Anthracnose on almond: epidemiology and characterisation of *Colletotrichum acutatum*

Suzanne F. Colmagro
B. Ag. Sc. (Hons) The University of Adelaide, Australia
B. App. Sc. (Physiotherapy) S.A.I.T., Australia

Thesis submitted for the degree of
Doctor of Philosophy
at The University of Adelaide

Discipline of Plant and Pest Science
Faculty of Sciences

July 2005

Almond blossom

I dedicate this thesis to my mum, Faye (8.04.1941-16.11.2002) who, with all her love and vibrancy, was always so proud of my achievements and interested in what I was doing. Not a day goes by without thinking of you. Also, I would like to remember my Nanna and Poppa Partington for all of their love and support in my formative years.

I miss you all.

Table of Contents

Abstract

In Australia, anthracnose on almond is caused by the ascomycete fungus *Colletotrichum acutatum*. Anthracnose has been confirmed throughout the major almond growing regions of Australia and significant economic losses have been reported. Fungicide trials carried out in Australia in 2000, prior to this project, yielded variable results that differed from those achieved in California. Two subpopulations of *C. acutatum* from almond have been reported in California, whereas one clonal population of *Colletotrichum* sp. from almond has been described in Israel, which differed from the subpopulations of *C. acutatum* in California. A collection of isolates of *C. acutatum* from almond in Australia was established. The isolates were characterised with respect to morphology, genetic variation and pathogenicity to detached plant tissues, and compared to representative isolates of *Colletotrichum* sp. from California and Israel. Plant material was cultured at regular intervals for isolation of *C. acutatum* to determine which tissues were likely to be the main sources of inoculum. The development of anthracnose on almond was monitored in the field for three successive growing seasons and relationships with weather data examined to elucidate the environmental conditions that are most conducive for disease.

There was considerable variation among isolates of *C. acutatum* from almond in Australia in terms of morphological and cultural characteristics. However, three main morphotypes were evident, namely pink, orange and cream colony colour. In general, isolates of *C. acutatum* from Australia were more similar morphologically to the pink subpopulation of *C. acutatum* from California than to the grey subpopulation from California and the isolates of *Colletotrichum* sp. from Israel.

Isolates of *Colletotrichum* sp. from almond in Australia were confirmed as *C. acutatum* by means of PCR with *C. acutatum*-specific primers. Subsequently, genetic variation was investigated using PCR with inter-simple sequence repeat primers, and the data were clustered using UPGMA. All isolates of *C. acutatum* from almond in Australia, except for one, shared 100% genetic similarity to one another, suggesting that the population of *C. acutatum* from almond was likely to be largely clonal. The isolates of *C. acutatum* from almond in Australia were genetically distinct from the isolates of the pink and grey subpopulation of *C. acutatum* from almond in California and from the *Colletotrichum* sp. from almond in Israel.

Pathogenicity experiments on detached leaves and fruit revealed pathogenic variation among representative isolates of *C. acutatum* from almond in Australia, California and Israel, however, all isolates tested caused disease symptoms. The susceptibility of the main almond cultivars grown in Australia was examined by inoculating detached leaves and fruit with isolates of *C. acutatum* from almond in Australia, California and *Colletotrichum* sp. from Israel. The results were inconclusive, and further research is needed to develop a rapid and reliable screening method to assess cultivar susceptibility.

The isolation of *C. acutatum* from almond tissues monthly for one year suggested that mummified fruit, peduncles and woody tissue were potentially significant sources of primary inoculum. These findings support the recommendation that the removal of mummified fruit and associated woody tissue may reduce inoculum potential and subsequent disease.

Correlating disease incidence in the field with weather data showed that rainfall early in the growing season appeared to be important in the development of anthracnose. Infection of almond tissues occurred when fruit was young, and disease incidence did not increase beyond November in two out of the three years during which disease was monitored. Disease

progress curves and relative area under the disease progress curve data showed significantly greater disease incidence on Price than Nonpareil, whereas the apparent rate of infection for Nonpareil and Price was similar for 2002 and 2003. On balance, these results suggested that there was little difference in susceptibility between Price and Nonpareil, but disease incidence may differ due to other factors, such as timing of fruit set, however, further investigation is needed to substantiate this. These results endorse the current recommendation that preventative fungicide sprays commence early in the growing season, however, sprays may not be necessary beyond November.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this thesis being made available for photocopying and loan when deposited in the University Library.

Signed: **Date:** 6-7-05

Acknowledgements

Naturally there are many people who I would truly like to thank.

Assoc Prof Eileen Scott, sincere thank-you for all of your guidance, support, interest, understanding and enthusiasm, and for letting me work in my own way. Thanks also to my other supervisors, Prof Margaret Sedgley, Dr Brent Kaiser and Dr Graham Collins for guidance, interest and assistance.

The Almond Board of Australia supported this project, both financially and in kind, and in particular, thanks to Chris Bennett and Andrew Lacey for their support and interest. Thank you to Leath and Sue Hunt, for the use of their almond orchard at Willunga for the duration of this project, and to all of the other almond growers who assisted.

I also sincerely thank Dr Stanley Freeman, of the Volcani Center, Bet Dagan, Israel, for collaboration, advice, and visiting us 'down under'. The following people provided fungal isolates, and I thank them: Dr Stanley Freeman, Dr Helga Förster, Dr Gavin Ash, Barbara Hall, Ru Huang, Nancy Cunningham, Andrew Watson and Dr Richard Lardner.

Thank you to:

- Dr Michelle Wirthensohn for advice and general assistance
- Dr Dani Shtienberg for extremely helpful epidemiological advice, greatly appreciated!
- The SARDI Horticulture Pathology crew, especially Barb Hall and Trevor Wicks, for advice, providing isolates and providing lots of fun on conferences, social nights etc!
- Heidi Schmidt, Amanda Benger, Sarah Swift, Grace Chan and Sky Ingram for assistance with field work and some lab work.
- Michelle Lorimer for assistance with statistics.
- Chris, Toni and Eva in the undergraduate teaching wing for all of their help, loan of incubators and the like.
- Dr Prue McMichael, Scholefield Robinson Pty Ltd, for advice and interest.
- Dr Richard Lardner for all his help in the lab and with computing, especially formatting.
- Nicki Featherstone, Gary Taylor, Geoff Clarke and Heather Fraser for general administrative help, much appreciated.

A sincere thanks to everyone in our great lab group, in particular, Richard Lardner (even though he is a Kiwi!), Tim Zanker, Evelina Facelli, Belinda Stummer, Belinda Brant, Don Gomez, Yvonne Smith, Carol Walker and Peter Crisp. Thanks to Dr Phil Davies and his lab group, especially Tanya Kovalenko, Parminder Sidhu and Bharati Das. Thankyou all, not only for your assistance in the lab, but for your friendship, support and patience over the last 4 years.

On to the 'personal' thanks, as without these people, I would never have kept going:-

- The biggest hug and thanks to (Dr!) Cas Collins who was always there for me, through the PhD and not-so PhD times. Thanks a million Cas, my brilliant friend.
- Michael who, despite it all, is still there for me, thank-you and love from the bottom of my heart.
- With love and thanks to my sister Di and brother Jay, and their families. We all went through the hardest of times, and I so wish mum could still be here to share our lives.
- Sue Coglan-McHugh and Richard McHugh…with love and gratitude for rescuing me when I really needed it, and providing me with a happy and comfortable house to live in whilst I almost completed this tome. You are truly part of this thesis, and I am sure you are not inspired to do one!!
- Fiona and Mark Roberston, for their welcoming and fun company and their wonderful home whilst I actually completed this thesis. Love and deepest gratitude.
- Nick Cave for the music.
- My other dear friends who have waited patiently for me to 'get a life' again!

Abbreviations

°C	degree Celsius
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
AUDPC	area under the disease progress curve
bp	base pair
ca	*circa*
m, cm, mm, nm, µm	metre, centimetre, millimitre, nanometre, micrometre
cv., cvs	cultivar(s)
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dd H_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphatases
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediamine *tetra* acetic acid
g, mg, µg, ng	gram, milligram, microgram, nanogram
h	hours
ISSR	inter-simple sequence repeat
ITS	internal transcribed spacer
L, mL, µL	litre, millilitre, microlitre
lsd	least significant difference
M, mM	molar, millimolar
MDS	multi-dimensional scaling
min	minute
NaCl	sodium chloride
NaOH	sodium hydroxide
PCA	principal components analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PVP-10	polyvinylpyrrolidone, molecular weight 10,000
RAPD	random amplified polymorphic DNA
RAUDPC	relative area under the disease progress curve
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RNAse A	ribonuclease A
RO	reverse osmosis
rpm	revolutions per minute
SCAR	sequence characterised amplified region
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
se	standard error
TAE	Tris-acetate-EDTA

TBE	Tris-borate-EDTA
TE	Tris-EDTA
UPGMA	unweighted pair group method, arithmetic average
UV	ultra violet
VCG	vegetative compatibility group
vs	versus, against
viz.	namely
V	volts
vol	volume(s)

Chapter 1 Introduction

Anthracnose on almond in Australia is caused by the ascomycete fungus *Colletotrichum acutatum* J.H. Simmonds. In Australia, anthracnose was first reported in 1996 (Hall *et al.*, 1998) in the Southern Vales region of South Australia and has since been confirmed in all of the major almond growing regions of Australia (McMichael, 2000). Significant economic losses, up to 80% of fruit infected, have been observed in some orchards. Anthracnose has been a serious problem in almond orchards in California and Israel also (Shabi and Katan, 1983; Förster and Adaskaveg, 1999; Heintz, 1997), with reports of crop losses of up to 25% (Stockwin, 1998). In Australia, control measures for this disease have been variable and inconsistent in efficacy, and it appears that season-long protection cannot be achieved (McMichael, 2000). The reasons for this variability and lack of consistent response to fungicides are not well understood.

Conventional taxonomy of fungi in the genus *Colletotrichum* is based on a limited number of morphological and cultural features (Cannon, 1998; Sutton, 1992), however, the relationships among several species, including *C. acutatum*, remain unresolved due to the overlapping morphological characteristics and wide host ranges. In recent times, DNA-based diagnostic methods have been used to identify and characterise *Colletotrichum* spp., including *C. acutatum* from almond in California (Förster and Adaskaveg, 1999), and *Colletotrichum* sp. from almond in Israel (Freeman *et al.*, 2000a). DNA-based methods have not yet been used to examine isolates of *C. acutatum* from almond in Australia.

Two distinct subpopulations have been identified among isolates of *C. acutatum* from almond in California on the basis of several morphological and cultural characteristics, and DNA fingerprints obtained by arbitrary-primed polymerase chain reaction (ap-PCR) methods

(Förster and Adaskaveg, 1999). In contrast, isolates of *Colletotrichum* sp. from almond in Israel are homogeneous with respect to morphology, cultural characteristics, vegetative compatibility group analysis and banding patterns following ap-PCR (Freeman *et al.*, 1996; Freeman *et al.*, 2000a). The isolates of *C. acutatum* from almond in Australia have not been characterised with respect to morphological and cultural features, pathogenicity and genetic variation and it is not known if these isolates differ from those of *C. acutatum* from almond in California and *Colletotrichum* sp. from almond in Israel. These factors may have implications for management and control measures undertaken in Australia.

The typical symptoms of anthracnose on almond are sunken lesions on young fruit, which shrivel, die and develop an orange colour. Affected fruit remain attached to the tree, and leaves, twigs, spurs and small branches may also be affected (Adaskaveg *et al.*, 1998). It appears that all above-ground plant tissues are susceptible to infection and may be sources of inoculum throughout the season, however, further research is required to substantiate these observations for anthracnose in Australia. There is some evidence to suggest that differences exist with respect to susceptibility of almond cultivars, however, this is based primarily on field observations (Adaskaveg and Förster, 2000; McMichael, 2000). Knowledge about cultivar susceptibility to anthracnose is important for the breeding and selection of new almond cultivars, for example, in the almond breeding program at the University of Adelaide, South Australia.

The optimal conditions for disease development are considered to be warm, wet weather, but evidence for this has been based primarily on field observations in California (Adaskaveg and Förster, 2000) and on findings from studies of *Colletotrichum* spp. on other crops (Denham and Waller, 1981; Dodd *et al.*, 1992). The optimal conditions

for anthracnose development on almond in orchards have not been adequately investigated in Australia, California or Israel. The disease cycle of anthracnose caused by *C. acutatum* on almond has not been documented.

The research described in this study was undertaken to (i) confirm the identity of *C. acutatum* as the causal organism of anthracnose on almond in Australia using PCR-based methods, (ii) characterise isolates of *C. acutatum* with respect to morphological and cultural features, pathogenic and genetic variation and (iii) explore epidemiological aspects of anthracnose. Such information will contribute towards the development of improved management strategies for Australian almond growers.

Chapter 2 Literature review

This review focuses on the characterisation of *C. acutatum* and epidemiological aspects of anthracnose on almond. Included are an overview of the almond industry and the history of anthracnose on almond.

2.1 Background

2.1.1 The almond industry

The almond (*Prunus dulcis* Miller [D.A. Webb]) is one of the oldest cultivated fruit tree crops in the world, having originated in Central Asia and spread to countries around the Mediterranean Sea where cultivation probably began around 3 BC (Zohary and Hopf, 1988; Kester *et al.*, 1991). Cultivation then spread to Europe, northern Africa and America in the mid to late 1800s, followed by smaller plantations in countries such as South Africa, Australia and South America (Kester *et al.*, 1991). Almond production is best suited to a Mediterranean climate of warm to hot, dry summers and mild, wet winters. Almonds belong to the *Rosaceae* family and produce a fruit with an edible seed or 'nut', which is used as food and as an ingredient in cosmetics and 'alternative' medicines (Zohary and Hopf, 1988; Kester *et al.*, 1991).

California is the world's major almond producer (Kester and Gradziel, 1996), with an estimated world market share of at least 37% (Pocock and Bennett, 1999). Spain, central and southwestern Asia are the next major production areas. Smaller production regions include Australia, South Africa, Chile and Argentina (Kester and Gradziel, 1996), with Australia contributing approximately 1% to world production (Pocock and Bennett, 1999). Although

Australia is a small producer by world standards, Australia's average productivity per tree is 25-30% greater than that of the United States (Bennett, 2004).

The Australian almond industry, currently worth $55 million (Bennett, 2004), continues to expand and P. Freeman (pers. comm., 2004), chair of the almond Industry Advisory Committee, estimated that within 10 years, Australia's annual production of kernel will increase from the current 10,000 tonnes to an estimated 25-35,000, which will equate to 5% of world production. The majority of the kernel produced in Australia is consumed domestically.

The almond was first introduced into South Australia in the 1830s-1840s (Quinn, 1928). The original production areas were located close to Adelaide in the Southern Vales, Central Adelaide Plains and the Barossa Valley. However, urban spread and a more suitable climate have resulted in most of the orchards being relocated to the Northern Adelaide Plains and the Riverland (Scholefield, 1995). In the 1960s, almond orchards were planted in areas around the Sunraysia region in Victoria and Padbury in Western Australia. Now, the main production regions are generally located in close proximity to the Murray River water supply, with some production remaining in the Willunga region and the Northern Adelaide Plains of South Australia (Fig 2.1). The following are the main almond growing areas; the Riverland (South Australia), Lindsay Point, Nangiloc, Robinvale, Redcliffs and Karadoc (Victoria) and Darlington Point (New South Wales) (Scholefield, 1995).

2.1.2 Anthracnose disease on almond: history, distribution and economic importance

Several ascomycete fungi, including those in the genus *Colletotrichum*, cause a range of symptoms which are collectively known as anthracnose. Anthracnose, caused by

Colletotrichum sp., occurs on a wide range of perennial and annual plant species and affects crops worldwide, as reviewed by Waller (1992). Anthracnose is characterised by dark coloured and sunken lesions that can occur on stems, foliage, fruits and flowers, affecting all stages of the plant life cycle (Agrios, 1997). *Colletotrichum* spp. cause preharvest and postharvest symptoms, and infections can be latent, which make anthracnose difficult to diagnose and control (Sutton, 1992; Cannon, 1998).

Fig 2.1 The main almond production regions in Australia are shown in red.

Anthracnose was first reported on almond in Italy in 1896 (Brizi, 1896) and the pathogen was identified and classified as a *Gloeosporium* species. Subsequently, anthracnose outbreaks were reported on almonds in California in 1916 (Czarnecki, 1916) and in 1925 (Heintz, 1997). Since then, outbreaks have occurred in the 1980s and, frequently, in the 1990s (Adaskaveg *et al.*, 1998), with the most serious epidemics following warm, wet weather in spring (Heintz, 1997). Serious crop losses due to anthracnose have also been reported in Israel and South Africa (Shabi and Katan, 1983).

Anthracnose was first documented in South Australian almond orchards in 1996, with the disease found to be widespread in the growing regions of the Southern Vales and Northern Adelaide Plains (Hall *et al.*, 1998). It has been suggested that the disease has been in these regions for a long period of time, having gone unrecognised and confused with other diseases, such as shot hole (Hall *et al.*, 1998) and brown rot (Hendricks, 1998). Anthracnose has been confirmed in all the major growing regions of Australia, and economically significant levels of infection (up to 80% of fruit) have been reported (McMichael, 2000).

The pathogen causing anthracnose on almonds in Australia and California has been identified as *Colletotrichum acutatum* J.H. Simmonds (Adaskaveg and Hartin, 1997; Hall *et al.*, 1998). In Israel, *C. gloeosporioides* (Penz.) Penz. & Sacc. was identified initially as the cause of anthracnose on almond (Shabi and Katan, 1983) but more recent evidence, using DNA-based identification methods, suggests that this pathogen is *C. acutatum* also (Förster and Adaskaveg, 1999; Freeman, 2000a). *C. acutatum* causes anthracnose on many other commercially important fruit crops (Dyko and Mordue, 1979), such as strawberry (Freeman and Rodriguez, 1995), avocado (Adaskaveg and Förster, 2000), citrus (Timmer and Brown, 2000), olive (Martin and García-Figueres, 1999), peach (Adaskaveg and Hartin, 1997), apple

(Guerber and Correll, 1997), pistachio (Ash and Lanoiselet, 2001) and mango (Afanador-Kafuri *et al.*, 2003).

2.2 The genus *Colletotrichum* Corda

The genus *Colletotrichum* was first described by Corda in 1831 and, although the genus has been well defined (Lu *et al.*, 2004), species concepts remain unresolved (Baxter *et al.*, 1985; Sutton, 1992), largely because many *Colletotrichum* species have overlapping host ranges, similar morphological characteristics and may have both an asexual (anamorph) and sexual state (teleomorph) (Cannon, 1998). In the past, around 750 'species' of *Colletotrichum* were recognised, and different species were described largely on the basis of 'assumed' host specificity (Cannon *et al.*, 2000). In 1957, von Arx revised *Colletotrichum* classification and described 11 taxa based on morphological characteristics rather than host specificity (von Arx, 1957; Cannon *et al.*, 2000). However, Sutton (1992), in the most recent review of the taxonomy of *Colletotrichum*, described 39 generally accepted taxa including *C. acutatum.* However, among these 39 taxa, there are currently three major species aggregates, based around *C. acutatum*, *C. gloeosporioides* and *C. orbiculare* (Sutton, 1992; Cannon *et al.*, 2000).

Currently, taxa are identified primarily on the basis of of morphological and cultural characteristics, in particular, conidium size and shape, appressorium characteristics, and the presence or absence of setae and of the sexual state (Cannon, 1998). However, other cultural features, including temperature response, growth rates, host range (Simmonds, 1965), fungicide sensitivity (Adaskaveg and Hartin, 1997; Jayasinghe and Fernando, 2004), vegetative compatibility group (VCG) analysis and pathogenicity (Smith and Black, 1990), are used also to identify and clarify relationships between *Colletotrichum* spp.

Within the last 10 years or so, DNA-based molecular methods have been increasingly used for characterisation and identification of many fungi, including *Colletotrichum* spp. Due to the similarity of morphological characters among species, it has been recommended that a combination of conventional and molecular methods be used to identify and characterise *Colletotrichum* species (Lardner *et al.*, 1999; Cannon *et al.*, 2000; Talhinhas *et al.*, 2002; Cook and Hughes, 2004).

2.3 Identification and characterisation of *Colletotrichum acutatum* by cultural methods

C. acutatum was first described by Simmonds in 1965 when studying ripe fruit rots in Queensland, Australia (Simmonds, 1965), and the species name was accepted in 1968 (Dyko and Mordue, 1979). Accurate identification of *C. acutatum* can be difficult, because *C. acutatum* is morphologically similar to *C. gloeosporioides* and *C. fragariae* and these species have overlapping host ranges (Smith and Black, 1990; Mills *et al.*, 1994). Additionally, there appears to be significant variation within the species, leading to the current classification of *C. acutatum* as a species complex, which comprises several subgroups (Johnston and Jones, 1997; Lardner *et al.*, 1999). Lardner *et al.* (1999) have suggested that *C. acutatum* J.H. Simmonds is a subspecific group within the broader *C. acutatum* complex and Freeman *et al.* (2001) referred to this group as *C. acutatum sensu* Simmonds. Furthermore, variation is apparent also within the *C. acutatum sensu* Simmonds group (Freeman *et al.*, 2001). The isolates of *Colletotrichum* obtained from almond in California and Israel are considered to belong to *C. acutatum sensu* Simmonds (Freeman *et al.*, 2001) and there is evidence of two subpopulations of isolates of *C. acutatum* from almond in California (Förster and Adaskaveg, 1999).

2.3.1 Morphology and cultural characteristics

The main morphological features that are considered diagnostic of *C. acutatum* J.H. Simmonds are: fusiform conidia; pink pigment in the culture medium; clavate appressoria; absence of setae and of the teleomorphic state (Simmonds, 1965; Dyko and Mordue, 1979; van der Aa *et al.*, 1990; Sutton, 1992; Cannon, 1998). These characteristics are summarised in Table 2.1, however, considerable variation has been reported among isolates of *C. acutatum* J.H. Simmonds. Additionally, the use of different culture media, age of the isolates and environmental factors can affect the morphology of *Colletotrichum* spp., including *C. acutatum* (Frost, 1964; Smith and Black, 1990; Gunnell and Gubler, 1992; Adaskaveg and Hartin, 1997). These factors complicate the classification of fungal taxa and they need to be taken into account when comparing published reports about the morphology of isolates of *C. acutatum*.

