80% of Australia’s cotton is produced in New South Wales, and 92% is watered by furrow irrigation (www.affa.gov.au, July 2006). Australian cotton growing regions are reported in Figure 1.1. Australian production is technologically sophisticated due to grower willingness for constant innovation (www.crdc.com.au, July 2006). Transgenic varieties, for example, account for around 90% of the total area planted to cotton in 2005, compared with a global average of 24% (Australian Bureau of Agricultural and Resource Economics, 2006; International Cotton Advisory Committee, 2006a).

Figure 1.1 Map of cotton growing regions in Australia (provided by Cotton Research and Development Corporation)
While Australia represents less than 2% of total global production, cotton yield is consistently high. When Australia’s cotton production peaked at 795,000 tonnes in 2000-01 (3.5 million 227kg bales) the exports generated from this crop were valued at approximately A$1.95 billion, making cotton Australia’s fourth largest rural export that year (Australian Bureau of Agricultural and Resource Economics, 2001), and placed Australia as the fourth largest exporter of cotton (International Cotton Advisory Committee, 2004b).

The worst drought in 100 years in 2002-2004 led to a 45% drop in area planted to cotton in Australia (International Cotton Advisory Committee, 2004a). Water availability will remain a key constraint on cotton plantings in the future (Australian Bureau of Agricultural and Resource Economics, 2006). But despite the world cotton yield reaching a record high in 2004/05, Australia’s yield was almost three times this figure, producing a world record 2,038kg/ha (www.cottonaustralia.com.au, June 2006).

Most of Australia’s 2004/05 crop was exported, mainly to Indonesia (131,210 tonnes), China (81,240 tonnes), Thailand (68,780 tonnes), Republic of Korea (63,000 tonnes), and Japan (43,770 tonnes) (International Cotton Advisory Committee, 2006b). Australia enjoys a reputation in the world market as a reliable supplier of high quality cotton (www.cottonaustralia.com.au, June 2006).

1.3 Diseases of cotton
The production of cotton requires intensive management of many pests. Of the US$32.4 billion spent worldwide on pesticides in 2004, almost 9% was applied to cotton.

<table>
<thead>
<tr>
<th></th>
<th>Herbicides</th>
<th>Insecticides</th>
<th>Fungicides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>777</td>
<td>1,618</td>
<td>70</td>
</tr>
<tr>
<td>All crops</td>
<td>14,849</td>
<td>8,635</td>
<td>7,296</td>
</tr>
<tr>
<td>Percentage of all crops</td>
<td>5.23</td>
<td>18.74</td>
<td>0.96</td>
</tr>
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</table>

Diseases of cotton are caused by a wide variety of biotic disease agents, with diverse mechanisms for entering, colonising and producing characteristic damage (Watkins, 1993). In his compendium of cotton diseases, Watkins (1993) describes the multitudes of fungi, nematodes, bacteria, viruses and mycoplasma-like organisms that can lead to disease, defined as “departures from the normal appearance, form, or functioning of the
plant.” Rather than documenting an exhaustive list of pathogens, this section describes cotton diseases of major global importance.

Seedling diseases (caused by *Rhizoctonia solani* Kuhn., *Pythium* spp., *Fusarium* spp.) may be visible as pre-emergent seed rot, post-emergent damping off, delayed germination, slow seedling growth or seedling root rot (Melero-Vara and Jimenez-Diaz, 1990). The causal agent may be a single pathogen, but more often is a complex of several fungi (Johnson *et al*., 1978; De Vay *et al*., 1982). These diseases are omnipresent in all cotton growing regions, and more predominant in marginal cool climates. If the management of soil moisture and temperature by careful selection of optimum sowing time is inadequate, they may be controlled through the effective use of fungicides.

*Fusarium* wilt (caused by *Fusarium oxysporum* Schlectend. f. sp. *vasinfectum* (Atk.) Snyd. & Hans., or “Fov”) was formally identified early in the history of plant pathology, and subsequently formed a basis for recognition of other wilts (Smith *et al*., 1981). Symptoms can appear at any stage of plant development. A patchy distribution of plants in the field often results from seedlings that wilt and die rapidly. Symptomatic older plants may be stunted and wilted. Chlorosis of the leaves and dieback that progresses from the top of the plant are accompanied by the formation of characteristic brown discolouration of the vascular tissue. Some plants die, while others re-shoot from the base, but these plants do not go on to produce bolls (Davis *et al*., 2006). *Fusarium* wilt affects all four domesticated cotton species (Armstrong and Armstrong, 1960, as cited by Davis *et al*., 2006; Fahmy, 1927). The disease is distributed globally and causes severe losses under conducive conditions.

*Fusarium* wilt caused significant losses in the US in the 1950’s, after which yield losses were minimised until 1990, when the incidence started to progressively increase (Kappelman and Smith, 1993). *Fusarium* wilt was nominated as the most important disease of cotton in the Peoples’ Republic of China in the late 70’s, but that country is now able to control losses with improved, more resistant cotton varieties (Jiang *et al*., 2000). On the other hand, *Fusarium* wilt continues to be Tanzania’s most important cotton disease, where it is spread rapidly by the feeding of gin waste to cattle and planting infected seed (Hillocks and Kibani, 2002).
In Tanzania, as in many other countries, infection of plants by both nematodes and Fov results in increased incidence and severity of wilt symptoms (De Vay et al., 1997; Smith, 2001; Katan et al., 1983). Infection by *Meloidogyne incognita* (Kofoid & White) Chit. and Fov – known as the Fusarium wilt-root knot nematode complex - is recognised as the most economically important disease complex of cotton worldwide (Starr et al., 2001). Fusarium wilt may occur in the absence of nematodes, but the disease severity is greatly increased by their presence (De Vay et al., 1997; Katsantonis et al., 2003). Understanding this association provides opportunities for disease control, and successful control in some locations depends on breeding for both Fusarium wilt and nematode resistance (Abd-El-Alim et al., 1999), and soil fumigation with nematicides (Colyer et al., 1997). Unique pathotypes of Fov in Australia, however, are extremely virulent and require no nematode association to produce severe disease in any cotton variety, and for which there is no control (Allen and Lonergan, 2000).

Several nematodes have long been known to independently cause significant damage in cotton, and the losses are steadily increasing in some countries (Overstreet and McGawley, 2001). These soilborne microscopic roundworms occur in all countries that grow cotton. Above-ground symptoms of nematode attack are non-specific and typical of plants with inhibited root function, and may include stunted or chlorotic plants that do not respond to normal crop management inputs. Plant roots may be damaged in particular ways, depending on the type of nematode: root-knot nematode (*Meloidogyne incognita* (Kofoid & White) Chit) causes galls (Shepherd and Huck, 1989); sting nematode (*Belonolaimus* spp.) causes bushy roots (Graham and Holdeman, 1953); and reniform (*Rotylenchulus reniformis* Linford & Oliveira) and lance nematodes (*Hoplolaimus* spp.) cause a general decline of the root system (Birchfield, 1962; Krusberg and Sasser, 1956).

The root-knot nematode is the most economically important nematode, due to its worldwide distribution and extensive host range (Overstreet and McGawley, 2001). Not only can it be found on all species of cotton, but it can infect many of the rotation crops and weeds associated with cotton production. The reniform nematode is most important in tropical and subtropical cotton growing regions, where production losses have sometimes been greater than that due to root-knot nematode. Sting and lance nematodes
are prevalent in the USA, and have a disjointed occurrence also in Egypt and India (Overstreet and McGawley, 2001).

Nematodes are managed by an area-dependent selection of cultural practices, including crop rotations and soil tillage, although few of these factors are especially effective due to nematodes’ persistence and wide host ranges (Westphal and Smart, 2003). Although a temporary, expensive and unsustainable form of management, chemical control is the most frequently used strategy (Lawrence et al., 2003; Noe, 1990).

Verticillium wilt is an important disease of cotton that occurs in most cotton-growing countries (Bell, 2001). The disease is more of a problem in temperate areas, and symptoms tend to occur in cool periods. The causal agent, *Verticillium dahliae* Kleb, penetrates the plant root from the soil, continuing inwards through the stele and eventually systemically invading the xylem (Gerik and Huisman, 1985). This vascular infection, along with the plant defence responses elicited, choke the xylem tissues and result in wilt symptoms. The vascular invasion can be seen in a stem cross section as brown discoloration, and is often associated with other symptoms include stunting, yellowing, necrosis of leaf margins and interveinal areas and characteristic leaf mosaic patterns which progress upwards from the base of the plant. Leaves are often shed, and if infected by one of the several devastating defoliating pathotypes, the plant is completely defoliated before terminal dieback sets in. Early infections cause young bolls to abscline or become malformed, but if infection is late in the season the lint yield and quality can remain unaffected. The losses attributable to Verticillium wilt have decreased dramatically in the last two decades due mainly to the development of resistant cultivars (Bell, 2001). Increased understanding of the disease has aided an integrated approach to control, including planting on raised beds and minimising irrigation to improve drainage and increase soil temperature, rotating crops and carefully managing nutrient requirements (El-Zik, 1985).

Alternaria leaf spot or leaf blight is caused by *Alternaria macrospora* A. Zimmerm. or *A. alternata* (Fr.:Fr.) Keissl. Brown lesions form on cotyledons, leaves and bracts and bolls, followed in severe cases by premature defoliation. While it is the most common foliar disease of cotton worldwide, yield losses are usually not substantial as the disease is generally associated with the late-season growth of cotton, after the crop has developed (Baird, 2001). The disease may also be associated with senescing leaves, or
environmental or nutritional stress. Pima cotton is particularly susceptible to Alternaria leaf spot, so the most substantial crop losses occur where this variety is grown. Annual outbreaks of leaf blight epidemics have been documented by Holguin and Bashan (1992) in Israel, accompanied by an increase in aggressiveness of the pathogen over several seasons. Alternaria leaf spot also causes losses to potential yields in Zimbabwe, USA and Mexico (Hillocks, 1991; Holguin and Bashan, 1992; Bashan, 1994). The minimisation of plant stress combined with fungicide application to seed and/or foliage followed by burying plant residues at season’s end provides effective prevention and control of Alternaria leaf spot.

*Thielaviopsis basicola* (Berk. & Broome) Ferraris (anamorph of *Chalara elegans* Nag Raj & Kendrick) causes black root rot of cotton, attacking roots and lower stems, causing root death, stunting, and sometimes plant death (Allen, 2001a). It occurs throughout the American cotton belt, causing most problems in irrigated regions. To avoid conducive cool moist conditions, planting is in raised seedbeds and is delayed.

Bacterial blight of cotton (also called angular leaf spot) is an important and potentially destructive bacterial disease. The disease is caused by a bacterium, *Xanthomonas axonopodis* pv. *malvacearum* (Smith) Vauterin [formerly *X. campestris* pv. *malvacearum* (Smith) Dye] (Hillocks, 1992a). Cotton yield losses in excess of 10% have been reported in the past, although breeding for resistance has provided cultivars with high resistance throughout the world (Thaxton and El-Zik, 2001).

Cotton root rot is a lethal disease of roots in US caused by the fungus *Phymatotrichopsis omnivora* (Duggar) Hennebert (Riggs, 2001). Infection results in symptoms of wilt, followed soon after by plant death. Its distribution is restricted to calcareous alkaline soils in warm climates. Management of cotton root rot is especially problematic in conducive weather for the disease, when severe plant losses are possible. The most effective control is to deep plough the field and plant early maturing varieties. However, this fungus produces persistent sclerotia that can survive in soil for years, making eradication difficult (Lyda, 1978).

Boll rots may be caused by a multitude of organisms, including *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *A. macrospora*, *Colletotrichum* spp., *Fusarium* spp. and *Phytophthora* spp. Some are primary pathogens, while others are opportunistic
wound pathogens or secondary invaders of diseased tissues. Symptoms are varied between pathogens, but always result in damage to bolls. The extent of losses to boll rots depends upon local climate, particularly humidity. Depending on the causal pathogen, management may involve the precise application of nitrogen fertilizer and plant growth regulators as well as careful management of crop density to control canopy humidity. The use of disease-free and fungicide-treated seed stock has dramatically improved the control of some boll rots of cotton (Batson, 2001).

1.3.1 Diseases of Australian cotton

The prevalence of diseases of cotton in Australia has changed with time. Cotton crops grown in NSW have been surveyed for disease distribution, incidence and severity since 1983 (Allen and Lonergan, 1998). Bacterial blight (caused by *X. campesritis* pv. *malvacearum*) and Verticillium wilt (caused by *V. dahliae*) caused significant losses in production in the 1980’s, but their impact has since been limited by the introduction of resistant cotton varieties (Nehl and Allen, 2002a).

Several non-lethal pathogens can reduce cotton yield. Alternaria leaf spot (caused by *A. macrospora* or *A. alternata*) and Phytophthora boll rot (caused by *P. nicotianae*) are considered “low-level threats” and their limited impacts are usually related to weather events (Nehl and Allen, 2002a). Cotton bunchy top is a recently named disease that characterises symptoms that have been observed in isolated field patches since the late 1990’s (Anonymous, 1999; Reddall et al., 2004). The disease is suspected to be caused by a biotic agent that is aphid- and graft-transmitted, but its occurrence appears to be declining.

Black root rot was discovered in Australian cotton fields in 1989 and the causal fungus, *T. basicola* has since escalated to pandemic levels, occurring in all cotton production regions (Allen, 1990; Nehl et al., 2004b). Control methods have focussed on the “Come Clean – Go Clean” farm hygiene initiative and crop management strategies have been devised to limit spread, but no resistant cotton varies are available (Anonymous, 2000; Allen et al., 2003; O’Neill, 1999).

Fusarium wilt also emerged in the 1990’s, and has spread rapidly to the current epidemic level (Kochman, 1995; Allen, 2004b). The absence of resistance to this
disease in any cotton variety led to the nomination of Fusarium wilt as one of the most
significant threats to sustainable cotton production in Australia (Kochman et al., 2000).

1.4 Fusarium wilt of cotton

Fusarium wilt of cotton was first formally described early in 1892, after years of
existence in the USA and India (Atkinson, 1892 as cited by Kochman, 1995). Having
finally been discovered in Australia in 1993, it has spread throughout all cotton growing
areas of the world (Smith et al., 1981; Colyer, 2001). In many countries it presents a
formidable constraint to production and in some cases even threatens the sustainability
of the industry (Hillocks and Kibani, 2002).

The Fusarium wilt pathogen, *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), was first
formally confirmed in Australia in 1993, when it was isolated from the vascular system
of wilted cotton plants from the Darling Downs in Queensland (Kochman, 1995). A
large proportion of this region's cotton fields became infested, causing an estimated
AUD$57 million loss in the 2000 season, and rendering many fields unsuitable for further
cotton production (Kochman et al., 2000). There was a rapid expansion of disease
occurrence, and the industry identified Fusarium wilt as a major industry issue and
priority in 1997 (Salmond et al., 1998). It is estimated that if the current rate of spread
continues, Fusarium wilt will be present in 90% of NSW farms by 2010 (Allen, 2004b).

Fusarium wilt can result a devastating set of disease symptoms. These symptoms may
appear at any stage of plant development, but most commonly when the plant is
vulnerable, such as during the seedling stage, after flowering, or following a cold-shock
event (Kochman et al., 2000). The leaves of infected seedlings become dull and flaccid,
and the young plant quickly displays chronic wilting and death. Older plants often
withstand infection for an extended period, usually display wilting, foliar yellowing,
leaf loss and stunting, before eventually dying from the top of the plant down. A
characteristic staining of the vascular system can be observed by cutting the stem in
cross section. Once symptomatic, the plant may go on to die or reshoot from the base of
the stem.
Figure 1.2 Cotton plants with advanced Fusarium wilt symptoms
Plants displaying: (A) desiccated leaves, and stem death progressing from the top down; and (B) extensive vascular browning.

1.4.1 Causal organism
Fusarium oxysporum is a genetically and morphologically diverse species with extensive pathogenic specialisation. It includes many pathogens of a broad range of ornamental and agricultural hosts, as well as saprophytes and benign endophytes. According to the system of Snyder and Hansen (1940) pathogenic isolates of F. oxysporum that share the same host range are separated into formae speciales (f. sp.), of which more than 120 have been described (Armstrong and Armstrong, 1981) including the forma specialis which causes vascular wilt in cotton, vas infectum.

This group is further divided into races by a variety of methods. Armstrong and Armstrong (1968, 1978) characterised six physiological races of Fov according to their pathogenicity on a differential set of cotton species and non-cotton hosts. These races incidentally also corresponded with their geographic origin. Based on these same techniques but with the addition of two additional non-cotton hosts, two further races (7, 8) from China were identified (Chen et al., 1985). Molecular characterisation confirmed the relatedness of race, virulence and geographic origin, although enlarging the geographic distribution previously described (Assigbetse et al., 1994). However, after differentiating Fov races by random amplified polymorphic DNA (RAPD) analysis Assigbetse et al. (1994) argued that races 1, 2 and 6 should be collapsed into one race A. These three races clustered into one RAPD group and were previously only distinguishable by their pathogenicity on the non-cotton differential hosts alfalfa
(Medicago sativa L.) and tobacco (Nicotiana tabacum L.). Various types of analyses have characterised Fov races in accordance with the convention of three races (A, 3 and 4) and many authors agree that it is preferable to confine the differential host set to Gossypium spp. (Nirenberg et al., 1994; Hering et al., 1999; Davis et al., 1996b; Bell et al., 2003).

The characterisation of Australian biotypes has not been completed: their pathogenicity on the differential hosts is similar to that of race 6, but vegetative compatibility grouping (VCG) and restriction fragment length polymorphism (RFLP) analysis indicate that they are distinct from all other biotypes (Davis et al., 1996b; Bentley et al., 2000). Australian isolates have been designated the unique VCG groups of 01111 and 01112 according to the methods of classification of Puhalla and Correll (Puhalla, 1985; Correll et al., 1987). A study of multigene genealogies of Fov found evidence of an evolutionary origin independent of all foreign biotypes (Kim et al., 2005).

The uniqueness of the Australian biotypes suggests that they arose indigenously and that some of Australia’s 17 endemic wild Gossypium species could be natural reservoirs of the pathogen (Davis et al., 1996b; Craven et al., 1994; Bentley et al., 2000). This theory is supported by a survey of native Gossypium species that found an association with wild Fov populations with a demonstrated ability to cause vascular wilt symptoms in cultivated cotton (Wang et al., 2004a). The occurrence of wild Fov in native Gossypium has led to the suggestion that the Australian biotypes are of indigenous origin, but the geographic origin of foreign isolates remains unknown (Smith et al., 1981; Davis et al., 2006).

Preliminary studies demonstrated that Australian Fov isolates were pathogenic to all Australian and overseas commercial cotton varieties that were tested (Davis et al., 1996b; Reid et al., 2002). Australian isolates also prosper in the heavy alkaline soils that are able to suppress isolates from other countries (Bell et al., 2003). This has alarmed countries importing Australian cottonseed as cattle feed, but as yet, there have been no reports of Australian Fov overseas (Kim et al., 2005; Kochman et al., 2002).

Although the Australian biotypes of Fov differ from their overseas counterparts in several ways, they are morphologically indistinguishable. In fact, the appearance of spores and cultures of all the races of Fov is typical for the species, F. oxysporum. The
fungus belongs to the Section Elegans according to the taxonomic system of Snyder and Hansen (Snyder and Hansen, 1940). It produces three types of asexual spores: macroconidia, microconidia and chlamydomospores. *F. oxysporum* has no known perfect state. Macroconida (3-4.5 × 40-50μm) are sickle-shaped to straight, thin walled and normally three septate (Figure 1.3A) (Colyer, 2001). They may be produced from monophialides either on branched conidiophores or hyphae (Figure 1.3C) and have a notched (foot-shaped) basal cell and a pointed apical cell (Burgess et al., 1994). Microconidia (2-4 × 5-12μm) are oval or reniform and are usually non-septate (Figure 1.3A) (Colyer, 2001). They are formed in false heads from short conidiophores on hyphae (Figure 1.3B) (Nelson et al., 1983). Single or pairs of thick-walled chlamydomospores (7-13μm diameter) are produced intercalary or terminal in the hyphae (Gerlach and Nirenberg, 1983). Cultured on carnation leaf agar, Fov forms a white mycelium and macroconidia are produced in pale orange sporodochia. On potato dextrose agar, the Australian isolates of Fov produce a violet pigment in the agar beneath a white mycelium, in addition to a strong volatile odour (Davis et al., 1996b).

Figure 1.3 Fov spores on potato dextrose agar plates: (A) microconidia (m) and macroconidia (M) on the surface of the agar; (B) microconidia formed in false heads on hyphae; and (C) macroconidia formed on branched conidiophores.
Fov is a soilborne fungus that can survive long periods in soil (Smith and Snyder, 1975; Smith et al., 2001). Fov is likely to persist in the soil mainly as chlamydospores, as in other formae speciales of this fungus (Nelson, 1981). These chlamydospores respond to exudates from germinating cotton seedlings, producing hyphae that grow through the soil towards the roots (Griffin, 1981; Mai and Abawi, 1987). Hyphae penetrate the roots, and continue towards the vascular tissue, eventually breaching the xylem. Once inside xylem elements, microconidia are produced and swept up the plant with the transpiration flow. When these microconidia are trapped by sieve plates at the end of the xylem elements, microconidia germinate, and the hyphae penetrate into the adjoining element. Thus, the sequence begins again and eventually results in systemic infection.

This scenario assumes the absence of plant resistance to infection. In reality, plants of all cotton varieties will mount some degree of defence in response invasion by Fov. This defence is evident when cutting through the stem of an infected plant and observing the brown discolouration caused by the plant’s attempts to block the vascular tissue. The plant responds to the presence of Fov by plugging xylem vessels with physical barriers, subsequently infusing these barriers with toxins (Shi et al., 1992). This response in some cotton varieties may be too small or too late, and infection will continue. The further colonisation will be accompanied by the response of further blocking of the vascular system until the plant wilts and dies. If, on the other hand, Fov growth is arrested by an initial successful defence response and colonisation is limited, the seedling can survive, although growth may be stunted. The plant is more susceptible to stresses with its compromised vascular system, and is likely to wilt and die after events such as a cold shock or flowering. The fungus, having proliferated throughout the plant is then returned to the soil in increased quantities, to survive saprophytically in the plant residues or as chlamydospores.

Fov may be introduced to a cotton field by planting seed harvested from an infected plant, or by a deposit of infested soil or infected plant debris (Wang et al., 1999c). Once present in the soil it is easily spread by the movement of contaminated soil by farm machinery or by irrigation water, both of which are integral to cotton production. While many details of Fov and Fusarium wilt of cotton have been documented, neither the life cycle nor disease cycle in Australia has been published. This paucity will be addressed
in the current study. Until now, the immediacy of this new and unique problem for the Australian cotton industry has dictated a focus of research on disease control.

1.4.2 Management strategies for Fusarium wilt of cotton

Many cotton growing regions throughout the world employ effective control methods for Fusarium wilt. A combination of factors including planting non-infested seed and the selection of cultivars with resistance to both Fov and nematodes is crucial for minimising losses to potential yield.

The situation in Australia, however, is made difficult by the virulence of the Fov isolates found in this country. Smith and Dick (1960) reported that the Fusarium wilt resistance found in *G. barbadense* is conditioned by two dominant genes with additive effects, one of which has been transferred to *G. hirsutum* (Smith and Dick, 1960). However, cultivar resistance that is valuable in other countries is not effective against Australian biotypes of Fov, and the disease lacks the association with nematodes. In a parallel case, Panama (or Fusarium) wilt of banana was effectively controlled by cultivar resistance until a new race of *Fusarium oxysporum* f. sp. *cubense* emerged and instigated a search for a new source of resistance (Stover, 1981; www.taiwanheadlines.gov.tw/20010904/20010904s4.html, September, 2001).

The development of Fusarium wilt resistant varieties has been a major component of the Australian cotton research effort, which also includes investigations into the effects on disease of a wide range of farming practices. Australian cotton breeders suggest that the development of resistant varieties may be ten or more years away, highlighting the importance of integrated disease management in the interim (Constable *et al.*, 2004).

1.4.2.1 Cultural management

*F. oxysporum* is one of the most common soil-borne Fusaria (Burgess, 1981) and is often the most prevalent fungus found on the surface of live cotton roots (Zhang *et al.*, 1996). Once Fov has infested soil, it is particularly difficult to eliminate (Burgess, 1981). The ability of *Fusarium* to grow on organic residues or to readily form chlamydospores ensures its long-term survival (Booth, 1971; Nash *et al.*, 1961; Schippers and van Eck, 1981). Persistence in the field is reinforced by the ability of the wilt Fusaria to colonise many alternate hosts (Armstrong and Armstrong, 1948; Hendrix and Nielson, 1958; Nash and Snyder, 1967).
Fov can grow saprophytically on organic matter, increasing the inoculum reservoir between crops (Smith 1975). Common features of Australian cotton farming systems favour the survival and spread of this pathogen (Allen, 2002). Preliminary findings indicate that cotton stubble mulched and left on the soil surface for up to six weeks before incorporation results in less disease the following planting. It is expected that management decisions such as immediate incorporation of mulched stubble or raking/burning stubble will lose favour as Fusarium wilt incidence increases (Kochman et al., 2000).

Several common Australian weed species have been identified as alternative hosts, many of which can grow symptomless in cotton production areas. Furthermore, high weed densities are associated with severe disease incidence (Baird and Carling, 1998; Kochman et al., 2002).

_Fusarium oxysporum_ is particularly difficult to eliminate from soil by rotation or long fallow (Burgess, 1981). Long-term research into non-host rotation crops including biofumigant crops is currently taking place in Australia (Swan and Salmond, 2005; Swan, 2004). The crop most commonly rotated with Australian cotton is wheat, and field experiments have shown that the incidence of Fusarium wilt was greater following winter cereals than following bare fallow. In fact, field trials have shown disease incidence to be the lowest after bare fallow compared with many other rotation crops, but this is not economically viable and may risk a reduction in valuable mycorrhizal fungi (Johnston, 2004). This persistence through rotations is evidence of the ability of the pathogen to infect non-hosts: Fov has been isolated in pot trials from asymptomatic crop plants including wheat, sorghum, maize, sunflower, mung bean, lucerne, field pea, chickpea, vetch and pigeon pea (Allen, 2004b; Swan, 2004).

Irrigation water is a vital management concern as it can carry Fov spores and often floods through multiple crop stands. Free from this source of inoculum, Australian dryland cotton farms remained uninfested by Fov until the 1999-2000 season, despite the rapid spread of the disease in irrigated areas. Producers are currently advised to allow water (which is often recycled) to settle before irrigation to precipitate out soil particles and Fov spores. Isolation of water from infested fields and the avoidance of waterlogging are encouraged as Fusarium wilt is both a stress-related and inoculum-dependant disease. At the time of writing, a project investigating Fov dispersal in farm
water residues and the significance of this mode of transmission was underway (Anderson, 2004).

Seedborne transmission of Fov has been long recognised as an important determinant of disease spread (Elliot, 1923). Acid de-linted seeds harvested from infected highly susceptible plants in Australian cotton fields have been observed to harbour viable Fov propagules (Allen, 2004b). The pathogen is not detectable after six months of seed storage, or after treatment with methyl bromide (Elliot, 1923; Kochman et al., 2002). Precaution was taken by Australian seed companies to ensure that seed production excluded diseased areas immediately after Fusarium wilt was identified in this country (Kochman, 1998).

1.4.2.2 Resistant cotton cultivars

1.4.2.2.1 Conventional

Host plant resistance has been identified as the primary strategy for long-term management of Fusarium wilt (Davis et al., 2006). Planting of less susceptible cotton varieties in infested fields was instigated quickly by the Australian cotton industry when it was discovered that Fov levels in the soil increased after a more susceptible crop had been grown (Wang et al., 1999b). Preferred cotton varieties will vary between regions and grower preferences according to many differing parameters (Reid et al., 1989), but these other considerations become incidental upon discovery of a Fusarium wilt incursion. Instead, the least susceptible variety to Fov is planted to maximise plant survival and limit pathogen proliferation and inoculum accumulation.

To facilitate growers’ selection of appropriate varieties, a standard protocol for describing relative cotton varietal resistance to Fusarium wilt was developed in 2002 (Cotton Seed Distributors, 2002). Based on the survival of plants in the presence of significant disease pressure, these “disease rankings” provide growers with a reference of the susceptibility of new or existing cotton varieties. The most resistant commercially grown variety at the time, Sicot 189, was nominated as the industry standard for Fusarium wilt resistance (Kochman et al., 2002). Rather than possessing complete resistance to Fov, Sicot 189 merely demonstrated less susceptibility to Fov, and still incurred substantial deaths under high disease pressure. In an especially severe outbreak of Fusarium wilt on the Darling Downs less than 10% of Sicot 189 plants survived in the 2003/04 season (Nehl et al., 2004a). However, the most susceptible variety, Siokra
1-4, will sustain more than six times more fatalities under high disease pressure (Cotton Seed Distributors, 2002) so the relative tolerance of Sicot 189 saw this variety replace many others in diseased areas (Allen, 2001b). Targeted breeding has resulted in a substantial advance in Fusarium wilt resistance with the commercial release of Sicot F-1 in 2004, which is twice a likely as its parent Sicot 189 to survive in a Fov-infested field (Reid et al., 2004; www.csd.net.au, December, 2004; www.affa.gov.au/content/pbr_database/docs/2004274.doc, December, 2004).

1.4.2.2.2 Transgenic

Despite screening thousands of local and exotic lines of cotton, no definitive resistance to Australian Fov has been found (Constable et al., 2004). The search for resistance has also led to a wider search for novel genes to introduce into existing cotton varieties (Becerra Lopez-Lavalle et al., 2002). Genetic transformation of cotton plants with a synthetic peptide gene has recently been shown to confer antifungal traits (Rajasekaran et al., 2005). The first Australian field trial with a defensin is currently underway (http://www.ogtr.gov.au/pdf/ir/dir063appsum.pdf, December 2006).

Australian cotton farms grow several genetically modified cotton varieties. Uptake of transgenic varieties is strong, with approximately 95% of growers planting Bollgard II® or Roundup Ready® varieties in the 2004-05 season (http://www.cottonaustralia.com.au/factSheets/resources/biotechnology2.pdf, Dec 2006). The gram-positive bacterium, Bacillus thuringiensis (Bt) produces insecticidal proteins toxic to the major pest of cotton, Helicoverpa spp. (Olsen and Daly, 2000). Transgenic Bt cotton expressing these proteins offer increased protection, and has been released in Australia as INGARD® and Bollgard® cotton. First grown commercially in 1996, this technology was appended and released as the two-gene transgene, Bollgard® II, in 2003 and has provided protection for crops with decreased pesticide inputs (http://www.monsanto.com.au/content/cotton/bollgard_ii_cotton/publicrelease.pdf, December, 2003). Roundup Ready® cotton has been available since 2000, and its tolerance to the herbicide glyphosate continues to provide benefits in weed management (CSD Extension & Development Team, 2005).

Many favoured varieties grown in Australian cotton fields at the time of the discovery of Fov were highly susceptible to Fov. It was initially observed that transgenic cotton varieties were particularly susceptible compared with their conventional counterparts.
(Kochman et al., 2002), a phenomenon also observed in the US (Colyer et al., 1999). This problem may be explained by the inadvertent reintroduction of susceptibility via crosses made to recover various transgenes (Bell, AA, unpublished in Bell et al., 2003), or simply due to the dependence of the transformation protocol on the susceptible Coker variety (Murray et al., 1993). Recent breeding efforts have focused on selecting for resistance to Fusarium wilt, resulting in the release of several relatively resistant Bollgard® varieties (http://www.afaa.com.au/news/news-1259.asp; January, 2006).

The introduction of foreign genes into cotton requires both a gene transfer mechanism and a method of regenerating plants from tissue culture. Several methods have produced transgenic cotton including particle bombardment (Finer and McMullen, 1990; Rajasekaran et al., 2000) and meristem transformation (Gould et al., 1991; Zapata et al., 1999) but the practicalities of these techniques remain problematic (John, 1997; Leelavathi et al., 2004). Somatic embryogenesis was first utilised in the regeneration of cotton by Davidonis and Hamilton (1983), and continues to provide the basis for regeneration of cotton plants (Finer, 1988; Kumria et al., 2003; Leelavathi et al., 2004; Sakhanokho et al., 2001). Gene transfer mediated by Agrobacterium vectors is useful in cotton transformation (Firoozabady et al., 1987; Gasser and Fraley, 1989) and is the most common method for cotton transformation (Leelavathi et al., 2004). However, regenerating transformed cotton is strongly influenced by genotype (Gawel et al., 1986) and the ‘Coker’ variety is most amenable to these techniques (Davidonis and Hamilton, 1983; Murray et al., 1993; Leelavathi et al., 2004). This superseded variety has limited commercial relevance to the Australian cotton industry due to its low agronomic value and level of susceptibility to Fusarium wilt (Allen, S. pers.comm). Research is underway to improve the efficiency of embryogenesis, thus allowing the direct transformation and regeneration of elite Australian cotton varieties (Cousins et al., 1991; Poon et al., 2004).

1.5 Disease resistance

Plants are invariably subject to persistent attack by numerous microbes throughout their life cycle. Although they may suffer some damage, many will survive and prosper, while the exceptional few will develop disease symptoms.

All plants respond to pathogen attack by activating a coordinated set of defence responses (Lamb et al., 1989). In a resistant host plant, this response will act to slow,
kill or contain the pathogen, whereas the susceptible plant’s defences will fail due to inadequate timing, location or quantity of the response (Dixon and Lamb, 1990). Contrary to susceptibility resulting from an absence or inactivation of defence mechanisms:

Resistance and susceptibility are...the extremes of a continuum upon which most host-parasite interactions sit...Resistance is quantitative – resistant hosts prevent or slow the development and reproduction of a higher proportion of pathogen propagules than susceptible hosts (Guest and Brown, 1997).

A pathogen will be equipped with strategies designed to avoid or inhibit the host’s recognition or defences. Pathogens have to overcome two types of plant defences to initiate disease: structural and chemical. These defences may be pre-existing, or induced upon the plant’s recognition of its attacker. Preformed defences such as thick cuticle or antimicrobial compounds may be present before infection (Jackson and Taylor, 1996; Osborne, 1996). Recognition of the pathogen induces active defence mechanisms, including physical barriers and the production of antimicrobial compounds such as β-1,3 glucans and phenolics (Benhamou et al., 1994; Hutcheson, 1998).

Systemic acquired resistance refers to the inducement of systemic defence responses that confer broad-spectrum protection against pathogens (Ryals et al., 1996; Durrant and Dong, 2004). The activation of the host plant’s natural defence mechanisms can be achieved by exposing the host to either a chemical or biological ‘activator’ prior to challenge by a pathogen. Defence responses are expressed more rapidly and intensely after the development of systemic acquired resistance (Hutcheson, 1998).

There are few examples of chemicals being used to induce resistance against soilborne and vascular diseases. The application of benzothiadiazole (BTH) to grapevines resulted in a reduced incidence of root knot nematodes (Owen, 1999) and has been used successfully in combination with fungicides to reduce Phytophthora cinnamomi root disease in nursery plants (Ali et al., 2000). Preliminary experiments involving BTH seed treatment of cotton demonstrated some control of seedling death due to Fusarium wilt at a high disease pressure site (Allen, 2001b). Seed treatment with has also been shown to induce resistance of cotton seedlings to black root rot (Mondal et al., 2000). Glasshouse trials involving the application 2,6-dichloroisonicotinic acid to seedlings
rendered the next emerging leaves less susceptible to *A. macrospora* (Brock *et al*., 1994). The use of isonicotinic acid also resulted in a significant reduction of verticillium wilt under field conditions (Colson-Hanks *et al*., 2000).

In addition to induced host plant resistance, biocontrol agents may operate through multiple mechanisms including the production of antifungal compounds, competition for nutrients, niche exclusion and hyperparasitism. Examples of biological activators of resistance being used in widespread cotton production are rare. A naturally occurring antagonistic bacterium was isolated from Australian cotton fields and subsequently tested as a biocontrol agent against Fusarium wilt of cotton (Putcha *et al*., 2000). Introduction of the bacterium to the cotton root zone in infested areas resulted in increased plant survival, reduced disease severity and a reduction in yield losses (Tingay *et al*., 2002), however the mechanisms responsible are unknown. Effective Microorganisms (EM) is a biological product that has been used in organic cotton production in the US and has reduced the incidence of several cotton diseases including Fov (Bill Worthey, pers.comm.). Many biological products have been tested in Australian field trials without resounding success, however research continues on the use of several products including non-pathogenic *F. oxysporum* (Allen, 2004b; Allen, 2004a).

Jorge *et al*. (1992) found that tomato plants inoculated simultaneously with an inducer (organism to which the plant is resistant) and a pathogen displayed a higher level of resistance than plants infected with the pathogen alone. The study found that the magnitude of the elevated resistance diminished as the ratio of inducer to pathogen was decreased. This is in line with other research involving Fusarium wilt of tomato that has found that the concentration of the inducer inoculum must be equal or greater than that of the challenger to achieve the highest levels of resistance (Davis, 1968; Wymore and Baker, 1982). As the time between inoculations of inducer and pathogen was increased 3-5 days, the increase in resistance was decreased or lost. Jorge *et al*. reported the optimum time of inoculation of the inducer was simultaneous to that of the pathogen (Jorge *et al*., 1992). This is contrary to work on Verticillium wilt protection in cotton (Schnathorst and Mathre, 1966) and Fusarium wilt of tomato (Davis, 1968; Wymore and Baker, 1982) that found plants needed to be inoculated with the inducer several days before the pathogens for increased resistance to be observed.
These characteristics may in fact describe direct competition between the inducer and pathogen, rather than the induction of resistance. More in-depth microscopic analysis is required before this type of protection can be attributed to induced resistance rather than other possibilities such as competitive exclusion at the root surface.

The colonisation of tomato roots by a pathogenic *F. oxysporum* f. sp. *lycopersici* (Fol) was compared to that of a non-pathogenic strain of *F. oxysporum* and found to have the same ability to colonise the root surface (Olivain and Alabouvette, 1999). Another study found that the intensity of root colonisation of Fol and another non-pathogenic strain was indeed initially similar, but divergent after the second day after inoculation, when the pathogen continued to grow while the non-pathogen diminished (Recorbet and Alabouvette, 1997). Olivain & Alabouvette (1999) found the penetration process was similar for both fungi, and that penetration occurred through both the well-differentiated and apical zones of the tomato root. Intensive defence responses such as cytoplasmic aggregation and cell wall thickening were observed after inoculation with both fungi, however the responses were more swift and intense after infection with the non-pathogen.

Knowledge of the intimate interactions between host and pathogen is necessary to understand why some varieties are able to fend off disease. Identification of plant responses essential to successful defence and weak points in pathogenesis are of great practical significance in the production of new disease resistant plants. Insights into host-pathogen interactions can help to focus conventional plant breeding or genetic engineering for disease resistance.

### 1.5.1 The infection process of *Fusarium oxysporum*

*Fusarium oxysporum* has a broad host range including many agricultural, horticultural and ornamental species. Its extensive pathogenic specialisation comes with disparate mechanisms for infection and colonisation of its varied hosts. The host-pathogen interactions of several plants and their respective *formae speciales* will be outlined.

The behaviour before penetration of *F. oxysporum* on the root surface of many hosts has been described. Several *formae speciales* including Fov form an extensive compact mycelium on the root surface of their respective hosts (Rodriguez-Galvez and Mendgen,
Conidia of Fov and *F. oxysporum* f. sp. *lilii* germinate 6 h after inoculation on the surface of cotton and lily roots, respectively (Baayen and Rijkenberg, 1999; Rodriguez-Galvez and Mendgen, 1995b). Hyphae of both pathogens commonly grow along the anticlinal wall junctions of epidermal cells. Although penetration is likely to be temperature-dependent (Rodriguez-Galvez and Mendgen, 1995b; Gardiner *et al.*, 1989), it occurs 6 hours after inoculation of lentil roots (Bhalla *et al.*, 1992), and within 24 hours in susceptible tomatoes and lilies (Baayen and Rijkenberg, 1999; Brammall and Higgins, 1988).

The meristematic and lateral root regions of asparagus are reported to be major infection sites for *F. oxysporum* f.sp. *asparagi* (Smith and Peterson, 1983). Rodriguez-Galvez and Mendgen (1995b) found that Fov penetration could occur in both the meristematic and elongation zones of cotton plants, but the majority of sites were close to the root tip, in agreement with previous work (Smith *et al.*, 1981; Nelson *et al.*, 1981). Likewise, *F. oxysporum* f. sp. *lilii* germ tubes have been observed to grow large distances before penetrating just behind the root cap (Baayen and Rijkenberg, 1999).

Direct intracellular penetration of *F. oxysporum* has been reported in flax, tomato and cotton (Turlier *et al.*, 1994; Olivain and Alabouvette, 1999; Rodriguez-Galvez and Mendgen, 1995b). Appressoria are sometimes associated with intracellular penetration, as is hyphal constriction at the point of cell wall penetration (Parry and Pegg, 1985; Bishop and Cooper, 1983; Beswetherick and Bishop, 1993). Both intercellular and intracellular penetration by *F. oxysporum* occurs in asparagus although the latter is dominant, particularly at root tips (Smith and Peterson, 1983). Localised plant cell wall dissolution has been observed at the point of penetration in the interactions between *F. oxysporum* f. sp. *lycopersici* and *pisi* and tomato, as well as in other wilt interactions (Robb *et al.*, 1975; Robb *et al.*, 1977). Cell wall-degrading enzymes are proposed to be responsible for wall degradation even in the absence of direct contact between the pathogen and wall substrate in Verticillium wilt of tomato (Cooper and Wood, 1980).

In contrast, the mode of penetration of *F. oxysporum* f. sp. *medicaginis* in lucerne roots most commonly observed by Parry and Pegg (1985) was intercellular. Likewise in *F.
oxysporum f. sp. lili, where some intracellular growth has been observed, but only as a result of the slow infringement of the predominant intercellular hyphae, culminating in a breach of the cell wall rather than aggressive direct penetration (Baayen and Rijkenberg, 1999). Penetration of vanilla roots by *F. oxysporum* f. sp. *vanillae* was almost exclusively associated with mechanical damage caused by insects, nematodes or other agents (Alconero, 1968). Similarly, a root-knot nematode-Fusarium wilt disease complex of cotton exists in the USA. Commercial cotton cultivars generally require infection by both *Meloidogyne incognita* and Fov for full expression of Fusarium wilt (Smith et al., 1981) and the management of both pathogens is co-dependant (Jeffers and Roberts, 1993). Furthermore, exposure to root-knot nematodes can induce susceptibility in cotton, tomato and squash plants normally resistant to Fusarium wilt (Starr et al., 1989). There is no evidence that Australian Fov requires such an association to infect Australian cottons, or the American cotton cultivars considered ‘resistant.’ This difference highlights the limitations of the findings of studies based on foreign isolates, and the need for detailed investigation into the domestic isolates of Fov.

### 1.5.2 Phenolic and terpenoid defence responses of cotton

The restriction of pathogen growth in vascular tissue is involved in wilt resistance in cotton (Wilhem et al., 1974b; Harrison and Beckman, 1982). It has been proposed that this restriction is a consequence of either the formation of physical barriers (Bugbee, 1970; Garber and Houston, 1967) or the accumulation of phytoalexins (Bell, 1969; Mace et al., 1976; Kaufman et al., 1981) or both (Mace, 1978; Mace et al., 1974a). Harrison and Beckman (1982) reported that accumulated terpenoid aldehydes had little effect on pathogen growth, and concluded that Fusarium wilt resistance may be solely dependant on physical localisation. However, supplemental work showed differential phytoalexin accumulation between resistant and susceptible cotton varieties within ten days of inoculation with Fov (Zhang et al., 1993) and *V. dahliae* (Mace et al., 1985). The physical and chemical responses of cotton to wilt pathogens are now widely considered to be complementary mechanisms of defence. Further research is necessary to confirm the presence and contribution of these defence responses to the resistance of Australian cotton to local isolates of Fov.

Limited work has been undertaken on the changes to host cells outside the vascular system. Prominent wall appositions surrounding invading Fov hypha were observed in both the meristematic and elongation zones of the cotton rhizodermis by Rodriguez-
Galvez and Mendgen (1995a), however no comparison was made between susceptible and resistant hosts. The same authors noted that Fov induced increased callose in host cell walls, although they found no evidence that these modifications stopped fungal growth (Rodriguez-Galvez and Mendgen, 1995a).

The responses of the vascular tissue of infected cotton seedlings have been studied in some detail, to test the assertion that “the restriction of fungal spread in the vascular tissue is a characteristic of the resistance of cotton plants to *Fusarium oxysporum* f.sp. *vasinfectum*” (Shi et al., 1993). The focus of ultrastructural studies has largely been xylem vessels and their immediately adjacent parenchyma cells, referred to as “contact cells” (Shi et al., 1991a; Mueller and Morgham, 1993).

The physical localisation of invading Fusarium pathogens by gels and tyloses has been described in many hosts. The sealing-off of xylem vessels by host production of unidentified gels and the balloon-like intrusion of a parenchyma cell’s protoplast into an adjacent xylem vessel (known as a tylose) has been documented in tomato (Beckman et al., 1972; Harrison and Beckman, 1987), broccoli (Beckman, 1968), banana (Beckman et al., 1961; Vandermolen et al., 1987) as well as cotton (Mace, 1978; Vandermolen et al., 1977). The occlusion of vessels by tylose-like structures was identified as a cotton resistance mechanism to *V. dahliae* after examination of fresh tissue by light microscopy (Harrison and Beckman, 1982; Mace, 1978). However, TEM and further histochemical testing elucidated the true nature of the globular structures that resembled tyloses (Shi et al., 1992; Mueller and Morgham, 1993). Their collapse and removal by treatment with organic solvent and histochemical tests suggested these structures were predominantly lipophilic, presumably terpenoids (Shi et al., 1991b; Shi et al., 1992). These accumulations in vessel lumens were secretion products from contact cells that took many shapes and forms, and could completely occlude vessels infected with *V. dahliae* and Fov.

Shi et al. (1991) observed that the majority of contact cells underwent cytoplasmic reorganisation and increased metabolic activity after inoculation with Fov. These events resulted in the secretion of osmiophilic materials into the adjoining vessel lumen. With further research, the same authors proposed these secretion products included toxic terpenoid aldehydes that may completely block vessel lumens, forming a barrier of chemical inhibition and physical restriction to the pathogen (Shi et al., 1992). Pathogen
expansion beyond these responses in a resistant cultivar was limited compared with a susceptible cultivar, as the responses were faster and more pronounced in the resistant cultivar. The authors concluded that resistance at the ultrastructural cell level was quantitative rather than qualitative.

Changes in host secondary metabolism are common plant responses to pathogen challenge. These defence responses may include the production and accumulation of phenolic compounds (Tan et al., 2004; Dixon and Paiva, 1995) and terpenoid phytoalexins (Hammerschmidt, 1999).

1.5.2.1 Phenolics
Disease resistance in plants sometimes involves phenolic metabolism and related oxidative enzymes (Hare, 1966; Anderson et al., 1991). Phenolic compounds whose oxidation products are fungitoxic can be released by the plant in response to pathogen challenge. Both phenol accumulation and the activity of the oxidative enzymes may be important in the host-pathogen interaction. Strong evidence of a link between polyphenol oxidase activity and disease resistance was established when an overexpression of the enzyme in tomato plants resulted in increased resistance to Pseudomonas syringae (Li and Steffens, 2002).

The role of phenolic compounds in resistance to Fusarium wilt in cotton has not been investigated, but phenolic compounds play a role in resistance of cotton to other pathogens. In the interaction between cotton and Alternaria macrospora, resistant plants exhibit higher phenolic accumulation and stronger polyphenol oxidase activity than susceptible plants (Bashan, 1986). The flavanol, catechin, accumulates in response to infection of cotton leaves by V. dahliae and acts to inhibit hyphal growth and sporulation of the pathogen (Howell et al., 1976). In fact, the infection-induced synthesis of the flavanols catechin and galloatechin was much more intense in Verticillium-wilt resistant cotton compared with the susceptible variety (Mace et al., 1978). The higher concentration of flavanols in younger leaves is also thought to be responsible for their greater resistance to colonisation by V. dahliae (Bell and Stipanovic, 1978; Howell et al., 1976). The presence of inhibitory flavonols in the endodermis of cotton presents an effective barrier to the colonising V. albo-atrum (Garber and Houston, 1966; Mace and Howell, 1974). Some of the phenol-related defence responses may involve terpenoid compounds.
1.5.2.2 Terpenoids

_Gossypium_ spp. produce a diverse array of terpenoid defence compounds that function to protect cotton from environmental and biological stress, including herbivore and pathogen attack (Bell, 1986). Many of these compounds are constitutive defences that accumulate in green tissues and roots of healthy unchallenged plants (Bell _et al._, 1978; Mace _et al._, 1974a) and provide barriers to insects, nematodes and pathogens (Bell, 1984). However defence compounds may also be elicited by pathogen attack, or by physical or chemical stress (Bell, 1967; Bell _et al._, 1975; Stipanovic _et al._, 1975a; Essenberg _et al._, 1990; Bell and Christiansen, 1968).

1.5.2.2.1 Biosynthetic pathway of cotton terpenoids

The large group of defence compounds found in cotton share a cadinane carbon skeleton. The sesquiterpene cyclase, cadinene synthase, catalyses the first committed step in cadinane biosynthesis (Benedict _et al._, 1995). Conversion of (E,E)-farnesyl diphosphate to (+)-δ-cadinene is catalysed by δ-cadinene synthase which is then metabolically converted to desoxyhemigossypol (dHG), desoxymethoxyhemigossypol (dMHG), hemigossypol (HG) and methoxyhemigossypol (MHG) (Davis _et al._, 1996a; Davis and Essenberg, 1995).

![Biosynthetic Pathway Diagram](image)

_Figure 1.4 Proposed biosynthetic pathway for cotton terpenoids within the acetate-mevalonate pathway._ Adapted from Liu _et al._ (1999) and Benedict _et al._ (2004).

The proposed biosynthetic pathway places dHG as the key intermediate (Figure 1.4). The enzyme desoxyhemigossypol-6-O-methyltransferases (dHG-6-OMT) specifically methylates dHG to dMHG (Liu _et al._, 1999). This effectively catalyses the key step leading to the biosynthesis of all of the methylated terpenoids; compounds in which the hydroxyl group at C-6 is methylated. Hydrogen peroxide (H₂O₂) initiates an oxidative process that readily decomposes dHG, resulting in the production of a series of free
radicals and eventually, HG (Mace and Stipanovic, 1995). The conversion of dMHG to MHG is likely to take place via a reaction analogous to the conversion of dHG to HG (Liu et al., 1999).

Gossypol (G) is formed by peroxidative dimerization of hemigossypol (Veech et al., 1976). Oxidation of the phenolic portion of gossypol leads to the generation of superoxide, which in turn can serve as a precursor of other active oxygen species (Kovacic, 2003). The sesquiterpene quinones, hemigossypolone (HGQ) and methoxyhemigossypolone (MHGQ), and the sesterterpenes, H, and B, are synthesised in plant tissues only when exposed to light (Bell et al., 1978). The synthesis or activity of the enzyme that catalyses quinone formation occurs only in tissues containing chlorophyll and is stimulated by light.

Another biosynthetic pathway is proposed to branch from (+)-δ -cadinene, which leads to cadalenes and lacinilenes (Wang et al., 2003). Antibacterial 2,7-dihydroxycadalene (DHC), lacinilene C (LC), lacinilene C 7-methyl ether (LCME) and 2-hydroxy-7-methoxycadalene (HMC) are localised in hypersensitively necrotic cells close to X. axonopodis pv. malvacearum colonies on cotton leaves (Essenberg et al., 1992b). These compounds accumulate in cotton specifically in response to inoculation with X. axonopodis pv. malvacearum (in addition to dHG and HG) (Abraham et al., 1999), but are not elicited by fungal pathogens.

Gossypol is a predominant cotton pigment and is distributed throughout the leaves, stem, root cortices and floral parts of cotton (Bell et al., 1978). Gossypol is constitutively located in sub-epidermal lysigenous glands of all green tissues and in epidermal and cortical cells in the young roots of healthy plants (Bell, 1969; Mace et al., 1974a). It is the predominant terpenoid found in cottonseed glands (Stipanovic et al., 1988). In addition to gossypol, foliage and floral glands also contain hemigossypolone and heliocides 1-4 (Stipanovic et al., 1988; Mahoney and Chan, 1985), and roots contain a mixture of other gossypol-related compounds (Stipanovic et al., 1975b).

The toxicity of these terpenoids to non-ruminants limits use of cottonseed and associated products as food (Reiser and Fu, 1962). The mammalian toxicity of gossypol stimulated the search for glandless, low-gossypol genotypes of cotton. “Glandless” cottons derived from G. hirsutum mutants completely lack lysigenous cavities, but are
still able to synthesise small amounts of gossypol and related terpenoids (Halloin and Bell, 1979; Beckmann and Heitefuss, 1998). The earliest production of glandless cotton did indeed lack the terpenoids normally contained within glands (McMichael, 1960), but it was subsequently shown that dHG and HG could still accumulate after infection with X. axonopodis pv. malvacearum (Abraham et al., 1999). Conversely, Brubaker et al. (1996) described an endemic Australian Gossypium subgenus with differentiated immature cavities, but no measurable terpenoid aldehydes. These examples indicated that terpenoid aldehyde biosynthesis and lysigenous cavity morphogenesis could be uncoupled and the authors suggest this could be the key to the development of cottonseed free of terpenoid aldehydes (Brubaker et al., 1996). More recently, this uncoupling of terpenoid aldehyde synthesis and gland formation was confirmed with a report of a cotton variant with mature glands but suppressed levels of terpenoid aldehydes (Benedict et al., 2004). This variant was transformed with antisense (+)-δ-cadinene synthase cDNA and produced seed with gossypol levels close to that fit for human consumption. However, foliar concentrations in resulting plants were also reduced, increasing plant vulnerability to insect attack. More recently, tissue-specific RNAi has been used to disrupt terpenoid biosynthesis in cottonseed tissue by interfering with δ-cadinene synthase expression (Sunilkumar et al., 2006). The result was to reduce gossypol in seed whilst retaining a full complement of gossypol and related terpenoids in the rest of the plant to maintain their function in plant defence.

1.5.2.2.2 Cotton terpenoids related to insect attack
Terpenoids are an integral component of herbivore resistance in cotton. Plants that lack terpenoids are subject to preferential feeding by insects and attacked by non-typical pests (Bottger et al., 1964; Jenkins et al., 1966; Lukefahr et al., 1966).

The devastating pest of cotton, Heliothis virescens avoided feeding on gossypol glands, and the growth of neonate larvae was decreased when fed squares of highly glanded cotton (Hedin et al., 1992). The glands of hosts resistant to H. virescens contained significantly higher amounts of HGQ and heliocides H1 and H2. The toxicity of gossypol, HGQ and the heliocides have been demonstrated against many cotton pests (Bottger et al., 1964; Stipanovic et al., 1977). However,methylation usually renders these compounds less toxic to insects and fungal pathogens (Stipanovic et al., 1977; Mace et al., 1985; Zhang et al., 1993).
1.5.2.3 **Cotton terpenoids related to pathogen challenge**

The production of terpenoids has been observed in response to several cotton pathogens including Fov (Mace *et al.*, 1974a) *V. dahliae* (Zaki *et al.*, 1972a), *R. solani* (Hunter *et al.*, 1978a) and *X. axonopodis pv. malvacearum* (Essenberg *et al.*, 1982; Essenberg *et al.*, 1990).

Gossypol was the first compound ascribed a defence role in cotton, however several more terpenoids have subsequently been described as more important in disease resistance due to their roles as phytoalexins (Bell, 1967; Mace *et al.*, 1985; Zhang *et al.*, 1993). Phytoalexins are defined as low molecular-weight plant antibiotics produced *de novo* at the infection site in response to pathogen attack or elicited by abiotic agents (Paxton, 1981). The term “gossypol” has been used to include all cotton terpenoid phytoalexins (Fryxell *et al.*, 1992), while other times gossypol has been excluded from phytoalexin studies (Zhang *et al.*, 1993). All of the cotton defence compounds including gossypol are encompassed by the terminology “antimicrobial terpenoids.”