C. acutatum is generally considered to be anamorphic (Dyko and Mordue, 1979; van der Aa *et al.*, 1990; Sutton, 1992), however there are brief references in the literature to the production of perithecia (Guerber and Correll, 1997; Lardner *et al.*, 1999). Recently, following a series of *in vitro* crosses between self-sterile strains of *C. acutatum* J. H. Simmonds obtained from various fruit in USA, Australia and New Zealand, fertile perithecia were produced (Guerber and Correll, 1997; Lardner *et al.* 1999; Guerber and Correll, 2001). The resultant teleomorph was described and named a new species, *Glomerella acutata* J.C. Guerber and J.C. Correll (Guerber and Correll, 2001). However, it should not be assumed that all groups of *C. acutatum* have a teleomorphic state (Guerber and Correll, 2001) as the teleomorph has not been observed *in planta*. Putative perithecial initials were observed in an Australian isolate of *C. acutatum* from almond (McMichael, 2000), however, perithecium production and the existence of the teleomorph have not been investigated for isolates of *C.*

acutatum from almond in Australia or California. The potential for sexual reproduction of *C. acutatum* has implications for increasing genetic diversity, which can affect the ability of an organism to adapt to changing conditions.

Table 2.1. A summary of the morphological and cultural features of *C. acutatum* as described by several authors

Morphological feature	Sutton (1992)	Cannon (1998)	Simmonds (1965)	Dyko and Mordue (1979)	van der Aa *et al.* (1990)
Conidia	Fusiform, occasionally medianly constricted, 8.5-16.5 x 2.5-4 µm	Fusiform, occasionally constricted in mid-portion, 8.5-16.5 x 2.5-4 µm	Short, narrow with pointed ends, some long spores, 8.3-14.4 x 2.5-4.0 µm	Fusiform, 8-16 x 2.5-4 µm	Fusiform or at least acuminate at one end
Colony colour	White becoming pinkish grey, reverse pink	Colonies produce pink exudates in nitrogen-rich media	White, developing a grey overlay, grey or greyish brown, reverse white/cream, with sometimes a creamy pink diffusion. Papaw isolates bright pink	White to pale orange, then greenish grey or black, reverse pink to reddish purple	Not reported
Appressoria	Clavate or slightly irregular, margins entire or slightly lobed, 8.5-10 x 4.5 –6 µm	Clavate, occasionally slightly irregular, 8.5-10 x 4.5-6 µm	Obovate and rarely lobed, 8-9 x 6-6.5 µm	Clavate to obovate, 6.5-11 x 4.5-7.4 µm	Not reported
Setae	Absent	Not reported	Occasional but poorly developed	Absent in most isolates	Absent in vitro
Teleomorph	Absent	Not reported	Not reported	Not reported	Absent

C. acutatum is morphologically similar to *C. gloeosporioides*, but isolates can be distinguished by mycelial growth rate in culture and sensitivity to the fungicide benomyl. The optimal temperature for mycelial growth of *C. gloeosporioides* is 26.0 -28.5°C, whereas it is 25.0-26.5°C for *C. acutatum* (Simmonds, 1965), and *C. gloeosporioides* grows faster than *C. acutatum* at their respective optimal temperatures for mycelial growth (Simmonds, 1965; Sutton, 1992). *C. gloeosporioides* is sensitive to benomyl *in vitro* whereas *C. acutatum* is not (Bernstein *et al.*, 1995; Adaskaveg and Hartin, 1997), however, Adaskaveg and Hartin (1997), found that this response is dependent on the benomyl concentration.

In culture, *C. acutatum* generally grows more slowly than *C. gloeosporioides* and other morphologically similar species (Smith and Black, 1990; Adaskaveg and Hartin, 1997) and this feature has been used to assist with the differentiation and identification of *Colletotrichum* spp.

2.3.2 Vegetative compatibility group analysis

VCG analysis is used to characterise a fungal population with respect to genetic diversity, in particular, genetic similarity among strains (Glass and Kuldau, 1992; Leslie, 1993; Kistler, 1997). Correll (1992) defined vegetative compatibility as the 'ability of the mycelia of two fungal strains to anastomose', which results in a cell that contains two genetically different nuclei. This is referred to as heterokaryosis, and strains that are capable of forming stable heterokaryons are said to be compatible and are classified as belonging to the same VCG. Correll (1992) stated that 'strains of a given species that are vegetatively compatible are usually identical or very similar with respect to genetic characteristics'. Hyphal anastomosis may be the first step in the parasexual cycle, which has the potential to increases the genetic diversity within a population (Glass and Kuldau, 1992). The outcome of the parasexual cycle can be likened to genetic recombination that occurs as a result of meiosis in fungi that reproduce sexually (Pontecorvo, 1956; Jennings and Lysek, 1996).

There are several methods for assessment of heterokaryon formation, however, discussions of these techniques are beyond the scope of this review. The main disadvantages of methods to determine VCGs are that they can be very time consuming, not all isolates are suitable for every method (Correll, 1992) and VCG testing does not elucidate the degree of genetic difference among strains (Kistler, 1997; McDonald, 1997). However, VCG analysis can be

useful for differentiating subgroups among a population, which may appear homogeneous with respect to other characters, e.g., morphology or genetic profile (Freeman *et al.*, 2000b).

VCG analysis has been conducted to characterise many plant pathogenic fungi, including *Fusarium oxysporum*, (for reviews see Kistler,1997; Leslie, 1993 and Correll, 1992), *Aspergillus* spp., (for review see Leslie, 1993) and *Verticillium* spp. (Elena, 1999; Korolev *et al.*, 2001). VCG experiments have been used for population analysis of *C. orbiculare* (Wasilwa *et al.*, 1993), *C. dematium* (Correll *et al.*, 1993a), and *C. acutatum* from pine and other hosts (Lardner *et al.*, 1999), strawberry (Freeman and Katan, 1997) and almond (Freeman *et al.*, 1998). Freeman *et al.* (1998) reported that the *Colletotrichum* sp. found on almond in Israel belonged to a single VCG, suggesting that the population is likely to be clonal. VCG analysis has not been carried out on Australian isolates of *C. acutatum* obtained from almond or on Californian isolates from almond.

2.4 Identification of *C. acutatum* using molecular methods

2.4.1 Immunological identification

The enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies has been one of the most commonly used immunological tests to detect plant pathogens (Miller and Martin, 1988). Organisms express unique and specific antigens that can be detected by immunological assays, which have been used to identify microbes at the genus, species and sub-species level (Correll, 1992). Diagnostic ELISAs have been used for the detection of several important plant pathogens, including *Rhizoctonia solani* (Fidanza and Dernoeden, 1995; Thornton *et al.*, 1999), *Pythium* spp. (White *et al.*, 1994; Yuen *et al.*, 1998) and *Phytophthora* spp. (Ellis and Miller, 1994). An ELISA test, using monoclonal antibody MAFF 26 (25/164.32), highly specific for *C. acutatum,* was developed to diagnose

anthracnose on strawberries in the United Kingdom (Barker *et al.*, 1994), and it is used to identify *C. acutatum* from other plants (Hughes *et al.*, 1997). Although results from this diagnostic procedure can be obtained within 24 hours, the test cannot detect latent infection or infection in the absence of conidia (Barker *et al.*, 1994). This may not be practical for use in almond as conidia of *C. acutatum* are not present at all stages of the disease cycle.

2.4.2 DNA-based identification

Oligonucleotide primers, based on sequences of the internally transcribed spacer (ITS) regions of ribosomal DNA (rDNA), have been developed and used in the polymerase chain reaction (PCR) to identify several species of plant pathogenic fungi, e.g., *Fusarium* spp. (Schilling *et al.*, 1996; Moricca *et al.*, 1998) and *R. solani* (Salazar *et al.*, 2000). Mills *et al.* (1994) and Sreenivasaprasad *et al.* (1994) developed *C. acutatum*- and *C. gloeosporioides*-specific primers, named *Ca*Int2 (GGGGAAGCCTCTCGCGG) and *Cg*Int (GGCCTCCCGCCTCCGGGCGG), respectively, based on the ITS regions 1 and 2 of the rDNA gene cluster. PCR with these primers, in conjunction with the universal primer ITS 4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990), has been used to identify and differentiate isolates of *C. acutatum* and *C. gloeosporioides* from one another and from other *Colletotrichum* spp. (Sreenivasaprasad *et al.*, 1996; Mills *et al.*, 1998; Förster and Adaskaveg, 1999; Freeman *et al.*, 2001; Peres *et al.*, 2002; Talhinhas *et al.*, 2002; Afanador-Kafuri *et al.*, 2003). Isolates of *Colletotrichum* spp. obtained from almond in California and Israel have been identified as *C. acutatum* following PCR with these primers (Förster and Adaskaveg, 1999; Freeman *et al.*, 2001), however, isolates of putative *C. acutatum* obtained from almond in Australia have not yet been tested.

2.5 DNA-based characterisation of fungal plant pathogens

DNA-based molecular markers have been developed for the identification of fungi at genus, species and sub-species levels, and for studying the genetic structure within and among fungal populations (McDonald, 1997). In the past, morphological, allozyme and isozyme makers were used, however, nucleic acid-based markers are now used extensively as they have several advantages over other markers. This section of the review will summarise the factors and mechanisms responsible for creating genetic diversity among anamorphic fungi and examine several DNA-based methods used to investigate genetic diversity, with particular reference to *Colletotrichum* spp.

2.5.1 Factors and mechanisms responsible for genetic diversity

Information about the genetic variation among and within populations of plant pathogenic fungi is essential for the understanding of the evolution of the pathogen and, to some extent, the host, and their interaction. For example: How will a pathogen population adapt to a change in environmental factors? How quickly will fungicide resistance develop in a pathogen population? The four processes that are considered to change the frequency of alleles in populations are mutation, random genetic drift, gene flow and natural selection, however, the initial source of all changes to genetic material is mutation (Ayala, 1983). Non-lethal mutations that occur in fungi with haploid mycelium will be expressed in the phenotype.

If the progeny has a mutation that is strongly deleterious, then this new phenotype is likely to be eliminated from the population by natural selection, resulting in no increase in the genetic diversity (Aquadro, 1992). In contrast, selectively neutral mutations, which do not affect the fitness of the fungus, and those which confer an increase in fitness, are less likely to be eliminated from a population, resulting in an increase in genetic diversity of a population over time (Aquadro, 1992). Mutation, genetic drift and migration

occur in populations regardless of whether the organism becomes more or less adapted to its environment, whereas natural selection results in adaptation of organisms to the environment (Ayala, 1983).

In fungi, both meiotic and mitotic processes pass on changes of the genetic material to subsequent generations, resulting in variation among populations. Sexual reproduction produces variation in the progeny mainly due to segregation and recombination of genes during meiosis. Many fungi, including *C. acutatum*, are anamorphic, however, despite the lack of apparent sexual reproduction, anamorphic fungi still display significant variation at the subspecies level (Michelmore and Hulbert, 1987). Although mutation is the initial source of variation, these changes to genetic material can be passed to the subsequent generations as a result of a specialised recombination process known as parasexuality (Pontecorvo, 1956). Parasexuality does not involve meiosis, but genetic material from the two 'parental strains' recombines during mitosis. Parasexuality involves hyphal fusion, heterokaryosis, karyogamy (nuclear fusion), recombination, or crossing over during mitosis, followed by reversion of the diploid nucleus to a haploid state (Tinline and MacNeill, 1969; Agrios, 1997; Debets, 1998). Parasexuality has been demonstrated for several fungi, including *Cryphonectria parasitica* (Rizwana and Powell, 1995), *Aspergillus* spp. (Pontecorvo, 1956; Brookman and Denning, 2000) and *Phytophthora parasitica* (Gu and Ko, 2000) in laboratory conditions, however, its importance in natural populations is still unclear (Millgroom, 1996; Debets, 1998).

The mating system of filamentous fungi, broadly, is heterothallic or homothallic. Heterothallism is where self-fertilisation cannot occur and cross-fertilisation is essential, whereas both self- and cross-fertilisation can occur in homothallic fungi (Bryson *et al.*, 1992). Bryson *et al.* (1992) noted that the "sexual mating system of *Colletotrichum* species, however,

does not fit simply into either of these categories", and many questions remain, including those involving taxonomy, evolution, origin of variation and host specificity.

An understanding of genetic diversity contributes to the knowledge of population structure, which has implications for the control of anthracnose, in particular, fungicide application regimes, and the breeding and selection of almond cultivars tolerant or resistant to anthracnose.

2.5.2 Development of molecular markers

A genetic marker is an easily scored character that can be used to investigate variation and population genetics, and to identify specific isolates. Nucleic acid-based markers have several advantages over phenotypic, immunological and isozyme markers, in that they can be extremely sensitive, have a broad range of specificity from genus to sub-species level, cost considerably less than the development of monoclonal antibodies (Henson and French, 1993), are selectively neutral, and do not vary with the developmental stage of the organism (Michelmore and Hulbert, 1987; McDonald, 1997). The choice of technique to be used for marker development and methods for fungal identification and investigating genetic diversity will depend on research aims, technical difficulty, cost and availability of equipment, materials and labour. However, regardless of the methodology chosen for marker development, in order to be effective, the marker system must be reliable, consistent (Horejsi *et al.*, 1999), precise and easy to interpret (Michelmore and Hulbert, 1987). Currently, two nucleic acid-based approaches, restriction fragment length polymorphism (RFLP) analysis and PCR-based methods, are commonly used to generate molecular markers for the characterisation of pathogenic fungi both at the species and sub-species levels.

2.5.2.1 Restriction Fragment Length Polymorphism analysis

RFLP analysis involves digesting DNA with restriction endonucleases and the resultant fragments are separated according to molecular weight by gel electrophoresis. The banding pattern can be compared among isolates, however, the banding pattern may be complex and difficult to interpret on an agarose gel, so the digested DNA can be denatured, transferred to a nylon or nitro-cellulose membrane, and hybridised to specific radioactive probes. This process is known as Southern hybridisation (Southern, 1975). The resultant banding pattern and number of fragments will vary depending on the type of probe used (Miller and Martin, 1988). Fragment size and banding pattern can be compared among isolates, and used to discriminate isolates at a species or strain level (Mills *et al.*, 1998). Polymorphisms result from differences in DNA sequence caused by base pair substitutions, deletions, additions and other chromosomal changes (Michelmore and Hulbert, 1987). RFLPs can be generated from total genomic DNA, mitochondrial DNA (mtDNA) and rDNA or from individual DNA fragments derived from restriction endonuclease digestion of PCR products. Depending on which part of the DNA is being subject to RFLP analysis, different banding patterns among isolates may result. Species and subspecies diversity may not be detected by using one type of DNA alone in RFLP analysis (Mills *et al.*, 1992). For example, Kistler *et al.* (1987) found no genetic diversity among 25 isolates of *Fusarium oxysporum* obtained from a wide geographical area and host plant range, following RFLP analysis of rDNA and total DNA, yet RFLP analysis of mtDNA was used to elucidate subspecies differences. In general, RFLPs are valuable for the identification of species, strains or populations and for the investigation of the population structure of fungal pathogens. However, differences in band patterns do not mean that there has been significant genetic divergence among isolates, so techniques such as RFLPs, which use band patterns for analysis, are not recommended for phylogenetic studies (Cannon *et al.*, 2000).

Drawbacks of RFLP analysis include; development of the probes can be difficult, large quantities of DNA are required, radioactivity is commonly used, and the methods are technically more difficult and time consuming than PCR-based methods (Pejic *et al.*, 1998). However, RFLP markers are co-dominant (heterozygotes can be distinguished from either homozygote) and non-radioactive methods are available (Rafalski and Tingey, 1993).

RFLP analysis has been used to investigate population structure and genetic diversity both within and among populations of various fungal plant pathogens (Edel *et al.*, 1995; Stummer *et al.*, 2000; Pryor and Michailides, 2002; Saha *et al.*, 2002), including *Colletotrichum* spp. (Liyange *et al.*, 1992; Correll *et al.*, 1993b; Freeman *et al.*, 1996; Buddie *et al.*, 1999).

2.5.2.2 PCR-based methods

The PCR technique was developed in the 1980s and, since then, several methods based on PCR have been used for the identification and characterisation of fungal plant pathogens. PCR amplifies very small quantities of a specific DNA sequence without prior knowledge of sequence data. In a relatively simple process, double stranded DNA is subjected to repeated cycles of denaturation of the DNA template, annealing of oligonucleotide primers to the template and synthesis of DNA in the region of interest by the activity of a thermostable DNA polymerase under suitable conditions. Theoretically, PCR results in an exponential increase of the target DNA (Newton and Graham, 1994).

Various techniques based on PCR are used to generate molecular markers, including randomly amplified polymorphic DNA (RAPD)-PCR, inter-simple sequence repeat (ISSR) primed-PCR and amplified fragment length polymorphism (AFLP).

Randomly amplified polymorphic DNA markers

Randomly amplified polymorphic DNA (RAPD)-PCR was independently described by Williams *et al.* (1990) and Welsh and McClelland (1990) in 1990. Single primers of random sequence (usually ten nucleotides in length) are used to amplify regions of genomic DNA. Low annealing temperatures are used in the PCR amplification to ensure that the primers anneal to several sites, which are randomly distributed throughout the genome, resulting in several amplification products (Rafalski and Tingey, 1993). The amplified DNA fragments are separated according to molecular weight by gel electrophoresis. Different banding patterns, commonly referred to as DNA fingerprints, may reveal polymorphisms between species, strains or populations, and are commonly used for identification of fungi at all of these levels (Cannon *et al.*, 2000), and for investigation of population structure and genetic diversity of fungal plant pathogens. However, as discussed above for RFLPs. RAPDS are not recommended for use in phylogenetic studies (Cannon *et al.*, 2000). RAPD-PCR is relatively simple, cheap and quick to perform, does not require the use of radiation, requires small quantities of template DNA and no prior knowledge of DNA sequences is necessary (Rafalski and Tingey, 1993). However, RAPD-PCR can lack reproducibility between (Jones *et al.*, 1997), and sometimes within, laboratories and only dominant markers are produced, so that heterozygotes and homozygotes cannot be distinguished without a progeny test (McDonald, 1997).

RAPD-PCR has been used extensively in fungal pathology to analyse population structure and genetic diversity for a range of species (Crowhurst *et al.* 1991; Wyss and Bonfante, 1993; Bentley and Bassam, 1996; Chiocchetti *et al.*, 1999; Vincente *et al.*, 1999), including *Colletotrichum* spp. (Correll, 1992; Vaillancourt and Hanau, 1992; Fabre *et al.*, 1995; Kurame-Izioka *et al.*, 1997; Yang and Sweetingham, 1998). DNA fragments obtained by

RAPD-PCR can also be used as the basis for the development of other genetic markers for species or strain identification (Williams *et al.*, 1990; Rafalski and Tingey, 1993), for example, RFLP markers and sequence characterised amplified region markers (Leclerc-Potvin *et al.*, 1999; Li *et al.*, 1999; Lardner *et al.*, 2005).

Inter-simple sequence repeat markers

Nucleotide primers, derived from the microsatellite regions of DNA, commonly referred to as inter-simple sequence repeats (ISSRs) are used in the PCR process, to amplify short regions of DNA containing a complementary repeat sequence. Microsatellite regions of DNA consist of tandem repeats of simple sequences, of generally one, two or three nucleotides, e.g., $(CAG)_n$, $(GC)_n$, usually less than 100 base pairs in length, but this often varies among individuals both within and among populations. Microsatellite DNA has been shown to be abundant and highly polymorphic in eukaryotic genomes (Tautz, 1989; Rafalski and Tingey, 1993). The DNA fragments are separated by gel electrophoresis, and different banding patterns among isolates constitute polymorphisms (Rafalski and Tingey, 1993). ISSR-primed PCR, first used as a technique to investigate genetic variation in plants (Zietkiewicz *et al.*, 1994), was then shown to generate polymorphic DNA markers for a range of fungi (Hantula *et al.*, 1996). ISSR-primed PCR has advantages and disadvantages similar to those of RAPD-PCR, but greater reproducibility of markers than those generated by RAPDs (Godwin *et al.*, 1997; Meng and Chen, 2001).

Polymorphisms obtained by ISSR-primed PCR have been used as genetic markers for the investigation of variation among individuals and populations of *Colletotrichum* spp. (Förster and Adaskaveg, 1999; Freeman *et al.*, 2001; Afanador-Kafuri *et al.*, 2003; Abang *et al.*, 2005)

as well as other plant pathogenic fungi (Tautz, 1989; Pejic *et al.*, 1998; Arenal *et al.* 1999; Stummer *et al.*, 2000; Meng and Chen, 2001; Menzies *et al.*, 2003).

Amplified fragment length polymorphism markers

The generation of genetic markers using the amplified fragment length polymorphism (AFLP) technique was originally developed by Vos *et al.* (1995) for detecting genetic diversity in plants. The technique involves digesting genomic DNA with selected restriction enzymes, then adapter molecules are ligated to the ends of the fragments generated by the restriction digest. The fragments are amplified using PCR with primers based on the adapter sequences with the addition of an arbitrary extension at the 3′ end. The number of fragments amplified depends on the combinations of primers and the length of the 3′ extension. Typically 50-70 fragments can be visualised on a denaturing polyacrylamide gel following a single PCR amplification (Brown, 1996; Sreenivasaprasad and Mills, 1998). AFLP analysis allows a large number of independent loci to be detected following a single PCR amplification and results are generally highly reproducible, which is an advantage over RAPD-PCR (Brown, 1996; Majer *et al.*, 1996). However, AFLP analysis is technically more difficult, more costly and the process takes longer than RAPD-and ISSR-primed PCR (Brown, 1996; Mills *et al.*, 1998).

AFLPs have been used to determine phylogentic relationships (Menzies *et al.*, 2003) and to detect genetic diversity (Majer *et al.*, 1996; Meng and Chen, 2001; Ansari *et al.*, 2004) of plant pathogenic fungi.

DNA sequence analysis

In contrast to the PCR methods discussed above, analysis of DNA sequence data compares the nucleic acid sequences of isolates to investigate genetic diversity. Dispersed throughout the fungal genome, there are regions of DNA that occur repeatedly in clusters and in high copy numbers, e.g., the nuclear rRNA genes (rDNA). This cluster of genes consists of DNA that codes for the ribosomal subunits, the internally transcribed spacer (ITS) regions and the non-transcribed inter-genic spacer (IGS) regions. The ITS regions are generally conserved, showing little variation among strains of a species within a genus. The ITS regions can be amplified, with a universal primer, using the PCR technique. The regions of DNA can then be sequenced and species-specific primers developed. The IGS regions have greater divergence among species and analysis of these regions is useful for strain identification within a species (Henson and French, 1993; Annamalai *et al.*, 1995; Bridge and Arora, 1998).