Antimicrobial terpenoids have been shown to be broadly involved in disease resistance in cotton, but their contribution to defence remains undefined. Their presence has been determined colourimetrically (Bell, 1967), histochemically (Mace *et al.*, 1974a) and by thin layer chromatography (Zaki *et al.*, 1972b). High pressure liquid chromatography analysis provides a more accurate measure of terpenoid aldehydes and the means of correlating observations with specific terpenoid aldehydes (Stipanovic *et al.*, 1988).

The phytoalexins hemigossypol (HG), desoxyhemigossypol (dHG), methoxyhemigossypol (MHG), and desoxymethoxyhemigossypol (dMHG) have been identified and implicated in the resistance of cotton to Fov and *V. dahliae* using HPLC techniques (Bell *et al.*, 1975; Stipanovic *et al.*, 1975a; Mace *et al.*, 1985; Zhang *et al.*, 1993).

Antimicrobial terpenoids elicited by the vascular wilt pathogens *V. dahliae* and Fov differ qualitatively depending on cotton species [*G. hirsutum, G. barbadense, G. arboreum* (Heinstein, 1985; Wang *et al.*, 2004c) or *G. herbaceum* (Bezemer *et al.*, 2004)], and variety (Khan *et al.*, 1999). Occurrence of antimicrobial terpenoids is most commonly reported in relatively resistant species: *G. hirsutum* varieties in the case of Fusarium wilt, and *G. barbadense* varieties in Verticillium wilt studies. Root and
vascular tissues interface with both of these soil-borne vascular pathogens, and the terpenoid content of these tissues vary.

1.5.2.3.1 Roots
Mace et al. (1974a) observed the localisation of gossypol and related terpenoids in cytoplasmic "granules" in the healthy cotton root epidermis and cortex. Their distribution was not unlike that of phenols in cytoplasmic particles in specialised parenchyma cells of banana roots (Beckman and Mueller, 1970). The identification of HG and MHG in healthy cotton root tissue was subsequently confirmed (Stapanovic et al., 1975b). The former study found an absence of terpenoids in the root cap zone of cotton plants (Mace et al., 1974a) which led the authors to suggest their absence may be causally related to the susceptibility of the root tip zone to nematode penetration (Minton, 1962). They proposed that this distribution would influence *V. dahliae* which infects primarily through the root cap zone and that the intensification of terpenoids where lateral roots ruptured the tap root epidermis was associated with the failure of these areas to serve as significant infection sites (Garber and Houston, 1966; Mace et al., 1974b).

1.5.2.3.2 Stele
Infection by *V. dahliae* induces the biosynthesis of phytoalexins in the stele tissue of all cotton, but more rapidly in resistant than susceptible varieties (Zaki et al., 1972a). While Mace et al. (1978) detected phytoalexins histochemically in both infected resistant and susceptible hosts, the response lagged behind the spreading pathogen in the susceptible host, but accumulated ahead of the advancing pathogen in the resistant host. In infected plants, terpenoid aldehyde phytoalexins initially occurred in scattered paratracheal parenchyma cells appressed to xylem vessels, before diffusion into sites of fungal colonisation (Mace et al., 1976). A similar process was observed through ultrastructural examination of cotton infected with *V. dahliae*. The cells adjacent to infected vessels responded with increased cytoplasmic activity and the direct secretion of osmiophilic materials, presumably terpenoids, into the vessel lumens to coat the invading fungal hyphae (Gaumann, 1957; Mueller and Morgham, 1993).

The antimicrobial terpenoids dHG, HG and MHG were localised in specialised paravascular parenchyma cells of *V. dahliae*-infected cotton (Mace et al., 1989; Stipanovic et al., 1975a; Mace et al., 1976). It has been proposed the different terpenoid
phytoalexins are synthesised and accumulate in the same cell (Mace et al., 1989), before DHG, the most water soluble (Mace et al., 1985) diffuses into the vessel lumen where it would act directly on the pathogen (Mace et al., 1989), or undergo oxidation to the relatively water insoluble HG (Stipanovic et al., 1975a). Indeed, terpenoids have been detected histochemically on the surface of V. dahliae mycelia and conidia in vessel lumens (Mace, 1978; Mace et al., 1976; Mace et al., 1984) where it was proposed they may exert fungicidal action (Mace, 1978).

Bugbee (1970) suggested that the formation of antibiotics following xylem vessel occlusion was also the cotton plant's main defence against Fov. More specifically, Kaufman et al. (1981) pointed to the rapid accumulation of gossypol-like compounds in infected cotton xylem as a factor in Fov resistance. A negative correlation between the terpenoid aldehyde content of the roots of 17 cotton strains and Fusarium wilt incidence has been described (Hedin et al., 1984). However, Harrison and Beckman's (1982) investigation into the dynamics of vascular colonisation by Fov found phytoalexin accumulation occurred more intensely in the resistant host, but no direct fungal inhibition could be observed. When the interaction was monitored every seven days, only physical localisation of the fungus by means such as vessel occlusion and apposition layer formation was demonstrated, but the sampling time proved inappropriate for observing the onset of phytoalexin accumulation, and led to misleading conclusions. Shi et al. (1993), however, focussed on the period of three to four days after inoculation and concluded that chemical inhibitors were indeed integral in resistance to Fov.

After specifically relating HG accumulation in different cultivars to their resistance to Verticillium and Fusarium wilts, Eldon and Hillocks (1996) concluded that HG production was the primary mechanism of resistance in Verticillium wilt, but not Fusarium wilt. This investigation, however, failed to assess the relative accumulation of any other phytoalexins, or the low solubility of HG in water (Eldon and Hillocks, 1996). Induced terpenoids other than HG accumulate at higher concentrations, and the solubility of HG under the conditions found in cotton stele was likely to limit its effect on V. dahliae (Mace et al., 1985). The identification of dHG, HG, dMHG, and MHG in the stem stele of plants inoculated with V. dahliae (Mace et al., 1985) and Fov (Zhang et al., 1993) by HPLC analysis confirmed their status as phytoalexins.
1.5.2.4 Effects of terpenoids

1.5.2.4.1 Gossypol

The drug and toxic effects of gossypol on animals have been well documented since it was described first as a pigment of cotton seed (Adams et al., 1960), then importantly as a male anti-fertility agent (Anonymous, 1978; Shi et al., 2003). It has consequently been found to have drug actions including anticancer, antipathozoan, antiparasitic and antiviral, while also being a carcinogen, reproductive toxin, carditoxin, and hepatotoxin (Shelley et al., 2000; Kovacic, 2003).

There is substantial evidence for the formation of free radicals from gossypol (Kovacic, 2003). In vitro studies of the effect of gossypol on mouse islet cells found cytotoxicity was by the generation of noxious free radicals (Grankvist, 1989). Gossypol causes an induction of superoxide in rat liver microsomes and a hydrogen peroxide-derived radical species in human sperm (De Peyster et al., 1984). Active oxygen species are also involved in the spermicidal activity of gossypol. Catalase allowed gossypol-treated sea urchin sperm to function normally, where fertilisation was otherwise inhibited by hydrogen peroxide (Coburn et al., 1980).

Free radicals react in the plasmalemma and subcellular sites with polyunsaturated fatty acid esters and inert cellular components such as proteins (Pryor, 1973). Gossypol has been shown to have an effect on rat membrane structure and membrane-associated function (De Peyster et al., 1986) and there is some indication that dHG also disrupts the membranes of V. dahliae conidia (Mace, unpublished results in Mace and Stipanovic, 1995).

1.5.2.4.2 Gossypol-related phytoalexins

All of the cotton terpenoid phytoalexins are toxic to Fov and V. dahliae (Mace et al., 1985; Zhang et al., 1993). In addition to being the most toxic, dHG is also the most water-soluble, making it the most likely terpenoid to reach high concentrations in the cotton stele (Mace et al., 1985). Mace et al. (1989) showed by histochemical localisation and chromatography of stele extracts that dHG is found on V. dahliae hyphae within vessel lumens.

Mace and Stipanovic (1995) investigated whether the actual decomposition of dHG might be involved in its fungitoxic action. The authors suggest that free radicals are
produced when dHG decomposes, including the extremely reactive hydroxyl and other free radicals of dHG (Mace and Stipanovic, 1995). The linkage between decomposition and its toxicity was demonstrated by stabilising the H$_2$O$_2$-mediated process by the decomposition of H$_2$O$_2$ by catalase or countering oxidation with glutathione, which had the effect of decreasing dHG toxicity. This suppression of toxicity provides evidence for the role of active oxygen species and/or free radicals in the process.

Active oxygen species production by plants in response to pathogens has been hypothesised to have many effects, from direct antimicrobial activity to broader roles in other defences including lignin and phytoalexin production and the hypersensitive response (Baker and Orlandi, 1995). Superoxide release has been found to be a necessary precursor for the hypersensitive response in Nicotiana tabacum (Able et al., 1998), and integral for phytoalexin accumulation in cells resistant to Phytophthora spp. (Perrone et al., 2003). The generation of active oxygen species is also important in local and systemic defence responses of cotton challenged by an avirulent race of X. axonopodis pv malvacearum (Martinez et al., 2000).

The implication of active oxygen species in the biological activity of gossypol in a diverse set of studies and publications, and the roles of free radicals in plant response to pathogen attack lends support to the hypothesis that they are ultimately responsible for the toxicity of dHG to V. dahliae (Mace and Stipanovic, 1995).

1.5.2.4.3 Toxicity

Crude stele extracts from V. dahliae-infected cotton plants have been shown to completely inhibit mycelium growth when incorporated into growth medium (Garas and Waiss, 1986). Ethyl acetate was used to extract terpenoids from the tissue three days after stem-puncture inoculation for use in V. dahliae growth assays. However, HPLC analysis of the same extract found no correlation between the total phytoalexin content and varietal resistance, indicating that the toxicity of the constituents might vary (Garas and Waiss, 1986). The toxicities of individual purified phytoalexins to V. dahliae and Fov were measured by direct turbidimetric analysis of conidia in a buffered nutrient solution to replicate the pH of xylem fluid (Mace et al., 1985; Zhang et al., 1993). All of the terpenoid phytoalexins were toxic to the pathogens. The ED$_{50}$'s of dHG, dMHG, HG, MHG were 8.8, 13.4, 29.3, 35 μg mL$^{-1}$ fresh tissue respectively, however concentrations well below those that were fungicidal inhibited the germination of
conidia. Only dHG had the water solubility apparently required to reach the concentration capable of killing all fungal propagules (30µg mL⁻¹) in the aqueous medium of infected xylem vessels.

While gossypol is present in root and stele tissues, its interaction with wilt pathogens has received little attention. Zhang et al. (1993) found gossypol in both infected and uninfected (but wounded) stele tissue extract, but performed no further analysis. Some of the related antimicrobial terpenoids are constitutively expressed in other cotton tissues (Mace et al., 1974a; Stipanovic et al., 1975b). The nomination of the antimicrobial terpenoids as phytoalexins is tissue-specific, and should not discount a role for gossypol in resistance to wilt pathogens. The toxic effects of gossypol on plant pathogens is not as widely reported as on insects and vertebrates (Abou-Donia, 1989). General antifungal activity has been documented (Abou-Donia, 1976) and there is evidence of toxicity to the phytopathogen, R. solani (Puckhaber et al., 2002).

1.5.3 The vascular wilt disease model

In “The nature of wilt diseases of plants,” Beckman (1987) draws together fragmentary information and details of wilt diseases of various plants to provide a generalised model of vascular wilt. Specific events, independently studied, are integrated to formulate a model where they are connected in time and space. This perspective on the complete dynamic interaction that results in wilt disease allows for speculation and hypothesis formulation where knowledge gaps exist, and also for teasing out areas requiring more intense investigation.

Beckman (1987) utilises a concept first proposed by Talboys (1957) where the host-pathogen interactions involved in wilt diseases fall within three phases:

1. the Primary determinative phase, which includes the infection and penetration of the outer tissues and events up to entry of the vascular system;

2. the Secondary determinative phase, which includes the interactions involved in vascular colonisation of the host, and finally;

3. the Expressive phase, in which the host develops symptoms of wilt.

The Primary determinative phase describes the dynamic interaction between the potential pathogen, Fov, and the roots of the cotton plant (Talboys, 1957; Beckman,
1987). These events determine whether the pathogen is able to: i) invade the root; ii) traverse the cortical tissues; and iii) gain entry to the vascular elements. The mode and circumstances of entry by Fov into the vascular system of cotton can be of help to minimise the impact of the disease through cultural practice.

The Secondary determinative phase describes the processes of defence within the vascular tissues of the infected plant to localise the pathogen (Talboys, 1957; Beckman, 1987). Failure to do so enables continued colonisation of the vascular system, and results in systemic distribution of the pathogen within the host. This advance involves hyphal growth as well as the production of spores that can quickly travel long distances in the transpiration stream, in an environment of inadequate plant defence responses.

1.5.3.1 Usefulness of the vascular wilt model
For clarity of the vascular wilt system, it is instructive to formulate the substantial body of research detailing Fusarium and Verticillium wilts of an extensive list of plant species into a single model. Studies of Fusarium wilt of cotton have been discontinuous and relatively limited in some areas, and our knowledge of the host-pathogen interaction relies substantially on inferences and speculation from related studies. Further is the unique situation of Australian cotton being attacked by a distinctive pathogen. The relevance of generalised models compiled of isolated observations are limited when investigating specific disease control measures. Rather than allowing us to draw conclusions, the generalised vascular wilt model highlights areas requiring more detailed understanding for future investigations into Australian Fusarium wilt of cotton. This review supports the use of the vascular wilt model to analyse and interpret our current understanding of Fusarium wilt of cotton, and I will use this model as a framework to guide the current study.

1.6 Aim and scope of this study
A thorough understanding of the underlying interaction between the plant and pathogen must complement a long-term solution to the devastating disease epidemic of Fusarium wilt of Australian cotton. Plant defence responses have been demonstrated to limit disease in many systems. Inadequate knowledge of the fundamental biology of the host-pathogen relationship can only act to limit the innovations required for disease control.
Thus, the aim of this research is to describe the host-pathogen interaction between a biotype of *Fusarium oxysporum* f. sp. *vasinfectum* found in Australia and the commercial cotton varieties grown in this country. It is hypothesised that the interaction of Fov with the hosts that are least and most susceptible to Fusarium wilt will differ.

The investigation will increase our current understanding of infection, colonisation and plant defence responses, and will focus on parameters previously identified in related systems as having influence on disease resistance. Comparisons will be made between the interactions of the pathogen and the cotton varieties least and most susceptible to Fusarium wilt to confirm the role of particular defence responses, and to ascertain their overall contribution to the plant’s susceptibility. An understanding of the methods of Fov infection and spread within different varieties of cotton and their subsequent defence responses can contribute to the development of more efficient cultural management practices and targeted breeding for resistance.

This chapter describes the uniqueness and severity of Fusarium wilt of cotton in Australia and has identified areas of knowledge requiring improvement to tackle this problem.

The next chapter describes the general materials and methods used to fulfil these aims. Many are drawn from methodologies presented in pivotal references surveyed in Chapter 1, while others were developed specifically for use in this investigation.

The development of a novel glasshouse bioassay for the investigation into Fusarium wilt of cotton is documented in Chapter 3. This bioassay provided the basis for subsequent studies.

Chapter 4 describes the infection process of *Fov* on cotton. Events along the infection pathway were described and quantified, including the differences in colonisation of the least and most susceptible cotton varieties.

Defence responses of cotton plants infected with Fusarium wilt are discussed in Chapter 5. Several microscopy techniques were used to examine the defence responses of Australian cotton. The structural and chemical changes involved in the host response to Fov infection were described, and shown to differ between hosts.
Chapter 6 focuses on the particularly important defence mechanism involving the production of antimicrobial terpenoids. These phytoalexins were located, identified and quantified in the host relative to the position of the invading pathogen. Substantial differences in the response in different cotton varieties were documented.

Knowledge gathered in this study is brought together in Chapter 7 to benchmark our current understanding of Fusarium wilt in Australia, and to place this into a global context. The contribution of this study to future disease control is discussed in conjunction with an analysis of its limitations and an assessment of the future work required.
CHAPTER 2  GENERAL MATERIALS AND METHODS

2.1 Host plants
2.1.1 Source and cultivar
Three commercial Australian cotton varieties (G. hirsutum) varying in their susceptibility to Fusarium wilt were studied: Siokra 1-4 (most susceptible), Sicala 40 (moderately susceptible), and Sicot 189 (least susceptible).

De-linted seeds free from fungicide treatment ("black" seed) were generously provided by Cotton Seed Distributors Limited each season. Seeds were stored in dry and dark conditions at 4°C.

2.1.2 Potting mix
The potting medium consisted of 5 parts Perlite, 5 parts Vermiculite, and 2 parts washed river sand. These ingredients were thoroughly mixed by hand and autoclaved at 121°C for 20 min. Slow release fertiliser (Osmocote®) was added at a rate of 4 g/L and mixed by hand.

2.1.3 Glasshouse conditions
Plants were grown in a glasshouse maintained at approximately 23°C. There was some temperature variation (±5°C) due to the necessity of conducting trials throughout different season conditions. Where possible, trials were avoided in winter (June-August) due to their unfavourable growth conditions. In June, for example, Melbourne receives an average of 3-5 hours of sunlight and the temperature averages a minimum of 7.2°C to a maximum of 16.5°C (www.bom.gov.au). During months of short day-length artificial lighting provided a 12 h photoperiod. Watering regimes depended on the type of assay.

2.1.4 Seed Germination
All seeds were surfaced sterilised by soaking in a hypochlorite solution (2% available chlorine) for 1 hr. Seeds were agitated several times throughout this period and rinsed thoroughly with sterile water before use.

Depending on the assay, seeds were either placed in a single layer onto water agar plates and incubated at approximately 23°C for four days, or placed approximately 2 cm under the surface of potting medium in pots and maintained at approximately 23°C for six weeks in the glasshouse. Four-day-old plants were 1.5-3.0 cm long, but 2 cm long plants
were selected for consistency. Six-week-old seedlings had approximately 3-4 true leaves and would reach late flowering / early boll set stage of development within a further six weeks.

2.2 Pathogen

2.2.1 Source and strain

Representative cultures of Australian Fov isolates were generously provided by the Farming Systems Institute, Department of Primary Industries, Queensland. Isolates were identified as: #24500 VCG 01111; #24595 VCG 01111; #24598 VCG 01112; and #24599 VCG 01112. Isolates were isolated from cotton fields and were supplied as fresh single spore cultures on carnation leaf agar plates.

2.2.2 Storage

Filter paper cultures were established using a method used by N. Moore (pers. comm., Farming Systems Institute, Department of Primary Industries, Queensland) adapted from the technique of Correll et al. (Correll et al., 1986). A plug (3×3mm) from the agar plate culture was placed onto the centre of a moist sterile Whatman #1 filter paper, and incubated at 25°C for approximately two weeks inside a sterile Petri dish. After this time the mycelium covered the filter paper that was by now dry. The paper was cut up aseptically, and placed in sterile, airtight, screw-top vials. Filter paper cultures were stored under dark and dry conditions at room temperature, and were found be unviable after four years.

Water cultures of all of the isolates were prepared for long-term storage under dark and dry conditions at room temperature, and were viable after four years. Plugs (5×5mm) of mycelium on water agar were placed into airtight screw-top vials containing sterile deionised water.

Large quantities of #24500 VCG 01111 glycerol stock were prepared due to its repeated use in assays. A single piece of filter paper culture was placed into 150 mL of fresh potato dextrose broth in a 250 mL Erlenmeyer flask and incubated for three days at 25°C on an orbital shaker (150 rpm). The liquid cultures were filtered through eight layers of sterile facial tissues. The microconidia concentration of the filtrate was quantified using a haemocytometer prior to being mixed with 20% v/v glycerol and stored at -70°C.
New #24500 VCG 01111 glycerol stocks were prepared each year, after passaging the fungal isolate through a cotton plant. Passaging involved the mechanical wounding of the plant stem at ground level with a needle, covering the wound with glycerol stock, and reisolating the fungus two weeks later by placing tissue from the plant stem (approximately 10 cm from point of inoculation) onto Fusarium-selective agar.

2.2.3  **Growth Media**

2.2.3.1  **Fusarium-selective (Peptone PCNB) agar**
This medium allows slow growth of Fusarium colonies (5-10mm diameter after 5-7 days) (Burgess *et al.*, 1994). It was used for the isolation of Fusarium from plant tissue.

To prepare 1 L of this agar, 15 g of Peptone, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, and 1.0 g of Terrachlor (PCNB 75%) was added to 20 g of powdered agar and 1 L of deionised water. This mixture was autoclaved at 121°C for 20 min and allowed to cool to 55°C before adding the mixture of 1.0 g of streptomycin sulphate and 0.12 g of neomycin sulphate in 10 mL of sterile deionised water. The agar was poured into sterile Petri dishes and allowed to dry in a laminar flow hood for 30 min before sealing and storing at 4°C.

2.2.3.2  **Potato dextrose broth / agar**
Potato dextrose media favours microconidia production in Fusarium spp. (Burgess *et al.*, 1994).

 Peeled and diced potato was placed in 1 L of deionised water and autoclaved at 121°C for 20 min. The resulting broth was strained through four layers of sterile muslin. Dextrose (10 g) and powdered agar (20 g) were added, and the liquid was again autoclaved at 121°C for 20 min. The agar powder was omitted in the preparation of broth. Broth was used fresh, but agar plates were sometimes sealed and stored at 4°C.

2.2.3.3  **Carnation leaf agar**
Carnation leaf agar is useful for the identification of *Fusarium* spp. as it favours the production of macroconidia (Burgess *et al.*, 1994).

Water agar was prepared by adding 2% v/v powdered agar to deionised water and autoclaving at 121°C for 20 min. The agar was poured into individual Petri dishes to
which 4-8 pieces of sterile gamma-irradiated carnation leaf pieces were immediately added while the water agar was molten.

2.2.4 **Inoculum Production**

2.2.4.1 **Solid inoculum**

Organic de-hulled millet was rinsed under running tap water, strained of free water, then 500 mL of moist millet was added to a 1 L Erlenmeyer flask. The millet was sterilised by autoclaving the flask at 121°C for 20 min. For glycerol stock culture (2 mL) was poured onto the sterile millet and incubated at 23°C for 10 days, with vigorous shaking undertaken each day.

Fresh solid inoculum was used immediately.

Powdered solid inoculum was obtained by oven-drying overnight in a paper bag before grinding, and was stored for short periods in an airtight container in the dark.

2.2.4.2 **Inoculum suspension**

A modified method of Wang *et al.* (1999a) was used to harvest microconidia for use as inoculum. Glycerol stock (1 mL) was added to potato dextrose broth in a 250 mL Erlenmeyer flask and incubated in an orbital shaker set at 150 rpm for one week at 25°C. The cultures were filtered through eight layers of sterile facial tissues. The conidial concentration of the filtrate was adjusted to \(5.0 \times 10^6\) conidia/mL using a haemocytometer.

2.3 **Statistical analyses**

Data were analysed using Minitab Release 14. Data were always subjected to a normality test (Anderson-Darling) before an analysis of variance (ANOVA) was performed. The resulting probability plot was checked for normal distribution.
CHAPTER 3  GLASSHOUSE BIOASSAY

3.1  Introduction

Glasshouse bioassays are useful tools for assessing disease resistance of plants in a controlled environment. A bioassay should be meaningful, consistent, rapid, and able to provide clear results. A reliable and useful bioassay must above all produce repeatable results. If these results are to be extrapolated to encompass disease resistance in field crops, then assay parameters should resemble field conditions as much as possible.

Establishing a protocol for a glasshouse bioassay requires repeated modification of many interdependent factors. Constant reference to the “disease triangle” is useful in which the pathogen, host and environment all interact to produce disease. By performing a bioassay under glasshouse conditions the environmental component of this interaction can be manipulated in favour of host or pathogen to ultimately determine disease outcomes. Environmental parameters must be precisely managed to avoid the skewing of results or the loss of relevance of the assay to the field.

Knowledge of disease epidemiology is integral to the design of a bioassay. In the case of Fov, the pathogen is thought to infect at any stage of plant development (Hutmacher et al., 2004) and Australian cotton succumbs to Fusarium wilt across different periods of the growing season (Anderson et al., 2004). While plants may initially survive infection, supplemental stresses such as flowering or cold shock increase plant vulnerability to Fov (Kochman et al., 2002). The triggers for symptom expression or the respective resistance of Australian cotton at different stages of plant development have not been thoroughly investigated.

The evaluation of Fusarium wilt resistance of Australian cotton varieties is routinely performed at the completion of the cotton season in naturally-infested field sites (Reid et al., 2002). This process is seasonal, lengthy, and logistically challenging. Field trials often rely on the goodwill of farmers for both access to and maintenance of infected sites, and data are gathered under highly variable and unpredictable environmental conditions. A rapid, reliable and informative glasshouse bioassay that could be undertaken intensively throughout the year would be valuable for cotton breeders to assess new varieties for resistance to Fov.
A technique which adequately predicts the field resistance of cotton varieties to Fusarium wilt has remained elusive. Researchers both throughout Australia and internationally utilise different bioassay protocols, and the lack of consistency prevents comparison between findings and limits the breadth of conclusions. The development and standardisation of a glasshouse bioassay was identified as one of the highest research priorities by the Fusarium Wilt Committee at Australian Cotton Cooperative Research Centre's Fusarium Workshop, held in Toowoomba in 2003.

3.1.1 Past bioassay protocols

A multitude of bioassay techniques to assess Fusarium wilt have been utilised by different researchers. Inoculation by dipping roots of cotton seedlings into a Fov conidial suspension is commonly used in glasshouse-based studies of Fov (Miller and Cooper, 1967), and is the most practiced inoculation method amongst Australian researchers (Kochman et al., 1996; Kochman et al., 1998; Wang et al., 1999a). An optimised procedure for screening Australian cotton germplasms against Fusarium wilt in the glasshouse was described by Wang et al. (1999a). Several parameters from this report were utilised in the current study.

Other methods of inoculation involve the severing of seedling taproots (Shi et al., 1993) or direct stem injection of inoculum (Bugbee and Sappenfield, 1968). A system involving seeds planted into pots containing naturally infested soil is used for studying other diseases at the Australian Cotton Research Institute in Narrabri, NSW, but quarantine restrictions prevent this system being used for Fusarium wilt. This approach would also be unfeasible in areas geographically distant from disease outbreaks.

This chapter documents the development of a glasshouse bioassay involving the selection of Fov isolate, the testing of inoculum types and methods of delivery, and plant growth conditions. The aim was to describe a bioassay that reliably correlated with field resistance and could be used in subsequent chapters of this current investigation into Fusarium wilt of Australian cotton.
3.2 Methods

3.2.1 Fov isolate selection

3.2.1.1 In vitro
Four Fov isolates (see 2.2.1) were grown on potato dextrose agar plates in order to select a representative of VCG’s 01111 and 01112 for further study on the basis of in vitro growth vigour. Plates were incubated at 25°C/20°C for 12 h each with a 12 h photoperiod.

3.2.1.2 In vivo
The following two published techniques were used to compare the relative aggressiveness of the selected isolates in the most susceptible Siokra 1-4 and the least susceptible Sicot 189.

3.2.1.2.1 Cut taproot
A method involving inoculation via a severed taproot (Shi et al., 1993) was used to quantify colonisation of the different cotton varieties by the two VCG’s.

Taproots of six-week-old Siokra 1-4 and Sicot 189 were severed in sterile water at approximately 15 cm below the cotyledons (equivalent to approximately 5 cm below ground level). The wound site and remaining roots were immediately transferred into a spore suspension of $5 \times 10^6$ mL$^{-1}$ microconidia for 30 min. Plants were placed into new pots in the glasshouse and lightly watered each day. Five replicates per treatment were harvested 0, 2, 4, 8, or 16 days later. Upon harvesting, lateral roots and leaves were excised and the plants were surface sterilised. Freehand lateral sections (approximately 1-2 mm long) at 1 cm intervals of each plant were incubated on Fusarium-selective agar (see 2.2.3.1) for one week at room temperature. The distance travelled by the pathogen was assessed by the growth of hyphae onto the agar from these sections. This experiment was performed twice.

3.2.1.2.2 Taproot puncture
Six-week-old Siokra 1-4 and Sicot 189 plants were removed from their pots and inoculated with VCG’s 01111 and 01112 by taproot puncture.

In a method based on the inoculation technique of Bugbee and Sappenfield (1968) a bead of microconidial suspension was formed at the tip of a 23-gauge needle. The needle was inserted into the taproot at a site approximately 3-5 cm below ground level
(approximately 15 cm below the cotyledons). The drop of inoculum was drawn into the injection point upon withdrawal of the needle. If this was not observed, the procedure was repeated.

Plants were incubated for 16 days in a moist chamber consisting of a sealed clear plastic box lined with moist sterile paper towel. Five replicates of each treatment were harvested 0, 2, 4, 8 and 16 days post-inoculation. Taproots were severed below the point of inoculation, and all lateral roots and leaves were removed before the plant was surface sterilised. Freehand lateral sections (approximately 1-2 mm long) were cut from successive 1cm intervals of each plant and placed onto Fusarium-selective agar (see 2.2.3.1). Agar plates were incubated at room temperature for one week before observations were made of hyphae growing out of sections onto the agar. The section furthest from the inoculation point from which hyphae grew was recorded as the distance travelled by the pathogen. This experiment was repeated once.

3.2.2 Glasshouse bioassays

Three methods of inoculation were investigated using solid inoculum and an inoculum suspension (see 2.2.4.1 and 2.2.4.2, respectively). Application of different inoculum types required modifications of the bioassay parameters, with possible variation in their abilities to initiate Fov infection.

3.2.2.1 Infested growth substrate assay

The addition of solid inoculum to the growth medium was investigated in an attempt to simulate the saprophytic growth of Fov on crop debris in field soil. Solid inoculum - either fresh or dried / powdered infested millet - was incorporated by hand into the potting medium before cotton seeds were sown. One seed was placed in each tube, and the distribution of seed variety within seedling trays was randomised. Water was provided by light misting (5 min) each day. Each inoculum type was initially subjected to a dosage experiment to optimise the application rate, followed by an assay to quantify seedling emergence and survival.

3.2.2.1.1 Fresh solid inoculum

Fresh solid inoculum of Fov VCG 01111 was added to potting medium at concentrations of 0, 1, 2, 5, and 10% v/v to assess the optimum dosage. It was incorporated by hand as the potting mix ingredients were homogenised (see 2.1.2).
Fifteen seeds each of Siokra 1-4 and Sicot 189 were sown approximately 3-5 cm below the surface into individual pots containing the different inoculum concentrations. Emergence was recorded by observing the percentage of seedlings that had emerged from the potting medium two weeks after sowing.

In a subsequent trial involving fresh solid inoculum, infested millet grains were added to potting medium at 1% v/v. Sterilised millet grains were added to the control potting mix at the same rate. Fifteen replicates per variety were sown as above, and both emergence and survival were recorded at two and four weeks, respectively. Sicala 40, a variety known to have moderate susceptibility to Fusarium wilt, was included in the assay to better assess the reliability of results. Emergence was recorded by observing the percentage of seedlings that emerged from the potting medium two weeks after sowing. Plants were considered dead when all leaves were completely dry or abscised. Survival values presented here were calculated by dividing the surviving number of plants by the number of plants that emerged before converting to a percentage. This experiment was performed twice.

3.2.2.1.2 Powdered solid inoculum

Powdered solid inoculum of Fov VCG 01111 was incorporated by hand into the potting medium at concentrations of 0.10, 0.25, 0.50, and 1.00% v/v to assess the optimum dosage. Siokra 1-4 and Sicot 189 seeds were sown approximately 3-5 cm below the surface into ten replicate pots per inoculum level.

Emergence was recorded at two weeks by observing the percentage of seedlings that had emerged from the potting medium. All seedlings were subsequently removed from their pots and surface sterilised by dipping into 70% ethanol, followed by sterile deionised water. The seedlings were blotted dry and placed onto Fusarium-selective agar (see 2.2.3.1). After incubating at room temperature for one week, the agar plates were macroscopically examined for the presence of Fusarium hyphae.

Powdered inoculum was subsequently incorporated into potting mix at a concentration of 0.1% v/v. The planting of Siokra 1-4, Sicala 40 and Sicot 189 seeds, seedling growth, and emergence / survival assessment was identical to the methods used for fresh inoculum (3.2.2.1.1).
3.2.2.2 Root dip assay
Six-week-old plants were removed from their pots and shaken gently to remove most of the potting medium from their roots. Whole root systems of 30 plants each of Siokra 1-4, Sicala 40 and Sicot 189 were submerged in an inoculum suspension (see 2.2.4.2) of Fov VCG 01111 for 30 min, before being re-potted into individual pots. Plants were watered lightly by hand each alternate day for a further six weeks before the number of surviving plants was recorded. Plants were considered dead if all leaves were completely dry or abscised. This experiment was repeated once.

3.2.2.3 Taproot puncture assay
To avoid the excessive disturbance of roots resulting from removing plants from their pots, six-week-old plants were injected with VCG 01111 in situ. Potting mix was carefully displaced from the base of the plant to allow access to the taproot. No roots were observed to grow in this area, and the plant tissue selected for inoculation was white and apparently devoid of large gossypol glands that are visible on the cotyledon. As described previously (3.2.1.2.2), a drop of inoculum was formed at the tip of the needle which was then inserted into the taproot. If the inoculum was not observed to be drawn into the wound, the needle was inserted again. At least nineteen plants of each variety were inoculated.

Each plant was watered until run-off escaped from the bottom of the pot, every fifth day for six weeks. After this time the respective survival rates of each of the varieties were recorded. Plants had flowered within this timeframe. Due to the tendency for wilt-affected plants to re-shoot after complete defoliation in this bioassay, only those plants with more than two leaves were recorded as “alive” in the survival data. Affected plants that re-shoot are severely stunted and do not go on to yield flowers or bolls and would be of little value in the field.

This experiment was repeated as above, with the addition of growth lamps to provide 12 h of sunlight regardless of the season. With this modification the assay was repeated twice with 17-32 plants per variety.
3.2.3 Statistical analysis
The infested growth substrate assays and root dip assays were single replicates and statistical analyses were not conducted. Data from three repeat taproot puncture experiments were combined and analysed by ANOVA.
3.3 Results

3.3.1 Fov isolate selection

3.3.1.1 In vitro

Fov isolates #24500 VCG 01111 and #24599 VCG 01112 (Figure 3.1A and B) displayed more vigorous growth on potato dextrose agar plates compared with the other isolates of the same VCG (Figure 3.1C and D). Consequently, these isolates were selected for use in further experiments.

3.3.1.2 In vivo

3.3.1.2.1 Cut taproot inoculation

The system of inoculation using a severed taproot resulted in no significant differences in the abilities of VCG’s 01111 and 01112 to colonise either Siokra 1-4 (Figure 3.2A) or Sicot 189 (Figure 3.2B). This technique resulted in trauma to most plants, with wilting evident soon after inoculation in both the most and least susceptible varieties. Due to the level of damage caused by severing the taproot, this technique was not used further.

3.3.1.2.2 Taproot puncture inoculation

The initial testing of the comparative abilities of two VCG’s to colonise cotton plants after taproot injection indicated little difference (Figure 3.3). Colonisation of Siokra 1-4 (Figure 3.3A) and Sicot 189 (Figure 3.3B) by both VCG’s is similar over eight days. VCG 0111 progresses further in Siokra 1-4 than VGC 12, but only at sixteen days. The similarities of the VCG’s colonisation ability lead to the use of only one representative VCG in subsequent assays. VCG 0111 was selected on the basis of widespread field distribution, and therefore greater industry relevance.

3.3.2 Glasshouse bioassays

3.3.2.1 Infested growth substrate assay

3.3.2.1.1 Fresh solid inoculum

All seeds planted in unamended potting medium had germinated and emerged as seedlings after two weeks (Figure 3.4). Conversely, there was no emergence of either cotton variety from potting medium amended with 10% solid inoculum. The greatest difference observed between the emergence of Siokra 1-4 and Sicot 189 was in potting mix with 1% solid inoculum (Figure 3.4).
High concentrations of the fresh inoculum caused the potting medium to become compacted and hydrophobic after two weeks, to the point where the surface could not be depressed and water beaded. To limit these unwanted effects on the soil characteristics, potting medium in subsequent trials were amended with 1% solid (fresh) inoculum (Figure 3.5). Plant emergence in the control medium was high, and all plants survived (Figure 3.5A). However, both emergence and survival was decreased in all varieties grown in the inoculated potting medium (Figure 3.5B).

When this experiment was repeated (Figure 3.5C, D) the overall results were inconsistent and there were no substantial differences in emergence and survival of the cotton varieties. Fresh solid inoculum added at this concentration again resulted in the formation of hydrophobic clumps of mycelium within pots two weeks after sowing, and substantially altered the physical characteristics of the potting medium.

Nonetheless, there was a trend in both experiments for the survival of Sicot 189 in Fov-infested potting medium to be greater than the survival of Siokra 1-4 and Sicala 40.

3.3.2.1.2 Powdered solid inoculum
The emergence of Siokra 1-4 and Sicot 189 seedlings from potting medium amended with 0.10, 0.25, 0.50, and 1.00% solid (powdered) inoculum revealed no identifiable trends (Figure 3.6).

At the conclusion of the trial, the incubation of the surface-sterilised seedlings on Fusarium-selective agar (see 2.2.3.1) resulted in hyphal growth from all plants in the inoculated treatments. Due to high infections irrespective of the inoculum concentration, the lowest concentration was used in subsequent experiments.

Siokra 1-4, Sicala 40 and Sicot 189 plants were grown in potting medium inoculated with 0.1% powdered inoculum. Seedling emergence and survival was reduced compared with the control (Figure 3.7A), and there was an exaggerated decrease in emergence and survival of Sicala 40 compared with the other varieties (Figure 3.7B). Approximately 80% of seedlings emerged and some of each variety of seedlings died during the following two weeks. This experiment was repeated (Figure 3.7C, D) but emergence and survival of Siokra 1-4 was again not substantially different to the other varieties tested.
When sections of stem from all plants in inoculated treatments of this assay were surface sterilised and placed on selective agar, 100% were found to be infected with Fov.

3.3.2.2 Root dip assay
There was little death in plants inoculated by dipping their roots into an inoculum suspension. Close to 90% of the most susceptible variety, Siokra 1-4, was still surviving six weeks after inoculation (Figure 3.8). Plant survival was very high both times this experiment was performed (Figure 3.8A, B).

3.3.2.3 Taproot puncture assay
Inoculation by taproot puncture resulted in between 50-80% death of cotton seedlings. The least susceptible variety, Sicot 189, recorded 50% survival, while only 20% of Siokra 1-4 seedlings survived (Figure 3.9). Surviving plants did not appear as healthy those grown at other times of the year with longer day lengths.

When plants were grown under supplemental lights, plant survival after inoculation by taproot puncture increased (Figure 3.10). The least susceptible variety recorded 80% survival, while 73% of the moderately susceptible Sicala 40 survived, and only 50% of the most susceptible Siokra 1-4 survived. An ANOVA test found the difference in survival between the most and least susceptible was statistically significant (p=0.001), and the survival of the moderately susceptible Sicala 40 was intermediate to Siokra 1-4 and Sicot 189.
3.4 Discussion

3.4.1 Fov isolate selection

The selection of one Fov isolate for use in bioassays was necessary for consistency. A previous inoculum protocol involved mixed inoculum made by combining spores of three different isolates (N. Moore, pers. comm.). Using this technique, the isolate responsible for any observed infection could not be easily identified. Infection by different isolates with undocumented pathogenicity could induce varied host responses.

Of the four isolates most commonly used in bioassays by other researchers, representatives of each VCG were chosen for their relatively prolific growth on agar under laboratory conditions. Growth assays using these two isolates demonstrated no observable difference in their abilities to colonise cotton plants. In their guidelines for the integrated disease management of Fusarium wilt, Allen et al. (2003) claim both VCG’s are equally capable of causing disease in all commercial cotton varieties. While experimental data to validate this have not been published, these assertions are supported by the results presented here. Therefore, the continued use of only one isolate can legitimately represent the behaviour of Fov. VCG 0111 was chosen due to its greater industry relevance, as it comprises by far the majority of Fov isolates in the field (Wang et al., 2004b).

3.4.2 Glasshouse bioassays

3.4.2.1 Infested growth substrate assay

Fov may persist in field soil by growing on organic matter such as crop residues. As is the case with other wilt Fusaria, chlamydospores are likely to be important to Fov survival in addition to acting as inoculum in the field (Hillocks, 1992b). The addition of Fov-infested millet grains to potting medium was investigated as an inoculation method broadly representing this natural system. However, the addition of the moist millet substrate resulted in the rapid proliferation of the pathogen throughout the potting medium in preliminary experiments (as described in 3.3.2.1.1). The consequences of such growth were an unquantifiable increase in the amount of infective Fov propagules, and a change in physical properties of the potting medium.

Such an immediate increase in the amount of secondary inoculum is problematic in a system which is dose-dependant (Bugbee and Sappenfield, 1968). The addition of 1% v/v inoculum inhibited the emergence of 40% of S1-4 seedlings, however the rapid
pathogen growth means the actual inoculum concentration is not known, adding an unquantifiable variable into the bioassay. Due to the substantial effect on emergence of this lowest inoculum concentration, 1% v/v inoculum was used in the successive trial.

At least 40% of all seedlings died two weeks after emergence (3.3.2.1.1) and repeated experiments resulted in inconsistencies that may be a consequence of several factors. A fluctuation in the ability of the pathogen to infect could be attributable to uncontrollable seasonal variation in temperature and/or light conditions in the glasshouse (see 2.1.3). Alternatively, the two week sampling period may have been inadequate to gather meaningful survival data from plants inundated by inoculum. Blanket infection of plants of all varieties might result from either an excessive inoculum load or an age-related effect of infecting very young plants. While extending the sampling period may have allowed relative susceptibilities of the varieties to emerge, continued deaths beyond 40% in the least susceptible variety would represent an extreme scenario with limited field relevance.

The same changes to the potting medium as a result of pathogen proliferation were observed in each experiment. The hard, compact and hydrophobic characteristics of the potting medium that developed as a result of adding this type of inoculum had unknown effects on plant growth and rate of infection. Fresh solid inoculum has been used successfully in other cotton/Fov glasshouse bioassays with different potting media (McKenna et al., 2004; Smith and O'Neill, 2004).

Lesser concentrations of powdered inoculum were incorporated into potting medium as it was a more concentrated source of Fov, having undergone drying and grinding. Concentrations up to 1% v/v resulted in emergence of no greater than 60% of Siokra 1-4, with no identifiable correlation between inoculum concentration and emergence (Figure 3.6). Further comparison of three varieties grown in 0.1% inoculum resulted in no significant differences in emergence or survival (Figure 3.7). Fov was reisolated from 100% of the plants at the conclusion of this assay, demonstrating there was infection of all plants regardless of variety. Differences between varieties may have become apparent if survival data were collected later, however, longer monitoring times were not investigated. Although there are no published data to support the notion of widespread latent Fov infection in the cotton field, anecdotal observations of early-
season infection followed by selective plant death after stress are widely accepted (Anderson et al., 2004).

3.4.2.2 Root dip assay

Use of the inoculum suspension facilitated the use of older cotton plants. The use of solid inoculum ensured immediate exposure to the pathogen after germination in infested potting medium, while the inoculum suspension allowed inoculation of six-week-old plants.

Glasshouse assays to evaluate resistance to the wilt pathogen, \textit{Verticillium albo-astrum}, have shown that assays of older cotton plants correlate more closely with field observations of wilt development than did younger seedlings (Bugbee and Presley, 1967). It is not uncommon for plants to develop resistance to disease as they age (Hammerschmidt, 2004). In the case of \textit{V. albo-astrum}, four to seven week-old inoculated cotton plants were old enough to demonstrate known varietal resistance.

As part of their published pathogenicity assay for screening cotton germplasms for resistance to Fov, Wang et al. (1999a) nominated two-week-old seedlings as the most suitable due to their high and stable susceptibility. They emphasised the importance of inoculating plants at their most susceptible stage of development when evaluating cultivar resistance, noting that resistance appears to increase with age. The authors assert that cultivar field resistance depends primarily upon their performance at this most susceptible age. If all plants encountered uniform dispersal of inoculum throughout the season this assumption of equal and persistent threat of infection may be valid. This however, is incongruous with plant deaths due to Fusarium wilt staggered through the cotton season. Further, studies of Fov have shown that Fov distribution is discontinuous in fields (Nehl and Allen, 2004) and heterogeneous in the soil profile (Allen, 2004b). Sowing seeds later in the season has also been shown to reduce Fusarium wilt incidence (Anderson et al., 2004). These studies indicate that the use of two-week-old seedlings as an indicator of Fov resistance is limited in such a complex system. In this study, six-week-old plants were found to flower within a six week sampling period after inoculation, which has the benefit of stressing infected plants and encouraging disease expression.
The ability of Fov microconidia to survive in a dry soil environment is limited (Burgess, 1981). The limited deaths observed in preliminary studies of six-week-old plants that had their roots dipped in the inoculum suspension before planting may be evidence of microconidia perishing in an unfavourable potting medium (Figure 3.8). Root-dipping in spore suspensions followed by incubation in MS medium (Dowd et al., 2004) and an unspecified “potting mix” (Wang et al., 1999a) has been used in the past. Different growth media may increase the longevity of microconidia, as might environmental conditions in different glasshouses. Despite inoculating with the very high concentrations of microconidia used in other Australian studies (Dowd et al., 2004; Wang et al., 1999a), in the current system this inoculation technique resulted in inadequate infection.

3.4.2.3 Taproot puncture assay

The rate of infection was appreciably improved through the use of taproot puncture inoculation. This may possibly be attributed to the increased survival of pathogen propagules after their direct placement into the favourable environment of cotton vascular tissue. Inoculation by stem puncture has been used in the study of several diseases of cotton including V. albo-atrum, V. dahliae, X. axonopodis pv. malvacearum and Fov (Bugbee and Presley, 1967; Mace et al., 1978; Essenber et al., 1982; Bugbee, 1970).

Inoculating tomato with Fol, Scheffer (1957) found that stem puncture inoculations resulted in the wilting of only those leaves traceable back to xylem elements at the site of infection, compared with widespread infection of xylem elements and wilting of all leaves of after inoculation by the severed taproot technique. Likewise, studies on cotton have shown that the severity of the wilt response to Fov inoculation can be increased by multiple stem punctures (Bugbee and Sappenfield, 1968). When the severed taproot method of inoculation was used in this present study, cotton plants became severely wilted (3.2.1.1). Single puncture inoculations resulted in obvious disease symptoms in the Australian cotton varieties tested.

In situ puncture inoculations were performed in plant taproots to minimise trauma to the root system. Plants were not removed from their pots, rather taproots were exposed by displacing only a small amount of potting medium. Disturbance-induced plant stress aside, the site of inoculation has been shown to exert no influence on the differential
varietal reactions of cotton to Fov (Bugbee and Sappenfield, 1968) and the resistance of
tomato to Fol (Schonberg, 1957). Both investigations concluded that factors for resistance
to these pathogens are located in the vascular system of both the stem and the root.

Taproot puncture inoculation resulted in substantial deaths of six week-old plants of all
varieties. Although the varietal trend for survival in this bioassay is in line with the
known field susceptibility to Fov, the least susceptible variety, Sicot 189, recorded only
50% survival. This is lower than would be expected in a naturally-infested field.
Pathogen distribution in a field would not be uniform resulting in more “escapes,” but
the extent of deaths indicates that the glasshouse conditions were perhaps favouring the
pathogen (Nehl and Allen, 2002b). Plants were not thriving during this trial due to
shorter day length. (approximately 9-10 h during winter in Melbourne).

The provision of 12 h photoperiods using artificial lighting in supplemental assays
resulted in healthier plants that flowered within the sampling period. Fewer deaths were
recorded in all varieties, with survival rates that could be reasonably expected to occur
in naturally infested fields.

The final bioassay involved minimal watering, with inoculated plants receiving water
approximately every five days. This increased the stress on the plant which encouraged
disease expression, and would better represent field moisture availability. Infrequent
watering was possible only after taproot puncture inoculations. The trauma of replanting
bare roots after root dip inoculations necessitated excessive hydration to avoid severe
wilting.

It has been suggested that Fusarium wilt severity of several hosts is affected more by
temperature than host genotype (Bell and Mace, 1981). Sub-optimum temperatures for
growth in culture cause severe Fusarium wilts in melon and pea (Bruehl, 1987), and
symptom development in cotton may be suppressed by higher temperatures (Wang et
al., 1999a). While Fov has an in vitro temperature optimum near 28°C (Davis et al.,
1996b), inoculated plants were maintained in the glasshouse at approximately 23°C in
line with previous research (Wang et al., 1999a).

3.4.3 Conclusions
A glasshouse bioassay protocol for the study of Fusarium wilt of cotton was developed.
The key factors considered in the development of this assay included plant age,
inoculum type, inoculum delivery, and the plant environment post-inoculation. The final
glasshouse bioassay was undertaken three times, but should be expanded for further
verification. The inclusion of a wider range of host varieties especially more recently
developed varieties with less susceptibility to Fov than Sicot 189 would be informative.
Comparisons to Fusarium wilt field susceptibilities are based on a wide range of data
sets from different trials (Australian varietal disease trial results and disease rankings:
www.csd.net.au), and may change in the future as more information is gathered.

Cotton seeds were surface sterilised and germinated in pots containing sterile potting
medium. Prior to inoculation seedlings were grown for six weeks at approximately 23°C
with a 12 h photoperiod, and watered daily. Plants were inoculated with a $5.0 \times 10^6$
spores/mL microconidia suspension by a single taproot puncture. Pots were watered
approximately every five days for six weeks after inoculation, while other growth
parameters remained unchanged. Plants with less than two leaves were considered
"dead."

The survival of cotton varieties in this rapid and reliable assay correlated with what is
known of Fusarium wilt field susceptibility. The bioassay provided an appropriate
technique for further evaluation in the context of this study and for wider use in
Fusarium wilt research in Australia.
Figure 3.1 Isolates of Fov on agar plates

Mycelium after ten days growth on PDA under 25° C, day/20° C, night incubation with 12 h photoperiod: A) #24500 VCG 01111; B) #24599 VCG 01112; C) #24595 VCG 01111; and D) #24598 VCG 01112.
Figure 3.2 Distance travelled by Fov in cotton stele after cut taproot inoculation

The distance from the point of inoculation of VCG’s 0111 and 0112 was measured within (A) Siokra 1-4 and (B) Sicot 189. Six-week-old plants were inoculated by the cut taproot method and re-potted. Five plants from each variety were harvested and surfaced sterilised 0, 2, 4, 8 and 16 days later. Successive sections were plated onto Fusarium-selective agar and incubated for one week. Fov grew from infected sections onto surrounding agar. Values represent the distance from the severed root tip of the furthestmost infected section. Bars represent the standard error of ten replicate plants.
Figure 3.3 Distance travelled by Fov in cotton stele after taproot puncture inoculation

The distance from the point of inoculation of VCG's 0111 and 0112 within (A) Siokra 1-4 and (B) Sicot 189. Six-week-old plants were inoculated by taproot puncture and repotted. Five plants from each variety were harvested and surfaced sterilised 0, 2, 4, 8 and 16 days later. Successive sections were plated onto Fusarium-selective agar and incubated for one week. Fov grew from infected sections onto surrounding agar. Values represent the mean distance from the severed root tip of the furthest infected sections. Bars represent the standard error of two experiments.
Figure 3.4 Emergence of seedlings in potting medium inoculated with different concentrations of solid (fresh) inoculum

Siokra 1-4 and Sicot 189 seeds were sown into potting medium amended with 0, 1, 2, 5, and 10% v/v solid (fresh) inoculum. Emergence was measured two weeks after sowing.
Figure 3.5 Emergence and survival of seedlings in potting medium inoculated with solid (fresh) inoculum.

Siokra 1-4, Sicala 40 and Sicot 189 seeds were sown into potting medium amended with: (A, C) 1.0% v/v uninoculated millet; or (B, D) 1.0% v/v solid (fresh) inoculum. Emergence was measured after two weeks, and survival was measured after four weeks. Experiment 1 (A, B) concluded 30/04/2003; Experiment 2 (C, D) concluded 14/05/2006.
Figure 3.6 Emergence of seedlings in potting medium inoculated with different concentrations of solid (powdered) inoculum.

Siokra 1-4 and Sicot 189 seeds were sown into potting medium amended with 0.10, 0.25, 0.50, and 1.00% v/v solid (powdered) inoculum. Emergence was measured two weeks after sowing.
Figure 3.7 Emergence and survival of seedlings in potting medium inoculated with solid (powdered) inoculum.
Siokra 1-4, Sicola 40 and Sicot 189 seeds were sown into potting medium amended with: (A, C) 0.1% v/v powdered uninoculated millet; or (B, D) 0.1% v/v solid (powdered) inoculum. Emergence was measured after two weeks, and survival after four weeks. Experiment 1 (A, B) concluded 07/11/2003; Experiment 2 (C, D) concluded 10/12/2003.
Figure 3.8 Survival of cotton plants after root-dip inoculation.
Siekra 1-4, Sicala 40, and Sicot 189 plants were taken from their pots at six-weeks-old, inoculated by the root-dip technique and re-potted. Survival was measured six weeks after inoculation. Experiment 1 (A) concluded 03/01/2002; Experiment 2 (B) concluded 29/04/2002.
Figure 3.9 Survival of cotton plants after inoculation by taproot puncture. Siokra 1-4, Sicala 40, and Sicot 189 plants were inoculated by taproot puncture at six-weeks-old. Survival was measured six weeks after inoculation. Experiment concluded 10/05/2005.
Figure 3.10 Survival of cotton plants after inoculation by taproot puncture with the provision of a 12 hour photoperiod.

Siokra 1-4, Sicala 40, and Sicot 189 plants were inoculated by taproot puncture at six-weeks-old. Survival was measured six weeks after inoculation. This data represents three experiments which concluded: 10/07/2005; 14/11/2005; and 10/12/2005.
CHAPTER 4 INFECTION PROCESS

4.1 Introduction

*Fusarium oxysporum* infects and colonises a diverse host range through a broad range of mechanisms. Independently studied mechanisms of related pathogens on different hosts, has been useful in directing paths of enquiry for further research since Fov was discovered in Australia.

Important studies of the infection process of Fov and cotton have been undertaken. Rodriguez-Galvez and Mendgen (1995b) described the primary determinative phase (see 1.5.3) including Fov penetration through the rhizodermis and the ensuing colonisation of the cortex and endodermis. Their study involved light and electron microscopy of the infection process of a Peruvian isolate of Fov and 2-day-old cotton seedlings.

The vast majority of studies of vascular wilts document the secondary determinative phase. Investigations into vascular colonisation using simple yet elegant assays and electron microscopy have shown that resistant cotton plants inhibit Fov growth in their vascular tissues (Shi et al., 1991b; Shi et al., 1992; Shi et al., 1993). These latter events in the infection process are heavily influenced by host defence responses, and will also be discussed in subsequent chapters.

Whilst specific research into Fov is valuable in providing understanding of discrete parts of the infection process, we may also borrow insights from other vascular wilts, such as Verticillium wilt of cotton, to formulate an overview (Garber and Houston, 1966). The limitations of this approach, however, are significant. The uniqueness of the Fov biotypes found in Australia compared with all foreign biotypes means that our understanding of Fusarium wilt in Australia is based not only on extrapolations from studies of different varieties of cotton and non-cotton hosts, but also distinctly different pathogens.