Primers based on sequences of the ITS regions of rDNA have been developed to identify several species of plant pathogenic fungi, including *Fusarium* spp. (Schilling *et al.*, 1996; Moricca *et al.*, 1998) and *R. solani* (Salazar *et al.*, 2000).

As discussed previously (Section 2.4.2), Mills *et al.* (1994) and Sreenivasaprasad *et al.* (1994) developed taxon-specific primers based on the ITS regions 1 and 2 of the rDNA gene cluster. PCR with these primers is used to differentiate isolates of *C. acutatum*, *C. gloeosporioides* and other *Colletotrichum* spp. (Sreenivasaprasad *et al.*, 1996; Mills *et al.*, 1998; Förster and Adaskaveg, 1999; Freeman *et al.*, 2001). Isolates of *Colletotrichum* spp. obtained from almond in California and Israel have been identified as *C. acutatum* using PCR with these primers, however, isolates of *C. acutatum* obtained from almond in Australia have not been examined using this method.

RFLP analyses of mitochondrial and ribosomal DNA were the predominant methods used to investigate species and population differences then, as technology advanced, analysis of genome sequences began and is now used frequently (Lu *et al.*, 2004) for the examination of phylogenetic relationships among fungi (Guerber *et al.*, 2003; Kohn, 2004). Portions of the genome used commonly in sequence analyses include the ITS regions and D1/D2 domain of rDNA (Johnston and Jones, 1997; Freeman *et al.*, 2001; Talhinhas *et al.*, 2002; Afanador-Kafuri *et al.*, 2003; Martínez-Culebras *et al.*, 2003; Lu *et al.*, 2004) and, increasingly, conserved protein genes, e.g., β-tubulin 2 gene and glyceraldehyde-3-phosphate dehydrogenase gene (Weeds *et al.*, 2000; Talhinhas *et al.*, 2002; Guerber *et al.*, 2003; Lu *et al.*, 2004).

2.5.3 Molecular analysis of *C. acutatum*

C. acutatum is considered a group species and, to reflect this, it has been referred to as *C. acutatum sensu lato* (broad sense) (Johnston and Jones, 1997; Lardner *et al.*, 1999). The isolates belonging to *C. acutatum sensu* lato were further divided into four distinct *C. acutatum sensu* stricto (narrow sense) groups based on morphology and sequence analysis of the rDNA D2 domain (Johnston and Jones, 1997). One of these four groups, group A (Lardner *et al.*, 1999) is referred to as *C. acutatum sensu* Simmonds (Freeman *et al.*, 2001), as the morphology of this group is typical of *C. acutatum* as described by Simmonds in 1965. Lardner *et al.* (1999) found that RAPD-PCR data supported these groupings. Furthermore, Freeman *et al.* (2001) examined isolates conforming to *C. acutatum sensu* Simmonds, obtained from diverse hosts and geographical regions, using various molecular approaches. They found evidence of genetic variability amongst the isolates and were able to identify four subgroups within *C. acutatum sensu* Simmonds: subgroup I included isolates of the pink subpopulation from almond in California, and apple, peach and pecan from the USA;

subgroup II included isolates from anemone, olive and strawberry; subgroup III contained isolates from almond in Israel and strawberry, subgroup IV consisted of a single isolate from anemone in the Netherlands. This evidence of genetic variation (Freeman *et al.*, 2001) supports the suggestion that, at some stage, sexual reproduction may have contributed to the development of the genetic diversity among isolates of *C. acutatum sensu* Simmonds (Freeman *et al.*, 2001; Guerber *et al.*, 2003).

Within populations of *C. acutatum* obtained from different hosts, there has been evidence for both heterogeneity and homogeneity among isolates from a single host. Based on various characteristics, e.g., genetic markers, morphology, pathogenicity, VCG analysis, nutrient assimilation and enzyme activity, diversity has been observed among isolates of *C. acutatum* from single hosts, e.g., strawberry (Smith and Black, 1990; Sreenivasaprasad *et al.*, 1992; Denoyes and Baudry, 1995; Freeman and Rodriguez, 1995; Buddie *et al.*, 1999; García-Muñoz *et al.*, 2000), anenome (Freeman *et al.*, 2000b) and almond (Förster and Adaskaveg, 1999). In contrast, based on various approaches, isolates of *C. acutatum* obtained from a single host have been shown to be identical, e.g., almond (Freeman *et al.*, 2000a), tamarillo (Afanador-Kafuri *et al.*, 2003) and strawberry (Freeman *et al.*, 1998; Freeman *et al.*, 2000a), suggestive of clonal populations and host specificity. However, there is considerable opportunity for further investigation of the diversity among isolates of *C. acutatum sensu* Simmonds and subgroups assigned within.

Förster and Adaskaveg (1999) identified two distinct subpopulations among 500 isolates of *C. acutatum* obtained from almond in California. These differed with respect to morphology, growth rate at a range of temperatures, sensitivity to benomyl, pathogenicity in laboratory assays, and DNA banding patterns using RAPD- and ISSR-primed PCR, and are referred to as the pink and grey subpopulations. Based on various molecular approaches, *viz.*, RAPD- and

ISSR-PCR, and RFLP analysis of rDNA, isolates within each subpopulation were shown to be identical (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a), which suggested that each population was clonal.

In contrast, there was no subspecific variation among isolates of the *Colletotrichum* sp. obtained from almond in Israel with respect to fungicide sensitivity (Shabi *et al.* 1994), VCG analysis (Katan and Shabi, 1996), pathogenicity (Freeman *et al.*, 1996), DNA fingerprints using RAPD- and ISSR-PCR (Förster and Adaskaveg, 1999), ISSR-PCR and A + T-rich-DNA analysis (Freeman *et al.*, 1996; Freeman *et al.*, 2000a), and sequence analysis of the ITS region (Freeman *et al.*, 2000a). These results suggested that the population of *C. acutatum* from almond in Israel was clonal (Freeman *et al.*, 1996; Freeman *et al.*, 2000a). Isolates of *Colletotrichum* sp. from almond in Israel have been shown to be genetically distinct from the pink subpopulation of *C. acutatum* from almond in California (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a), yet genetically similar to the grey subpopulation from almond in California (Förster and Adaskaveg, 1999).

B. Hall (pers. comm., 2000), of the South Australian Research and Development Institute, observed morphological variation among several isolates of *C. acutatum* from almond in Australia, which was supported by Colmagro (unpublished data) in a study of 26 isolates of *C. acutatum* from almond in Australia. However, genetic variation has yet to be investigated for isolates of *C. acutatum* from almond in Australia, nor have isolates been compared to isolates of *C. acutatum* from California or *Colletotrichum* sp. from Israel. It is important to determine whether the genotype(s) in Australia differ from those in California and Israel, as different genotypes may show variation in characters such as pathogencitiy and fungicide responses. In addition, this may provide information regarding the origin of almond anthracnose in Australia.

2.6 Epidemiology of anthracnose on almond

The development of an epidemic of plant disease depends on several factors relating to the host, the pathogen, the environment and their interaction (Agrios, 1997). The epidemiology of diseases caused by *Colletotrichum* spp. on a variety of hosts has been studied, including citrus (Denham and Waller, 1981; Timmer and Zitko, 1996), mango (Fitzell and Peak, 1984; Dodd *et al.*, 1991), rubber (Wastie, 1972a; 1972b) and cocoa (Chandra Mohanan *et al.*, 1989). The development of anthracnose on almond appears to be favoured by warm (20-25°C), wet conditions (Adaskaveg and Förster, 2000), however, the optimal conditions for disease occurrence and development on almond have not been thoroughly investigated. Factors contributing to the development of an epidemic will be discussed in relation to the host, the pathogen and the environment.

2.6.1 The disease cycle

In the literature reviewed to date, a disease cycle for anthracnose caused by *C. acutatum* has been published for blueberry only (Wharton and Diéguez-Uribeondo, 2004), however, there are many features of the disease cycle which appear to be common to all *Colletotrichum* spp. Conidia of *Colletotrichum* spp. are dispersed primarily by rain splash (Bailey *et al.*, 1992; Waller, 1992) and occasionally by wind (Nicholson and Moraes, 1980). Waller (1972) found that conidia of *C. gloeosporioides* were spread within and between coffee trees by human activity, but it has not been determined if other agents, e.g., pruning tools, insects, contribute to the spread of inoculum for anthracnose of almond.

The usual stages of infection for *C. acutatum* are as follows: in warm, wet conditions, the conidia germinate on susceptible tissues, producing melanised appressoria. An infection peg grows from the underside of an appressorium, penetrating the plant cell wall and the hyphae

invade the underlying tissue, where the fungus survives on living tissue for a variable period of time. Following this period, the underlying tissue collapses to produce the characteristic sunken lesions, and the fungus becomes necrotrophic (Bailey *et al.*, 1992). Subcuticular acervuli develop within the lesions and produce conidia, which burst through the cuticle to be dispersed, and new infection of susceptible tissues takes place under suitable conditions.

Mycelium and appressoria on infected host tissue are considered to be the main overwintering structures of *C. acutatum* (Waller, 1992). However, not all conidia of *C. acutatum* follow these usual stages of development (for review see Wharton and Diéguez-Uribeondo, 2004), e.g., some conidia germinate to produce secondary conidia without producing a germ tube and appressorium, germ-tubes from different conidia undergo anastomosis. Further detail of these stages is beyond the scope of this review, but it is important to note that conidial germination and germ tube differentiation have been shown to occur within a few hours following contact of conidia with the plant surface (Zulfiqar *et al.*, 1996; Curry *et al.*, 2002; Diéguez-Uribeondo *et al.*, 2003; Wharton and Schilder, 2003), which suggests that infection by *C. acutatum* can occur rapidly in suitable conditions (Wharton and Diéguez-Uribeondo, 2004).

Many *Colletotrichum* spp., including *C. acutatum*, can live as saprophytes and this may provide a source of inoculum throughout the season (Waller, 1992). Mummified fruit are considered to be the main source of primary inoculum for anthracnose on almond in California (Förster and Adaskaveg, 1999). Apparently healthy tissue can also be infected, and the fungus can remain dormant until conditions are suitable for growth. This ability to cause latent infection can make early detection and disease diagnosis difficult.

It has been reported that blueberry and almond fruit are susceptible to infection by *Colletotrichum* sp. at all stages of development (Hartung *et al.*, 1981; Adaskaveg and Förster,

2000), but Shabi and Katan (1983) found that new infection of almond fruit occurred only in spring in Israel. This has not been investigated for almond fruit in Australia.

There are several aspects of the disease cycle of anthracnose on almond in Australia that are poorly understood and warrant further investigation. The main site(s) of overwintering which are the potential sources of primary and secondary inoculum have not been thoroughly investigated.

2.6.2 Symptoms

Anthracnose symptoms have been observed on all above-ground plant parts of almond including blossom, fruit, leaves, peduncles, spurs and branches (Adaskaveg and Förster, 2000; McMichael, 2000). In California, infection early in the growing season causes blossom blight (Adaskaveg *et al.*, 1998), which has not been observed in Australia, although the fungus has been recovered from asymptomatic blossoms (McMichael, 2000). Pinkish to orange, circular lesions appear on young infected fruit (Fig 2.2), which generally die, shrivel and typically remain on the tree as mummified fruit throughout the season (Adaskaveg *et al.*, 1998) (Fig 2.3). In Australia, McMichael (2000) reported that orange-tinged, dead fruit, evident within 4 weeks of petal fall, were the earliest reliable indicator of anthracnose. Fruit aborted due to other reasons generally are buff-coloured and fall from the tree (McMichael, 2000). Profuse exudate of tan-coloured gum is characteristic of infection on older fruit.

Infected leaves initially show water-soaked lesions, yellowing and wilting, then marginal necrosis (Adaskaveg *et al.*, 1998) (Fig 2.2). Leaves may fall to the ground, or may remain on the tree if they are attached to an infected twig, spur or branch (Adaskaveg *et al.*, 1998). Infected twigs, spurs and branches up to 5 cm in diameter die back, resulting in the loss of fruitful wood and tree decline (McMichael, 2000). Adaskaveg and Förster (2000) suggested

that the death of woody tissues was associated with the production of phytotoxic substances rather than direct colonisation by the fungus, and stated that research is continuing in an attempt to confirm this hypothesis.

2.6.3 Cultivar susceptibility

There is some evidence that almond cultivars vary in susceptibility to infection by *C. acutatum*. It appears from field observations in California, carried out by Adaskaveg and Förster (2000), that most almond cultivars are affected by anthracnose, although they appear to differ in susceptibility. Anthracnose has been confirmed on cultivars commonly grown in Australia, including Fritz, Carmel, Price, Ne Plus Ultra, Peerless, Baxendale, Chellaston, Mission and Nonpareil (McMichael, 2000). Based primarily on field observations, Nonpareil appears to be one of the least affected cultivars both in Australia and California (Hendricks, 1998; Adaskaveg and Förster, 2000; McMichael, 2000; Diéguez-Uribeondo *et al.*, 2002), but further data are required to substantiate this, in particular for the cultivars grown in Australia. Several studies were conducted in Western Europe in the 1930s and 1970s to assess the susceptibility of some European almond cultivars to anthracnose (Striem *et al.*, 1989), but it is difficult to relate these findings to Australian conditions as these varieties are not grown commercially either in Australia or California.

2.6.4 Main source(s) of inoculum

It is important to know on which plant tissues *C. acutatum* survives throughout the year, as this may have implications for disease management. Mummified almonds are considered to be the main source of primary inoculum (Förster and Adaskaveg, 1999), and anecdotal observations suggest that *C. acutatum* can survive for several seasons in mummified almond fruit. *C. acutatum* was isolated also from infected blossom, peduncles, spurs, twigs and

leaves collected during the growing season in California (Förster and Adaskaveg, 1999), suggesting that these plant tissues all have a role as sources of primary and secondary inoculum during the growing season. It is not known if *C. acutatum* survives in other plant parts, e.g., peduncles or woody tissue, during the autumn and winter months, which may be sources of primary inoculum when conditions become suitable in spring. Other studies on fungal diseases of perennial fruit tree crops, e.g., peach, have found that peduncles were important overwintering sites and provided significant sources of inoculum (Kable, 1965). Kable (1965) also found that blighted and apparently healthy woody tissue provided sources of inoculum for *M. fructicola* in peach in Australia. Stensvand *et al.* (2001) found that mummified fruit and previously infected fruit spurs from sweet cherry were able to produce conidia of *Monilinia laxa* for the entire growing season, but mummified fruit produced more conidia than did fruit spurs, and conidia were produced for 2-3 years after initial infection. This study highlights the important role of mummified fruit and associated woody tissue as the predominant sources of primary inoculum for fungal diseases of sweet cherry.

The amount of inoculum available affects disease development (Wastie, 1972b; Timmer and Zitko, 1996). Identifying the main inoculum sources and quantifying the amount of inoculum available may assist with disease forecasting and the development of effective control measures, such as the removal of inoculum sources and the application of fungicide treatments at the time of greatest inoculum production (Waller, 1972). In addition, both theoretically (Van der Plank, 1963) and in practice, the use of anti-sporulant chemicals during dormancy in winter on stone fruits (Kable, 1970; Wicks, 1981) and on almonds (Ramsdell and Ogawa, 1973) was found to decrease the amount of primary inoculum of *Monilinia* spp. before the growing season, which led to subsequent decreases in disease.

Fig 2.2. Anthracnose symptoms on almond. **A.** Tan-coloured lesions on infected young fruit and water-soaked lesions on leaves. Asymptomatic leaf on left (arrow) and asymptomatic fruit shown right (arrow). **B.** Gum exuding from infected fruit on left (arrow).

Fig 2.3. Anthracnose symptoms on almond, cv. Price, caused by *Colletotrichum acutatum*. Mummified fruit remain attached to the twigs and twig dieback is evident. Photo taken in March 2000. Leaf symptoms in this picture are not due to anthracnose.

2.6.5 Pathogenicity

Pathogenic variation has been reported among isolates of *Colletotrichum* spp., including *C. acutatum* from strawberry (Denoyes and Baudry, 1995; Denoyes-Rothan *et al.*, 2003) and *C. gloeosporioides* from citrus (Denham and Waller, 1981), avocado (Freeman *et al.*, 1996) and *Stylosanthes* spp. (Davis *et al.*, 1987). Förster and Adaskaveg (1999) assessed the pathogenicity of selected isolates of *C. acutatum* from almond in California on the cultivar Butte and found that isolates from the pink subpopulation caused larger lesions on detached fruit than did grey isolates. However, these findings did not translate to the field, as both subgroups caused damage that was rated as severe. It is not known whether pathotypes of *C. acutatum* from almond in Australia exist, as the pathogenicity of isolates of *C. acutatum* from

almond in Australia has not been determined. Nor is it known, if there are differences in pathogenicity among isolates of *Colletotrichum* sp. from almond in Australia, California and Israel. If isolates of *Colletotrichum* sp. from almond in California and Israel were pathogenic to almond cultivars in Australia, then it is possible that, if introduced, these could lead to disease in Australian almond orchards. This may be a problem if opposite mating types or genotyps more pathogenic than those already in Australia were introduced.

Understanding pathogenic variation among isolates is necessary for the breeding and selection of almond cultivars resistant to infection by *C. acutatum*. As previously mentioned (Section 2.6.3), there is evidence that Nonpareil appears to be less affected by anthracnose than several other cultivars, including Price and Carmel, however, it is unclear if this perceived lack of susceptibility is due to genetic resistance or other factors, e.g., tree architecture or inoculum potential. First, it needs to be determined if different pathotypes of *C. acutatum* from almond in Australia exist, then various cultivars can be assessed for susceptibility using selected pathotypes.

2.6.6 Environmental factors

Environmental factors, such as moisture, temperature and light, can have direct effects on the host, the pathogen and their interaction, thus influence the incidence and development of disease (Colhoun, 1973; Agrios, 1997). The effects of environmental factors on disease development have been documented for many plant pathogens, e.g., *Phytophthora* spp. (Grove and Boal, 1991), *Puccinia* spp. (Hovmøller, 2001; Pfender, 2001), *Monilinia fructicola* (Koball *et al.*, 1997), *Venturia inaequalis* (Penrose and Nicol, 1996), *Uncinula necator* (Jailloux *et al.*, 1999), *Uromyces* sp. (Joseph and Hering, 1997), *Fusarium* sp.

(Swanson and van Gundy, 1985) and *Colletotrichum* spp. (Wastie, 1972a; Denham and Waller, 1981; Dodd *et al.*, 1992; King *et al.*, 1997; Fernando *et al.*, 2000).

All phases of the cycle of disease caused by *Colletotrichum* spp. that occur on the external surface of the host tissue are dependent on water. Sporulation requires high relative humidity, whereas spore release and dispersal require free water. Spore germination and infection can occur with less than 4 hours of wetness at optimal temperatures (Waller, 1992). The critical importance of moisture for the disease cycle has been demonstrated for *C. gloeosporioides* (Wastie, 1972a; Wastie, 1972b; Fitzell and Peak, 1984; Chandra Mohanan *et al.*, 1989; Chakraborty *et al.*, 1990) and for *C. acutatum* on strawberry (Wilson *et al.*, 1990; Fernando *et al.*, 2000) and rubber (Wilson *et al.*, 1990; Fernando *et al.*, 2000). In a brief report, Diéguez-Uribeondo *et al.* (2002) found that longer wetness duration was needed for infection to occur on leaves of almond than on blossom at 10°C following *in vitro* inoculation studies with *C. acutatum*, yet at 15°C, the reverse was true. Whereas the exact water requirements for the various stages of the disease cycle of anthracnose on almond have not yet been determined, in the field, it has been observed that major outbreaks of anthracnose have followed extended periods of rain during spring in California (Adaskaveg and Förster, 2000) and Israel (Shabi and Katan, 1983).

With respect to *Colletotrichum* spp., temperature has been shown to affect sporulation and the duration of latent infection (King *et al.*, 1997), spore germination, appressorium formation (Miehle and Lukezic, 1972) and mycelial growth rate (Wastie, 1972a; Wilson *et al.*, 1990; Förster and Adaskaveg, 1999), but it appears that germination can occur over a wide temperature range (Wastie, 1972a). Diéguez-Uribeondo *et al.* (2002) found that, for both almond leaves and blossom, 10°C was a critical low threshold for disease development following inoculation *in vitro* with *C. acutatum*. Major outbreaks of anthracnose occurred in

California in the late 1990s following spring rains when warm (20-25°C) (Adaskaveg and Förster, 2000).

In contrast to moisture and, to a lesser extent, temperature, it does not appear as though the life cycle of *C. acutatum* has specific light requirements, although exposure to sunlight may be detrimental to particular phases of the life cycle of the fungus. Wastie (1972a) found that exposure to sunlight led to rapid inactivation of conidia of *C. gloeosporioides* from rubber. Almond trees less than 5 years old do not appear to be affected by anthracnose and it has been speculated that the open canopy of these young trees results in greater air flow than older trees and thus dry conditions which are not conducive for infection by *C. acutatum* (McMichael, 2000). Although this is a reasonable hypothesis, it could also be that more sunlight penetrates the open canopy of young trees and promotes desiccation of conidia of *C. acutatum*. Also, the distance between tree canopies is greater in younger than in older orchards, which may reduce the efficiency of spore dispersal.

Other factors, such as tree spacing, canopy density, pruning methods and types of irrigation are all cultural practices that may affect the disease cycle (Waller, 1992), due to their effects on the microclimate within the orchard and canopy, respectively. The effects of environmental factors, such as moisture and temperature, and cultural practices on the disease cycle of anthracnose and on the life cycle of *C. acutatum* from almond in Australia have not yet been adequately investigated *in vitro* or *in planta*.

2.6.7 Disease management

Fungicide applications and cultural practice are the two main approaches used to control anthracnose (Waller, 1992), however, the efficacy of these strategies is variable (Heintz, 1997; Adaskaveg *et al.*, 1998; McMichael, 2000).

In California, a range of fungicides, with various modes of action, is recommended, with the aim of preventing growth, reproduction and survival of the fungus. Likewise, alternating chemicals with different modes of action to delay fungicide resistance is recommended (Adaskaveg *et al.*, 1998; Hendricks, 1998). In Australia, fungicide efficacy trials conducted in 1998/99 and 2000 by McMichael (2000) of Scholefield Robinson Horticultural Consulting Services, showed that early (pink bud stage) fungicide applications were essential for disease control. McMichael (2000) found that all new tissue must be continuously protected, with spray intervals not exceeding 12 days from pink bud until the end of the season, particularly if wet and warm conditions occurred. None of the fungicide treatments eradicated the disease, which concurs with the findings of Adaskaveg and Hartin (1997), and control varied among the trial locations, namely Willunga, Angle Vale and Renmark (South Australia), and Nangiloc and Lindsay Point (Victoria) (McMichael, 2000). However, there were several limitations to these trials, including variation in experimental design and different disease incidence and severity among field sites. Notwithstanding the limitations, the recommended fungicides to apply for management anthracnose in Australia are Bravo® (chlorothalonil) and Captan (McMichael, 2000).