Clarification of the infection process of Australian isolates of Fov on local cotton varieties is required. A generalised wilt model comprising many host-pathogen interactions has helped to orient the focus of the current investigation into this poorly understood system (Beckman, 1987). However, in-depth knowledge of the mechanisms of infection and colonisation will be necessary for disease control.
This chapter aims to describe the infection process of cotton by Australian Fov through histopathological examination and simple assays.
4.2 Materials and Methods

4.2.1 Histopathology of Fov-infected cotton

4.2.1.1 Plant and pathogen

Four-day-old and six-week-old Sicot 189 plants and liquid microconidial inoculum of #24500 VCG 01111 were prepared as previously described in Chapter 2.

Four-day-old seedlings were used to investigate the behaviour of Fov on the outside of cotton roots. Seedlings were inoculated by dipping the whole radicle into a microconidial suspension (2.5×10^6 conidia/mL), then placed into a petri dish containing water agar and incubated at room temperature.

Six-week-old plants were used in longer-term investigations. Seedlings were removed from their pots by inverting the pots and gently shaking the root mass free of potting medium. The seedlings were inoculated by placing their roots into 1 L of water. One millilitre of microconidial suspension (glycerol stock of concentration 2.5×10^6 conidia/mL) was added and plants were incubated on an orbital shaker set at 20 rpm/25°C.

4.2.1.2 Histochemical techniques

Several methods were utilised to observe Fov in a range of tissues.

4.2.1.2.1 Cleared tissue / lactophenol cotton blue stain

Whole roots were placed into saturated chloral hydrate for 48 h. The samples were then placed in lactophenol cotton blue for five minutes, and mounted on a microscope slide in chloral hydrate.

Sections were observed using an Olympus BH2 light microscope and images were captured using an attached Olympus DP10 camera system.

4.2.1.2.2 Embedded tissue / toluidine blue stain

Tissue was embedded according to a modification of the methods of Rodriguez-Galvez and Mendgen (1995b) and Kalc-Wright (1993).

Roots were rinsed in water, cut into 2 mm long sections and fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature. Samples were placed under vacuum for 3×10 min before being rinsed in same buffer three times for 15
min. Samples were dehydrated in increasing ethanol concentrations (20, 40, 60, 80, 100, 100, 100%) for 15 min each.

Root sections were placed in an increasing concentration of LR White resin (hard grade) as follows: 25% for 2 h; 50% for 24 h; then 75% for 2 h. The samples were then placed in 100% resin and infiltrated for five days with daily changes of fresh resin.

Finally, the samples were placed in fresh resin in gelatin capsules (No. 1 size) and polymerised at 60°C for 12 h.

The resulting resin blocks were trimmed using a Leica EM TRIM before 0.4 µm sections were cut with a glass knife on a Reichert-Jung ultra microtome. Sections were collected in distilled water on a microscope slide and dried on a hotplate. The slides were stained with 0.05% toluidine blue in 0.1 M phosphate buffer at pH 6.8 (Ghemawat, 1977) for 2 min (at 60°C), rinsed with water and dried.

Images were taken after the addition of a coverslip and immersion oil with an Olympus BH2 light microscope fitted with an Olympus DP10 digital camera system.

4.2.1.2.3 *Fresh tissue / DioC₆(3) stain*

Tissue was stained with the vital fluorescent stain, DioC₆(3), which non-specifically dyes live hyphae, using a modified method of Duckett and Read (1991).

Whole roots were immersed for 1-2 min in 50 µg mL⁻¹ 3,3′dihexyloxacarbocyanin iodide DioC₆(3) (Duckett and Read, 1991). They were mounted in water on a microscope slide fitted with a well to allow viewing of thick segments of roots. The samples were viewed under UV epifluorescence using 480 / 501 nm filters fitted to a Leica MZFL III dissection microscope.

Images were recorded using a Leica DC300F camera and processed using Leica IM50 Image Manager V1.20 software.

4.2.1.2.4 *Scanning electron microscopy*

Tissue was dehydrated using the same method described in Section 4.2.1.2.2. Critical point drying of the samples was then performed by a Sandri PVT3 Critical Point Dryer. The tissue was mounted on aluminium stubs with carbon adhesive tabs, and specimen
edges were painted with Acheson Electodag 914 high conductivity paint. The stubs were sputter coated with gold using an Edwards S150B Sputter Coater.

Samples were observed with a Philips XL30 FEG SEM.

4.2.2 *Fov growth assays*

4.2.2.1 *Growth in root diffusate*

In order to quantify some characteristics of the behaviour of *Fov* prior to penetration, root diffusate was collected for use in an assay.

Four-day-old seedlings were germinated on agar as described previously (see 2.1.4). Four whole plants of each variety measuring approximately 2 cm were placed into 4 individual centrifuge tubes containing 0.5 mL of sterile deionised water each and sealed. After 1 h the liquid was collected and passed through a 0.2 μm syringe filter.

Of this diffusate, 50 μL was pipetted onto a glass microscope slide, and 10 μL of #24500 VCG 01111 microconidia (glycerol stock) was added. Microconidia were also added to a sterile deionised water control. Four replicate slides were prepared for each treatment (one slide per plant).

Slides were placed into a moist chamber and incubated at 21°C for either 1, 12, 24 or 36 h. At the nominated time, microconidial germination and germ tube length were measured in three random fields of view at ×400 magnification using an Olympus BH2 light microscope. Mean germination and germ tube length values were obtained by averaging the observations from three fields of view. This experiment was performed five times.

4.2.2.1.1 *Treatment of diffusate*

In another experiment, diffusate was treated in two different ways before the addition of microconidia: heated or extracted with ethyl acetate. Diffusate was collected using the method described in 4.2.2.1.

In order to denature proteins within the diffusate, the centrifuge tubes containing the diffusate samples were placed into boiling water for 10 min.

In order to extract lipophilic compounds, 100 μL of ethyl acetate was added to the centrifuge tube containing the diffusate. The tube was vortexed and centrifuged on a
low setting for 2 min before the supernatant was pipetted off. Another 50 μL of ethyl acetate was added, before the sample tube was again vortexed and centrifuged. The supernatant was combined with the original extract.

Microconidia were then added to these treated diffusates and observed, as described in 4.2.2.1 but at 12 h only. Mean germination and germ tube length values were obtained by averaging the observations from three fields of view. This experiment was performed five times.

4.2.2.2 Growth in stele tissue

4.2.2.2.1 Root boxes

In situ root inoculation was made possible by growing cotton plants from seed in specialized pots similar to those designed for root observation (Grant and Byrl, 1984) or *V. dahliae* inoculation (Wilhem et al., 1974b). This allowed for the direct introduction of inoculum into the stele at a known point without trauma to the root mass, enabling the accurate tracking of the pathogen’s progression through tissue of an undisturbed plant.

The polyvinylchloride boxes had a removable panel that allowed direct access to the root system (See Figure 4.1A). A rectangular tube with one side removed was made into a pot by enclosing the open side with a tight fitting lid and securing with tape. A piece of mesh was fixed to one end to prevent loss of potting mix whilst allowing water drainage.

Tubes were filled with potting medium and seeds were planted close to the removable lid. The tubes were placed at a 20° angle during plant growth, which resulted in plant roots growing down the removable panel. Roots had usually grown to the bottom of the pots after six weeks and were accessible for inoculation by removing the tape and gently lifting the removable panel.

4.2.2.2.2 Taproot puncture in root boxes

Inoculation by taproot puncture has been described previously (see 3.2.2.3). However, the site of inoculation was more easily accessed in this assay due to the use of root boxes.
The six-week-old seedling was infected by opening the removable panel of the root box and inserting a fine needle into the taproot (see Figure 4.1A, B). Prior to insertion, a drop of inoculum was squeezed out of the needle, which was drawn into the injection point upon withdrawal of the needle. The site of inoculation was approximately 5 cm below ground level (approximately 15 cm below the cotyledons) and was marked with red ink for later reference. The root box panel was reattached and the plants were lightly watered by spraying until water ran freely from the bottom of the pots.

Four replicates of each treatment were harvested 0, 1, 3, 5, 7 and 14 days post-inoculation. Taproots were severed below the point of inoculation, and all lateral roots and leaves were removed. The remaining taproot/stem was surface sterilised by dipping into 70% ethanol, then sterile deionised water, and blotted dry using sterile paper towel. Freehand lateral sections (approximately 1 mm long) were cut from successive 1 cm intervals of each plant and placed onto Fusarium-selective agar (see Figure 4.1C, D).

Agar plates were incubated at room temperature for one week before observations were made of hyphae growing out of sections onto the agar. By recording the original location of the tissue from which the pathogen grew into the agar, the distance travelled up the tap root/stem by the fungus could be quantified. The section taken furthest from the inoculation point displaying hyphal growth was recorded as the distance travelled by the pathogen. This experiment was repeated three times, and the results analysed by an ANOVA.
4.3 Results

4.3.1 Histopathology

4.3.1.1 Root surface
Inoculation by root dipping resulted in abundant microconidia on the root surface of four-day-old seedlings (Figure 4.2A). Microconidia germination and hyphal growth along anticlinal cell wall ridges was observed 12 h after inoculation (Figure 4.2B). Continued growth formed a hyphal net that ensheathed the root 24 h after inoculation (Figure 4.2C). The production of macroconidia was observed in this mycelium (Figure 4.2D).

4.3.1.2 Penetration of root epidermis
The hyphal net was partially removed for observation of the root surface using scanning electron transmission microscopy (Figure 4.3A).

Three days after inoculation, microconidia associated with the root surface had germinated, and penetration was observed predominantly in the root hair zone. Hyphae either grew along anticlinal cell wall ridges or immediately penetrated the root epidermis. Penetration was both intercellular (Figure 4.3B-E) or by direct penetration of root hairs at the tip (Figure 4.4A) or base (Figure 4.4B). Germ tubes were also observed growing between root hair and other epidermal cell walls (Figure 4.4C).

4.3.1.3 Colonisation of root epidermis and cortex
Observations made five days after inoculation showed colonisation of discrete epidermal cells (Figure 4.5A) including root hair cells (Figure 4.5B). Intensely colonised root epidermal cells were observed beneath the hyphal net seven days after inoculation (Figure 4.5C).

As hyphae colonised the cortex, growth was both intra- and inter-cellular (Figure 4.6A). Hyphae appeared to exert mechanical pressure on cell walls (Figure 4.6B). Intracellular penetration resulted in the colonisation of adjacent cortical cells (Figure 4.6C) and resulted in densely colonised areas (Figure 4.6D).

4.3.1.4 Colonisation of vascular tissue
Hyphae were observed seven days after inoculation entering adjacent xylem vessels by either directly penetrating vessel walls (Figure 4.7A) or by weaving through pits (Figure 4.7B-C). Constriction is evident in the hypha penetrating the thick cell wall in Figure
4.7A, indicative of intracellular penetration. The hyphae passing through pits in Figures 4.7B and C appear to deviate in their direction of growth to enter the adjacent cell via the path of least resistance. That is, the hyphae appear to change their course to favour growth in the direction of pit openings.

4.3.1.5 Heavily infested tissue
Observation of infested root tissue after 14 days revealed widespread colonisation of most cortical cells near the inoculation site (Figure 4.8A). Chlamydospores formed in tissue that had degraded and collapsed (Figure 4.8B).

A figure representing the Fusarium wilt disease cycle on cotton is presented that incorporates microscopic observations of the infection process (Figure 4.9).

4.3.2 *Fov* growth assays
4.3.2.1 Growth in root diffusate
Germination of microconidia was significantly higher in both types of cotton root diffusate than in water at 12-36 h (p≤0.05) (Figure 4.10A).

More microconidia germinated in Siokra 1-4 diffusate than Sicot 189 diffusate at each of the sampling periods after 1 h. The number of germinated microconidia increased with time until 24 h in each of the treatments, when the percentage that had germinated started to plateau. A second generation of microconidia was observed after 24 h, borne from hyphae that grew from the original microconidial inoculum.

The length of germ tubes growing from microconidia placed in water and Sicot 189 diffusate was similar (Figure 4.10B). The average length of germ tubes in these treatments increased to approximately 7 μm over 24 h. However, germ tubes grew significantly longer in Siokra 1-4 diffusate at 12-24 h. Germ tubes in this treatment grew an average of approximately 22 μm in 24 h.

Diffusates were subjected to heating or extraction with ethyl acetate to assess whether protein or lipophilic factors affected the growth of *Fov* (Figure 4.11). Untreated diffusates were included as standards, which resulted in similar microconidia germination and germ tube length as previous experiments (Figure 4.10).

Germination in Sicot 189 was unaffected by both diffusate treatments (Figure 4.11A). However, germination was reduced in Siokra 1-4 diffusate that had been extracted with
ethyl acetate. Both treatments had little effect on length of germ tubes when applied to Siokra 1-4 diffusate (Figure 4.11B). Extracting Sicot 189 diffusate with ethyl acetate resulted in a dramatic increase in the average germ tube length. In summary, removing lipophilic compounds from diffusate by extracting with ethyl acetate resulted in a decrease in microconidia germinating in Siokra 1-4 diffusate (Figure 4.11A) and an increase in the germ tube length in Sicot 189 diffusate (Figure 4.11B).

4.3.2.2 Growth in stele tissue
Fov progressed significantly further within Siokra 1-4 stele than in Sicot 189 (Figure 4.12). Pathogen growth was similar in both varieties until three days after inoculation, after which growth rates diverged. There was significantly less growth in Sicot 189 than in Siokra 1-4 (measured by distance from inoculation point) from Day 5-14 (p≤0.05).
4.4 Discussion

In terms of Beckman’s vascular wilt model (discussed in section 1.5.3), the “primary determinative phase,” consisting of root penetration and cortex colonisation, has received little research attention (Beckman, 1987). Studies into the “secondary determinative phase” constitute by far the majority of investigations, and focus on the colonisation and establishment of the pathogens in vascular tissues and the associated plant defence responses. The results presented in this chapter constitute an histopathological tracking of the pathogen from the root surface through to systemic vascular colonisation, and report on Fov growth in root exudate (primary determinative phase) and vascular tissue (secondary determinative phase). This Discussion aims to provide an overview of both phases of the Fov infection process.

4.4.1 Histopathological insights

The extensive colonisation of the root surface by Fov after inoculation with microconidia resulted in a hyphal net that ensheathed the root. A compact mycelium covering the root surface has also been observed on cotton seedlings inoculated with a Peruvian isolate of Fov (Rodriguez-Galvez and Mendgen, 1995b). The authors of that study suggested a dense surface mycelium was an essential precursor to the production of penetration hyphae, and therefore, infection. It is possible that this external growth phase is also characteristic of the infection of cotton by the Australian biotypes of Fov. An extensive mycelium has been noted on roots of cotton seedlings grown in sterile culture pots after inoculation with Australian Fov (Dowd et al., 2002). Each of these studies reported that this pre-penetration development of a thick mycelium on the root surface occurred within a period of approximately one day.

The mode of entry of Fov into the root epidermis was variable. Penetration was by both intercellular and intracellular penetration of the root, predominantly in the root hair zone. Hyphal growth along anticlinal cell wall ridges resulted in the ingress of hyphae between adjoining epidermal cells. Alternatively, direct penetration of epidermal cells took place soon after conidial germination. The subsequent passage through the root cortex was similarly by both inter- or intra-cellular growth. The ability of Fov hyphae to penetrate the root by either mechanism has been documented previously (Rodriguez-Galvez and Mendgen, 1995b). Due to vascular colonisation dominating the focus of past
studies of wilt pathogens, inoculations were most often directly into the xylem tissue, so data regarding initial penetration of the epidermis are rare.

As discussed previously, the entry point for *F. oxysporum* into different hosts is diverse. Rodriguez-Galvez and Mendgen (1995b) lamented that while observations of this pathogen were numerous there was "no quantitative data available indicating in which root zone penetration occurs." In response to this paucity, the authors documented that infection of 2-day-old cotton roots took place predominantly in the meristematic zone, to a lesser extent in the elongation and root hair zones, but not in the lateral root zone (Rodriguez-Galvez and Mendgen, 1995b). Using methods described in the current study (see 2.1.4), seeds incubated for two days consisted of a radicle of approximately 1-3 cm long, and division into functional zones would be extremely subjective.

For this reason, quantification was deemed inappropriate in this current study. In line with Rodriguez-Galvez and Mendgen's work, penetration was observed at various locations on the root, however infections in the root hair zone appeared to predominate. Rather than inferring where penetration is common in natural field infections, this observation may simply be due to the inoculation technique, involving dipping the roots into an inoculum suspension. Hyphae of Fov appeared to enter the roots with ease, with non-specific mode of penetration evident in all regions. A higher incidence of infection in the root hair zone may simply arise from a more concentrated source of inoculum after the entrapment of conidia by the root hairs. This is another reason why the results presented here are limited to qualitative observations.

It is well documented that during the colonisation of the cotton xylem tissues, the conidia of vascular pathogens travel in the transpiration flow until they become trapped at vessel end walls (Beckman *et al.*, 1976; Shi *et al.*, 1991a). The pathogen escapes these trapping sites by germinating, penetrating the end walls, and producing microconidia in the next vessel (Garber and Houston, 1966; Presley *et al.*, 1966). While this mechanism is not involved in the spread of *F. oxysporum* within all of its hosts (Baayen and De Maat, 1987; Kale-Wright, 1993), it facilitates colonisation of bananas (Beckman *et al.*, 1961), tomato (Beckman, 1987) and cotton (Shi *et al.*, 1991a).

It was assumed in this study that once Fov gained entry to the vascular system, passive transport of free microconidia in the transpiration stream was involved in vascular
colonisation. Evidence supporting this mode of colonisation was presented. Hyphae were observed growing within xylem vessels, and entering adjacent xylem vessel by either directly penetrating vessel walls, or by growing through pits in end walls. A distinctive constriction of hyphae penetrating xylem cell walls was seen, in line with the specialised Fov “penetration hyphae” described by Rodriguez-Galvez and Mendgen (1995b). The images depicting hyphae passing through pit openings presented in this chapter are all but identical to those presented by Shi et al. (1991) which show the penetration and growth of Fov hyphae through pits from an initially-infected vessel into a second vessel.

The appearance of Fov chlamydospores in my study was always associated with significant degradation of host tissues. A consequence of this damage to plant tissues would be the disruption of water transport that would ensure the onset of wilt symptoms. In this system, chlamydospores are merely indicators of an existing severe infection, rather than an infective propagule, which is perhaps why their formation in cotton has received so little attention. However, the potential consequence of the accumulation of these resting spores is an increase in the soil inoculum load for a consecutive crop.

It has been shown using an Australian Fov isolate that higher pathogen populations are supported by soils planted to the most susceptible cotton cultivar, Siokra 1-4, compared with a less susceptible cultivar (Wang et al., 1999b; Wang et al., 1999c). Differential chlamydospore formation in the cultivars may explain the increase in inoculum over a growing season, however, this has not been investigated. Previous glasshouse trials demonstrating an increase in incidence and severity of Fusarium wilt of cotton following incorporation of plant residues may also support a role for chlamydospores in increasing soil inoculum (Wang et al., 1999c). Chlamydospores were not quantified or compared in different host varieties in the current study, but warrant further investigation.

The formulation of the Fusarium wilt of cotton disease cycle within this study documents many detailed insights arising from this work. The disease cycle proposes several types of Fov propagules are present in the soil: chlamydospores, macroconidia, microconidia and hyphae. Infection of the roots is by both intracellular and intercellular penetration, frequently but not exclusively, in the root hair zone. Continued intra- and
inter-cellular growth through the root leads to colonisation of the vascular tissue. Proliferation in the xylem causes vascular browning, which is observed as brown flecks or a dark ring in a cross-section of the root. Hyphae and microconidia are observable in xylem vessels. Rapid colonisation of the xylem is by movement of microconidia with the transpiration flow of the plant. Microconidia become trapped at vessel end walls, where they germinate and either penetrate the wall or grow through pits. The production of microconidia in the adjacent vessel continues the new cycle of colonisation. Spread of the pathogen in a susceptible host leads to systemic infection and symptoms of wilt appear, firstly at the uppermost leaves, then extending down to include the rest of the plant. The plant loses its leaves and may either re-shoot (but never fully recover), or more often, die. Saprophytic growth continues until plant material is heavily infested. The pathogen is returned to the soil and spread via the use of machinery and/or by irrigation water.

While publications presenting abbreviated disease cycles for Fusarium wilt have been informally distributed amongst Australian cotton farmers and researchers for the purposes of Fusarium wilt disease management and investigation, this is the first Fov disease cycle to be published (Allen et al., 2003) (Smith, L., pers. comm. Department of Primary Industries and Fisheries, Queensland, Australia). A general lifecycle of F. oxysporum, including its saprophytic and parasite growth and successive phases of colonisation and pathogenesis within the generalised “host” plant has until now provided limited insights into the Fov-cotton interaction (Beckman, 1987). A basis of microscopic observations specific to this pathosystem adds value to the detailed Fusarium wilt of cotton disease cycle presented in this chapter.

4.4.2 Fov growth assays
4.4.2.1 Growth in root diffusate
Root diffusates were investigated under the assumption that they share similarities with root exudates in the field. Root exudates are substances that are released by plant roots and leak out into the surrounding medium (Curl and Truelove, 1986). Importantly, they contain carbohydrates and amino acids that influence the surrounding rhizosphere, including soilborne microorganisms. They may promote the growth of fungi and bacteria via the non-specific provision of nutrients, or contain highly-specific triggers for particular pathogens (Rovira, 1969). They may also contain toxic compounds that adversely affect spore germination or hyphal growth of pathogens (Katan, 2002).
Root exudates may be collected for study from plants grown in different mediums, including soil or solution culture (Rovira, 1969). This current investigation used water to collect diffusates, similar to a technique published for collecting cottonseed exudates (Hayman, 1970). A simple non-specific comparison of the effect on Fov of diffusates from least susceptible (Sicot 189) and most susceptible (Siokra 1-4) Australian cotton varieties demonstrated differences. Germination of microconidia was not only greater in cotton root diffusate compared with water, but more germination occurred in the diffusate of the most susceptible variety, Siokra 1-4. In addition to this effect, the germ tubes were longer in Siokra 1-4 diffusate.

The differential germination and growth of other formae speciales of *F. oxysporum* in host exudate of differing susceptibilities has been documented. For example, pea root exudates from cultivars resistant to *F. oxysporum* f. sp. *pisí* depress both the germination of conidia and hyphal growth (Buxton, 1957). The relevance of varietal differences in exudate to chlamydospore germination has been questioned in the past, considering the non-specific germination of these resting spores in the presence of a carbon/nitrogen source (Schroth and Hildebrand, 1964). However Youssef and Heitefuss (1983b) showed the influence of differential cotton root exudates on all Fov propagules. In addition to increased mycelial growth, germination of both conidia and chlamydospores was greater in media containing exudates from a susceptible cultivar than from a resistant one (Youssef and Heitefuss, 1983a).

Contradictory findings on the effects of pea exudates on *F. oxysporum* f. sp. *pisí* highlight the complexity of the interaction. While Buxton (1957) postulated that the lower conidial germination in root exudate of a resistant host was due to inhibitory compounds, others suggested the basis for the difference was nutritional (Whalley and Taylor, 1973). Further studies reported a stimulatory effect of pea root exudates on the germination of conidia in sterile water and chlamydospores in soil, but found this was not related to host cultivar susceptibility (Schippers and Voetberg, 1969; Whalley and Taylor, 1973; Whalley and Taylor, 1976).

The effects of root diffusates on Fov described in this chapter may be attributed to varietal differences operating in two complementary ways. The simple provision of substrate may result in the promotion of fungal growth, and the presence of antifungal
compounds in diffusate may act as a growth-inhibitive preinvasive defence mechanism. Each of these possibilities will be dealt with in turn.

Many soil-borne pathogens are supported by nutrition from root exudates (Snyder, 1970). A wide range of compounds exude from roots, including carbohydrates, amino acids, peptides and enzymes (Rovira, 1969). Depending on variety, the composition of cottonseed and cotton root exudates can vary (Hayman, 1970; Sulochna, 1962).

Varietal differences, such as the exudation of more amino acids from the roots of some cotton varieties may affect their interaction with pathogens (Sulochna, 1962). Nitrogen has been shown to have multiple effects on Fusarium wilts of cotton (Nakamura et al., 1976), chrysanthemum (Woltz and Engelhard, 1973) and tomato (Woltz and Jones, 1973). Increasing soil nitrogen is also correlated with increased root disease of bean caused by Fusarium solani f. phaseoli (Weinke, 1962). The influence of cotton root exudate on Fov in the rhizosphere has not been studied.

The components of root exudates can be affected by environmental parameters. Hayman (1970) documented qualitative and quantitative differences in exudates from two varieties of cottonseed over a range of temperatures. An increase in seedling infection by R. solani was correlated with increased exudation at low temperatures. He suggested that the increase in infection may be due primarily to the greater accumulation of nutrients at low temperatures (Hayman, 1970). The exudate of cotton seeds and roots also change in response to other environmental factors such as exposure to herbicide (Youseff and Heitefuss, 1983b).

There is evidence of the inhibition of spore germination of F. oxysporum f. sp. capsici by pea and chilli exudate (Naqvi and Chauhan, 1980). Cotton roots have been shown to exude terpenoid compounds, which may then accumulate on the root surface (Hunter et al., 1978b). The amount of antifungal terpenoid compounds that are exuded into the soil by cotton plants is increased in response to inoculation with R. solani (Hunter et al., 1978a). Hedin et al. also documented a greater accumulation of terpenoids in soils surrounding the roots of cotton resistant to Fusarium wilt and root-knot nematode compared to a susceptible variety (Hedin et al., 1984).

An inhibitory terpenoid or similar compound may be present in root diffusate of Sicot 189. Treatment involving the removal of lipophilic compounds from the diffusates
resulted in a dramatic increase in Fov germ tube length in Sicot 189. This indicates the presence of a lipophilic inhibitory factor in the diffusate, potentially a terpenoid. Germ tube length in Siokra 1-4 diffusate remained constant after the same treatment, perhaps indicating the absence of any lipophilic inhibitor. Interestingly, extracting with ethyl acetate resulted in a decrease in microconidia germination in Siokra 1-4 diffusate, which may point to a lipophilic component in Siokra 1-4 diffusate that promotes, rather than inhibits, the germination of Fov conidia.

4.4.2.2 Growth in stele
The progression of Fov through the stele tissue was different depending on the host variety. The pathogen travelled significantly further within Siokra 1-4 stele than in Sicot 189. The spread of Fov was initially similar in the two varieties, but after three days pathogen growth through the least susceptible Sicot 189 stele appeared to be restricted. This finding is consistent with earlier research that showed the first four days after inoculation was the critical period in which a resistant host plant restricts Fov colonisation (Harrison and Beckman, 1982; Shi et al., 1991a; Shi et al., 1992; Shi et al., 1993).

While inhibition in the current study became evident within a similar timeframe to studies of US Fov isolates, the magnitude of both pathogen progression and inhibition was not comparable. In the susceptible US cotton variety, the fungus was detected up to 34 cm from the inoculation site after six days, while there was no spread in the resistant hosts (Shi et al., 1993). However, the Australian Fov isolate progressed approximately ten cm in the seven days after inoculation in Siokra 1-4, while there was still approximately six cm spread in Sicot 189.

Due to the lack of a variety resistant to Australian Fov, it is not unexpected that the differences between the least and most susceptible would be reduced. The comparatively greater spread of Fov through the susceptible host in US studies may also be attributed partly to the inoculation method used. The taproots were severed from plants before they were immersed into a spore suspension. This technique ensures the immediate entry to the vascular system of a vast amount of spores, and can also place the plant under extreme stress, as documented previously (see 3.3.1.2.1). It is possible that these factors facilitated the passage of the pathogen through the stele tissue. The taproot puncture method of inoculation used in this current study is believed to be more
representative of a natural infection, but may have resulted in less striking differences between the least and most susceptible host varieties. Nevertheless, the statistical significance of the differences between the varieties after 3 days provides evidence of some resistance. The expression of resistance found in Sicot 189 plants in this study conforms to the notion that “the restriction of fungal spread in the vascular tissues is a characteristic of the resistance of cotton plants” (Shi et al., 1993).

The mechanism responsible for the putative inhibition of fungal growth in cotton stele is likely to be the production of antifungal compounds. The production and accumulation of terpenoid compounds are well documented responses of cotton to infection by *V. dahliae* (Garas and Waiss, 1986; Mace, 1983), *V. albo-astrum* (Zaki et al., 1972b), and Fov (Harrison and Beckman, 1982). They accumulate in appropriate locations and in adequate concentrations for their antifungal properties to act as a factor in resistance to wilt diseases (Mace et al., 1974a; Shi et al., 1991a; Shi et al., 1991b; Shi et al., 1992; Mace et al., 1985; Zhang et al., 1993). These compounds will be further investigated in subsequent chapters.

### 4.4.3 Conclusions

The infection process of Fov on cotton shares many similarities with other Fusarium wilts and other cotton wilt pathogens. Its description here, including the presentation of a disease cycle, is the most complete survey to date of the interaction of Australian Fov with commercial cotton varieties.

VARIetal differences were found in the diffusates of cotton roots. The diffusate from the most susceptible Siokra 1-4 appeared to contain one or more lipophilic compounds that promoted the germination of Fov microconidia. There is also evidence to show that another lipophilic compound inhibited the growth of germ tubes in the least susceptible Sicot 189 diffusate. Cotton produces abundant terpenoids with antimicrobial activities that are also lipophilic. Evidence for the identity of the inhibitory compound will be gathered in subsequent chapters.

Colonisation by Fov of vascular tissues of Sicot 189 was inhibited. This restriction is characteristic of host resistance and is likely to be associated with the terpenoid defence mechanism.
The growth assays presented in this chapter document the behaviour of Fov in discrete circumstances, removed from the dynamic infection process as a whole. Whilst these assays have a very specific focus, the observations made under these limitations provide preliminary insights into defence responses of host plants. A more comprehensive investigation of the respective defence mechanisms of the two cotton varieties is reported in Chapter 5.
Figure 4.1 Method used for quantification of *in vivo* pathogen growth.

(A) Growth of plants in rootboxes; (B) inoculation by taproot puncture; (C) sectioning of stele; and (D) incubation of sections on selective agar plates.
**Figure 4.2 Colonisation of the root surface.**

Inoculated whole fresh roots were stained with DioC₃(3) and viewed with UV epifluorescence to reveal: (A) abundant microconidia on the root epidermis immediately after inoculation; (B) hyphal growth along anticlinal cell wall ridges after microconidia germination on the root surface 12 h after inoculation; (C) formation of a hyphal net that encased the root 24 h after inoculation; and (D) the production of abundant macroconidia in the hyphal net.
Figure 4.3 Penetration of the root epidermis

(A) Observation by SEM of a whole inoculated root revealed a thick hyphal net that was partially removed from the surface of the root (Day 3). UV epifluorescence of tissue stained with DioC<sub>6</sub>(3) showed hyphal growth along cell wall ridges and intercellular penetration of the root surface underneath the hyphal sheath. (B) Whole cleared roots stained with lactophenol cotton blue three days after inoculation also showed intercellular penetration of the root epidermis (C-E).
Figure 4.4 Penetration of root hairs.
Whole cleared roots stained with lactophenol cotton blue showed microconidia germinating and: (A) directly penetrating a root hair tip; or (B) root hair base; or (C) penetrating via the intercellular space between root hair and other epidermal cell walls. Images taken three days after inoculation.
Figure 4.5 Colonisation of host cells.
Whole cleared roots stained with lactophenol cotton blue 5 days after inoculation showed: (A) intense colonisation of discrete epidermal cells including (B) root hair cells. Root epidermal cells packed with hyphae were observed underneath the hyphal net (C). Sections of fixed tissue were stained with toluidine blue seven days after inoculation.
Figure 4.6 Colonisation of the cortex.

(A) Hyphal growth was both intra- and intercellular seven days after inoculation. (B) Hyphae appeared to exert mechanical pressure on cell walls and (C) colonise adjacent cortical cells after intracellular penetration. Densely colonised cells were observed (D). Thin sections were fixed and stained with toluidine blue. Arrowheads = Fov hyphae; small arrows = intracellular penetration; large arrows = intercellular penetration.
Figure 4.7 Colonisation of the xylem.
Hyphae transgressed xylem vessel walls, by either: (A) directly penetrating vessel walls where no pits were observable; or (B-C) entering through pit openings between adjacent vessels. Thin sections were fixed and stained with toluidine blue. Images taken seven days after inoculation.
Figure 4.8 Roots heavily infested with *Fov*.
Colonisation of tissues resulted in: (A) widespread infection of taproot cortical cells near the inoculation site; and (B) the loss of cellular integrity and formation of chlamydospores. Whole cleared roots were stained with lactophenol cotton blue 14 days after inoculation.
Figure 4.9 Disease cycle of *Fusarium oxysporum* f. sp. *vasinfectum* on cotton.
Colonisation of xylem tissue results in vascular browning.

Colonisation of xylem by movement of microconidia in transpiration flow. When trapped at vessel end walls, spores germinate, penetrate end walls & produce microconidia.

Colonisation continues causing systemic infection in susceptible host.

Intra- & intercellular penetration of root hair zone.

Plant wilts from top down and dies.

Germinated conidia or chlamydospires attack roots.

Saprophytic growth on plant debris increases soil inoculum. Dispersed by machinery & irrigation water.

Fov in soil (I-r): Chlamydospore, macroconidia, microconidia & hypha.
Figure 4.10 The growth of Fov in root diffusate.

(A) Germination of microconidia, and (B) length of germ tubes. Germination was measured over 36 h. Due to the production of a second generation of microconidia in Siokra 1-4 after 24-36 h, germ tube length was measured up to 24 h. Values are the mean of five experiments and error bars represent the standard error.
Figure 4.11 The growth of Fov in treated root diffusate.

(A) Germination of microconidia, and (B) length of germ tubes. Measurements were made 12 h after microconidia were added to the diffusates. Values represent the mean of three experiments and error bars represent the standard error.
Figure 4.12 The colonisation by Fov of cotton stele tissue.
Distance travelled by Fov VCG's 0111 within the stele of six-week-old plants inoculated by taproot puncture and grown under glasshouse conditions (see 2.1.3). Four plants from each variety were harvested and surfaced sterilised 0, 1, 3, 7 and 14 days later. Successive sections were plated onto Fusarium-selective agar and incubated for one week. Fov grew from infected sections onto surrounding agar. Values represent the mean distance from the severed root tip of the furthestmost infected sections. Bars represent the standard errors of four experiments. \( P \)-values: Day 5 = 0.023; Day 7 = 0.018; Day 14 = 0.001.
CHAPTER 5 HISTOLOGICAL RESPONSES TO INFECTION

5.1 Introduction
The restriction of pathogen growth in vascular tissue is involved in the resistance of cotton to several wilt diseases (Wilhem et al., 1974b; Harrison and Beckman, 1982). Shi et al. (1993) more specifically found that "the restriction of fungal spread in the vascular tissue is a characteristic of the resistance of cotton plants to Fusarium oxysporum f.sp. vas insetum."

Resistant plants infected with the cotton wilt pathogens V. dahliae and Fov are able to restrict colonisation to the lower stem, whereas spread is extensive throughout the susceptible varieties (Wilhem et al., 1974a; Harrison and Beckman, 1982). The occlusion of infected vessels and the production of terpenoid phytoalexins has been compared in resistant and susceptible hosts. A physical localisation of V. dahliae occurred rapidly in resistant hosts, followed or accompanied by phytoalexin production that appeared to kill pathogen propagules (Mace, 1978). Terpenoid aldehyde accumulation was monitored histochemically, to reveal a more intense response that extended beyond the region of infection and in advance of the spreading pathogen in the resistant host (Harrison and Beckman, 1982).

This integrated sequence of physical and biochemical defences whereby infected vessels are occluded with phytoalexins, is akin to defence responses associated with wilt resistance in cocoa (Cooper and Williams, 2004), peas (Bishop and Cooper, 1984; Tessier et al., 1990) and tomato (Beckman et al., 1972; Beckman et al., 1991).

All of the cotton terpenoid phytoalexins are toxic to Fov and V. dahliae (see 1.5.2.4.3) (Mace et al., 1985; Zhang et al., 1993). Crude stele extracts from V. dahliae-infected cotton plants have been shown to completely inhibit mycelium growth when incorporated into growth medium (Garas and Waiss, 1986). The differential accumulation of antimicrobial terpenoids between resistant and susceptible cotton varieties after inoculation with Fov provides further evidence for their role in inhibiting pathogen colonisation in resistant varieties (Zhang et al., 1993).

The accumulation of antimicrobial terpenoids in response to infection does not directly infer a role in resistance. Phytoalexin studies demonstrate correlations between disease
resistance and the presence of compounds with *in vitro* toxicity. Good evidence for the role of phytoalexins in defence exists (Keen, 1981), however no direct proof of the cessation of pathogen growth in plant tissue due to phytoalexin toxicity has been published (Hammerschmidt, 1999).

As discussed previously (see 1.5.2.1), the accumulation of phenolic compounds has been shown to influence resistance of cotton to fungal pathogens. Higher concentrations of phenolic compounds accumulate in cotton resistant to *A. macrospora* and *V. dahliae* compared to the more susceptible varieties (Bashan, 1986; Mace *et al.*, 1978). The role of phenolics in Fusarium wilt has not been investigated.

The responses of two cotton cultivars of differing susceptibility to Australian Fov will be presented in this chapter. Different microscopic methods and two histochemical stains were used to compare the defence responses of the two host cultivars. A technique using antimony trichloride (SbCl$_3$) was developed by Mace *et al.* (1989) after the reagent originally designed for steroid detection in plants (Hardman and Safowora, 1972) was found to have a high degree of specificity for cotton terpenoids. Toluidine blue has previously been used to visualise colonisation by *Fusarium oxysporum* (Brammall and Higgins, 1988) and staining reactions of plant structures and defence responses have been defined (O'Brien and McCully, 1981). The hypothesis that the least susceptible cultivar responds to infection with a rapid accumulation of antimicrobial compounds that restrict pathogen growth was investigated.
5.2 Materials and Methods

5.2.1 Plant and pathogen

Six-week-old cotton plants and the inoculum suspension were prepared as previously described in Chapter 2.

5.2.1.1 Inoculation

Two techniques were used to inoculate cotton with Fov in order to study the host defence responses.

Initially six-week-old Sicot 189 plants were inoculated by placing roots into 1 L of water to which 1 mL of microconidial suspension (2.5 × 10⁶ conidia/mL) was added. Plants were harvested at seven days. This ‘root-dip’ inoculation method was previously described (see 4.2.1.1) and was used only for initial qualitative assessment of plant responses. Stem tissue was cut approximately 5 cm above the root mass and embedded and stained with toluidine blue (see 4.2.1.2.2).

The second technique allowed comparison of the different cotton varieties’ responses to infection of the vascular tissue. Inoculation of taproots of Siokra 1-4 and Sicot 189 plants grown in root-boxes was performed using the taproot puncture method as described in Chapter 4 (Section 4.2.2.2.2). Sterile deionised water was used in place of microconidial suspension for the mock-inoculated control plants. After three or five days incubation in root-boxes, a 0.2 cm length of the stem/taproot 2 cm above the point of inoculation was cut, and processed using one of the three methods described below.

5.2.2 Histochemical techniques

Various techniques were used to visualise responses of cotton tissues to Fov invasion.

5.2.2.1 Embedded tissue / toluidine blue stain

The method used for embedding Sicot 189 and Siokra 1-4 tissue and staining with toluidine blue has been described previously (see 4.2.1.2.2).

Images were captured with an Olympus BH2 light microscope fitted with an Olympus DP10 digital camera system.

5.2.2.2 Transmission electron microscopy

Tissue samples from Sicot 189 plants were embedded as described in 4.2.1.2.2. Resin blocks were trimmed using a Leica EM TRIM, then ultrathin (90 nm) sections were cut
with a DiATOME diamond knife on a Leica UltracutR microtome. Sections were collected into pioloform-coated copper grids (100 mesh). Grids were sequentially stained with 2.5% uranyl acetate for 15 min and triple lead stained for 10 min (Sato, 1968). Sections were viewed using a Philips CM120 biotwin TEM at 120 kV and imaged with a Gatan MultiScan CCD camera (Model 791).

5.2.2.3 Fresh tissue / toluidine blue or SbCl₃ – HClO₄ reagent
Fresh free-hand sections (approximately 100 µm thick) were cut from six-week-old Sicot 189 and Siokra 1-4 plants that had been inoculated by taproot injection in root boxes. Samples were taken 1 cm from the point of inoculation.

To allow for the direct comparison of observations of embedded and fresh tissue, fresh hand sections were mounted in 0.05% toluidine blue stain at pH 6.8.

Alternatively, a saturated solution of antimony trichloride (SbCl₃) in 40% perchloric acid (HClO₄) was used to histochemically localise terpenoids in fresh tissue (Mace et al., 1989). A few drops of the SbCl₃-HClO₄ reagent were added to several sections on a glass slide. A coverslip was added and sections were photographed within 5 min. Images were captured using an Olympus light microscope equipped with an Olympus U-PMTVC adapter and a Leica DC300F camera and processed by Leica IM50 Image Manager V1.20 software.
5.3 Results

5.3.1 Embedded tissue / toluidine blue

Responses were observed in cotton tissue harvested seven days after Fov inoculation by root-soaking. There was an apparent accumulation of materials that stained with toluidine blue around the site of invading hyphae. There were two distinct staining reactions: one material became a vivid green colour; the other stained a pale aqua blue, similar in colour to xylem vessel walls. These reactions were observed in both Siokra 1-4 and Sicot 189 tissue samples.

The green-staining compound was observed within epidermal cells (Figure 5.1A) and parenchyma cells of both the cortex (Figure 5.1B and C) and vascular tissue (Figure 5.1D). An example of this response in primarily infected epidermal cells can also be observed in micrographs presented in Chapter 4 (Figures 4.5C and 4.6D). This response occurred within cells containing hyphae (Figures 5.1A and B) and also in uninvaded cells adjacent to cells containing hyphae (Figures 5.1C and D).

The other compound was observed coating hyphae growing both between (Figure 5.2A) and inside vascular parenchyma cells (Figs. 5.2B). This material stained a similar colour to vessel walls, and accumulated in intensely-stained parenchyma cells immediately adjacent to vessels containing hyphae (Figure 5.2C).

Uninfected plants’ contact cells had a single large central vacuole, with the remainder of the cytoplasm adpressed to the cell wall. The vessel lumen is generally devoid of contents that stain with toluidine blue (Figure 5.3A).

When infected Sicot 189 tissue was harvested at Day 3, green-staining compounds were not visible. There were however accumulations of materials in xylem vessels of infected plants that stained a similar colour to xylem vessels walls, and these were associated with physical changes to contact cells (Figure 5.3B).

However, in infected Siokra 1-4 and Sicot 189 plants, contact cells appeared to have an increased cytoplasmic content in conjunction with a partitioned vacuole. The cytoplasm of these cells stained densely, and contained several small vacuoles. In the vicinity of these cells there was frequently an accumulation of amorphous materials in vessel lumens (Figure 5.3B). The amorphous material in vessels lined vessel walls (Figure...
5.4A) and typically appeared to stream in from a single point to form a globular structure (Figure 5.4B).

These cellular changes were visible in infected tissue of both cotton varieties at Days 5 and 7 after inoculation, although in a greater magnitude in Sicot 189. At Day 3, however, changes to contact cells were observed in Sicot 189, but Siokra 1-4 displayed little difference to the mock-inoculated control plants (Figure 5.5).

**5.3.2 Transmission electron microscopy**

Ultrastructural images of Sicot 189 vascular tissue further illustrate discernable cellular changes in response to inoculation with Fov. Uninfected vessel lumens appeared empty, and contact cells had a single large central vacuole, with the remainder of the cytoplasm adpressed to the cell wall (Figure 5.6A). However, the vessel lumens of infected plants often contained electron-dense granular materials, while in adjacent contact cells the cytoplasm became electron-dense and granular and filled up the cell (Figure 5.6B).

In infected tissue, the electron-dense granular amorphous materials in vessels had a similar appearance to the some of the granular material in the contact cells. This amorphous material appeared in vessel lumens adjacent to the contact cells that had changed in response to infection, and was visible in the pits between cells (Figure 5.6B). Small quantities of this material were sparsely distributed in the lumen, and were not associated with any tyloses.

The freely-moving materials lined vessel walls (Figures 5.7A and B) and passed through pits between vessels (Figures 5.7C and 5.8A). The materials sometimes clung together to form a globular mass (Figure 5.7C) similar to the accumulations observed in embedded (Figure 5.4) and fresh tissue (Figure 5.9B). The amorphous materials passed through cell wall pits (Figure 5.8A) and vessels containing a small amount of material were observed immediately adjacent to cells where the vessel lumen was full (Figure 5.8B).

**5.3.3 Fresh tissue stained with toluidine blue**

Toluidine blue stain was also applied to free-hand sections of Sicot 189 fresh tissue. Amorphous materials that stained a similar colour to vessel walls lined the inside of xylem vessels (Figure 5.9A) and formed globular structures (Figure 5.9B) within fresh
tissue, as had been observed in embedded samples. This material was similar to that observed with toluidine staining of embedded sections.

5.3.4 Fresh tissue stained with SbCl₃-HClO₄

Fresh tap root tissue was observed after treatment with SbCl₃-HClO₄ reagent. Resulting orange, pink and red products indicated there was a heterogeneous distribution of different terpenoids in the vascular tissues.

Mock-inoculated plants displayed faint pink staining of the phloem in Siokra 1-4 (Figure 5.10A) and Sicot 189 (Figure 5.10B). There were no detectable differences between the varieties. Infected plants of both varieties had a pink-staining product in the phloem, and dark orange staining areas in xylem tissue, however there was substantially less orange-coloured product in Siokra 1-4 (Figure 5.10C) than in Sicot 189 (Figure 5.10D).

Infected Sicot 189 xylem stained in discrete areas, with some xylem elements appearing dark orange while others in close proximity remained clear (Figure 5.11). Both vessel walls and the surrounding parenchyma cells stained orange (Figure 5.11A and B). The intensity of staining of vessel walls varied between individual cells. Cell walls that stained very dark orange could be found immediately adjacent to pale orange or unstained walls (Figure 5.11C).

Intensely-stained vessels occurred in the immediate area around Fov hyphae, while more distant vessels often contained no orange-staining product (Figure 5.12A). Hyphae within vessels also took on an orange colour (Figure 5.12B and C).

Orange-staining materials were observed lining the inside walls of vessels (Figure 5.13A) and accumulating within vessel lumens (Figure 5.13B and C). The colour of the materials in vessels sometimes varied and incorporated red tones (Figure 5.13C). Some individual vessel lumens contained large amounts of these materials (Figure 5.13D).
5.4 Discussion
This chapter described defence responses observed in two Australian cotton varieties after inoculation with Fov. These responses included changes in the structure and chemistry of the host cells.

5.4.1 Structural changes
Significant structural changes in cotton xylem tissue were observed in response to Fov inoculation. They were identified using light and transmission electron microscopy (TEM) that allowed detailed observation and comparison to published findings using similar techniques. In the present study, infection induced the reorganisation of contact cells in vascular tissue. Infection induced an increase in cytoplasmic content and a partitioning of the vacuoles, corresponding with the appearance of materials apparently secreted through pits into adjacent vessel lumens. These changes were observed using both light microscopy and TEM.

In a series of TEM studies on the changes of the contact cells of US cotton cultivars, Shi et al. documented varied responses to Fov infection (Shi et al., 1991a; Shi et al., 1991b; Shi et al., 1992). The nature of the response depended on the location of the cells relative to infected vessels. In the most common type of response, contact cells showed evidence of cytoplasmic reorganisation and increased metabolic activity. Cytoplasm disintegration was observed in some contact cells, with more rapid degeneration in those cells directly penetrated by hyphae. These changes resulted in the development of osmiophilic droplets in the cytoplasm that were secreted through the plasmalemma into the apposition layer. However this layer was not observed in contact cells surrounding the secondarily infected vessels into which the fungus had penetrated from initially infected vessels. The increase in cytoplasmic content and concomitant decrease in the size of the central vacuole in this case was linked to the direct secretion of osmiophilic materials into the vessels that consequently coated the fungus. The responses described in this chapter resemble those of contact cells adjacent to the secondarily infected vessel. It is feasible that Fov could have spread, either laterally or longitudinally, from one infected vessel to another in tissue collected on Day 3 from 2 cm from the point of inoculation.

Globular structures accumulated in vessels superficially resembling tyloses when viewed with light microscopy. However, when viewed with TEM no tyloses were
detected in line with past research into cotton’s response to *V. dahliae* (Mueller and Morgham, 1993) and Fov (Shi et al., 1992). It is probable, as in previous work, that these globular structures were in fact terpenoid phytoalexin accumulations being secreted into the vessel lumen.

The changes to the structures of contact cells, and the associated accumulation of materials in vessels were observed in both varieties in the current study. However, the responses were greater and faster in the less susceptible variety, Sicot 189. Likewise, the cellular responses observed in both resistant and susceptible US cultivars were produced faster and more pronounced in the contact cells of the resistant cultivar (Shi et al., 1991a). This was also the case with vessel occlusion by the secretion products of contact cells (Shi et al., 1992).

TEM allowed the confirmation that the globular structures in vessels were not bound by a membrane, and were therefore not tyloses. Histochemical staining of sections was undertaken to better understand the nature of the materials that accumulated in vessels after inoculation.

### 5.4.2 Chemical defences

Staining reactions with toluidine blue and the SbCl₃-HClO₄ reagent indicated the presence of phenolic and terpenoid defence responses.

#### 5.4.2.1 Phenolics

The occurrence of a green product after staining sections with toluidine blue indicated the presence of phenolic compounds. Staining indicative of phenolic accumulation was observed within epidermal, cortical and vascular cells seven days after inoculation. However phenolics were not present three days after inoculation in either embedded or fresh tissue, suggesting a limited effect on the outcome of the host-pathogen interaction. Phenolics have been found to be more likely to be present at a later time after inoculation than terpenoids (Mace et al., 1978), but the outcome of infection has been shown in this and another study (Shi et al., 1993) to be determined between three and four days after inoculation.

The high concentrations of tannins and other polyphenols associated with host resistance to *V. dahliae* are thought to have minor importance in the initial defence of cells in the vascular tissue (Bell, 1969). Although it has been suggested that phenols
participate in resistance of cotton to *Alternaria macrospora*, the extent of their role was not quantified (Bashan, 1986).

Not only are phenolics absent in the early stages of infection, but their broader interaction with other organisms may also limit their usefulness as a tool for disease control. For instance, plant phenolic oxidases in both cotton and tomato have a profound ability to modulate disease in insects caused by baculoviruses, which has serious implications for integrated pest management (Hoover *et al.*, 1998).

### 5.4.2.2 Terpenoids

The location and appearance of the compound that stained pale blue after the application of toluidine blue corresponded with the orange product resulting from SbCl₃-HClO₄ staining, identifying it as a terpenoid.

Staining with toluidine blue allowed the visualisation of materials present seven days after inoculation in both Siokra 1-4 and Sicot 189. This aqua-blue staining compound was located between and inside vascular parenchyma cells, and in association with hyphae. At Day 3 however, these materials lined vessel walls and formed globular structures in vessel lumens of Sicot 189, but not in Siokra 1-4.

Through the use of the terpenoid-specific reagent, SbCl₃-HClO₄, these materials were identified as terpenoids. Several different staining reactions occurred in the cotton xylem tissue of infected plants which stained varying shades of orange or red, particularly in Sicot 189. This indicates the presence of more than one terpenoid compound. Thin layer chromatography of different purified cotton phytoalexins has shown the production of a range of red- and orange-coloured products after application of the SbCl₃-HClO₄ reagent depending on the terpenoid (Mace *et al.*, 1974a).

Cotton parenchyma cells surrounding vessels were the sites of terpenoid accumulation in this study, supporting a previous description of these “scattered, usually solitary, paratracheal parenchyma cells adpressed to infected xylem vessels” as the site of phytoalexin synthesis (Mace *et al.*, 1976). Intense accumulation of terpenoids around the site of infection were reported in this chapter, which sometimes coated the Fov hyphae within vessels. The movement of terpenoids from contact cells to infected vessel lumens facilitated the direct impact of the phytoalexins on fungal hyphae (Shi *et al.*, 1992; Mueller and Morgham, 1993). Terpenoids lined the inside walls of vessels and
accumulated within vessel lumens in the same fashion as the amorphous materials documented in this chapter after toluidine blue staining, and may act to establish a preformed barrier to invading hyphae (Shi et al., 1992).

Further detail of the accumulating terpenoids and correlation with past research was obtained using TEM. The appearance of granular materials in vessel lumens adjacent to the contact cells that had changed in response to infection has been described in previous studies of terpenoid accumulation (Shi et al., 1991a; Mueller and Morgham, 1993). The sparse distribution or globular accumulation of the terpenoids in the lumen not only correlated with materials suspected to be terpenoids in embedded and fresh tissue in this study, but was identical to previous descriptions of the terpenoid response of cotton to Fov and V. dahliae based on TEM (Shi et al., 1991a; Mueller and Morgham, 1993). The freely-moving terpenoids lined vessel walls, passed through pits between vessels, and occluded vessel lumens, as described in a previous ultrastructural study of the phytoalexin response to Fusarium wilt of cotton (Shi et al., 1992).

Healthy uninoculated cotton plants have previously been shown to contain terpenoids (Bell, 1967; Bell, 1969; Zaki et al., 1972b). Small quantities of terpenoids were observed in the current study, as indicated by faint staining reactions of the phloem of mock-inoculated control plants of both varieties which lends support to their involvement in constitutive defence of cotton plants.

5.4.3 Conclusion
The results presented in this chapter provide evidence for a differential terpenoid response in Australian cotton varieties. The occurrence of a phenolic response seven days after Fov inoculation precludes the involvement of this response in the determination of susceptibility to Fusarium wilt. While the nature of the structural and terpenoid responses in Siokra 1-4 and Sicot 189 were similar, they were more intense and rapid in the latter. The rates of response could not be determined due to the sampling of only one time point and location, so characterisation and quantification of the terpenoid response will be reported in Chapter 6.
Figure 5.1 Sicot 189 tissues seven days after root-dip inoculation with Fov showing a green-staining compound.

Samples embedded in LR White resin were stained with toluidine blue. A green-staining compound (large arrows) was observed in (A) epidermal cells and (B) cortical parenchyma cells invaded by Fov hyphae (small arrows). The compound was also found in uninjured parenchyma cells of both the (C) cortical and (D) vascular tissue. Small arrow = Fov hypha, V = xylem vessel, C = contact cell.
Figure 5.2 Sicot 189 tissues seven days after root-dip inoculation with Fov indicating a pale blue-staining compound.

Samples embedded in LR White resin were stained with toluidine blue. The pale blue-staining compound (large arrows): (A) covered intracellular hyphae; and (B) intercellular hyphae; and (C) accumulated in contact cells adjacent to infected xylem vessels with visible Fov hyphae. Small arrow = Fov hypha, V = xylem vessel, C = contact cell.
Figure 5.3 Xylem tissue of Sicot 189 plants three days after taproot-puncture inoculation with Fov.

Samples embedded in LR White resin were stained with toluidine blue. Longitudinal sections of xylem tissue of (A) uninfected and (B) infected plants. Amorphous materials (arrows) appear in xylem vessel (V) lumens of infected plants. Contact parenchyma cells (C) in infected plants stained darker, had an increased cytoplasmic content, and a partitioned vacuole.
Figure 5.4 Sicot 189 xylem vessels three days after taproot-puncture inoculation with Fov.

Samples embedded in LR White resin were stained with toluidine blue. Amorphous materials (arrows) lined walls and formed globular structures (X) in xylem vessel (V) lumens. (A) Whole vessel lumen lined with materials which stain a similar colour to xylem wall. (B) Discrete globular collection of material in lumen which appeared to originate from a single point of the cell wall.
Figure 5.5 Changes in contact cells and vessel lumens three days after Fov taproot-puncture inoculation.