Mummified fruit represent the main source of inoculum, according to Shabi and Katan (1983) and Adaskaveg and Förster (2000), and the removal of mummified fruit and infected branches has been recommended to reduce potential inoculum and improve air circulation (Shabi and Katan, 1983; Adaskaveg *et al.*, 1998). The efficacy of this method has not been assessed either in California or in Australia.

Another possible management strategy is breeding and selection of almond cultivars with resistance to or tolerance of infection by *C. acutatum* (Waller, 1992). However, there is no evidence to suggest that any almond cultivar is resistant to anthracnose, as was discussed in

Section 2.6.3. Information about the relative susceptibility of cultivars would be useful for the almond breeding program, based at the University of Adelaide, South Australia, for the selection of cultivars resistant to or tolerant of infection by *C. acutatum*.

Further work is needed to improve disease control strategies and to understand why measures currently applied with the aim of controlling anthracnose gave variable results in the field.

2.6.8 Disease development models

Models to forecast an epidemic have been developed for many diseases of horticultural crops, e.g., late blight of potato (Krause *et al.*, 1975), brown spot of pear (Llorente *et al.*, 2000), fire blight of apple (Van der Zwet *et al.*, 1994), apple scab (Buhler *et al.*, 1993; Butt and Xu, 1994) and downy mildew of grapevine (Magarey and Western, 1997; Madden *et al.*, 2000). Models may vary from simple to complex and can be used to predict whether the disease is expected (positive forecast) or is not expected (negative forecast). Simple models have been developed empirically, by correlating weather data and qualitative records of disease. More complex models use meteorological data, crop weather data, data from laboratory and controlled experiments and quantitative measures of disease (Bourke, 1970; Fitzell *et al.*, 1984).

'Meteopathological forecasting' aims to predict the onset and development of disease over time, and is based on the relationship between the weather conditions and disease development (Bourke, 1970). The intended benefit of forecasting is generally to reduce the number of chemical sprays and improve the timing of such treatments to minimise loss to disease. There are several reports of a decrease in the number of fungicide applications in response to the use of a forecasting model, for example, brown spot of pears (Montesinos and Vilardell, 1992), downy mildew of lettuce (Scherm *et al.*, 1995) and post-bloom fruit drop in

citrus (Timmer and Zitko, 1996). However, Bourke (1972) suggested that four requirements must be met if a disease forecasting model, based on weather conditions, is to be of any use to a grower. These are: the disease causes economically significant damage; the disease varies throughout and between seasons due to weather factors; cost effective control measures are available and data from laboratory and glasshouse experiments are available, which outline the nature of the dependence of the disease on weather factors.

As discussed previously, anthracnose has caused significant economic damage in Australia, with growers having to spray early and regularly throughout the growing season, with variable results. Further research is needed to understand pathogen variation and the conditions that are most conducive for disease development. This will assist with the development of effective and reliable control measures.

In order to monitor disease development, it is essential to have a quantitative measure of the disease. This can be done in a variety of ways, including the measurement of the number of spores (Waller, 1972a; Wastie, 1972b), the number of infected plant parts (Timmer and Zitko, 1996), disease incidence (Denham and Waller, 1981), visual assessment of disease severity (3Wastie, 1972a ; Chakraborty *et al.*, 1990) and yield (Timmer and Zitko, 1996). Spore measurements may be useful for those pathogens that have spores that are easy to trap, identify and measure, however, this process may be difficult and time consuming for *C. acutatum*, which has relatively nondescript conidia.

Wastie (1972a, 1972b) measured combinations of environmental factors that were thought to influence secondary leaf fall caused by *C. gloeosporioides* in rubber crops. Measurements were made of temperature, relative humidity, light and free water, and the effect of these factors on infection, spore germination, viability and production. Wastie (1972a) concluded that the development of a disease forecasting system was not possible as the interaction of

factors was too complex. In contrast, Timmer and Zitko (1996) developed a predictive model for post-bloom fruit drop in citrus caused by *C. acutatum*. Their linear regression model is based on the relationship between the total number of diseased flowers and the rainfall (mm) for the past 5 days. The use of this model led to a decrease in the number of fungicide applications, an increase in fruit count, and an overall improvement in disease control. However, this model was not useful on days when only light rain fell or when there was a lack of inoculum.

In general, several environmental factors interact to influence the development of an epidemic, so research programs should aim to study a combination of these factors (Fitzell *et al.*, 1984; Wilson *et al.*, 1990) in both laboratory, glasshouse and field experiments to gain the greatest understanding of the development of a disease.

2.7 Summary

Anthracnose is found on almonds in California and Israel, and has recently been discovered in almond crops in Australia. Control measures vary in effectiveness and little is known about the morphological, pathogenic and genetic diversity of the pathogen, *C. acutatum*, in Australian conditions. The development of an epidemic of anthracnose is believed to be favoured by warm, wet conditions, however, this has yet to be investigated thoroughly in Australia, California or Israel. Mummified almond fruit are thought to be the main source of primary inoculum and the overwintering site for *C. acutatum*, however, this and the role of other plant tissues as inoculum sources and overwintering sites have not been investigated in Australia. Such information will assist growers in the management of anthracnose.

2.8 Research objectives

The objectives of this research were to;

1 characterise isolates of *C. acutatum* from almond in Australia with respect to morphological and cultural characteristics, genetic diversity and pathogenicity, and compare these isolates with those of *Colletotrichum* sp. from almond in California and Israel,

2 investigate several epidemiological aspects of *C. acutatum* on almond in the field, including monitoring anthracnose progression, examining the environmental conditions most conducive for disease and determining which plant tissues are potential sources of primary inoculum and the main overwintering sites.

The information gained from this study will contribute knowledge about the pathogen and the disease, which will assist in improving control measures of anthracnose for the Australian almond industry.

Chapter 3 General materials and methods

3.1 Collection and establishment of isolates of *C. acutatum*

Almond plant material suspected of having anthracnose was collected from all major almond growing regions of Australia. An initial collection was carried out in the Southern Vales and Northern Adelaide Plains regions close to Adelaide, South Australia (Fig 2.1, Section 2.1.1) in July 2001. A survey was conducted in October 2001, in which material was collected from orchards in the Riverland region of South Australia, and the Lindsay Point and the Sunraysia regions of Victoria (Table 3.1), from the trees that growers considered were affected by anthracnose. Where possible, dead almond fruit attached to peduncles was collected, aiming at five mummified fruit per aspect (north, south, east and west) per tree per cultivar. Where this was not possible, any fruit that may have been affected by anthracnose was collected. Plant material was stored on ice for transport to the laboratory and then stored in a cool room (4°C). Material suspected of having anthracnose was also provided by several almond growers.

Plant material was surface sterilised with a 25% solution of commercial bleach (White King®, sodium hypochlorite 42 g/L) for 3 min, washed twice in sterile distilled water (SDW) for 2 min each time and excess water was removed by blotting with a sterile tissue.

Plant material was placed into plastic trays (42 x 26 x 5 cm), which were lined with two layers of sterile Chux® cloth over two layers of sterile paper towel and moistened with 200 mL of SDW. The trays were sealed in a plastic bag, incubated in the laboratory at room temperature (approx 22°C) in natural daylight and checked regularly for mycelium and/or spore masses, which may indicate the presence of *C. acutatum*. Fungal material, putatively identified as *C. acutatum*, was transferred aseptically, using a needle or scalpel, onto Potato Dextrose Agar (PDA, Difco, USA) (39 g/L) acidified with lactic acid (LAPDA, 1 mL/L),

incubated at 25°C in darkness, and subcultured onto PDA until a pure culture was established. After incubation for approximately 2 weeks, 1 mL of sterile double distilled (dd) H_20 was pipetted onto the surface of the culture, which was rubbed with a sterile glass rod to dislodge the spores. One hundred µL of the spore suspension was pipetted onto the surface of 7.5 % tap water agar (TWA, Oxoid, No. 3) in a 90-mm diameter Petri dish, and spread with a sterile bent glass rod. The plates were incubated in the laboratory at room temperature in diurnal light for 12-24 h. With the aid of a binocular microscope, single germinating spores were excised using a sterile scalpel, transferred to PDA and incubated in the dark at 25°C. Single spore-derived cultures were prepared for all isolates used in this study.

Table 3.1. The almond grower, area and cultivar from which plant material was obtained, with date of collection.

Grower	Area	Cultivar	Date
M. and D. Clements	Langhorne Creek, South Australia	Johnstone, Bruce	July 2001
G. Keene	Munno Para Downs, South Australia	Fritz, Chellaston, Nonpareil, Price, Somerton	August 2001
L. Hunt	Willunga, South Australia	Nonpareil, Carmel, Fritz, Price	July 2001, throughout 2002
Jubilee Almonds	Waikerie, South Australia	Ne Plus Ultra, Price, Nonpareil, Carmel	October 2001
Simarloo Almonds	Lyrup, South Australia	Ne Plus Ultra	October 2001
P. Martin	Lindsay Point, South Australia	Ne Plus Ultra, Price	October 2001
A. Lacey	Lindsay Point, South Australia	Price, Johnstone, Mission, Fritz, Peerless	October 2001
P. Thompson	Renmark, South Australia	Price, Carmel, Nonpareil	October 2001
Mildura Agricultural Contractors	Red Cliffs, Victoria	Ne Plus Ultra, Fritz	October 2001
I. Keen	Nangiloc and Colignan, Victoria	Price, Carmel, Mission, Price, Ne Plus Ultra	October 2001
L. Phillips	Red Cliffs, Victoria	Ne Plus Ultra, Fritz	October 2001
B. Hengsen	Red Cliffs, Victoria	Fritz and various unknown	October 2001
D. Taplin	Red Cliffs, Victoria	Ne Plus Ultra, Nonpareil	October 2001
Msrs. Whitelegg	Red Cliffs, Victoria	Ne Plus Ultra	October 2001

3.2 Fungal cultures

The isolates of *C. acutatum* and other fungi used in this study are listed in Table 3.2. These include isolates of *C. acutatum* obtained from almond and other host plants, which were provided by colleagues in Australia, Israel and California, USA.

Fungal cultures were routinely maintained on PDA in the dark at 25°C. For long-term storage, approximately 10 mycelial plugs (5 mm diameter) were excised from the growing margin of a colony, transferred to a McCartney bottle containing 20 mL of sterile dd$H_2$0 and stored at 4°C. In addition, 750 μL of conidial suspension were added to a 1.5 mL Eppendorf tube containing 150 μL of sterile glycerol, vortexed for 5 sec and stored at -70°C.

Table 3.2. The origin of the fungal isolates used in this study.

Isolate code	Species	Year of isolation	Site	Host
W1	*C. acutatum*	2000	Willunga, SA	leaf, *P. dulcis*
W2	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
W4p	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
W4m	*C. acutatum*	2000	Willunga, SA	mummified fruit, *P. dulcis*
W5p	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
W5L	*C. acutatum*	2000	Willunga, SA	leaf, *P. dulcis*
W5m	*C. acutatum*	2000	Willunga, SA	mummified fruit, *P. dulcis*
W6	*C. acutatum*	2000	Willunga, SA	mummified fruit, *P. dulcis*
W7	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
CSL-1689*	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
W16	*C. acutatum*	2000	Willunga, SA	peduncle, *P. dulcis*
W17	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W26	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W29	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W31	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W32	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W33	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*

Isolate code	Species	Year of isolation	Site	Host
W34	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W36	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W38	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W39	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W42	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W43	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W44	*C. acutatum*	2001	Willunga, SA	twig, *P. dulcis*
W45	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W47	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W55	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W64	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W65	*C. acutatum*	2001	Willunga, SA	twig, *P. dulcis*
W66	*C. acutatum*	2001	Willunga, SA	leaf, *P. dulcis*
W76	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W77	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W87	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W93	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W100	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W107	*C. acutatum*	2001	Willunga, SA	peduncle, *P. dulcis*
W117	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W122	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W128b	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W129	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W136	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W143	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W147	*C. acutatum*	2001	Willunga, SA	mummified fruit, *P. dulcis*
W153	*C. acutatum*	2002	Willunga, SA	peduncle, *P. dulcis*
W154	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W155	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W156	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W157	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W158	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W159	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W160	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W162	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W162a	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W163	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W164	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*

Isolate code	Species	Year of isolation	Site	Host
W165	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W166	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W167	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W168	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W169	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W169a	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W169b	*C. acutatum*	2002	Willunga, SA	leaf, *P. dulcis*
W170	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W171	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W172	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W173	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W174	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W176	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W177	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W178	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W179	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W180	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W181	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W183	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W184	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W184b	*C. acutatum*	2002	Willunga, SA.	bark, *P. dulcis*
W185	*C. acutatum*	2002	Willunga, SA	bark, *P. dulcis*
W186	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W188	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
W189	*C. acutatum*	2002	Willunga, SA	peduncle, *P. dulcis*
W190	*C. acutatum*	2002	Willunga, SA	mummified fruit, *P. dulcis*
MPD1	*C. acutatum*	2001	Munno Para Downs, SA	mummified fruit, *P. dulcis*
MPD2	*C. acutatum*	2001	Munno Para Downs, SA	mummified fruit, *P. dulcis*
MPD3	*C. acutatum*	2001	Munno Para Downs, SA	mummified fruit, *P. dulcis*
MPD4	*C. acutatum*	2001	Munno Para Downs, SA	peduncle, *P. dulcis*
MPD5	*C. acutatum*	2001	Munno Para Downs, SA	mummified fruit, *P. dulcis*
CSL-1691*	*C. acutatum*	2001	Munno Para Downs, SA	mummified fruit, *P. dulcis*
MPD7	*C. acutatum*	2001	Munno Para Downs, SA	peduncle, *P. dulcis*
CSL-1690 [a]*	*C. acutatum*	2002	Angle Vale, SA	*P. dulcis*
S43[a]	*C. acutatum*	1998	Angle Vale, SA	*P. dulcis*
S44[a]	*C. acutatum*	1998	WA	fruit, *P. dulcis*
CSL-1314 [a]*	*C. acutatum*	1998	SA	fruit, *P. dulcis*
CSL-1687 [a]*	*C. acutatum*	1998	Willunga, SA	fruit, *P. dulcis*
CSL-1315 [a]*	*C. acutatum*	1997	Munno Para Downs, SA	fruit, *P. dulcis*
DAR-72407 [a]**	*C. acutatum*	1996	Willunga, SA	*P. dulcis*
S53[a]	*C. acutatum*	Unknown	The Adelaide Hills, SA	*Fragaria* sp.

Isolate code	Species	Year of isolation	Site	Host
CSL-1317[a] *	*C. acutatum*	1997	Unknown, Australia	*P. dulcis*
CSL-1318[a]*	*C. acutatum*	1997	Angle Vale, SA	*P. dulcis*
CSL-1298[a]*	*C. acutatum*	1998	South Australia	*Persea* sp.
S66[a]	*C. acutatum*	1998	Angle Vale, SA	*P. dulcis*
CSL-1688[a]*	*C. acutatum*	1998	Nangiloc, Vic	*P. dulcis*
S68[a]	*C. acutatum*	1998	Unknown, Australia	*P. dulcis*
ALM-NRB-3DK[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-BZR-8L[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-KSH-10[b]	*C, acutatum*	Unknown	Israel	*P. dulcis*
ALM-GZT-1F[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-GVA-6A[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-GOZ-42B[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-NA-3P[b]	*C. acutatum*	Unknown	Israel	*P. dulcis*
ALM-US-3[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
ALM-US-4[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
ALM-US-6B[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
ALM-US-9[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
ALM-US-11[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
ALM-US-12[b]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
US-1732[c]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
US-1813[c]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
US-1776[c]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
US-1796[c]	*C. acutatum*	Unknown	California, USA	*P. dulcis*
P1[d]	*C. acutatum*	2001	NSW	*Pistacia* sp.
NSW1[f]	*C. acutatum*	Unknown	Hunter Valley, NSW	*Olea europea*
SE37[a]	*C. acutatum*	2001	SA	*O. europea*
OL2[a]	*C. acutatum*	2002	SA	*O. europea*
OL3[a]	*C. acutatum*	2003	SA	*O. europea*
OL4[a]	*C. acutatum*	2003	SA	*O. europea*
DAR-75574[g]**	*C. acutatum*	Unknown	NSW	*Vitis vinifera*
Bb1[g]	*C. acutatum*	Unknown	NSW	*Vaccinium* sp.
Or1[e]	*C. gloeosporioides*	2002	SA	*Citrus* sp.
S54[a]	*C. gloeosporioides*	1989	Unknown, Australia	*Citrus* sp.
TUT-5954[b]	*C. gloeosporioides*	Unknown	Israel	*Fragaria* sp.
AVO-58	*C. gloeosporioides*	Unknown	Israel	*Persea* sp.
AVO-37-4B[b]	*C. gloeosporioides*	Unknown	Israel	*Persea* sp.
cc 40[a]	*Chondrostereum purpureum*	Unknown	Unknown	*Pyrus communis*
cc 147[a]	*Fusarium oxysporum*	1996	Unknown	*Vitis* sp.
cc 296[a]	*Verticillium sp.*	1980	Unknown	*P. dulcis*
cc 318[a]	*Colletotrichum coccodes*	2000	Unknown	*Solanum tuberosum*
B 58[a]	*Botrytis cinerea*	1997	Unknown	*P. dulcis*
1765[h]	*Botryosphaeria ribis*	1997	Vic	*V. vinifera*
M280[h]	*Eutypa lata*	1999	Vic	*V. vinifera*

South Australia (SA), Victoria (Vic), New South Wales (NSW), Western Australia (WA), The United States of America (USA)

[a] Isolate provided by Barbara Hall, SARDI, South Australia
[b] Isolate provided by Dr Stanley Freeman, The Volcani Center, Bet Dagan, Israel
[c] Isolate provided by Dr Helga Förster, University of California, Riverside, USA
[d] Isolate provided by Dr Gavin Ash, Charles Sturt University, NSW
[e] Isolate provided by Nancy Cunningham, SARDI, South Australia

f Isolate provided by Ru Huang, Charles Sturt University, NSW
g Isolate provided by Andrew Watson, Yanco Agricultural Institute, NSW
h Isolate provided by Dr Richard Lardner, the University of Adelaide, South Australia
* Isolate lodged with the Central Science Laboratory, Sand Hutton, York, UK
** Isolate lodged with the Agricultural Scientific Collection Unit, Orange, NSW, Australia

3.3 Identification of *C. acutatum* using PCR

To confirm the identity of putative isolates of *C. acutatum* (Table 3.2) used in experiments in this study, genomic DNA was amplified by PCR with species-specific primers (Section 2.4.2).

3.3.1 Isolation and purification of fungal DNA

Isolates listed in Table 3.2 were grown on PDA and prepared for DNA extraction as follows: 250-mL conical flasks containing 100 mL of sterile potato-dextrose broth (24 g/L, Difco, USA) (PDB) were inoculated with 4-5 plugs of mycelium (2 x 2 mm) excised from the margin of colonies and incubated on a rotating disk in natural light at room temperature for 5-7 days. The mycelium was filtered through sterile Whatmann® no. 4. filter paper using a Buchner funnel and washed with 300 mL sterile dd H_2O. Excess moisture was removed by blotting with filter paper, then mycelium was frozen in liquid nitrogen and stored at -70°C until required.

The mycelium was ground to a fine powder in liquid nitrogen using a mortar and pestle. Fifty mg of ground mycelium was transferred to a 2-mL Eppendorf tube and 1 mL of lysis buffer (150 mM EDTA, 50 mM Tris pH 8, 1% lauroyl sarkosine, 500 µg/mL pronase E) added. Genomic DNA was extracted based on the protocols of Rodriguez and Yoder (1991) and Freeman *et al.* (2001) with the following modification: 10 µL of RNAse A (10 mg/mL) was added to the 1 mL of lysis buffer. The resultant slurry was vortexed for 5 sec, incubated for

15 min at 65°C then centrifuged at 14 000 rpm for 20 min. The supernatant (600 μL) was transferred to a clean Eppendorf tube and 0.7 vol of PEG/NaCl (20% polyethylene glycol 6000, 2.5 M sodium chloride) was added. Materials were incubated on ice for 10 min then centrifuged at 14 000 rpm for 5 min. The supernatant was discarded and the DNA pellet was suspended in 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA). Ammonium acetate was added to a final concentration of 2.5 M, the extracts were incubated on ice for 20 min, then centrifuged at 14 000 rpm for 15 min. The supernatant was transferred to a clean Eppendorf tube and 0.6 vol of ice-cold isopropanol added. The solutions were incubated overnight at 4°C, then centrifuged at 14 000 rpm for 5 min. The isopropanol was decanted and the DNA pellet dissolved in 250 μL of TE. The DNA was precipitated in 0.1 M NaCl and 2 vol of 95% ice-cold ethanol, incubated on ice for 10 min then centrifuged at 14 000 rpm for 5 min. Ethanol was removed with an aspirator and the DNA pellet was re-suspended in 80 μL of TE buffer.

DNA quality and quantity were assessed by subjecting a 2-μL aliquot to electrophoresis in a 1% agarose gel (Roche Diagnostics, Germany) in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 65 V for 1.5 h. Gels were stained with ethidium bromide (0.5 μg/mL), viewed under UV light and photographed using Polaroid 667 film (Polaroid Corporation, UK). DNA quantity was estimated by comparison with a λ *Hin*d III ladder (Roche Diagnostics, Germany), containing known quantities of DNA. The DNA was diluted with TE buffer to a concentration of approximately 20 ng/μL, then stored at -20°C until required.

3.3.2 PCR amplification

3.3.2.1 Identification of *C. acutatum* using genomic DNA

Genomic DNA was amplified by PCR using the universal primer ITS 4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990) coupled with specific primers for *C. acutatum* (*Ca*Int2) (GGGGAAGCCTCTCGCGG) or *C. gloeosporioides (Cg*Int) (GGCCTCCCGCCTCCGGGCGG) (Sreenivasaprasad *et al.*, 1996). Amplification was expected to result in amplicons of 490 bp and 450 bp for *C. acutatum* and *C. gloeosporioides*, respectively (Freeman *et al.*, 2000a). Reference isolates of *C. acutatum*, CSL-1689, CSL-1691, DAR-72407, CSL-1688, US-1796 and US-1776, and *C. gloeosporioides*, AVO-58 and AVO-37-4B (Table 3.2), were used as positive controls in the PCR. Genomic DNA of fungi other than *Colletotrichum* spp., listed in Table 3.2, was included to assess the specificity of the PCR.