Representative sections of mock-inoculated (A) Siokra 1-4 and (B) Sicot 189, and Fov-inoculated (C) Siokra 1-4 and (D) Sicot 189. Taproot xylem tissue was sampled 2 cm from the inoculation point. Small arrows indicate contact cells containing unaltered cytoplasm, large arrows indicate contact cells containing altered cytoplasm. Tissue samples embedded in LR White resin were stained with toluidine blue.
Figure 5.6 Longitudinal TEM views of the Sicot 189 vascular tissue.
The vessels (V) and contact parenchyma cells (C) of: (A) an uninfected Sicot 189 plant and (B) a Sicot 189 plant 3 days after Fov taproot-puncture inoculation.
Figure 5.7 TEM images of Sicot 189 xylem vessels three days after *Fov* taproot-puncture inoculation.

Amorphous materials associated with vessels (V): (A) lined vessel walls; (B) accumulated in vessel lumens; and (C) passed through pits in vessel walls to form globular structures (X) in adjoining lumens.
Figure 5.8 TEM images of Sicot 189 xylem vessels three days after Fov taproot-puncture inoculation.

Amorphous materials associated with vessels (V): (A) passed through pits in vessel wall until; (B) the accumulation occluded vessel lumens.
Figure 5.9 Accumulation of amorphous materials in Sicot 189 xylem vessels three days after Fov taproot-puncture inoculation.

Cross-section of fresh tissue stained with toluidine blue showing amorphous materials (arrows): (A) lining vessel walls; and (B) forming globular structures in vessel lumens.
Figure 5.10 Radial sections of uninfected and Fov-infected cotton.
Representative sections of uninfected Siokra 1-4 (A) and Sicot 189 (B) and infected Siokra 1-4 (C) and Sicot 189 (D) plants are shown. Fresh tissue sampled three days after taproot-puncture inoculation and stained with SbCl₃-HClO₄.
Figure 5.11 Accumulation of orange-staining materials Sicot 189 plant three days after taproot-puncture inoculation.

Fresh tissue stained with SbCl₃-HClO₄. Localised differential accumulation of orange-staining material in: (A) a radial cross-section; (B) a longitudinal section; and (C) latitudinal section. Intensely stained xylem vessel walls (small arrows) are in close proximity to unstained vessels (large arrows).
Figure 5.12 Intense orange staining in Sicot 189 xylem tissue three days after Fov taproot-puncture inoculation.

Fresh tissue samples were stained with SbCl₃-HClO₄. A longitudinal section (A) shows intense staining of xylem vessel wall (small arrow) adjacent to a Fov hypha (arrowhead) and in close proximity to an unstained vessel (large arrow). Hyphae in some intensely stained areas are also stained orange (B and C).
Figure 5.13 Accumulation of orange-staining materials in Sicot 189 xylem tissue three days after Fov taproot-puncture inoculation.

Fresh tissue was stained with SbCl₃-HClO₄. A longitudinal section of infected xylem shows orange-staining materials lined the walls of vessels (A). The accumulation of orange-staining materials in a xylem vessel and several parenchyma cells was evident in longitudinal (B) and latitudinal (C) sections. A xylem vessel lumen in longitudinal section contained large amounts of orange-staining material (D).
CHAPTER 6  ANTIMICROBIAL TERPENOIDS

6.1 Introduction

The defence compounds produced by *Gossypium* spp. include a diverse array of terpenoids (Bell, 1986). These chemical defences can provide constitutive protection from herbivores and pathogens (Bell et al., 1978; Mace et al., 1974a), or act as phytoalexins synthesised in response to challenge (Bell, 1967; Bell et al., 1975; Stipanovic et al., 1975a; Essenbergs et al., 1990; Bell and Christiansen, 1968). The diversity of these compounds is outlined in Table 6.1.

The compounds present depend on the site of synthesis and the type of stimulus. The phytoalexins formed in response to Fov infection have been identified as sesquiterpenoids, terpenoid aldehydes and naphthofurans (Zhang et al., 1993).

While the presence of antimicrobial terpenoids in cotton has been documented widely, their exact contribution to defence remains undefined (Bell, 1967; Mace et al., 1974a; Zaki et al., 1972b). Specific compounds with known toxicities have been identified by HPLC analysis, but their individual accumulation has not been correlated directly with observations of Fov as it colonises its host (Stipanovic et al., 1988; Bell et al., 1975; Stipanovic et al., 1975a; Mace et al., 1985; Zhang et al., 1993). Importantly, the effect of some of the compounds may be limited by their solubility within infected xylem vessels, suggesting that only dHG will have any effect (Mace et al., 1985; Zhang et al., 1993).

The phytoalexins hemigossypol (HG), desoxyhemigossypol (dHG), methoxyhemigossypol (MHG), and desoxymethoxyhemigossypol (dMHG) have been identified and implicated in the resistance of cotton to Fov (Bell et al., 1975; Stipanovic et al., 1975a; Mace et al., 1985; Zhang et al., 1993). The circumstantial evidence is strong, correlating the ability of resistant cotton varieties to inhibit Fov growth in their vascular tissues (Shi et al., 1993) with their accumulation of higher concentrations of antimicrobial terpenoids, which are known to be toxic to Fov (Zhang et al., 1993). Histochemical monitoring of terpenoids has shown the response is more intense in the resistant host, where it extends beyond the region of infection and in advance of the spreading pathogen (Harrison and Beckman, 1982). Microscopic studies of the ultrastructure of resistant and susceptible cotton plants documented the accumulation of
a lipoidal compounds in infected vessels, which appeared to restrict Fov growth (Shi et al., 1991b; Shi et al., 1991a).

There is good evidence of a terpenoid phytoalexin response of cotton to infection by Fov. However, no knowledge exists about the influence of this defence mechanism on the host-pathogen interaction in the Australian context. The aim of this chapter is to quantitatively document this response in order to test the hypothesis that antimicrobial terpenoid accumulation in elite Australian cotton varieties correlates with resistance to Fusarium wilt.
Table 6.1 Synonyms for cotton terpenoids

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<th>Triterpenes</th>
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<td>Gossypol (G)</td>
<td>C_{30}H_{39}O_{8}</td>
<td>8,8'-dicarboxaldehyde, 1,1',6,6',7,7'-hexahydroxy, 5,5'-diisopropyl, 3,3'-dimethyl, 2,2'-binaphthalene</td>
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<td>1,1',6,6',7,7'-Hexahydroxy-3,3'-dimethyl-5,5'bis(1-methylethyl)[2,2'binaphthalene]-8,8'dicarboxaldehyde</td>
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<td></td>
<td></td>
<td>Isohemigossypol [name correction in (Veech et al., 1976)]</td>
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<tr>
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</tr>
<tr>
<td>Naphthofurans</td>
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<td>Desoxyhemigossypol (dHG)</td>
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<td></td>
<td></td>
<td>desoxyhemigossypol-6-methyl ether</td>
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<tr>
<td></td>
<td></td>
<td>Misidentified as vergosin (Zaki et al., 1972b)</td>
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6.2 Materials and Methods
Cotton stele extracts were subjected to HPLC analysis to describe the timing, magnitude and location of antimicrobial terpenoid accumulation relative to the pathogen’s progression through the plant stele. The following section documents the techniques used in addition to the general methods described in Chapter 2.

6.2.1 Pathogen and host
Cotton plants were grown in root boxes for six weeks (see 4.2.2.2.1). Plants were inoculated by stem puncture approximately 5 cm below soil level and control plants were “mock-inoculated” by using sterile distilled water in place of microconidial suspension. These techniques have been previously described in detail (See 4.2.2.2.2).

6.2.2 Extraction of antimicrobial terpenoids
Samples were extracted using a modified method of Benson et al. (2001). Whole plants were harvested one, three, and five days after inoculation. The cortex of each plant was split and peeled off, and the stele was dissected into sequential 1 cm-long sections above the site of inoculation. Sections were collected up to 10 cm above the site of inoculation from plants harvested on Day 1, and up to 20 cm for plants harvested on Days 3 and 5. This experiment was repeated, however in Experiment 2 harvesting took place only on Days 3 and 5, and the sections sampled were 1-5, 7, 9, 11, 13, and 15 cm from the inoculation point.

Corresponding sections from four plants (approximately 50 mg tissue) were pooled in each sample. That is, the first 1 cm-long sections of stele from the inoculation point of four plants was pooled, then the second 1 cm-long sections from four plants, and so on. Three replicate samples (each made up of four pooled sections) were collected for each treatment. These samples were then lyophilised and ground to a powder using a Retsch MM300 Mixer Mill. To each sample (approximately 30 mg dry weight), 0.5 mL of HPLC-grade acetone with 1.0 mg mL⁻¹ ascorbic acid filtered through a 0.45 µm nylon filter was added (Zhang et al., 1993). The suspension was ultrasonicated using a Unisonics Ultra™Sonic K42 for 3 min, then centrifuged for 3 min at 14.5 × 1000 rpm before the supernatant was pipetted off (Benson et al., 2001). Another 0.5 mL of extraction solvent was added, the sample vortexed, and centrifuged as above. The supernatants were pooled and filtered using a 0.2 µm syringe filter nylon membrane into an amber glass vial.
6.2.3 **HPLC analysis**

Analysis was performed on a Dionex HPLC fitted with a Dionex ASI-100 autosampler and Dionex PDA-100 photodiode array detector. Standard curves were calculated for G (Sigma Aldrich Gossypol acetic acid G4382) and for dHG, HG, dMHG, and MHG (generously provided by R. Stipanovic, US Department of Agriculture, Agricultural Research Service). Absorbance was measured at 235 nm, and the UV spectra were also captured. Extracts were separated on a SGE 150 × 4.6 mm SS WAKOSIL C18 (5 μm) RS column maintained at 40°C.

Mobile phase consisted of methanol and water that were both acidified by adding orthophosphoric acid (0.07% H₃PO₄). The solvents were delivered using a gradient starting with 20% methanol and changing to 60% after 5 min, then shifting to 87% after 22 min, decreasing to 80% at 27 min, which continued until data acquisition stopped at 30 min. The solvent flow rate was 1mL min⁻¹ (Figure 6.1).

Data collection and integration were performed using Chromeleon Version 6.50 software.

6.2.4 **Fov growth in stele**

The growth of Fov in the stele of every plant used for HPLC analysis was quantified by measuring the distance from the inoculation point from which the pathogen could be reisolated. This was to confirm that the behaviour of Fov conformed to observations made in previous assays.

Thin cross-sections of stele corresponding to each sequential 1 cm-long sample (described in 6.2.2) were placed onto agar plates containing *Fusarium*-selective media (see 2.2.3.1) that were incubated at room temperature for one week. The original location in the intact plant stele was noted for sections that had Fov growing out into the agar. This gave an indication of the extent of Fov growth up the plant stele from the point of inoculation. Sections were taken from all of the four plants that went into each pooled sample, for each of the three replicates.

6.2.5 **Statistical analysis**

All statistical analyses of data were performed using Minitab 14 software. Data from single days, or in single stele samples were subject to ANOVA tests.
6.3 Results

6.3.1 Identification
The antimicrobial terpenoids dHG, HG, dMHG, MHG and G were identified in stele extracts. No antimicrobial terpenoids were detected in plants prior to mock- or Fov-inoculation (Figure 6.2), but dHG, HG, dMHG and G were detected in all treatments after inoculation (Figure 6.3). Their identities were confirmed by comparison of retention times and UV spectra with purified samples and the published characteristics of each compound (Stipanovic et al., 1974; Stipanovic and Bell, 1975). The terpenoid compounds eluted at the following intervals after injection: 16.7 min (HG); 17.5 min (dHG); 19.5 min (dMHG); 19.6 min (MHG); and 27.7 min (G) (Figure 6.3).

6.3.2 Quantification
6.3.2.1 Timing and magnitude of antimicrobial terpenoids
No antimicrobial terpenoids were detected at the time of inoculation (Day 0). Sicot 189 produced more dHG, HG, and dMHG than Siokra 1-4 in response to both wounding (mock-inoculation) and Fov-inoculation. There was, without exception, more of each of the antimicrobial terpenoids detected in plants inoculated with Fov (Figures 6.4, 6.5).

The presence of dHG at low levels was static in the wounded control plants. In infected plants, Sicot 189 reached its maximum dHG content at Day 3. Siokra 1-4 reached its maximum at Day 5, however this was only marginally higher than Day 3. By Day 5 the dHG content of infected Sicot 189 plants had decreased, but was still more than double that of infected Siokra 1-4 (Figure 6.4A).

There was more HG detected than any other antimicrobial terpenoid. Infected Sicot 189 produced significantly more HG at Days 3 and 5 than Siokra 1-4, conforming to a general trend in concentration: Siokra 1-4 < Sicot 189 < inoculated Siokra 1-4 < inoculated Sicot 189 (Figure 6.4B).

Very small amounts of dMHG were detected. More of this antimicrobial terpenoid was detected in inoculated plants than the controls, but the varietal response was similar (Figure 6.5A).

No MHG was detected in the stele extracts of any treatment.
The only treatment that recorded a progressive increase in G content over the sampling period was inoculated Sicot 189. Compared with the other antimicrobial terpenoids, the G response was quick, with substantial amounts produced on Day 1 (Figure 6.5B).

At Day 3 the concentrations of dHG, HG, dMHG, MHG and G in inoculated Siokra 1-4 were 20, 73, 9, 0 and 50 µg g⁻¹ fresh tissue, respectively. In inoculated Sicot 189 the concentrations measured at Day 3 of dHG, HG, dMHG, MHG and G were 94, 233, 37, 0, and 78 µg g⁻¹ fresh tissue, respectively.

6.3.2.2 Total antimicrobial terpenoid production relative to Fov spread

Figures 6.6-6.10 display the distance travelled up the stele tissue by Fov relative to antimicrobial terpenoid content. Bars represent the antimicrobial terpenoid contents of 10 consecutive 1 cm-long samples, taken above the point of inoculation. Data relating to control plants have not been included.

The relative total antimicrobial terpenoid contents of both cotton varieties were comparable one day post inoculation (Figure 6.6). The content significantly increases in Sicot 189 at Day 3 (p=0.035), to a level significantly higher than Siokra 1-4 (p=0.025) (Figure 6.7). No increase in Siokra 1-4 was statistically significant over the sampling period.

The corresponding distance travelled by Fov up the stele was measured. One day after inoculation, Fov progressed approximately 6 cm up the stele of both varieties (Figure 6.6). Pathogen progression started to diverge after this time, and by Day 5 Fov had travelled almost 12 cm in Siokra 1-4 - significantly further than in Sicot 189 (p=0.002)(Figure 6.8). By Day 5 Fov could be found in the Siokra 1-4 stele at a distance approximately three times further than in Sicot 189.

In summary, the total amount of antimicrobial terpenoids detected in whole plants peaked at Day 3 in Sicot 189. There were significantly more total antimicrobial terpenoids in Sicot 189 at Day 3 (p=0.025), after which there was a significant reduction in the distance travelled by the pathogen in this variety compared with Sicot 1-4 (p=0.002).
The second repeat of this experiment was harvested only on Days 3 and 5, and demonstrated similar trends in the detection of total antimicrobial terpenoids (Figures 6.9, 6.10). Total antimicrobial terpenoid content was greatest on Day 3 in Sicot 189, and on both days significantly more antimicrobial terpenoids were detected in Sicot 189 than Siokra 1-4 (Day 3: p=0.041; Day 5: p=0.034).

The progression of Fov in Experiment 2 was greater in Sicot 189 than in Experiment 1 at Day 3 (Figure 6.9), however the outcome remained unchanged. By Day 5 pathogen progression appeared to have ceased in Sicot 189, but colonisation continues in Siokra 1-4. At Day 5 Fov had travelled significantly further up the stele tissue of Siokra 1-4 plants than Sicot 189 (p=0.00)(Figure 6.10).

6.3.2.3 Location of antimicrobial terpenoids
Figures 6.6-6.8 indicate the precise location within the Fov-infected stele of each of the antimicrobial terpenoids on Days 1, 3 and 5, respectively.

On Day 1, similar small quantities of antimicrobial terpenoids were detected in the stele extracts from both cotton varieties (Figure 6.6). They were localised about the point of inoculation, and extended approximately 5 cm up the stele.

By Day 3 there was a significant increase in the total antimicrobial terpenoid content in Sicot 189 (p=0.035), and an approximate doubling of the distance from the inoculation point that they are present (Figure 6.7). These increases were much smaller in Siokra 1-4. The proportion of dHG was also higher in Sicot 189. For example, in the first 1-cm section from the point of inoculation dHG represents 24% of the total antimicrobial terpenoids in Sicot 189, compared with only 13% in Siokra 1-4.

Five days after inoculation antimicrobial terpenoids could be detected up to approximately 15 cm from the inoculation point in both cotton varieties (Figure 6.8). The majority were detected within the first 5 cm, decreasing with distance up the stele. Sicot 189 produced approximately three times the quantity of antimicrobial terpenoids in the first 1-cm section compared to Siokra 1-4. Although the terpenoid content differs greatly between the two cotton varieties, the proportion that dHG comprises of the total is similar.
The results of the second replication of this experiment are presented in Figures 6.9 and 6.10. These data differ from Experiment 1 due to a summarised sampling regime. Harvesting took place only on Days 3 and 5, and fewer sections were collected. This repeat confirmed the same trends.

Sicot 189 had significantly more antimicrobial terpenoids than Siokra 1-4 at Day 3 (p=0.041)(Figure 6.9). In the first 1-cm section, for example, there were more than double the antimicrobial terpenoids in Sicot 189. They extended further up the stele than in Siokra 1-4, and diminished with distance from the inoculation point. Again, there were much greater quantities of antimicrobial terpenoids measured in S189, detected approximately double the distance up the stele on Day 5 (Figure 6.10).

### 6.3.2.4 Desoxyhemigossypol content of stele extract

Due to the importance to resistance attributed to dHG by other researchers, the content of this specific terpenoid aldehyde in stele extracts is presented (Figure 6.11).

Desoxyhemigossypol was detected in all plants in Experiment 1, localised about the inoculation point (Figure 6.11A). The amounts were small at Day 1, and there were no significant differences between treatments.

At Day 3, inoculated Sicot 189 had a dHG content in the first 6 cm that was significantly greater than any other treatment (p<0.05)(Figure 6.11B). Desoxyhemigossypol was detected the furthest away from the inoculation point in inoculated Sicot 189, but was detected in only the first 2 cm away from the mock-inoculation point in control plants.

By Day 5, the dHG content of inoculated Sicot 189 had decreased, while other plants maintained similar levels (Figure 6.11C). Inoculated Sicot 189 had a significantly greater dHG content than the control plants only in the first cm from the inoculation point by this time (p=0.049).
6.4 Discussion

6.4.1 Qualitative analysis

HPLC analysis confirmed that Australian cotton varieties respond to Fov infection by producing antimicrobial terpenoids. The compounds identified, dHG, HG, dMHG and G, have been previously described in the phytoalexin response of cotton plants with resistance to Fov (Zhang et al., 1993). In the previous study, these antimicrobial terpenoids were identified in stele extracts of infected cotton, in addition to MHG.

MHG was not detected in the present study. According to the proposed biosynthetic pathway of the antimicrobial terpenoid (refer to Figure 1.4) it is indeed possible for the other compounds to be present without MHG (Liu et al., 1999; Benedict et al., 2004). Very low concentrations of MHG in stele extracts were recorded in previous assays in response to Fov infection (Zhang et al., 1993). The low detection of MHG may be due to the compound's relatively low water solubility and the use of aqueous mobile phase in HPLC analysis. Experiments using an alternate mobile phase have recorded much higher MHG concentrations in Verticillium wilt resistant plants (Mace et al., 1985).

However, due to the nature of MHG, the impact of such an experimental underestimation is negligible. Compared with dHG, HG and dMHG, MHG is by far the least toxic to V. dahliae and Fov (Mace et al., 1985; Zhang et al., 1993). The compound's water solubility at the estimated pH of infected stem xylem was so low as to prevent the determination of its ED$_{50}$. For these reasons, it is unlikely that MHG would make a substantial contribution to defence.

Analyses presented in this chapter include gossypol, where other investigations of terpenoid phytoalexins have disregarded this compound. In their HPLC investigation into terpenoids and Fusarium wilt, Zhang et al. (1993) observed that G appeared in both control and inoculated cotton plants. This perhaps was the justification for excluding G from any further analysis in this paper, as in others (Mace et al., 1985). Importantly, their control plants were mock-inoculated by injecting with sterile distilled water. The G detected does not necessarily assign the compound a role in constitutive defence, but determines that it is produced in response to wounding. The production of G in response to injury is well documented (Bell, 1967; Bell and Christiansen, 1968).

This characteristic should not exclude gossypol from the investigations into phytoalexins, as results in this chapter demonstrate that in addition to G, dHG, HG and
dMHG are also produced in stele tissue of mock-inoculated control plants. Histochemical studies have shown that G is often present at sites of the other antimicrobial terpenoids (Mace et al., 1974a). In the present study, these compounds were not detected in unwounded plants, and although there are quantitative differences, de novo production was recorded in response to both wounding and Fov inoculation. Although the toxicity of G to Fov has not been documented, there is evidence of broad antifungal activity (Abou-Donia, 1976; Medentsev and Akimenko, 1999; Mellon et al., 2002; Puckhaber et al., 2002). Gossypol falls within Paxton's phytoalexin definition (Paxton, 1981), as do dHG, HG and dMHG, and was therefore included in my investigation.

6.4.2 Role in defence

Results presented here indicate that the rapid accumulation of antimicrobial terpenoids is associated with the restriction of colonisation. Evidence for a role in defence is provided by data that quantifies these compounds specifically at the infection site and relates their accumulation to the inhibition of pathogen development.

The timing of the antimicrobial terpenoid response in cotton has not been investigated in such detail previously. The pivotal HPLC studies harvested cotton stele ten days after inoculation with Fov or V. dahliae, effectively taking an average of what is likely to be a dynamic and site-specific response (Zhang et al., 1993; Mace et al., 1985). Results presented in this chapter demonstrate that the phytoalexin response varies with time and physical proximity to the pathogen. The differences in phytoalexins between host varieties are greatest at Day 3. The relevance of this finding becomes clear when considering the pathogen is in direct contact with the accumulated phytoalexins within the plant in this timeframe and that colonisation of Sicot 189 stopped after three days (Chapter 4, Figure 4.12).

I demonstrated that when the two Australian cotton varieties were infected with Fov, the least susceptible Sicot 189 was able to restrict pathogen growth more than the most susceptible Siokra 1-4. This growth inhibition occurred after Day 3, when significantly greater quantities of phytoalexins were detected in Sicot 189, including dHG. Shi et al. (1993) also found that the resistant cotton plant restricted vertical growth three days after inoculation with Fov, whereas the pathogen continued to spread within the susceptible host. This confirmed the authors' earlier assertion that the first four days
after inoculation is a critical period in which the resistant host restricts fungal colonisation (Shi et al., 1991a; Shi et al., 1992).

The differential abilities of the cotton varieties, Siokra 1-4 and Sicot 189, to restrict fungal spread in the stele tissues were demonstrated in Chapter 4. Furthermore, a correlation between this ability and the accumulation of antimicrobial terpenoids was demonstrated in Chapters 5 and 6. Overseas studies show that the restriction of wilt pathogen spread in cotton vascular tissues is characteristic of resistance (Wilhem et al., 1974b; Wilhem et al., 1974a; Shi et al., 1993), and so too is the production of antimicrobial terpenoids (Mace et al., 1985; Zhang et al., 1993).

Until this current study, the differential accumulation of individual antimicrobial terpenoids at numerous sites in Fov infected cotton stele tissue had not been measured. The broad time/space relationship was investigated through the histochemical detection of a bulk terpenoid aldehyde response relative to the Fov distribution in cotton plants (Harrison and Beckman, 1982). Previous HPLC investigations obtained the mean concentration in stele tissue up to 10 cm from the inoculation point (Zhang et al., 1993). Cotton cell suspension cultures were tested but readily lost the ability to produce G and HG after repeated subculture (Heinstein and El-Shagi, 1981; Heinstein, 1985).

It is important to quantify the phytoalexin response in different parts of the plant in order to assess the specific concentration where direct contact occurs with the colonising pathogen. Results presented in this chapter demonstrate the strong localisation of antimicrobial terpenoids around the site of inoculation, and a decline in concentration with distance from the inoculation point. The faster progression of the pathogen up the stele tissue of Siokra 1-4 effectively allows Fov to escape, by outpacing, direct contact with the antimicrobial terpenoid response. The distance of the colonising pathogen from the phytoalexins tightly localised about the inoculation point in the most susceptible host demonstrates that by Day 3 Fov has already outgrown the site of accumulation. This renders the response ineffectual and possibly allows unmitigated pathogen development. A similar escape of the pathogen from the site of terpenoid aldehyde accumulation was observed histochemically in cotton by Harrison and Beckman (1982). The authors describe a more intense phytoalexin response in the resistant host, which extended beyond the region of infection and importantly, in advance of the spreading pathogen (Harrison and Beckman, 1982).
Antimicrobial terpenoids have now been detected in extracts from the stele tissue of Australian cottons as part of a response to Fov inoculation. In order to assess this response’s contribution to defence, it is important to confirm that the phytoalexins accumulate in a location within stele that would allow them to directly act on the invading pathogen. That is, their location needs to be determined on a cellular level before a defensive role can be attributed to their accumulation. The extraction and subsequent localisation of phytoalexins in and around vessels in resistant cocoa infected with *V. albo-atrum* has provided compelling evidence of their role in defence (Cooper et al., 1996). This linkage between the accumulation of phytoalexins to inhibitory concentrations and their cellular localisation at the site of pathogen development has been established between cotton and *Xanthomonas axonopodis* (Essenberg et al., 1992a; Pierce et al., 1996) and *V. dahliae* (Mace et al., 1989), but not Fov. Histochemical identification of terpenoids within close proximity of Fov hyphae in and around xylem vessels was presented in Chapter 5.

Desoxyhemigossypol, dMHG, HG, and MHG are all toxic to *V. dahliae* and Fov, but the terpenoids in which the hydroxyl group at C-6 is methylated (dMHG, MHG) are less toxic than their non-methylated counterparts (Mace et al., 1985; Zhang et al., 1993). The gene encoding the enzyme responsible for the methylation of the key intermediate terpenoid, dHG, has been characterised and cloned (Liu et al., 1999; Liu et al., 2002). The potential exists to down regulate desoxyhemigossypol-6-O-methyltransferase and thus preserve the more potent antimicrobial terpenoid compounds.

Several authors have proposed that only the most toxic dHG will have an effect on pathogens in planta (Mace et al., 1985; Zhang et al., 1993). The pH of infected cotton xylem vessels was initially measured at 6.3 after inoculation with *V. dahliae* (Mace et al., 1985), but this figure has also been used as the basis for studies relating to Fov (Zhang et al., 1993). The low solubility of HG and dMHG at the estimated pH of infected cotton xylem vessels probably excludes them from involvement in the inhibition of pathogen development. The results presented in this chapter demonstrate that at Day 3 the least susceptible host, Sicot 189, produces significantly greater amounts of dHG, and that its distribution is broader than in Siokra 1-4. That is, the accumulation of dHG correlates with a decrease in varietal susceptibility to Fov. Zhang et al. (1993) predicted that a dHG concentration of 45.1 μg g⁻¹ fresh stele tissue would
kill all Fov propagules. Concentrations more than 10 times greater than this amount in Sicot 189 in several cm from the inoculation point were observed. The most susceptible host, Siokra 1-4, only produces dHG concentrations greater than this amount in the first 1-cm from the point of inoculation. The inhibition of pathogen development after Day 3 corresponds to a significant accumulation of dHG in Sicot 189, providing sound evidence for its role in defence. This finding supports the hypothesis that the accumulation of antimicrobial terpenoids in Australian cotton varieties correlates with their degree of resistance to Australian Fov.

Further support for a role of dHG in defence could be obtained by the assessment of its effect on different isolates of Fov. The relationship between Fov virulence and dHG tolerance may elucidate a clearer role for dHG in resistance and might also reveal why Australian Fov isolates are so virulent compared with overseas isolates.

Results presented in this chapter, like a large proportion of phytoalexin research, have been based on well-defined non-field based experiments (Hammerschmidt, 1999). Clarification of the accumulation of antimicrobial terpenoids in field grown cotton is an essential component of future studies.