Amplification by PCR was performed in a total volume of 20 μL, comprising 10-20 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Promega, USA) and 0.5 μM ITS 4 primer coupled with either 0.5 μM *Ca*Int2 or 0.5 μM *Cg*Int. The DNA was amplified in a thermocycler (PTC-100, MJ Research, Watertown, MA), starting with 5 min of denaturation at 95°C, followed by 30 cycles consisting of 30 sec at 95°C, 30 sec at 60°C and 1.5 min at 72°C (Freeman *et al.*, 2000a). Negative controls, using sterile dd H_2O instead of DNA, were included in each experiment.

Amplification products were separated in agarose gels (1.5% w/v) in 1 x TAE buffer and subjected to electrophoresis at 80 V for 2 h. Gels were stained in ethidium bromide (0.5 μg/mL), and amplification products were viewed under UV light, then photographed as

described previously in Section 3.3.1. Amplification products were compared to a 100-bp ladder (Geneworks, Australia). Amplification reactions were performed twice.

All isolates of *Colletotrichum* sp. obtained from almond in Australia, California and Israel, including the reference isolates of *C. acutatum* from Australia and California, produced a 490-bp fragment using the primers *Ca*Int2 and ITS 4. No PCR product was obtained from isolates of *C. gloeosporioides* (S54, Or1, AVO-58 and AVO-37-4B) or of fungi other than *Colletotrichum* spp. Fig 3.1 shows representative isolates of *C. acutatum*, *C. gloeosporioides* and *Fusarium* sp. following PCR amplification with *Ca*Int2 and ITS 4. Isolates obtained from plants other than almond, which had been identified as *C. acutatum* based on morphological criteria, also produced an amplicon of 490 bp following PCR with the *Ca*Int2 and ITS 4 primers. This group included isolates from olive, pistachio, wine grape, strawberry, blueberry and avocado. *C. gloeosporioides* reference isolates (AVO-58 and AVO-37-4B) and isolates of putative *C. gloeosporioides* (S54 and Or1) produced a fragment of 450 bp following PCR with the primers *Cg*Int and ITS 4, whereas none of the isolates of *C. acutatum* or other fungi produced an amplicon. Fig 3.2 shows representative isolates of *C. gloeosporioides* and *C. acutatum* following amplification with *Cg*Int and ITS 4.

3.3.2.2 Identification of *C. acutatum* using mycelial extract

Mycelial extract, instead of genomic DNA, of representative isolates of *C. acutatum*, *C. gloeosporioides* and other fungi, was used in PCR amplification with the primers *Ca*Int2 and ITS 4. The following isolates were tested; CSL-1690, ALM-KSH-10, P1, OL2, S43, S44, CSL-1687, DAR-72407, CSL-1688, CSL-1689, W16, W42, W77, W107, W153, W155, US-1776, US-1796 and US-1732 (*C. acutatum*) and AVO-37-4B and S54 (*C. gloeosporioides*) (Table 3.2). Other fungi tested included *Fusarium* sp., *Botryosphaeria* sp., several

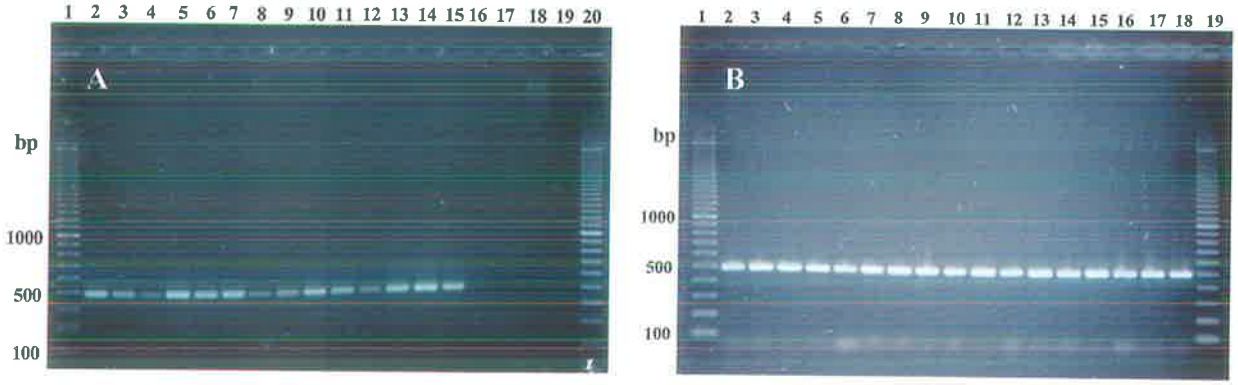


Fig 3.1. Amplification products of DNA extracted from isolates of *Colletotrichum* spp. obtained following PCR with the *C. acutatum*-specific primers *Ca*Int2 and ITS 4.
A. Lanes 1 and 20, 100-bp ladder; lanes 2-13, *C. acutatum* from almond in Australia, (CSL-1690), *Colletotrichum* sp. from almond in Israel (ALM-NRB-3DK and ALM-BZR-8L), *C. acutatum* from almond in Australia (MPD2, S44, DAR-72407, CSL-1317 and CSL-1688), *C. acutatum* from almond in California (ALM-US-3 and ALM-US-4), *C. acutatum* from almond in Australia (W2 and W154); lanes 14 and 15, *C. acutatum* from pistachio (P1) and from avocado in Australia (CSL-1298); lanes 16 and 17, *C. gloeosporioides* from avocado in Israel (AVO-58) and orange in Australia (Or1); lane 18, *Fusarium oxysporum* (CSL-147); lane 19, dd H_2O.
B. Lanes 1 and 19, 100-bp ladder, lanes 2-17, *C. acutatum* from almond in Australia (W5L, W136, W156, W158, W162a, W166, W167, W169, W174, W176, W177, W178, W179, W180, W184); lane 18, *C. acutatum* from olive in Australia (OL2).

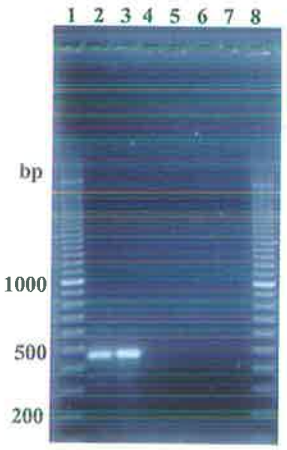


Fig 3.2. Amplification products of DNA extracted from isolates of *Colletotrichum* spp. obtained following PCR with the *C. gloeosporioides*-specific primers *Cg*Int and ITS 4. Lanes 1 and 8, 100-bp ladder; lanes 2-7, *C. gloeosporioides* from avocado in Israel (AVO-58), *C. gloeosporioides* from orange in Australia (Or1), *C. acutatum* from almond in Australia (DAR-72407), *C. acutatum* from almond in California (ALM-US-3), *C. acutatum* from almond in Australia (W154) and dd H_2O.

unidentified fungi and a yeast, which were frequently recovered following incubation of almond plant material (Section 7.3.2). Based on the protocol of Hamelin *et al.* (2000), approximately 3 x 3 mm of mycelium was removed from the growing margin of cultures on PDA using a sterile pipette tip, and added to 100 μL of extraction buffer (0.5 M Tris-HCl, pH 9.0, 1 % Triton® X-100). The mycelium was gently macerated using the pipette tip and the mixture vortexed for 5 sec. Materials were boiled at 100°C for 5 min, then immediately placed on ice for 5 min. A 2-μL aliquot of the extract was used in each 20-μL reaction and amplified as previously described, with the inclusion of polyvinyl-pyrrolidone, MW 10 000 (PVP-10) in the reaction mixture to a final concentration of 2 % (Koonjul *et al.*, 1999). A positive control, comprising genomic DNA of isolate DAR-72407 instead of mycelial extract, and a negative control, sterile dd H_2O instead of DNA, were included in each experiment. Two replicates from each extract were amplified per experiment.

Amplification products were separated, stained and viewed as described (Section 3.3.2.1) and compared to a 100-bp ladder (Geneworks, Australia).

Following PCR amplification with *C. acutatum*-specific primers, a 490-bp fragment was produced for the representative isolates of *C. acutatum* from almond, whereas isolates other than *C. acutatum* did not produce an amplicon of this size following PCR with these primers. The intensity of the fragment was occasionally variable and a false negative result was produced infrequently. However, results were consistent and reliable when adequate amounts (3 x 3 mm) of mycelium was available for use in PCR.

3.3.3 Conclusions

All of the isolates of *Colletotrichum* sp. obtained from almond in Australia, as well as those from California and Israel, were confirmed as *C. acutatum* using PCR. These results were

consistent with those of Freeman *et al.*, (2000a), who reported that the Israeli almond isolates, which had been identified previously as *C. gloeosporioides* using morphological criteria, produced fragments of expected size following PCR with the *C. acutatum*-specific primers. The results of this study were also consistent with those of Förster and Adaskaveg (1999) and Freeman *et al*, (2000a), who found that isolates from the pink and grey subpopulation of *C. acutatum* from almond in California were positive with the *C. acutatum*-specific primers and not with the *C. gloeosporioides*-specific primers. The isolates of *C. gloeosporioides* from orange in Australia and from avocado in Israel were positive with the *C. gloeosporioides*-specific primers and not with the *C. acutatum*-specific primers, confirming that these isolates were *C. gloeosporioides*.

Identification of *C. acutatum* using mycelial extract in PCR amplification demonstrated that the common process of growing the fungus in culture media and subsequent extraction of total genomic DNA may not be necessary for identification of *C. acutatum* using PCR with species-specific primers. PCR using mycelial extract was efficient and may be used for rapid identification of *C. acutatum*, in conjunction with morphological criteria. However, this method was tested with a small range of isolates of *C. acutatum* and microorganisms other than *C. acutatum*, and should be evaluated with a larger range of isolates of *C. acutatum* and other microorganisms before this method could be recommended as a procedure for rapid and accurate identification of *C. acutatum*.

3.4 Source and maintenance of potted plants

Five cultivars of almond; Carmel, Price, Nonpareil, Ne Plus Ultra and Fritz, were used in this study. All cultivars had been grafted onto Nemaguard® rootstock, and the grafted trees were obtained from the registered almond nursery, Laxton Nursery, Lindsay Point, Victoria, in

May 2002. The trees were grown in 30-cm plastic pots containing a mixture of 50 % perlite and 50 % UC soil mix (Baker, 1957), fertilised regularly with Osmocote® as per the manufacturer's instructions, and kept in a shadehouse at the Waite Campus. Plants were watered as required and no pesticide sprays were used.

3.5 Confirmation of identity of almond cultivars

There was uncertainty about the identity of trees in the almond orchard, situated at Willunga, South Australia, used in several experiments in this study. To confirm the identity of these cultivars, genomic DNA was amplified by PCR using two types of primer and amplification products were compared against reference specimens.

DNA from the following trees was used as reference material; Carmel, Price, Fritz, Ne Plus Ultra and Nonpareil, which were supplied from Laxton almond nursery. In addition, genomic DNA of Carmel, extracted from trees used in the almond breeding program at the University of Adelaide, was supplied by Dr Michelle Wirthensohn. DNA was extracted from the leaves of representative trees in rows four and five of the almond orchard at Willunga, South Australia (Section 7.2.1.1) and, following PCR amplification, the amplicons were compared against the reference material.

DNA was extracted from reference material and trees in rows four and five (as mentioned above) from 50 mg of leaf tissue, which had been ground to a fine powder in liquid nitrogen, using the Dneasy® (Qiagen, Germany) plant mini kit.

DNA quality and quantity were assessed by subjecting a 2-μL aliquot to electrophoresis in a 1% agarose gel (Roche Diagnostics, Germany) in 1 x TAE buffer at 65 V for 1.5 h. Gels were stained with ethidium bromide (0.5 μg/mL), viewed and photographed as described in

Section 3.3.1. DNA quantity was estimated by comparison with a λ *Hin*d III ladder (Roche Diagnostics, Germany), containing known quantities of DNA. The DNA was diluted with TE buffer to a concentration of approximately 20 ng/μL, then stored at -20°C until required.

3.5.1 PCR using anchored dinucleotide primers

PCR amplification was performed in a total volume of 20 μL, comprising 10-20 ng of genomic DNA, 50 mM KCl, 10mM Tris-HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Promega, USA) and 0.5 μM primer $(AG)_8YC$, $(CA)_8T$, $(CT)_8G$, $(CA)_8G$, $(AG)_4YT$ or $(AG)_8YA$. The DNA was amplified in a thermocycler (PTC-100, MJ Research, Watertown, MA), using two cycling profiles as follows; 2 min of denaturation at 95°C, followed by 34 cycles consisting of 30 sec at 95°C, 45 sec at 48°C [for $(AG)_8YC$, $(CA)_8T$, $(CT)_8G$ and $(CA)_8G$] or 55°C [for $(AG)_4YT$, and $(AG)_8YA$] and 1 min at 72°C, and a final extension of 10 min at 72°C (M. Wirthensohn, pers. comm., 12 August 2004). Amplification products were separated in agarose gels (1.5% w/v) in 0.5 x TBE buffer (45 mM Tris-borate, 1 mM EDTA), subjected to electrophoresis at 65 V for 2.5 h. Gels were stained in ethidium bromide (0.5 μg/mL), and amplification products were viewed under UV light, and photographed with Polaroid 667 film (Polaroid Corporation, UK). Amplification products were compared to a 100-bp ladder (Geneworks, Australia), and banding patterns compared against the reference material.

For each PCR, a negative control containing all reagents but with dd H_20 instead of DNA was included. Two amplifications of each sample were performed and banding patterns compared.

The DNA banding patterns obtained following PCR with the primers $(AG)_8YC$, $(CT)_8G$, $(CA)_8G$, $(CA)_8T$, and $(AG)_8YT$ were identical for Carmel, Price and Fritz. The banding

patterns for Ne Plus Ultra and Nonpareil were different from the other cultivars and from one another. The banding pattern for trees in row four from the Willunga orchard was the same as that for the Carmel, Price and Fritz standard and the banding pattern for trees in row five was the same as that for the Nonpareil standard. The banding pattern using primer $(AG)_8YA$ was the same for Carmel and Price, but different for each of Fritz, Ne Plus Ultra and Nonpareil. Trees from row four of the Willunga orchard had the same banding pattern as Carmel and Price, and trees from row five had the same pattern as Nonpareil using primer $(AG)_8YA$.

Overall, banding patterns obtained using anchored dinucleotide-primed PCR showed that Ne Plus and Nonpareil could be differentiated from one another and from Carmel, Price and Fritz, however, it was not possible to differentiate Carmel from Price. Therefore, it was not possible to determine if the trees in row four were Carmel or Price. DNA from trees in row five had the same fingerprint as Nonpareil using primers $(AG)_8YC$, $(CA)_8G$, $(CA)_8T$, and $(AG)_8YA$, which supported the identification of the trees from row five as Nonpareil.

3.5.2 PCR using *S*-allele primers

PCR amplifications were performed on DNA from Nonpareil, Price and Carmel, trees from the Willunga orchard rows four and five, using primers designed to target specific regions on the introns of the *S*-alleles (Channuntapipat *et al.*, 2003). The sequences of the *S*-allele primers and size of expected amplicons (bp) are given in Table 3.3. Amplification was carried out in a total volume of 20 μL, comprising 10-20 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Promega, USA) and 0.3 μM each of the forward and reverse *S*-allele primers. The *S*-alleles, *S*1 and *S*7 are found in Price, *S*7 and *S*8 in Nonpareil, and *S*5 and *S*8 in Carmel.

The DNA was amplified in a thermocycler (PTC-100, MJ Research, Watertown, MA), using three cycling profiles as follows: 3 min of denaturation at 95°C, followed by 34 cycles consisting of 30 sec at 95°C, 45 sec at either 60°C for *S*1 and *S*8, 56°C for *S*5 or 54°C for *S*7, and 1 min at 72°C, and a final extension of 10 min at 72°C (Channuntapipat *et al.*, 2003). Amplification products were separated as previously described. For each PCR, a negative control containing all reagents but with sterile dd H_2O instead of DNA was included. Two amplifications of each sample were performed, the size of amplicons determined and bands amplified from DNA of trees in Willunga orchard rows four and five were compared against Carmel, Price and Nonpareil standards.

Table 3.3. Specific primers designed from the introns of genomic DNA for *S*-allele identification from Channuntapipat *et al.* (2003).

Specific allele	Primer	Nucleotide sequence (5′-3′)	Amplicon (bp)
*S*1	*S*1F *S*1R	CTC TTT AGC ATT TTA GTT TTT AGCTG AGA CAT CCA AGC AAT ATA G	488
*S*5	*S*5F *S*5R	GGC TCT TTG TTT TTC TAG TTA C GCA ACA TCC AAG CAA TAA ATC	75
*S*7	*S*7F *S*7R	ACC ATA TAA CAT CGT GTT GC GAG GAT AAT ATG GTA CAT TC	425
*S*8	*S*8F *S*8R	CAA ATG GTC CTT ACT CAC TCT CCC AAA TCG CAG ACT CAC TCT	648

A 488-bp amplicon was produced following PCR using the *S1* primer pair for DNA from Price and for trees from Willunga orchard row four, but not for DNA from Carmel, Nonpareil or trees from row five. A 75-bp amplicon was produced using the *S*5 primer pair for DNA from Carmel, but not for Price, Nonpareil or trees from row four. A 425-bp amplicon was produced using the *S*7 primer pair in PCR for DNA from Price, Nonpareil, trees in row four and row five, but not for Carmel. A band of 648 bp was evident following PCR with the *S*8

primer pair for DNA from Carmel, Nonpareil and trees from row five. Fig 3.3 shows an example of amplification products following PCR using the *S*1 and *S*8 primer pairs.

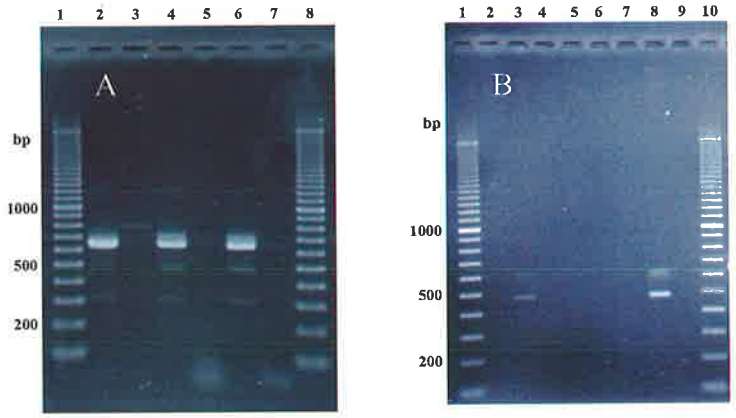


Fig 3.3. PCR amplification of genomic DNA extracted from leaves of almond trees from reference material of Carmel, Price and Nonpareil, and from trees in rows four and five in an almond orchard at Willunga, South Australia.
A. PCR with the *S8*-allele primer pair. Lanes 1 and 8, 100-bp ladder; lanes 2-6, the almond cultivars Carmel, Price, Nonpareil, trees from row four and row five; lane 7, dd H_2O.
B. PCR with the *S*1-allele primer pair. Lanes 1 and 10, 100-bp ladder; lane 2, Carmel; lane 3, trees from row four; lanes 4-7, Carmel; lane 8, Price; lane 9, dd H_2O.

3.5.3 Conclusions

The results from PCR amplification using anchored-dinucleotide and *S*-allele primers suggested that trees in Willunga orchard row four were the cultivar Price and trees in row five were Nonpareil.

Chapter 4 Morphological and cultural variation of *Colletotrichum* spp.

4.1 Introduction

Fungi of the genus *Colletotrichum* Corda have been examined extensively and described with respect to morphological characteristics in culture (Section 2.3.1), however, due to the inherent morphological plasticity and overlapping of features among species (Cannon *et al.*, 2000; Wharton and Diéguez-Uribeondo, 2004), the taxonomy of *Colletotrichum* spp. has not yet been resolved. Recent advances in molecular biology are helping to resolve some of the taxonomic confusion (Johnston and Jones, 1997; Lardner *et al.*, 1999; Freeman *et al.*, 2001; Guerber *et al.*, 2003), but morphology remains important for the identification and description of *Colletotrichum* spp., particularly for plant pathologists who work in the area of applied pathology and may have limited access to facilities for molecular biology.

C. acutatum is currently considered a 'group species' (Section 2.3) (Johnston and Jones, 1997; Cannon *et al.*, 2000), with variation of morphology and cultural characteristics evident among the groups (Lardner *et al.*, 1999). One subgroup, referred to as '*C. acutatum sensu* Simmonds' (Freeman *et al.*, 2001; Guerber and Correll, 2001), contains isolates of *Colletotrichum* sp. from almond in California and Israel (Freeman *et al.*, 2001).

The isolates of *C. acutatum* from almond in California and *Colletotrichum* sp. in Israel have been characterised with respect to morphological and cultural characteristics (Förster and Adaskaveg, 1999; Freeman *et al.*, 1996). Two subpopulations of *C. acutatum* from almond from California have been described (Section 2.6.3) and are referred to as the pink and grey subpopulations (Förster and Adaskaveg, 1999). These subpopulations differ in colour when viewed from above and below the culture plate, conidial shape, the amount of sporulation and

mycelial growth rate at various temperatures (Förster and Adaskaveg, 1999). The isolates of *Colletotrichum* sp. from almond in Israel are grey in culture, and have been identified as *C. gloeosporioides* based on morphological characteristics (Freeman *et al.*, 1996), but as *C. acutatum* using PCR-based methods (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a). Isolates of *C. acutatum* from almond in Australia have not been characterised with respect to morphological and cultural characteristics, nor have they been compared to the isolates of *Colletotrichum* sp. from almond in California and Israel. The aims of the research presented in this chapter were to characterise the isolates of *C. acutatum* from almond in Australia using morphological and cultural characteristics, and to compare these with isolates of *Colletotrichum* sp. from almond in California and Israel.

4.2 Materials and methods

4.2.1 Morphological variation

4.2.1.1 Colony characteristics and conidial morphology

Macroscopic and microscopic characteristics were examined for 132 isolates of *Colletotrichum* spp. (Table 3.2, Section 3.2), which included 101 isolates of *C. acutatum* from almond in Australia, 10 isolates of *C. acutatum* from almond in California, seven isolates of *Colletotrichum* sp. from almond in Israel, 10 isolates of *C. acutatum* from plants other than almond in Australia, one isolate of *C. acutatum* from strawberry in Israel, two isolates of *C. gloeosporioides* from avocado in Israel and two isolates of *C. gloeosporioides* from orange in Australia. All isolates were subcultured onto PDA from spore suspensions which had been stored at -70°C (Section 3.2). Cultures were grown in the dark at 25°C for 10 days, then a 5-mm diam plug of mycelium was taken from the margin of each colony and placed in the centre of a 90-mm Petri dish containing PDA. Three replicate plates were prepared for each

isolate. All isolates were incubated in the dark at 25°C for 14 days and the following morphological and colony characters examined; colony colour-upper, colony colour-reverse, mycelial texture, mycelial density, mycelial elevation, colony edge, colony diameter (mm), spore length/width (l/w) ratio and spore shape. Several of the characters were scored using a different number of categories within each characteristic and these will be referred to subsequently as maximum (max), moderate (mod) and minimum (min) number of categories and are described in detail in Table 4.1.

Colony colour, upper and reverse of the culture plate, was determined by visual observation based on the mycological colour chart of Raynor (1970). The main colour categories of the colonies, both upper and underside (reverse), were pink, orange, grey and cream (min number of categories). However, within these categories, cultures could be further differentiated into pink, orange, grey, orange-pink, cream and orange-grey for colour-upper, and vinaceous, pink, cream, brick, orange, grey and rose-grey for colour-reverse (max number of categories). Mycelial elevation was classified as low, moderate or high and mycelial density was classified as sparse, moderate or dense. Mycelial texture was described using a minimum number of two categories; 1. smooth, fine, flat with or without crystalline surface, with or without granular appearance, or 2. feathery, fluffy, irregular tufts, woolly or wispy. However, within these two categories, the mycelial texture was further divided into five categories; 1. smooth, 2. granular, 3. furry/velvety, 4. tufted, 5. feathery/wispy (maximum number of categories). Colony edge was classified as regular or irregular.

Colony diameter (mm) was measured for each replicate and the mean diameter calculated for each isolate. Colony diameter of each isolate was categorised as shown in Table 4.1.

Table 4.1. Colony characteristics and categories which were used in principal components analysis, nonmetric multi-dimensional scaling and clustering analysis of 132 isolates of *Colletotrichum* spp. from almond and other plants from Australia, California and Israel.

Colony characteristic	'Maximum' number of categories	'Moderate' number of categories	'Minimum' number of categories
Colour- upper	1. Pink 2. Orange 3. Grey 4. Orange-pink 5. Cream 6. Orange-grey	As for maximum number of categories	1. Pink 2. Orange 3. Grey 4. Cream
Colour- reverse	1. Vinaceous 2. Pink 3. Cream 4. Brick 5. Orange 6. Grey 7. Rose-grey	As for maximum number of categories	1. Pink 2. Orange 3. Grey 4. Cream
Mycelial elevation	1. Low 2. Moderate 3. High	As for maximum number of categories	As for maximum number of categories
Mycelial density	1. Sparse 2. Moderate 3. Dense	As for maximum number of categories	As for maximum number of categories
Mycelial texture	1. Smooth a (fine, regular mycelium, generally crystalline) 2. Smooth b (fine mycelium underneath with small granular mycelial tufts) 3. Furry (soft, velvety, dense and fluffy) 4. Tufted (tufts of mycelium, irregular height and density) 5. Feathery/fluffy (sparse and wispy feathery with tufts of upright mycelium)	1. Smooth 2. Feathery/fluffy (includes furry, irregular tufts, velvety cobwebby, woolly, wispy mycelium)	1. Smooth 2. Feathery/fluffy
Colony edge	Regular or irregular	As for maximum number of categories	As for maximum number of categories
Colony diameter	1. 21-30 mm 2. 31-40 mm 3. 41-50 mm 4. 51-60 mm 5. 61-70 mm 6. 71-80 mm 7. 81-90 mm	As for maximum number of categories	As for maximum number of categories
Conidial shape	1. One end rounded and the other pointed 2. Both ends rounded 3. Both ends pointed	As for maximum number of categories	As for maximum number of categories
Conidial length/width ratio	1. 2.0-2.19 2. 2.2-2.39 3. 2.4-2.59 4. 2.6-2.79 5. 2.8-2.99 6. 3.0-3.19 7. 3.2-3.39 8. 3.4-3.59 9. 3.6-3.79 10. 3.8-3.99 11. 4.0-4.19 12. 4.2-4.39	As for maximum number of categories	1. 2.0-2.49 2. 2.5-2.99 3. 3.0-3.49 4. 3.5-3.99 5. 4.0+

On completion of colony descriptions, conidia were removed using a sterile wire loop, mounted in lactoglycerol and viewed using the x 100 objective with oil immersion. Fifty conidia, chosen at random, per isolate were examined and measured for length, width and shape. The conidia were assigned to one of three shape categories (Smith and Black, 1990); 1. one end rounded and the other pointed, 2. both ends rounded and 3. both ends pointed (fusiform). The mean length/width ratio was calculated for each isolate, and isolates were categorised as shown in Table 4.1. Cultures of US-1776, an isolate from the grey subpopulation of *C. acutatum* in California, did not produce conidia during this experiment, therefore data for spore size and shape were not available.

4.2.1.2 Statistical analysis

To determine if there were significant differences among isolates, with respect to conidial length and width, data were subjected to analysis of variance (ANOVA) (Genstat® for Windows, 6th edn). Results were considered significant if $P<0.001$, unless otherwise stated.

Second, to determine if discrete groups could be elucidated among the isolates examined, statistical analysis using principal components analysis (PCA), nonmetric multi-dimensional scaling (MDS) and cluster analysis were carried out based on the morphological and colony characteristics described previously. As noted above, several of the morphological and colony characteristics were scored using a different number of categories, *viz,* max, mod and min, within each characteristic. PCA, MDS and cluster analyses were performed on the max, mod and min data sets. The mode of three replicates was used for further analysis for colour-upper, colour-reverse, mycelial elevation, mycelial density, mycelial texture, colony edge and spore shape, whereas the mean of three replicates was used for colony diameter and spore l/w ratio. Data for spore size and shape of US-1776 were deemed 'missing values' for PCA, MDS and cluster analysis.

PCA was performed on the max, mod and min data sets using Multi-variate Statistical Package© software (Kovach Computing Services, Wales). PCA is an ordination method used to determine which variables, morphological characters in this case, accounted for the maximum amount of variance of the data. Principal components with an eigen value of ≥ 1 were retained for use in further analysis. PCA can decrease the number of variables that account for the maximum variance of the data. PCA has been used for analysis of disease variables in other pathosystems (Chakraborty *et al.*, 2002; Vernière *et al.*, 2003), however, reference to the use of PCA analysis of morphological data for fungi was not found in the literature. PCA and similar techniques have been used for analysis of morphological characters in plants (Keiper and McConchie, 2001).

The retained principal components were then subjected to nonmetric MDS analysis using NT-SYSpc (Numerical Taxonomy and Multivariate Analysis System, version 2.02k, Exeter Software, Setauket, New York). MDS is an ordination technique used for representing similarity among individuals (Kruskal, 1964a, 1964b). Two and three-dimensional MDS were performed, and stress statistics were calculated as part of these analyses. The stress statistic measures the goodness of fit of the distances among the individuals in the multi-dimensional space to the distances calculated in the original similarity matrix. Stress values can be interpreted, as suggested by Kruskal (1964b), as follows:

Stress	Goodness of fit
0.40	Poor
0.20	Fair
0.10	Good
0.05	Excellent
0.00	Perfect

To determine if isolates formed discrete clusters for either the max, mod or min data sets, a phenogram of relative distance among isolates was created using the retained principal components. The relative distance among isolates was determined using the average 'taxonomic' distance coefficient, resulting in a dissimilarity matrix. The matrix was clustered by UPGMA (unweighted pair-group method, arithmetic average) using the SAHN (sequential, agglomerative, hierarchical and nested cluster analysis) algorithm, generating a tree matrix, which was then displayed as a phenogram (Rohlf, 1997). A cophenetic coefficient was calculated (Mantel, 1967) to determine goodness of fit of the cluster analysis to the data. Values of ≥ 0.9 indicate that clusters within the data are highly likely to be real, thus the clusters are a very good fit to the data (Rohlf, 1997).

4.2.2 Growth rate at a range of temperatures

A 5-mm plug was excised from the margin of 7-day-old colonies of the following isolates on PDA; CSL-1689, W16, W153, DAR-75574, P1, S43, S44, CSL-1687, S53, S54, CSL-1318, CSL-1298, CSL-1688, CSL-1690, MPD1, Or1, OL3, ALM-KSH-10, US-1813 and US-1796. The mycelial plug was placed in the centre of a 90-mm Petri dish containing PDA and incubated in the dark at 4, 10, 15, 20, 25, 30 or 35°C for 7 days. Colony diameter was measured from day three until day seven and the mean radial growth rate (mm/day) determined for each isolate at each temperature. There were three replicates of each isolate at each temperature and the experiment was repeated twice. The experiment was a split-plot design, with blocks being the replicates in time, temperatures the whole plots and isolates the subplots. The data were analysed using a two-way ANOVA and, as there was no significant difference between the two replicates of the experiments (blocks), the data from each experimental replicate were combined. The growth rate of each isolate, using the combined data, was determined using simple linear regression. Subsequently, in order to determine if

there were significant differences in the mean mycelial growth rate among isolates, ANOVA was performed for isolates at each temperature and results were considered to be significant if $P<0.001$ unless stated otherwise. Tukey's test at the 5% level was used to determine which isolates were significantly different from one another. All analyses were carried out using Genstat® for Windows, 6th edn.

4.3 Results

4.3.1 Morphological variation

4.3.1.1 Cultural characteristics

There was considerable variability in the morphological characteristics amongst the 132 isolates of *Colletotrichum* spp. examined, as well as among the isolates of *C. acutatum* from almond in Australia.

General cultural characteristics

Based on general colony appearance, four main groups could be distinguished among the 132 isolates of *Colletotrichum* spp. examined. These will subsequently be referred to as 1. pink, 2. orange, 3. cream and 4. grey. The morphology and cultural characteristics of each group are described in Table 4.2. Fig 4.1 shows isolates representative of the four groups. The groups were determined based primarily on colony colour and on general mycelial characteristics. Colony diameter, spore shape and size were not used to determine these groups. The pink group contained the majority of isolates of *C. acutatum* from almond in Australia and those from the pink subpopulation in California, except for ALM-US-12, which was cream. Isolates Se37, OL3 and OL4 from olive in Australia were also included in this

group. The orange group contained six isolates from almond in Australia, *viz.* CSL-1315, CSL-1318, CSL-1688, S68, W169b and W184b, as well as CSL-1298 from avocado in Australia, P1 from pistachio in Australia, NSW1 from olive in Australia and TUT-5954 from strawberry in Israel. The cream group contained 10 isolates of *C. acutatum* from almond in Australia, *viz.* CSL-1314, CSL-1687, W76, W107, W117, W157, W162a, W169a, W171, W180, as well as ALM-US-12. The grey group consisted of isolates of *Colletotrichum* sp. from almond in Israel and from the grey subpopulation of *C. acutatum* from almond in California. In addition, the following isolates were included in the 'grey' group, S53 (*C. acutatum* from strawberry in Australia), AVO-58 and AVO-37-4B (*C. gloeosporioides* from avocado in Israel), S54 and Or1 (*C. gloeosporioides* from orange in Australia), DAR-75574 (*C. acutatum* from wine grape in Australia), Bb1 (*C. acutatum* from blueberry in Australia) and OL2 (*C. acutatum* from olive in Australia). No isolates of *C. acutatum* from almond in Australia were classified as grey. Although four main groups were evident, there was considerable variation among isolates within each group, and the placement of several isolates was equivocal, because the general colony appearance overlapped between groups. Examples of this were; ALM-US-12 had an upper colour that was cream-pink and orange in reverse, CSL-1298, NSW1 and TUT-5954 had colony colours of orange-grey.

In this experiment, colony characteristics were examined after incubation for 14 days at 25°C, however, some characteristics changed during and after this time. For example, some cream coloured colonies became pale grey with increasing age or a pink pigmentation developed in the centre of the culture, whereas some orange colonies developed a grey aerial mycelium. In general, the characteristics of the pink isolates changed less than the isolates from the cream and orange groups. In addition, there was some variation among replicates of the same isolate and, in a few instances, among single spore isolates that were derived from the same bulk spore suspension (Section 3.1).

4.3.1.2 Conidial morphology

The isolates from almond in Australia and from the pink subpopulation in California sporulated profusely, whereas the isolates from the grey subpopulation in California, from almond in Israel, and from the cream group produced few conidia.

There was significant variation in the conidial length and width among isolates of *Colletotrichum* spp.and also among Australian isolates of *C. acutatum* from almond. The mean length for isolates from almond in Australia was 8.7 μm (+/- 0.17), and the range was 5.3-21.2 μm (Table 4.3). The mean width of isolates from almond in Australia was 3.18 μm, and the range was 2.12-4.24 μm. The majority of conidia from isolates from almond in Australia were rounded at one end and pointed at the other. However, many conidia were fusiform, some were rounded at both ends, and several intermediate forms occurred. Fusiform conidia were more common among isolates from the orange group than for the other groups, however, the majority of conidia from the orange group were rounded at one end and pointed at the other. There were no obvious differences among the pink, orange or cream groups with respect to conidial morphology. The range of sizes and shapes overlapped among the groups, and these characters did not assist in assigning the isolates of *C. acutatum* from almond in Australia to groups.

Conidia of the isolates from the pink subpopulation in California had a mean length of 9.39 μm (+/-0.1), which was larger than the mean conidial length of the Australian isolates. Although conidia of the isolates from the pink subpopulation in California, with a mean width of 3.37 μm, tended to be wider than Australian isolates (mean width of 3.18 μm), the range was the same, 2.1-4.2 μm (Table 4.3). In general, conidia of the isolates from the pink subpopulation from almond in California were rounded at one end and pointed at the other, however many were fusiform. There appeared to be a larger proportion of conidia that were

fusiform and fewer intermediate conidial forms than for the conidia of Australian isolates from almond. Conidia from isolate US-1813, representative of the grey subpopulation from California, were generally longer than conidia of the isolates of *C. acutatum* from Australia and the pink subpopulation from California (Table 4.3). All of the conidia of isolate US-1813 examined were rounded at one end and pointed at the other. Although there was a trend for conidia of the isolates of *C. acutatum* from almond in California to be larger than the isolates of *C. acutatum* from almond in Australia, the Californian isolates were within the range of that for the isolates from Australia.

The conidia from isolates of *Colletotrichum* sp. from almond in Israel, with mean length of 12.4 μm, were generally longer than conidia of the isolates of *C. acutatum* from almond in Australia (mean length, 8.7 μm) and of the Californian pink and grey subpopulation (9.39 and 10.77 μm, respectively) (Table 4.3). In general, the conidia of isolates from almond in Israel were distinctly rounded at one end, yet somewhat intermediate between rounded and pointed at the other. It was difficult to classify these isolates as being either distinctly rounded at both ends, or rounded at one end and pointed at the other. However, they appeared different, in shape and size, from those of isolates from Australia and from those of the pink subpopulation from California, as the latter groups had, in general, one end distinctly pointed.

Table 4.4 summarises the mean conidial length and width for isolates of *Colletotrichum* spp. from plants other than almond in Australia and Israel. Conidia from isolates of *C. gloeosporioides* from orange in Australia and from avocado in Israel were, in general, longer and wider than conidia of *C. acutatum* from almond or other host plants. The size range of conidia for isolates of *C. acutatum* from other plants was consistent with that of *C. acutatum* from almond in Australia. However, the size range of the isolates of *C. acutatum* from almond in Australia also overlapped with that of the isolates of *C. gloeosporioides*. Conidia

Table 4.2. Appearance, on PDA[a], of morphological groups recognised among isolates of *Colletotrichum* spp. from almond and other plants in Australia, California and Israel.

	Pink	**Grey**	**Orange**	**Cream**
Colony colour - upper	Pale to bright pink, some colonies dark pink and occasionally orange-pink. Colonies had concentric rings of different shades of pink.	Light to dark mouse grey, occasionally cream-grey.	Bright orange, occasionally cream-orange and orange with greyish surface mycelium with increasing age for some isolates.	Cream to off-white, occasionally with pale to dark pink concentric ring in centre. Some colonies with pink sectors.
Colony colour - reverse	Medium to dark pink, light to dark vinaceous and some colonies brick.	Light to dark mouse grey, occasionally cream-grey and rose-grey.	Bright orange, occasionally cream-orange.	Cream to light-brown, occasionally pink concentric bands and pink sectors.
Mycelial texture	Smooth, fine, usually crystalline.	Smooth, fine, usually crystalline, occasionally slightly fluffy and velvety.	Smooth, fine, crystalline.	a. Furry, fluffy, velvety and tufted or b. Wispy, feathery and not at all tufted.
Mycelial elevation	Low	Low, occasionally moderate	Low	a. Moderate to high or b. Low.
Mycelial density	Moderate	Moderate, occasionally dense	Moderate, occasionally sparse	a. Dense or b. Sparse to very sparse.
Colony margins	Regular	Regular, occasionally irregular	Regular	a. Irregular or b. Regular.
Spore masses	Clear, colourless spore masses arranged in concentric rings. With increasing age, occasionally orange spore masses on inoculum plug.	Generally no visible spore masses, but if present, clear and colourless.	With increasing age, abundant orange, opaque and slimy spore masses generally in concentric rings.	Generally no visible spore masses.

[a] Cultures grown for 14 days at 25°C in dark

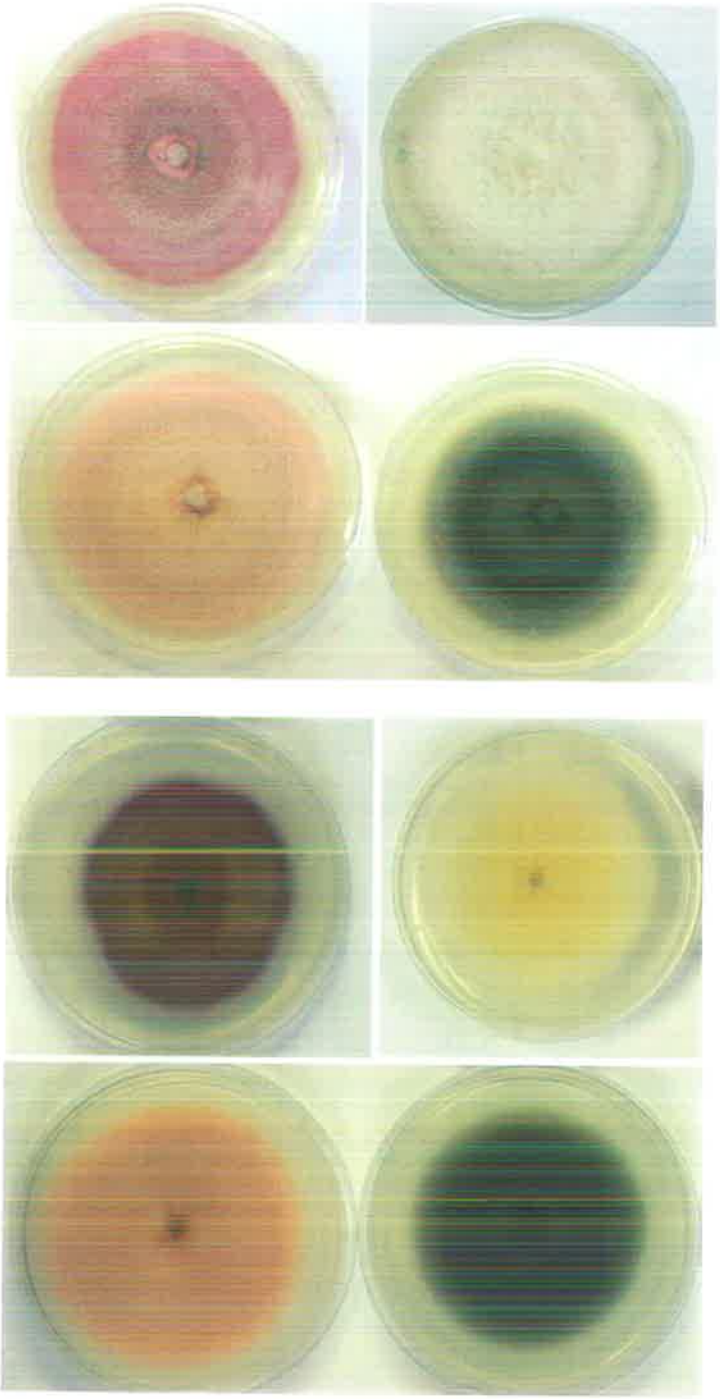


Fig. 4.1. Cultures, on PDA, after 14 days incubation at 25°C in the dark, viewed from **A.** the upper surface and **B.** the underside, representative of the main colony types observed among isolates of *Colletotrichum* spp. from almond and other plants in Australia, California and Israel.
Clockwise from bottom left; CSL-1688 (orange group), W64 (pink group), CSL-1314 (cream group) (All *C. acutatum* from almond in Australia) and AVO-37-4B (grey group) (*C. gloeosporioides* from avocado in Israel).

Table 4.3. Conidial size for isolates of *Colletotrichum* sp. from almond in Australia, California (USA) and Israel[a].

Country, *Colletotrichum* species (number of isolates)	**Conidial length (μm)**			**Conidial width (μm)**		
	Mean (standard error)	Min	Max	Mean (standard error)	Min	Max
Australia, *C. acutatum* (99)	8.7 (0.17)	5.3	16.9	3.18 (*)	2.12	4.24
Pink isolates (84)	9.0 (0.01)	4.24	14.84	3.0 (*)	2.12	4.24
Orange isolates (6)	8.6 (0.1)	5.3	16.96	3.2 (*)	3.18	4.24
Cream isolates (9)	9.2 (0.1)	5.3	21.2	3.2 (*)	3.18	4.24
California, USA, *C. acutatum*						
Pink subpopulation (8)	9.39 (0.1)	6.36	14.84	3.37 (0.02)	2.12	4.24
Grey subpopulation (1)	10.77 (0.32)	7.42	16.96	3.18 (0)	3.18	3.18
Israel, *Colletotrichum* sp. (6)	12.42 (0.1)	7.42	19.08	3.29 (*)	3.18	4.24

* standard error ≤0.005
[a] conidia harvested from colonies grown on PDA for 14 days at 25°C in dark.

of *C. gloeosporioides* from isolate Or1 (from orange in Australia) and AVO-58 and AVO-37-4B (from avocado in Israel), were predominantly rounded at both ends, whereas, for S54 (*C. gloeosporioides* from orange in Australia) approximately half of the conidia were rounded at one end and pointed at the other. However, it was often difficult to define this character, as several intermediate forms were evident. Shape of conidia of isolates of *C. gloeosporioides*, in general, was more similar to that of conidia of *Colletotrichum* sp. from almond in Israel and *C. acutatum* from the grey subpopulation from almond in California, than to *C. acutatum* from almond in Australia and the pink subpopulation from almond in California. The isolates of *C. acutatum* from strawberry (S53 and TUT-5954), avocado (CSL-1298), pistachio (P1), wine grape (DAR-75574) and one olive isolate (NSW1) had fusiform conidia, whereas the isolate from blueberry (Bb1) and four olive isolates (Se37, OL2, OL3 and OL4) had conidia that were predominantly rounded at one end and pointed at the other.