Cumulative data from both previous research and this current study strongly indicate a role for phytoalexins in defence, and perhaps ultimately, resistance to Fov. In order to confirm and utilise this contribution to resistance, cotton plants with enhanced or suppressed phytoalexin production could be engineered for experimental analysis. This approach has been initiated, with the cloning of a sesquiterpene cyclase required for phytoalexin production, (+)-delta-cadinene synthase (Chen et al., 1996; Davis et al., 1996a). Although this sesquiterpene cyclase appears to be phytoalexin pathway-specific, care must be taken to evaluate the effect of such a transformation on the expression of other defences and additional factors that may alter host resistance (Hammerschmidt, 1999).

6.4.3 Conclusions
Although the absolute contribution of phytoalexins to disease resistance has not been established, good evidence for the role of phytoalexins in defence exists (Keen, 1981). Of the criteria that Hammerschmidt (1999) proposed to determine if phytoalexins are an important part of defence, this chapter has demonstrated that phytoalexins accumulate to
levels toxic to Fov at the infection site at the time that pathogen development was stopped. The less susceptible Sicot 189 was able to restrict pathogen growth more than the most susceptible Siokra 1-4. Furthermore, this growth inhibition occurred after Day 3, when significantly greater quantities of phytoalexins were detected in Sicot 189. This infers that the phytoalexin response in Australian cotton varieties influences its susceptibility to Fusarium wilt.
Figure 6.1 Graph showing mobile phase gradient used for HPLC analysis.
Figure 6.2 Chromatograms of cotton stele extracts taken prior to inoculation
Siokra 1-4 (A) and Sicot 189 (B) stele tissue harvested at Day 0.
Figure 6.3 Representative chromatograms of cotton stele extracts 5 days after mock- and Fov-inoculation

Stele tissue harvested five days after: (A) mock inoculation of Siokra 1-4 (control); (B) mock inoculation of Sicot 189 (control); (C) Fov inoculation of Siokra 1-4; and (D) Fov inoculation of Sicot 189. HG= hemigossypol; dHG= desoxyhemigossypol; dMHG= desoxymethoxyhemigossypol; G= gossypol.
Figure 6.4 Antimicrobial terpenoid contents of cotton stele extracts: dHG, and HG. Each bar represents the mean antimicrobial terpenoid content of extracts from the first 10 cm of stele tissue from the inoculation point. Stele extract was harvested at Day 0, 1, 3, and 5 after mock inoculation, or inoculation with Fov (+): (A) dHG; and (B) HG. Error bars represent the standard error of the mean of three replicates.
Figure 6.5 Antimicrobial terpenoid contents of cotton stele extracts: dMHG and G
Each bar represents the mean antimicrobial terpenoid content of extracts from the first 10 cm of stele tissue from the inoculation point. Stele extract was harvested at Day 0, 1, 3, and 5 after mock inoculation, or inoculation with Fov (+): (A) dMHG; and (B) G. Error bars represent the standard error of the mean of three replicates.
Figure 6.6 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 1.

Experiment 1. Stele tissues were harvested one day after inoculation of (A) Siokra 1-4 and (B) Sicot 189 with Fov. The distance travelled by Fov from the inoculation point (red bar) was measured by plating consecutive cross sections of stele tissue onto selective media. Stele extracts from the remaining sections were processed for HPLC analysis. Error bars represent the mean standard error of three replicates.
Figure 6.7 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 3.

Experiment 1. Stele tissues were harvested three days after inoculation of (A) Siokra 1-4 and (B) Sicot 189 with Fov. The distance travelled by Fov from the inoculation point (red bar) was measured by plating consecutive cross sections of stele tissue onto selective media. Stele extracts from the remaining sections were processed for HPLC analysis. Error bars represent the mean standard error of three replicates.
Figure 6.8 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 5.

Experiment 1. Stele tissues were harvested five days after inoculation of (A) Siokra 1-4 and (B) Sicot 189 with Fov. The distance travelled by Fov from the inoculation point (red bar) was measured by plating consecutive cross sections of stele tissue onto selective media. Stele extracts from the remaining sections were processed for HPLC analysis. Error bars represent the mean standard error of three replicates.
Figure 6.9 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 3.

Experiment 2. Stele tissues were harvested three days after inoculation of (A) Siokra 1-4 and (B) Sicot 189 with Fov. The distance travelled by Fov from the inoculation point (red bar) was measured by plating consecutive cross sections of stele tissue onto selective media. Stele extracts from the remaining sections were processed for HPLC analysis. Error bars represent the mean standard error of three replicates.
Figure 6.10 Antimicrobial terpenoid content of stele extract relative to Fov growth on Day 5.

Experiment 2. Stele tissues were harvested five days after inoculation of (A) Siokra 1-4 and (B) Sicot 189 with Fov. The distance travelled by Fov from the inoculation point (red bar) was measured by plating consecutive cross sections of stele tissue onto selective media. Stele extracts from the remaining sections were processed for HPLC analysis. Error bars represent the mean standard error of three replicates.
Figure 6.11 Desoxyhemigossypol content of stele extracts on Days 1(A), 3(B) and 5(C).

Siokra 1-4 (S1-4) and Sicot 189 (S189) plants were either mock-inoculated, or inoculated with Fov (+). Graphs representing Days 3 and 5 (B and C) have been restricted to 15 cm distance from the inoculation point for clarity. Error bars represent the standard error of the mean of three replicates.
CHAPTER 7 GENERAL DISCUSSION

Fusarium wilt of cotton recently arose as a serious threat to the Australian cotton industry (Kochman, 1995; Salmond et al., 1998). Soon after the first isolations from cotton fields in this country, the new Fov biotypes were shown to be unique and highly virulent (Davis et al., 1996b; Kim et al., 2005). Significant advances have been made in the breeding of varieties that are less susceptible to Fusarium wilt and in the management of Fov in the field (Reid et al., 2002; Swan and Salmond, 2005). Despite notable progress, the Fusarium wilt epidemic that followed has prompted predictions of widespread distribution throughout the majority of cotton farms in Australia. (Allen, 2004b; Kim et al., 2005; Kochman et al., 2002; Kochman, 1995).

In addition to causing significant losses in production, the disease has rendered many fields unsuitable for further cotton production and the risk of spread has alarmed countries importing Australian cottonseed products (Kochman et al., 2000; Kim et al., 2005; Kochman et al., 2002). Fusarium wilt is deservedly considered one of the most serious threats to sustainable cotton production in Australia (Kochman, 1995; Salmond et al., 1998).

Considering the aggressiveness of the novel Fov biotypes found in Australia, and the lack of resistance in G. hirsutum it is important to understand the fundamental biology of this pathogen. Knowledge of how Fov infects and colonises its host, and how plant defences may mitigate disease development are essential components of disease control. Until this current study, research into the underlying interaction between the plant and pathogen has been limited. Our working knowledge of the interaction between Fov and Australian cotton relied substantially on inferences and speculation from studies conducted overseas and in different environments. The unique biotypes of the pathogen found in this country limited the usefulness of this approach and warranted individual study.

My research has described the interaction between an Australian biotype of Fov and two commercial cotton varieties grown in this country. Important differences have been identified in the host-pathogen interaction between Fov and host genotypes with different susceptibilities to Fusarium wilt.
Each part of this study has advanced our understanding of Fov, and progressed our ability to manage Fusarium wilt of cotton in Australia. The contributions of the present study will be discussed and interpreted. Priorities for future research will be defined.

7.1 Bioassay

Large-scale pathogenicity assays will remain an essential component of disease resistance research into the future (Davis et al., 2006). Bioassay techniques to assess Fusarium wilt vary between researchers, limiting the accuracy of comparisons between study findings. Inoculation by dipping roots of cotton seedlings into a Fov conidial suspension is the most commonly used method amongst Australian researchers in their glasshouse-based studies of Fov, although details of the technique and many other parameters can differ between studies (Kochman et al., 1996; Kochman et al., 1998; Wang et al., 1999a).

My aim was to develop a simple bioassay that reliably correlated with field resistance, not only for use in this investigation, but to form a common basis for the study of Fusarium wilt of Australian cotton.

A rapid, reliable glasshouse bioassay was developed and described in Chapter 3. Cotton plants were grown from surface-sterilised seeds in pots containing sterile potting medium. Plants were grown for six weeks at approximately 23°C with a 12 h photoperiod before inoculation with $5.0 \times 10^6$ spores/mL microconidia suspension by a single taproot puncture. Glasshouse conditions remained unchanged and pots were watered every five days for six weeks after inoculation. Importantly, reshooting plants were excluded by scoring plants with two leaves or less “dead” at the end of the trial. The survival of cotton varieties in this Taproot Puncture Assay correlated with what is known of Fusarium wilt field susceptibility, and provided a reliable basis for further investigation in this study.

While the cotton cultivars used throughout this research were both G. hirsutum accessions, the use of Siokra 1-4 and Sicot 189 varieties meant that comparisons were made between varieties with somewhat dissimilar genetic backgrounds. This genetic diversity provides broad insight into the determinants of susceptibility to Fusarium wilt. Using Siokra 1-4 and Sicot 189 throughout this work allowed for the observation of many similarities in the interactions between Fov and the two host varieties, but it also
highlighted points of difference requiring further investigation. Selection of host
genotypes on the basis of disease resistance is common in this type of research, and
often results in comparisons between hosts of different species. Studies of Fov
commonly compare commercial US varieties representing different Gossypium species:
G. hirsutum is highly susceptible to Fusarium wilt, while G. barbadense is resistant
(Harrison and Beckman, 1982).

For convenience in pathogenicity bioassays, the inoculum used may differ widely from
that found in the field in terms of type, quantity and delivery. While it is likely that Fov
survives long-term in the soil as chlamydospores, they are difficult to use in glasshouse
bioassays. Their production is relatively sparse compared with microconidia and
macroconidia, they are frequently embedded in their growth medium, and germination
is often irregular and staggered. The use of solid inoculum such as infested millet grains
may incorporate an immeasurable quantity of chlamydospores, but this inoculation
technique had unwanted side effects. The use of microconidia as inoculum lent itself to
direct inoculum injection and predictable infection, for reasons discussed in Chapter 3.

It is estimated that one gram of Fov-infested soil from the field may contain up to 5000
spores (Smith et al., 1981). The use of such a high microconidial concentration (5.0 ×
10^6 spores/mL) in the Taproot Puncture Assay may appear inappropriate. Highly
concentrated inoculum is a common feature of glasshouse experiments, where “it
appears that much higher inoculum levels are required to produce disease symptoms in
pot experiments than in conditions of natural infection in the field” (Hillocks, 1992b).
Evidence exists of a correlation between the inoculum concentration in the soil and the
subsequent development of Fusarium wilt symptoms in cotton, celery and bean (Elmer
and Lacy, 1987; Salgado and Schwartz, 1993; Wang et al., 1999a). Furthermore, the
density of penetration hyphae produced by Fov on the root surface is directly influenced
by the number of conidia used for inoculation (Rodriguez-Galvez and Mendgen,
1995b). In a detailed comparison of inoculum concentrations for pathogenicity testing
under glasshouse conditions, Wang et al. (1999a) found 5.0 × 10^6 spores/mL to be the
optimum concentration for Australian cotton and Fov. It is important that researchers
maintain uniformity in inoculum concentrations to enable comparison between findings
and that inoculum pressure reflects that faced by plants in the field as much as possible.
7.1.1 Future applications and further questions

A defined protocol was required not only for use in this study, but also for consistent use around Australia in research into Fusarium wilt. The standardisation of Fov pathogenicity testing has long been a goal, as "variations in environmental conditions, inoculation levels and subjective evaluation methods may lead to variable results" (Kim et al., 2005). The Taproot Puncture Assay technique describes a simple method that is easy to replicate in other laboratories, and results in clear differentiation of the cotton varieties tested. The assay will provide a basis for future studies, increasing both the reliability of bioassay results and the confidence of conclusions drawn from isolated studies.

The direct Fov inoculation into the root reduces the time required for the completion of the bioassay whilst still resulting in varietal survival that correlated with field observations of Fusarium wilt susceptibility, and may therefore constitute a useful screen for use in breeding.

Although this taproot puncture technique produced reliable infections, direct injection of spores into the plant bypasses constitutive and facultative defences that may be present in the roots. The exact location of inoculation provided by the Taproot Puncture Assay was required in this work, however in future work natural infection through roots may be more informative, depending on the experimental goals. Techniques using infested potting medium are used with success by some researchers, and would include defences present at the point of entry into the root (McKenna et al., 2004; Smith and O'Neill, 2004). Infested potting medium can sustain plant growth in long-term assays, however if the duration of an assay is relatively short, inoculation by placing infested agar on bare roots may provide insights to defences at the root surface (Rodriguez-Galvez and Mendgen, 1995b; Rodriguez-Galvez and Mendgen, 1995a).

The Taproot Puncture Assay was performed three times, but would benefit from further verification. The Fusarium wilt field susceptibilities referenced in this study are based on a wide range of data sets from trials over different seasons and locations (Australian varietal disease trial results and disease rankings: www.csd.net.au), and are likely to change as more information is gathered. Sicot 189 was nominated some years ago as the "industry standard" for low Fusarium wilt susceptibility, but 2004 saw the release of
“Sicot F1,” with significantly lower field susceptibility (Reid et al., 2004). Future glasshouse trials should include a wider range of host varieties, particularly Sicot F1.

The Taproot Puncture Assay developed as part of this work utilised a simple and efficient glasshouse protocol that provided reliable and repeatable results. By controlling as many environmental variables as possible, this bioassay should produce consistent results in each of the geographically diverse locations that Fov research is undertaken. Many components of this bioassay are already in use by other research groups, but the reliability of the protocol described has not yet been verified elsewhere.

7.2 Infection process

The planting of less susceptible cotton varieties is a key strategy for Fusarium wilt disease control, as is exclusion by farm hygiene practices. An understanding of the basic biology of the host-pathogen interaction is required for effective exclusion, and identification of potential mechanisms of resistance is essential for the development of new varieties.

The infection process of Fov, as described here, represents the most complete survey to date of the interaction of Australian Fov with commercial cotton varieties. The mode of entry of Fov into the root epidermis was by both intercellular and intracellular penetration of the root, as was the subsequent growth through the root cortex. The ability of Fov hyphae to penetrate the root by either mechanism has been documented previously (Rodriguez-Galvez and Mendgen, 1995b). The extensive proliferation of the fungus on the root surface and within root tissue is evidence of its ability to aggressively colonise by either mechanism.

The presence of both hyphae and microconidia within xylem vessels in micrographs presented here confirmed their role in the rapid colonisation of the vascular system as reported by Beckman et al. (1976) and Shi et al. (1991a). Active latitudinal xylem colonisation was also documented here in micrographs showing the constriction of “penetration hyphae” breaching xylem cell walls, and hyphae passing through pit openings in vessel walls confirming previous observations in a foreign cotton variety (Rodriguez-Galvez and Mendgen, 1995b).

The restriction of pathogen growth in vascular tissue is involved in the resistance of cotton to several wilt diseases including Fusarium wilt (Wilhem et al., 1974b; Harrison
and Beckman, 1982; Shi et al., 1993). Resistant plants infected with the cotton wilt pathogens, *V. dahliae* and Fov, have been shown to restrict colonisation to the lower stem, as opposed to the extensive spread observed in susceptible varieties (Wilhem et al., 1974a; Harrison and Beckman, 1982). Until the present study, this restriction had not been investigated in the Australian context, where host resistance does not exist.

The characteristic restriction of Fov by resistant varieties was confirmed in the less susceptible Australian variety, Sicot 189. Colonisation was initially similar in the two varieties, but after three days Fov growth in the least susceptible Sicot 189 stele was restricted. This resulted in the pathogen travelling significantly further within Siokra 1-4 stele than in Sicot 189. These results are consistent with previous investigations that have shown that resistant host plants restrict Fov colonisation within the first four days after inoculation (Harrison and Beckman, 1982; Shi et al., 1991a; Shi et al., 1992; Shi et al., 1993). This restriction has been widely related to the accumulation of terpenoids (Stipanovic et al., 1988; Bell et al., 1975; Stipanovic et al., 1975a; Mace et al., 1985; Zhang et al., 1993).

The production of Fov chlamydospores in degraded tissues of heavily infested plants has not been documented in Australian cotton previously. Their presence has significant implications for consecutive crops, as these hardy resting spores could readily accumulate in plant debris and rapidly increase the soil inoculum load. The formation of chlamydospores might explain the increased incidence and severity of Fusarium wilt of cotton in glasshouse trials observed by Wang et al. (1999c) following the incorporation of plant residues. The rapid increase in field Fov populations and resulting disease incidence with successive growth of highly susceptible varieties (Hillocks, 1992b; Allen, 2004a) may be influenced by the quantity of chlamydospores produced within particular cotton varieties.

Cotton root exudates may impact on Fov even before infection. Exudates can promote the growth of plant pathogens by providing nutrients or triggers for particular pathogens (Rovira, 1969). They can also contain toxic compounds that inhibit spore germination and hyphal growth of pathogens (Katan, 2002). Varietal differences in the diffusates of cotton roots were documented in Chapter 4. The diffusate from the most susceptible Siokra 1-4 contained one or more lipophilic compounds that promoted the germination of Fov microconidia. Cotton exudates from a variety susceptible to Fusarium wilt have
previously been shown to increase mycelial growth and germination of both conidia and chlamydospores, however the mechanism is not known (Youseff and Heitefuss, 1983a).

Results presented in this chapter also showed that a lipophilic compound present in Sicot 189 diffusate inhibited the growth of Fov germ tubes. Future characterisation by HPLC analysis could confirm the nature of this compound, but there is a high likelihood it is a terpenoid. Cotton roots have previously been shown to exude and accumulate terpenoid compounds (Hunter et al., 1978b) and the amount of terpenoids is greater in soil surrounding the roots of cotton resistant to Fusarium wilt compared to a susceptible variety (Hedin et al., 1984).

7.2.1 Future applications and further questions

While the infection process described here shares similarities with other wilt diseases, it is important that a unique Fov biotype has now been documented. The description of the Fov infection process and related Fusarium wilt disease cycle will provide researchers with in-depth information on which to build further studies. The survival and infection strategies of Fov in plant and soil environments should be accounted for in the cultural management of Fusarium wilt in the field. The identification of differences in the interaction of Fov with different host varieties can perhaps be used to target future cotton breeding efforts.

Field trials should be carried out to confirm the glasshouse findings. A study of the progression of the infection process in the field, from natural infection through to plant defence responses, would validate the assumption that the observations made here are representative of what occurs in the field. Heavily infested Sicot 189 has anecdotally been noted in the field, and can exhibit severe Fusarium wilt symptoms. Considering that infection may occur at multiple sites in a root system, unlike in the Taproot Puncture system used here, inoculum density and temperature are likely to be factors requiring field experimentation. An investigation into the infection process of Fov on Sicot F1, the variety with significantly improved resistance to Fusarium wilt, would provide an interesting comparison to Sicot 189, and may elucidate the basis for its improved resistance.

The presence of chlamydospores in plant debris was documented, but their importance in the disease cycle was not quantified. A comparison of chlamydospore production in
different host varieties warrants further investigation due to their potential role as inoculum.

The varietal differences found in the diffusates of cotton roots may be an important consideration when determining which cotton variety to plant in a Fov-infested field. Further study is required to confirm the identities of the lipophilic compounds that were found to promote the germination of Fov microconidia and inhibit the growth of germ tubes. Their effect on Fov in the soil environment must first be quantified in order to assess their contribution, if any, to varietal resistance.

7.3 Defence responses

Antimicrobial terpenoids have been widely shown to accumulate in cotton after inoculation with Fov (Bell et al., 1975; Stipanovic et al., 1975a; Mace et al., 1985; Zhang et al., 1993). Their greater accumulation in resistant cotton varieties compared with susceptible varieties provides evidence for their role in inhibiting pathogen colonisation (Zhang et al., 1993). These terpenoid phytoalexins are toxic to both Fov and V. dahliae (Mace et al., 1985; Zhang et al., 1993).

The responses of two cotton cultivars of differing susceptibility to Australian Fov were compared in this study. The hypothesis that the least susceptible cultivar responds to infection with a rapid accumulation of antimicrobial compounds that restrict pathogen growth was investigated.

Evidence for differential terpenoid and phenolic responses was observed histochemically and described in Chapter 5, however the phenolic response (only observed seven days after inoculation) was too slow to account for the restriction of the pathogen seen after three days. Infection induced the reorganisation of contact cells in vascular tissue. An increase in cytoplasmic content and a partitioning of the vacuoles was documented, corresponding with the appearance of materials apparently secreted through pits into adjacent vessel lumens. These changes were observed using both light microscopy and TEM.

In line with previous studies, the globular structures accumulating in vessels were suspected to be terpenoid phytoalexins, secreted into the vessel lumens from adjacent contact cells (Mueller and Morgham, 1993; Shi et al., 1992). Confirmation of their
terpenoid identity was obtained using the terpenoid-specific histochemical reagent, SbCl₃-HClO₄ (Mace et al., 1974a).

The structural and terpenoid responses in Siokra 1-4 and Sicot 189 were similar, however, they were more intense and rapid in the latter, less susceptible variety. Thus, a correlation was demonstrated between the rapid and intense induction of structural and biochemical responses with decreased susceptibility.

Until this current study, the differential accumulation of individual antimicrobial terpenoids at numerous sites in Fov infected cotton stele tissue had not been measured. These factors, as well as the timing of the antimicrobial terpenoid response in cotton, were described in a level of detail not published previously.

The quantification of the terpenoid response was reported in Chapter 6 in relation to the invading pathogen. By three days after inoculation, antimicrobial terpenoids accumulated at the infection site in Sicot 189 to concentrations previously shown to be toxic in vitro (Mace et al., 1985; Zhang et al., 1993). This less susceptible variety restricted pathogen growth from this point in time. The timely accumulation of toxic terpenoids that confronted the invading Fov hyphae in the less susceptible Sicot 189 was in contrast to the weak response of the highly susceptible Siokra 1-4.

It was important to quantify the phytoalexin concentration in different locations relative to the invading pathogen in order to assess the specific site where direct contact occurs. High concentrations of antimicrobial terpenoids were localised around the site of infection, and decreased with distance from the inoculation point. The rapid progression of the pathogen up the Siokra 1-4 stele allows Fov to escape direct contact with the antimicrobial terpenoid response. However, in Sicot 189, Fov remains in the immediate vicinity of higher concentrations of the most toxic phytoalexin, dHG. This supports earlier work that revealed resistant cotton hosts produced a more intense terpenoid response that extended beyond the region of infection and in advance of the spreading pathogen (Harrison and Beckman, 1982). Fov outgrows the site of terpenoid accumulation 3 days after inoculation in Siokra 1-4, which renders the defence response ineffectual.

In summary, growth assays confirmed that Fov colonisation was restricted in the less susceptible variety after 3 days. Histochemical analysis showed a lipophilic compound
rapidly accumulated about the walls and (as globular structures) in lumens of Sicot 189 vessels within the same time period. Using a selective stain, these accumulations were identified as terpenoids. The terpenoids were quantified through HPLC analysis, which showed that they accumulated in stele tissue more rapidly and to higher concentrations in the less susceptible cotton variety. Evidence has been presented in this report that demonstrates that antimicrobial terpenoids accumulate at a time and location within the plant tissue, and at adequate concentrations, to enable the less susceptible cotton variety to inhibit the growth of Fov.

These findings establish that the susceptibility of Australian varieties to Fov is influenced by their ability to accumulate terpenoid phytoalexins, in line with a substantial body of research into Fusarium and Verticillium wilts of international varieties (Mace et al., 1985; Zhang et al., 1993). The results presented here provide strong circumstantial evidence that antimicrobial terpenoids are involved in the determination of Fusarium wilt susceptibility of Australian cotton varieties. Furthermore, the experimental approaches used in this study support a wider role for phytoalexins in plant defence (Hammerschmidt, 1999). By revealing the correlation between the rate and location of terpenoid phytoalexin accumulation in cotton in relation to the cessation of Fov development, this work has contributed to our broader understanding of the role of phytoalexins in disease resistance.

7.3.1 Future applications and further questions
This study has advanced our knowledge of the terpenoid cotton defence response and its influence on the susceptibility of Siokra 1-4 and Sicot 189 to an Australian biotype of Fov. I have demonstrated a correlation between Fusarium wilt resistance and the presence of terpenoid compounds with in vitro toxicity. This new insight may influence future breeding for disease resistance, which should aim to improve varietal accumulation of antimicrobial terpenoids.

Further confirmation of the role of terpenoid phytoalexins in the defence of cotton against Fov is achievable. Staining the compounds with osmium for viewing by TEM would allow correlation with the observations of cotton terpenoids identified by Mueller and Morgham (1993) as implicated in Verticillium wilt resistance and provide finer detail about the compounds' origins in contact cells.
Direct observations could also be made using a technique developed for estimating the concentration of cotton phytoalexins in situ after *X. axonopodis* pv. *malvacearum* infection (Essenberg et al., 1992a). Owing to the auto-fluorescence of the phytoalexins lacinilene C and lacinilene C 7-methyl ether, the location and concentration can be determined in relation to the pathogen. The possibility of using this technique for observing other cotton phytoalexins at the cellular level remains unexplored. Such insights into the phytoalexins induced by Fov would help to confirm their role in defence.

The application of several inducers of systemic acquired resistance, including non-pathogenic *Fusarium oxysporum*, to stimulate a phytoalexin response in Australian cotton is currently being investigated (Allen, 2004a; Smith and O'Neill, 2004). Preliminary trials of soluble silicon application show some potential for *Fusarium* wilt control, although the mechanism in not yet known (Smith and O'Neill, 2004). Signalling pathways in systemic acquired resistance has been the focus of considerable research (Hammerschmidt, 1993; Hutcheson, 1998). However, the hypersensitive response and the related generation of reactive oxygen species and salicylic acid in cotton have received relatively little attention. For example, the role of salicylic acid as a signal in plant defence responses is well established (Klessig and Malamy, 1994; Chen et al., 1993; Malamy and Klessig, 1992), but its function in cotton is not clear. While salicylic acid has been described as a key molecule in the systemic response of cotton to *X. axonopodis* pv *malvacearum* (Martinez et al., 2000), its role in the systemic acquired resistance of cotton to pathogens and insects has been questioned by others (Bi et al., 1997). The basis for systemic acquired resistance in cotton warrants further study due to its fundamental importance to disease resistance.

Detailed studies of the biology of Fov could be informative for disease control. An investigation, for example, into the tolerance of Fov biotypes to terpenoids, particularly dHG, may provide insight into the determinants of Fov virulence. The highly pathogenic Australian biotypes may have an elevated ability to tolerate or even degrade cotton phytoalexins. The ability of the various biotypes to produce the phytotoxin, fusaric acid, has been shown to be important in cotton wilt, however its role in the Australian context has not been determined (Gaumann, 1957; Smith and O'Neill, 2004).
The direct injection of inoculum into the vascular system has resulted in the study of post-infection defence responses. For a more complete overview of the host-pathogen interaction, it would be necessary to also quantify specific host responses at the point of entry into the root. Such methods are difficult in soil environments, but inoculations involving infested agar on bare roots surfaces have been described in previous investigations (Rodriguez-Galvez and Mendgen, 1995b; Rodriguez-Galvez and Mendgen, 1995a).

Although the development of cotton resistance to Fusarium wilt has been moderately successful in some parts of the world, an adequate level of resistance to all biotypes of Fov has not been found (Davis et al., 2006). The use of resistant cotton varieties is widely viewed as the most likely strategy for effective long-term management of Fusarium wilt (Davis et al., 2006). Research efforts must be directed towards the development of stable resistance in new varieties, from a solid basis of knowledge of the infection process of Fov and the subsequent defence responses of cotton.
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Title:
The infection process of Fusarium oxysporum f. sp. vasinfectum in Australian cotton and associated cotton defence mechanisms

Date:
2007

Citation:

Publication Status:
Unpublished

Persistent Link:
http://hdl.handle.net/11343/35364

File Description:
The infection process of Fusarium oxysporum f. sp. vasinfectum in Australian cotton and associated cotton defence mechanisms

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