Table 4.4. Conidial size for isolates of *Colletotrichum* spp. obtained from blueberry, olive, orange, pistachio, avocado, strawberry and wine grape in Australia and Israel[a].

Host plant (number of isolates)	***Colletotrichum* species (country of origin)**	**Conidial length (µm)**			**Conidial width (µm)**		
		Mean (standard error)	Min	Max	Mean (standard error)	Min	Max
Blueberry (1)	*C. acutatum* (Australia)	9.2 (0.2)	7.42	12.72	3.39 (*)	3.18	4.24
Olive (5)	*C. acutatum* (Australia)	9.4 (0.1)	4.24	14.84	3.2 (0.006)	3.18	3.24
Orange (2)	*C. gloeosporioides* (Australia)	14.2 (0.2)	9.54	18.02	4.91 (0.03)	4.24	5.3
Pistachio (1)	*C. acutatum* (Australia)	9.92 (0.16)	7.42	12.72	3.18 (0)	3.18	3.18
Avocado (1)	*C. acutatum* (Australia)	10.68 (0.15)	7.42	13.78	4.24 (0)	4.24	4.24
Strawberry (1)	*C. acutatum* (Australia)	9.71 (0.21)	6.36	11.66	3.22 (*)	3.18	4.24
Strawberry (1)	*C. acutatum* (Israel)	8.37 (0.11)	8.48	9.54	3.45 (*)	3.18	5.3
Wine grape (1)	*C. acutatum* (Australia)	8.27 (0.16)	8.48	10.6	3.18 (0)	3.18	3.18
Avocado (2)	*C. gloeosporioides* (Israel)	14.5 (0.2)	10.6	18.02	5.02 (*)	4.24	5.3

* standard error is ≤ 0.005

[a] conidia harvested from colonies grown on PDA for 14 days at 25°C in dark.

Determination of groups

To determine if distinct groups or clusters could be revealed among isolates based on scoring of all of the morphological and cultural characteristics described in Section 4.2.1.1, the data were subjected to PCA, MDS and cluster analyses.

Based on an eigen value greater than 1, PCA on the max data set revealed three principal components, which accounted for 69% of the variance in the data. In this analysis, all variables or morphological characters were deemed important and, therefore, retained. MDS was performed on the three principal components, and two-dimensional MDS revealed that the majority of isolates of *C. acutatum* from almond in Australia tended to be similar to each other and, in general, discrete groups were not evident (data not shown). The stress value was 0.14, suggesting that the distances between the isolates in the two-dimensional space were a fair to good fit to the original similarity matrix. The distances were better represented by the three-dimensional MDS, with a stress value of zero, suggesting that the distances depicted in

the three dimensions were a perfect fit to the distances calculated in the original similarity matrix. The three-dimensional MDS plot (Fig 4.2) showed considerable variation among isolates, but there was a trend toward many of the isolates from almond in Australia being close together in the three-dimensional space. The isolates that were the greatest relative distance from the majority of isolates from almond in Australia were those of *Colletotrichum* sp. from almond in Israel. Cluster analysis of the three principal components using the max data set revealed many clusters, as shown in the phenogram in Fig 4.3. The cophenetic coefficient was 0.91, suggesting that the clusters were highly likely to be real. There were 13 clusters in which all of the isolates were identical, and each of these contained isolates that were from almond in Australia, as well as two isolates from olive in Australia. However, many isolates from almond in Australia were not within these clusters and were not identical using the max data set. In this analysis, no isolates from almond in Israel or in California were found to be identical.

PCA on the mod data set, using an eigen value greater than 1, showed that two principal components were most important and accounted for 66% of the variance in the data. This analysis found that the variables, elevation and density, did not contribute significantly to the two principal components, therefore they were removed. MDS was performed on the two retained principal components and seven retained variables, i.e., colour-upper, colour-reverse, mycelial texture, spore l/w ratio, colony edge, colony diameter and spore shape. Two-dimensional MDS revealed considerable variability among isolates, with a trend toward most of the isolates from almond in Australia being close together and six of the isolates from almond in Israel being relatively close together, yet relatively distant from the other isolates in the two-dimensional space (data not shown). The stress value was zero, which suggested that the distances depicted in the two-dimensional plot were a perfect fit to the original similarity matrix. The cluster analysis, as depicted by the phenogram (data not shown) found many

small clusters among isolates, and the cophenetic coefficient was 0.93, indicating that the clusters were highly likely to be real. As for the max data set, there were 13 clusters in which isolates were identical and, within these, isolates were from almond and two from olive in Australia. However, the clusters were not the same as those found following cluster analysis of the max data set. This analysis revealed that isolates from the pink subpopulation in California, ALM-US-3 and US-1732, were considered to be identical, as were isolates ALM-GVA-6A and ALM-GOZ-42B, from almond in Israel. None of the isolates from almond in Australia were identical to isolates from almond in California or Israel.

PCA, when performed on the min data set, showed that three principal components with nine variables or morphological characters, accounted for 70% of the variance in the data. Two-dimensional MDS was carried out on the three retained principal components, and the stress value was 0.12, suggesting a good fit of the distances depicted in the two-dimensional space to the original similarity matrix (data not shown). The stress value for the three-dimensional analysis was zero, suggesting a perfect fit of the distances in this analysis to the original similarity matrix, and the three-dimensional MDS plot is shown in Fig 4.4.

As with the max and mod data sets, distinct groups were not evident among isolates, however a similar trend was observed, with isolates of *C. acutatum* from almond in Australia tending to be close to each other in three-dimensional space, and five of the isolates from almond in Israel being relatively close together yet distant from the majority of isolates from almond in Australia (Fig 4.4). In contrast to the MDS analysis of the max and mod data sets, MDS on the min data set revealed that there were several isolates from the pink subpopulation from almond in California which were relatively close to the majority of isolates from almond in Australia (Fig 4.4). UPGMA cluster analysis of the min data set revealed many clusters (Fig 4.5), with a cophenetic coefficient of 0.95, which were highly likely to be real. Eleven

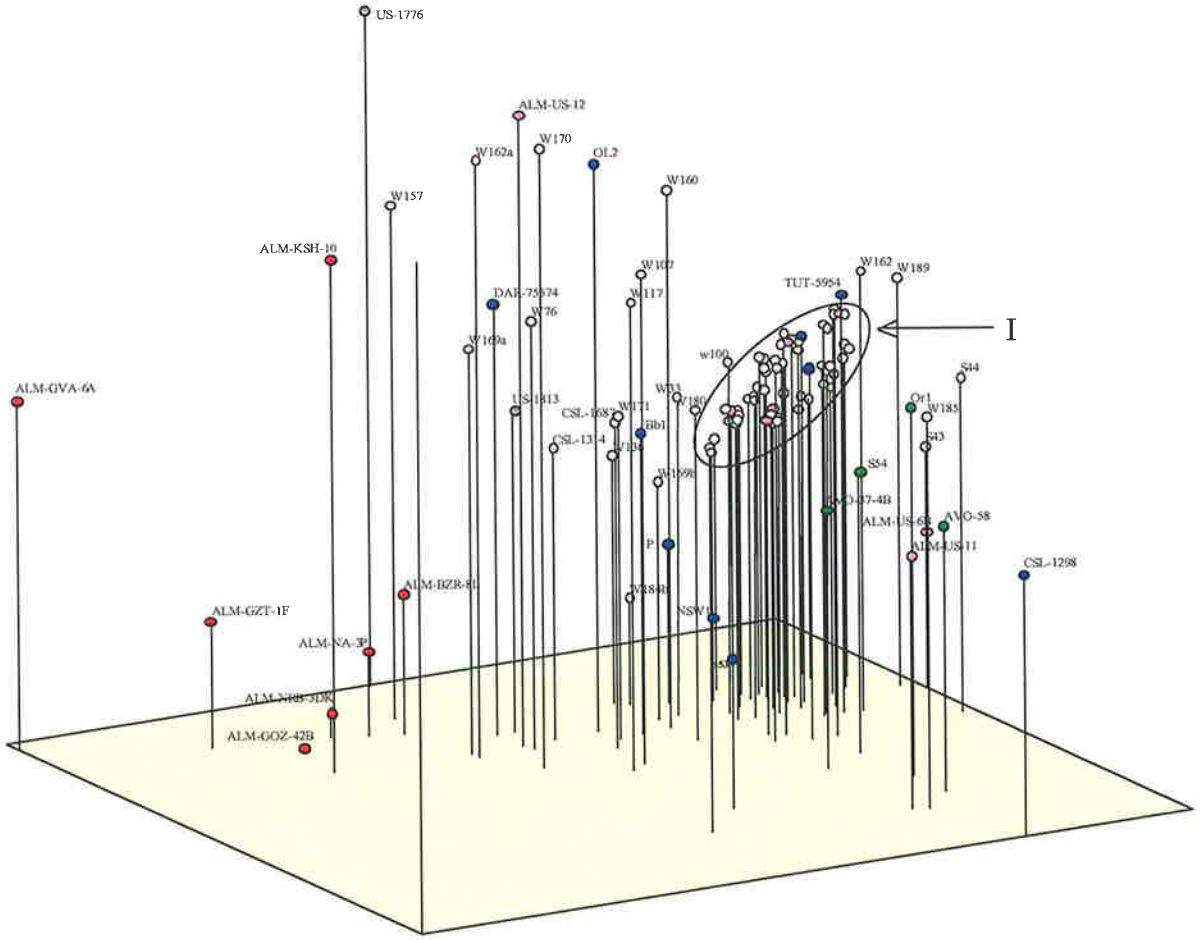


Fig 4.2 . Three-dimensional nonmetric multi-dimensional scaling (MDS) analysis of 132 isolates of *Colletotrichum* spp. from almond in Australia, California and Israel, and other plants, based on the max data set. Nine morphological characters (Table 4.1) were scored and subjected to principal components analysis, then data were clustered using MDS with NTSYSpc (v. 2.02k).
Isolates of *C. acutatum* from almond in Australia are shown as a white data points; *C. acutatum* representative of the pink and grey subpopulations from almond in California are pink and grey data points, respectively; *Colletotrichum* sp. from almond in Israel are red data points; *C. acutatum* from other plants are blue and *C. gloeosporioides*, green.
The following isolates are found in the area referred to as 'I'; ALM-US-3, ALM-US-4, ALM-US-9, CSL-1315, CSL-1317, CSL-1318, CSL-1688, CSL-1689, CSL-1690, CSL-1691, DAR-72407, MPD1, MPD2, MPD3, MPD4, MPD5, MPD6, MPD7, OL3, OL4, S66, S68, Se37, W1, W2, W4m, W4p, W5L, W5m, W5p, W6, W7, W16, W17, W24, W26, W29, W31, W39, W64, W32, W32a, W34, W36, W38, W43, W44, W45, W47, W55, W65, W66, W77, W87, W122, W128, W129, W147, W153, W154, W155, W156, W158, W159, W163, W164, W165, W166, W167, W168, W169, W172, W173, W174, W176, W177, W178, W179, W181, W183, W184, W186, W188 and W190.

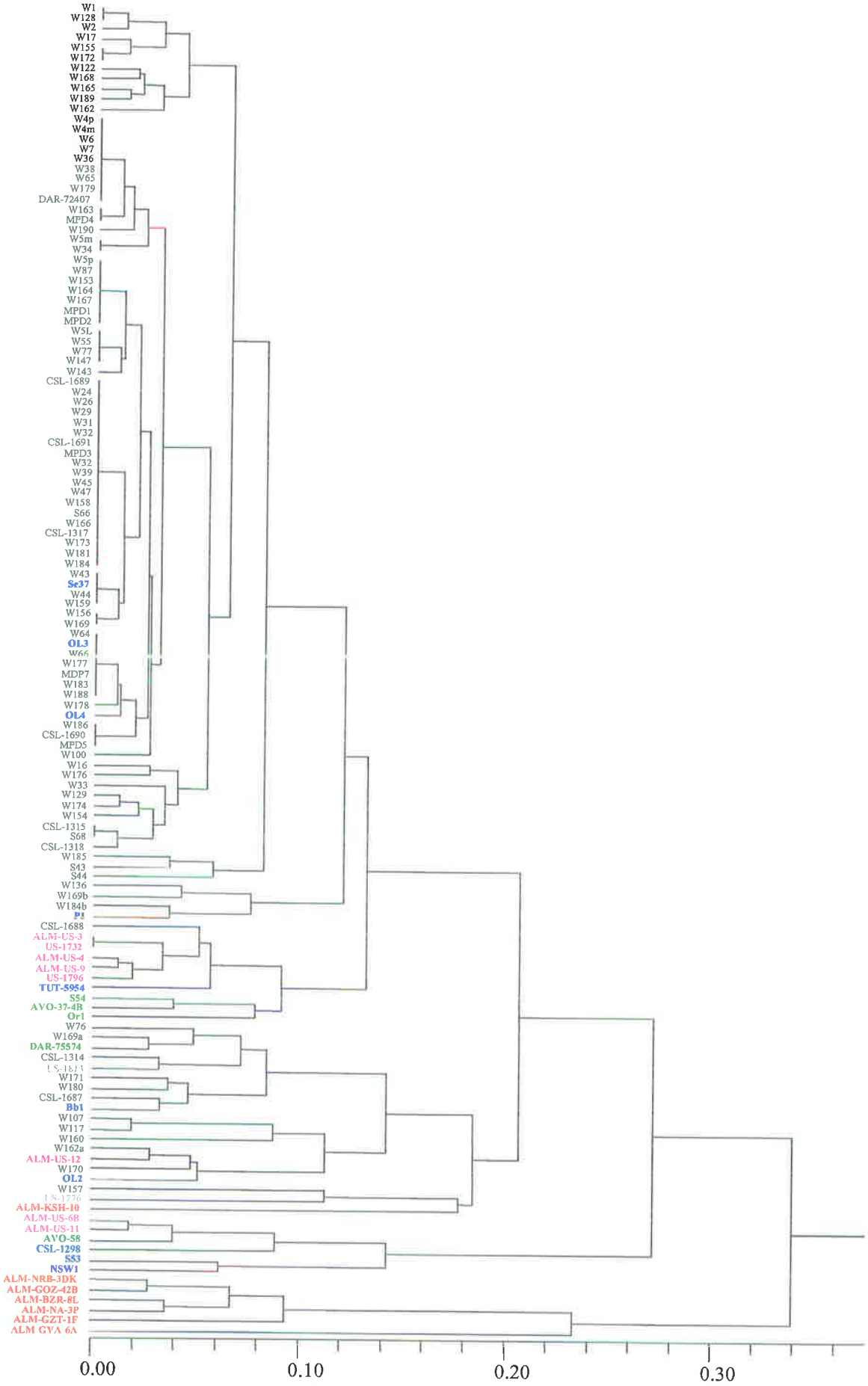

W1
W128
W2
W17
W155
W172
W122
W168
W165
W189
W162
W4p
W4m
W6
W7
W36
W38
W65
W179
DAR-72407
W163
MPD4
W190
W5m
W34
W5p
W87
W153
W164
W167
MPD1
MPD2
W5L
W55
W77
W147
W143
CSL-1689
W24
W26
W29
W31
W32
CSL-1691
MPD3
W32
W39
W45
W47
W158
S66
W166
CSL-1317
W173
W181
W184
W43
Se37
W44
W159
W156
W169
W64
OL3
W66
W177
MDP7
W183
W188
W178
OL4
W186
CSL-1690
MPD5
W100
W16
W176
W33
W129
W174
W154
CSL-1315
S68
CSL-1318
W185
S43
S44
W136
W169b
W184b
P1
CSL-1688
ALM-US-3
US-1732
ALM-US-4
ALM-US-9
US-1796
TUT-5954
S54
AVO-37-4B
Or1
W76
W169a
DAR-75574
CSL-1314
US-1811
W171
W180
CSL-1687
Bb1
W107
W117
W160
W162a
ALM-US-12
W170
OL2
W157
US-1776
ALM-KSH-10
ALM-US-6B
ALM-US-11
AVO-58
CSL-1298
S53
NSW1
ALM-NRB-3DK
ALM-GOZ-42B
ALM-BZR-8L
ALM-NA-3P
ALM-GZT-1F
ALM GVA 6A
0.00
0.10
0.20
0.30
Average taxonomic distance coefficient

Fig 4.3. Morphological similarity among 132 isolates of *Colletotrichum* spp. from almond in Australia, California and Israel, and other plants. Based on the max data set, nine morphological characters were scored, subjected to principal components analysis, then similarity was determined using the average taxonomic distance coefficient and UPGMA cluster analysis.
Isolates of *C. acutatum* from almond in Australia are shown as black text; *C. acutatum*, representative of the pink and grey subpopulations from almond in California are pink and grey text, respectively; *Colletotrichum* sp. from almond in Israel are red text; *C. acutatum* from other plants are blue and *C. gloeosporioides*, green.

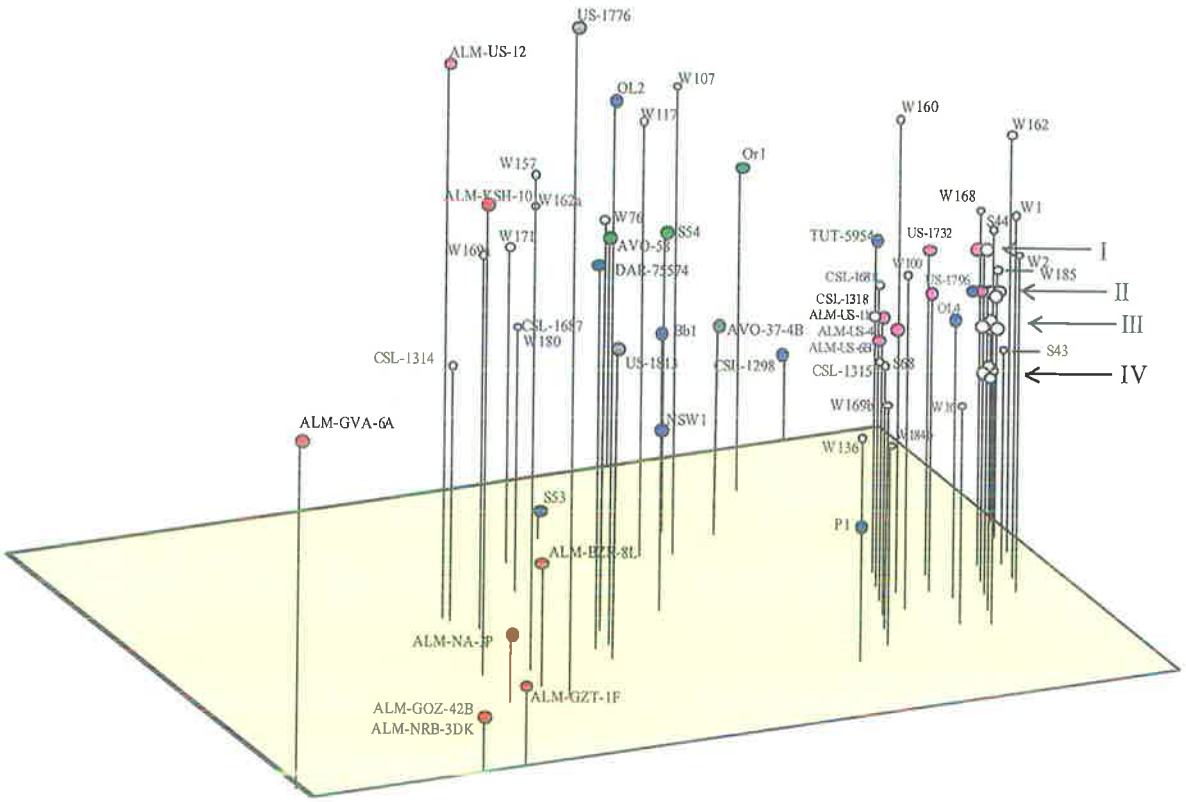


Fig 4.4 . Three-dimensional nonmetric multi-dimensional scaling (MDS) analysis of 132 isolates of *Colletotrichum* spp. from almond in Australia, California and Israel, and other plants, based on the min data set. Nine morphological characters (Table 4.1) were scored and subjected to principal components analysis, then data were clustered using MDS with NTSYSpc (v. 2.02k).

Isolates of *C. acutatum* from almond in Australia are shown as white data points; *C. acutatum* representative of the pink and grey subpopulations from almond in California are the pink and grey data points, respectively; *Colletotrichum* sp. from almond in Israel are red data points; *C. acutatum* from other plants are blue; *C. gloeosporioides* are green.

The following isolates are found in the groups 1-4, *viz.*: group I, ALM-US-3, W155 and W172; group II, ALM-US-9, CSL-1690, MPD5, MPD7, OL4, Se37, W44, W55, W64, W77, W86, W128, W147, W154, W174, W177, W183, W186 and W188; group III, CSL-1689, CSL-1317, CSL-1691, DAR-72407, MPD1, MPD2, MPD3, S66, W4m, W4p, W5p, W6, W7, W24, W26, W32, W32a, W33, W36, W38, W39, W45, W47, W65, W87, W129, W153, W156, W158, W164, W167, W169, W170, W173, W176, W178, W179, W181, W184 and W190; group IV, MPD4, W5m, W34 and W163.

clusters contained isolates that were identical, but most of these were not the same clusters as revealed by cluster analysis of the max or mod data set. Two isolates from the pink subpopulation in California, ALM-US-3 and ALM-US-9, were identical to isolates from almond in Australia. Two isolates from almond in Israel, ALM-NRB-3DK and ALM-GOZ-42B, were considered identical, and two from the pink subpopulation in California, ALM-US-6B and ALM-US-11, were identical. None of the isolates from Israel or from the grey subpopulation in California were identical to isolates from almond in Australia.

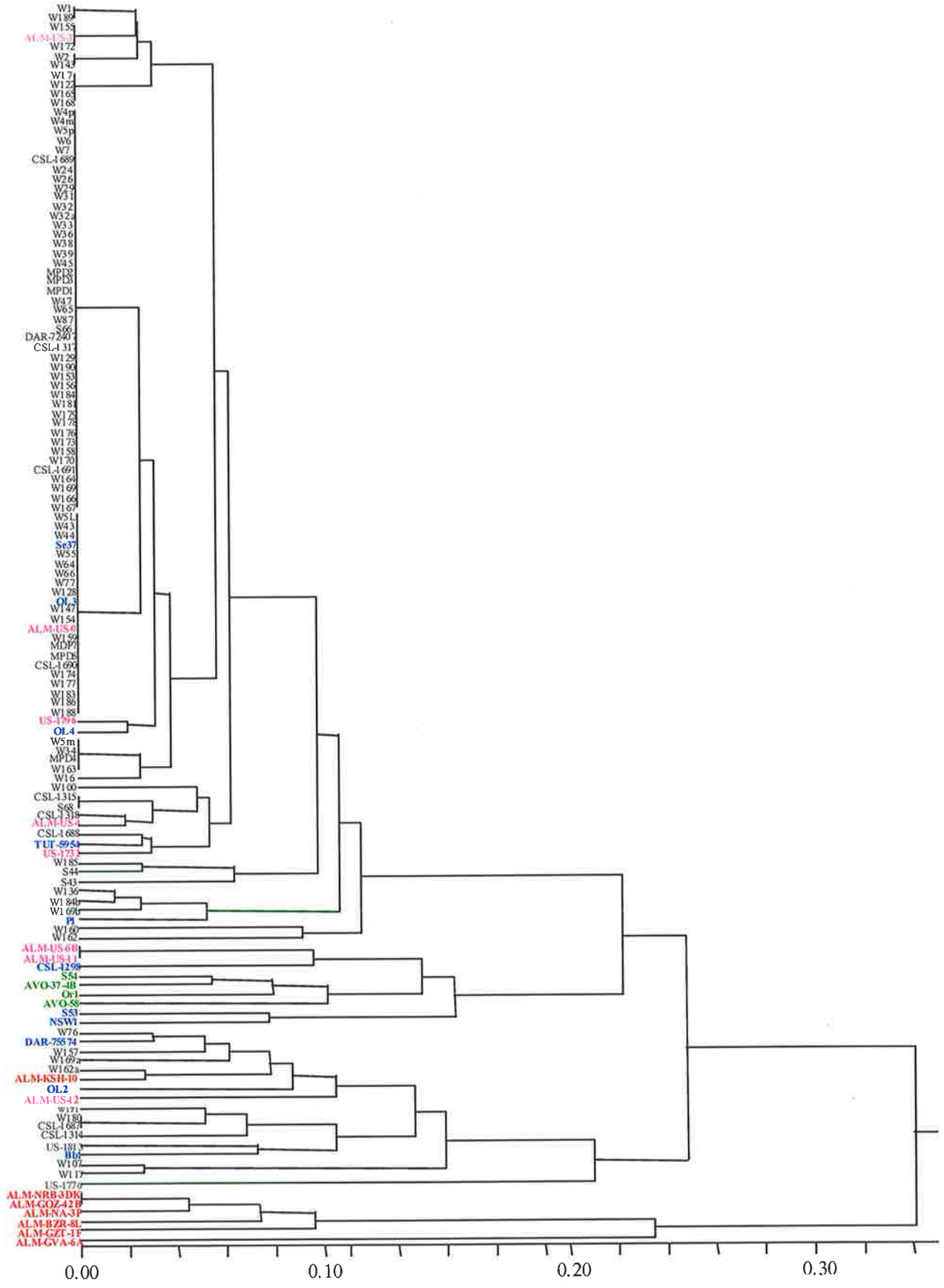
4.3.2 Growth rate at a range of temperatures

Mycelial growth rate (mm/day) at temperatures from 5 to 35°C was determined for selected isolates of *Colletotrichum* spp. from almond in Australia, California and Israel, and from plants other than almond in Australia. Figs 4.6 and 4.7 show the mycelial growth rate for representative isolates of *C. acutatum* from almond in Australia in this range.

The maximum mycelial growth rate for isolates of *C. acutatum* from almond in Australia generally occurred at 25°C (Fig 4.6A), however, there was significant variation among isolates at this temperature (Fig 4.7D). Isolate CSL-1318 differed from the other Australian isolates, in that the mycelial growth rate at 20°C was the same as at 25°C (Fig 4.6A). At 25°C, the mean mycelial growth rate for isolates of *C. acutatum* from almond in Australia was 3.5 mm/day and the range was 3.0 mm/day (W16 and CSL-1688) to 4.3 mm/day (CSL-1318) (Figs 4.6A and 4.7D).

The maximum mycelial growth rate for isolates representative of the pink and grey subpopulations of *C. acutatum* in California, US-1796 and US-1813, respectively, occurred at 25°C (Fig 4.6A) and there was no significant difference between the two isolates at this temperature (Fig 4.7D). The growth rates of isolate ALM-KSH-10, representative of

Fig 4.5. Morphological similarity among 132 isolates of *Colletotrichum* spp. from almond in Australia, California and Israel, and other plants. Based on the min data set, nine morphological characters were scored, subjected to principal components analysis, then similarity was determined using the average taxonomic distance coefficient and UPGMA cluster analysis.
Isolates of *C. acutatum* from almond in Australia are shown as black text; *C. acutatum*, representative of the pink and grey subpopulations from almond in California are pink and grey text, respectively; *Colletotrichum* sp. from almond in Israel are red text; *C. acutatum* from other plants are blue and *C. gloeosporioides*, green.


0.00
0.10
0.20
0.30
Average taxonomic distance coefficient

Colletotrichum sp. from almond in Israel, were 3.0 mm/day at 20°C and 3.1 mm/day at 25°C (Figs 4.6A and 4.7D), which were not significantly different from each other. The mycelial growth rates for US-1796, US-1813 and ALM-KSH-10 were not significantly different at 25°C, and they were within the range for isolates of *C. acutatum* from almond in Australia (Fig 4.7D).

Among isolates of *Colletotrichum* spp. from plants other than almond, the maximum growth rate for isolates from avocado (CSL-1298) and olive (OL3) occurred at 25°C (Fig 4.6B) and the mean mycelial growth rate was 3.8 mm/day and 3.5 mm/day, respectively, within the range for isolates of *C. acutatum* from almond. The maximum growth rate for isolates of *C. acutatum* obtained from strawberry (S53) and *C. gloeosporioides* from citrus (Or1 and S54) occurred at 30°C (Fig 4.6B). Isolates from pistachio (P1) and wine grape (DAR-75574) grew fastest at 25 and 30°C (Fig 4.6B).

There was significant variation in mycelial growth rate among isolates of *Colletotrichum* spp., and among isolates of *C. acutatum* from almond in Australia, at 10, 15, 20, 25, 30 and 35°C (Fig 4.7). Isolates of *C. gloeosporioides* from orange in Australia, S54 and Or1, had the fastest mycelial growth rate at 10 to 35°C (Fig 4.7), and this was statistically significant at 10, 25 and 30°C (Fig 4.7A, D and E). With respect to isolates of *C. acutatum* from almond in Australia, the only obvious trend was that isolate CSL-1318 consistently grew faster than the other isolates of *Colletotrichum* sp. from almond in Australia, California and Israel at 10 to 30°C. As the temperature decreased from 25°C to 10°C, mycelial growth rate of all isolates of *Colletotrichum* spp. tended to decrease, and no isolate grew at 5°C. However, after the completion of this experiment, the isolates that had been incubated at 5°C were returned to room temperature for several days, and all grew.

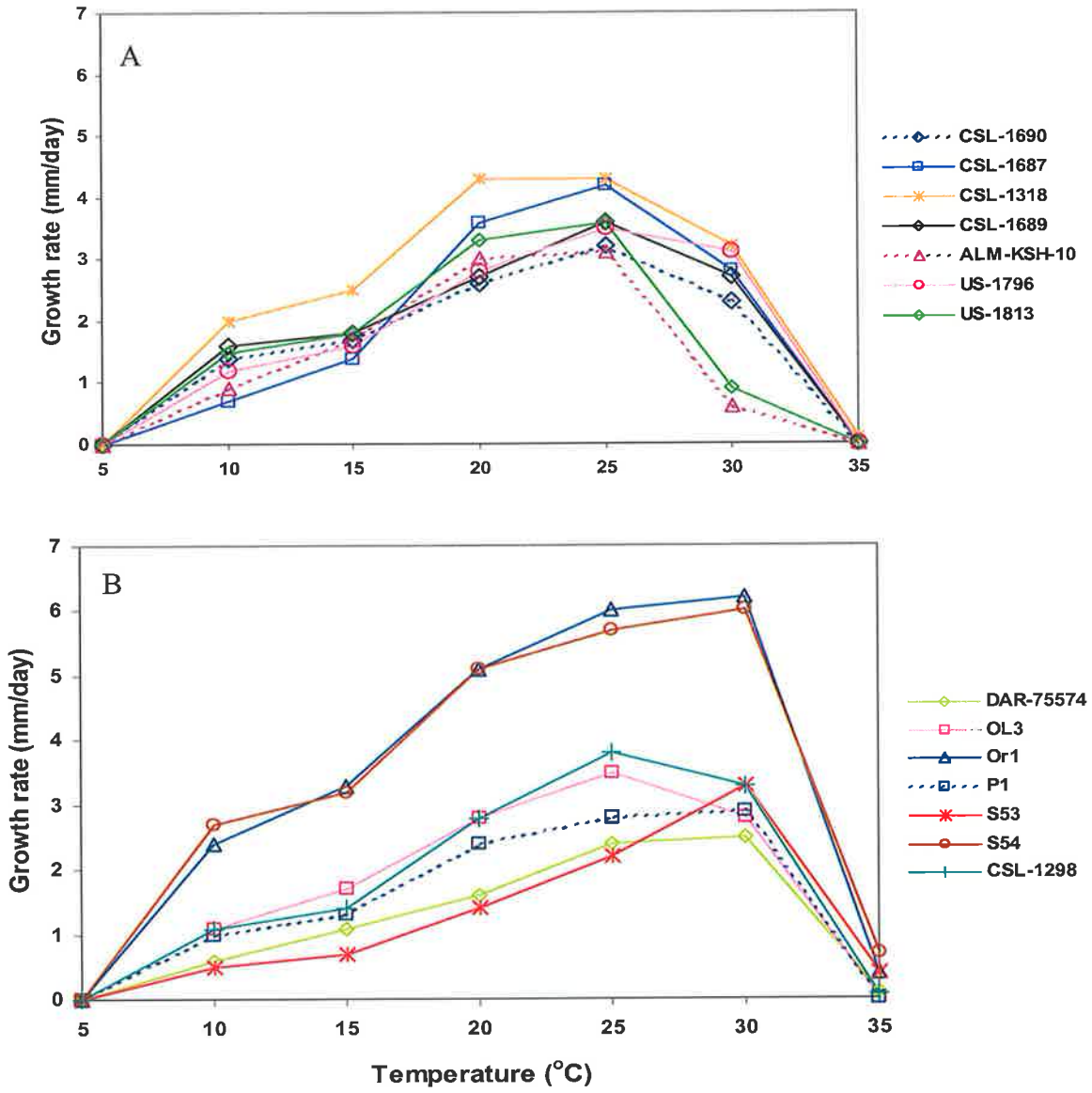


Fig 4.6. The mean mycelial growth rate (mm/day) of representative isolates of *Colletotrichum* spp. following incubation for 7 days in the dark at 5-35°C.
A. *C. acutatum* from almond in Australia, CSL-1687, CSL-1688, W153, CSL-1690, MPD1, S43, W16, CSL-1689, S44 and CSL-1318; *C. acutatum* from almond in California, US-1796 and US-1813; *Colletotrichum* sp. from almond in Israel, ALM-KSH-10;
B. *C. acutatum* from strawberry, S53, from wine grape, DAR-75574, from pistachio, P1, from olive, OL3 and from avocado, CSL-1298; *C. gloeosporioides* from orange, S54 and Or1.

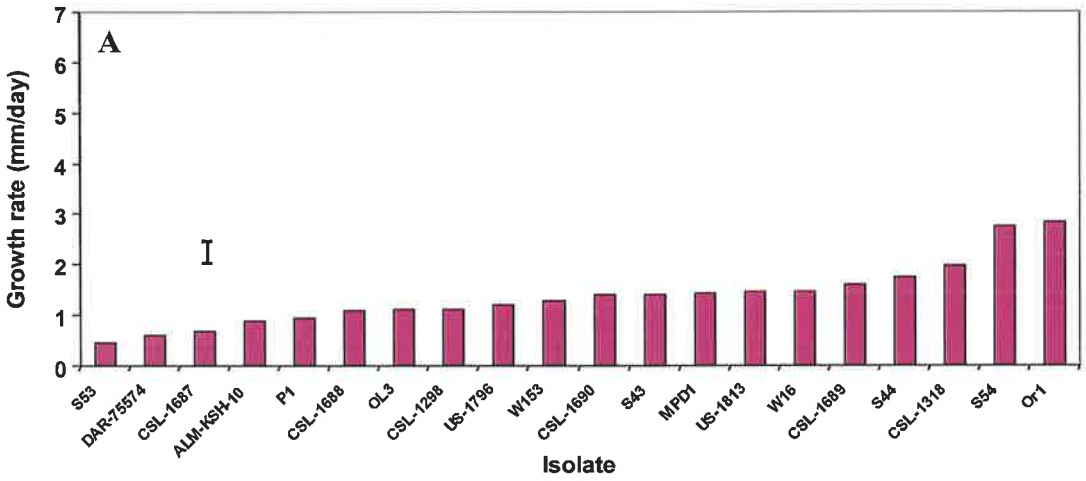
Although not statistically significant, the isolates from strawberry and wine grape, S53 and DAR-75574, respectively, grew more slowly than the other isolates at 10-25°C, however, but not at 30 or 35°C. At 30°C, the isolates from almond in Israel and from the grey subpopulation in California, ALM-KSH-10 and US-1813, respectively, grew more slowly than all of the other isolates (Fig 4.7E).

The only isolates of *C. acutatum* to grow at 35°C were those from strawberry (S53), avocado (CSL-1298), wine grape (DAR-75574) and almond (CSL-1318), as did *C. gloeosporioides* from orange in Australia (Or1 and S54) (Fig 4.7F). CSL-1318 was the only isolate of *C. acutatum* from almond in Australia to grow at 35°C and there was no growth from either of the isolates obtained from almond in California or Israel at this temperature. S54 grew significantly faster, 0.7 mm/day, than the other isolates and CSL-1298 was the slowest, 0.08 mm/day. Following the 7-day period, the isolates were placed at room temperature for several days and all, except for ALM-KSH-10 and US-1813, grew.

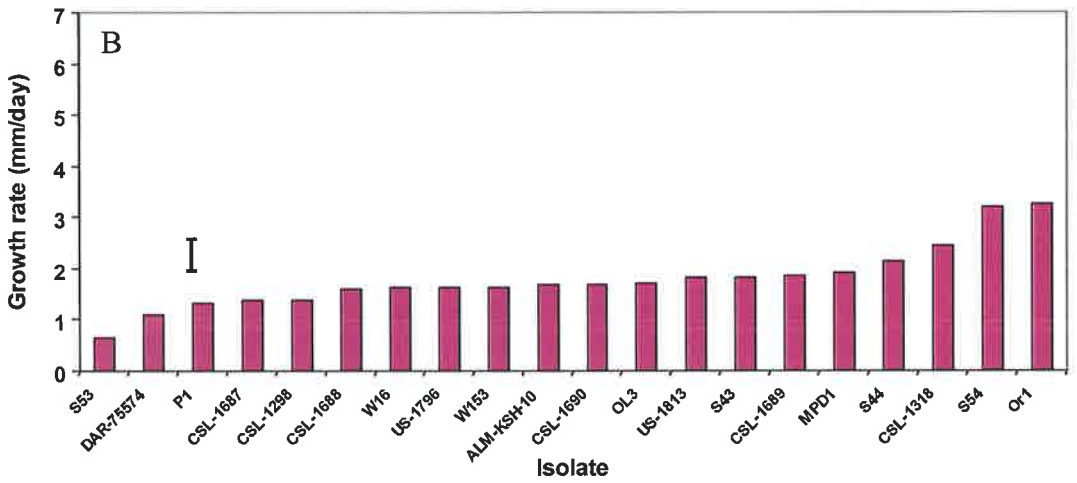
The mycelial growth rate for isolates from pistachio (P1), avocado (CSL-1298) and olive (OL3) was within the range of that for the isolates of *C. acutatum* from almond in Australia at 10 to 30°C (Fig 4.7).

4.4 Discussion

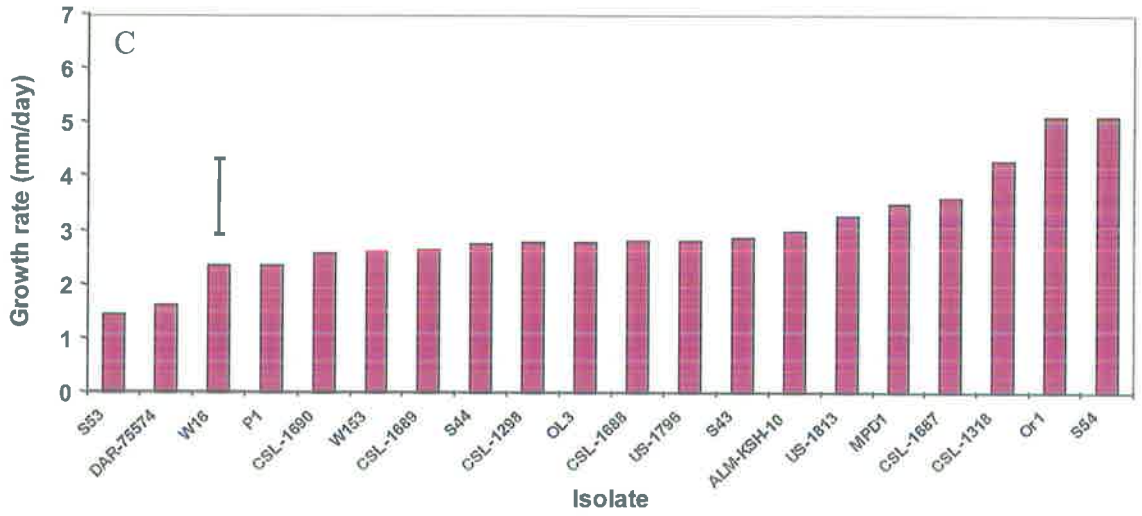
The purpose of the experiments described in this chapter was to characterise the isolates of *C. acutatum* from almond in Australia with respect to morphological and cultural characteristics, and compare them to isolates of *Colletotrichum* spp. from almond in California and Israel, as well as to those obtained from plants other than almond. Although there was considerable variation among isolates of *C. acutatum* from almond in Australia, based on the



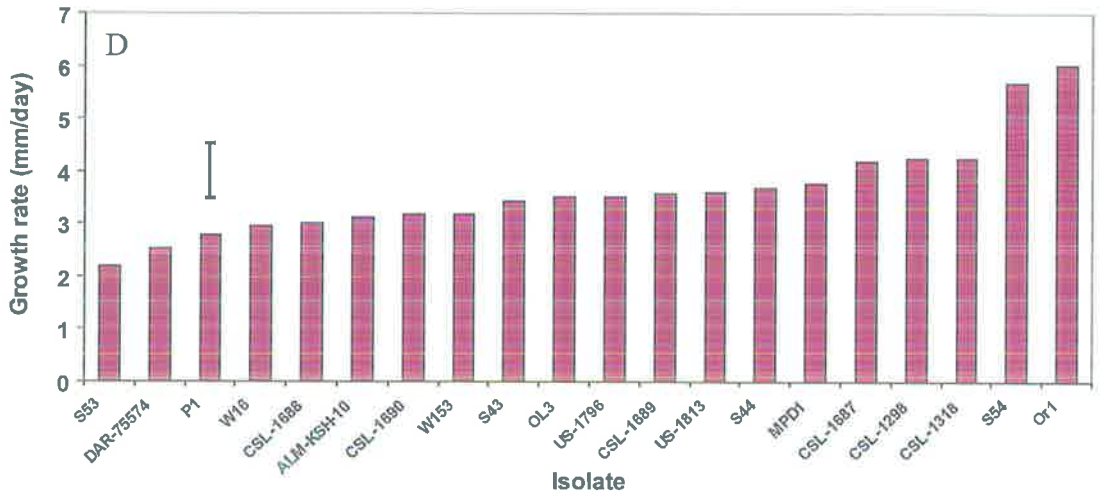
A. Mean mycelial growth rate at 10°C, bar represents Tukey's sd of 0.7.



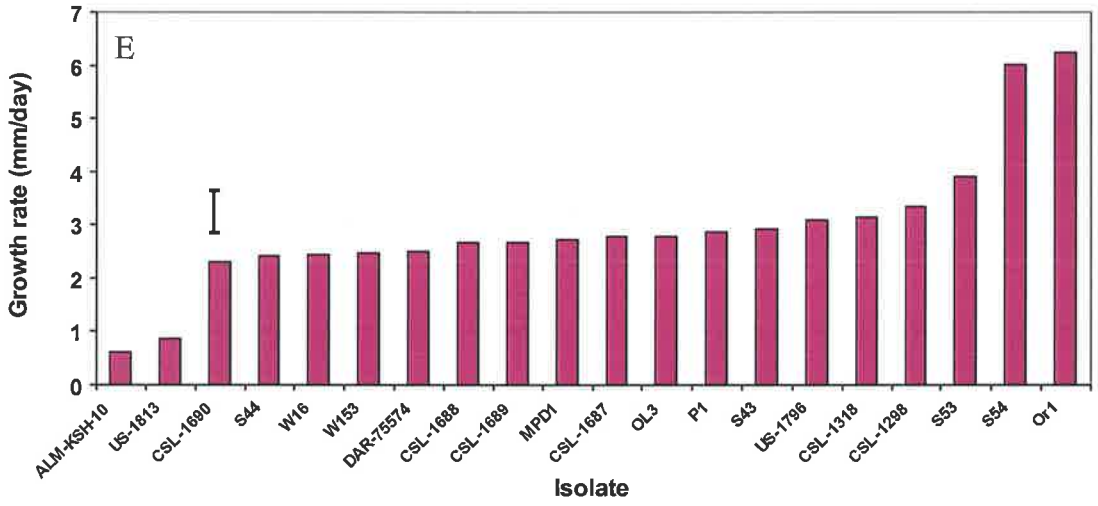
B. Mean mycelial growth rate at 15°C, bar represents Tukey's sd of 0.9.



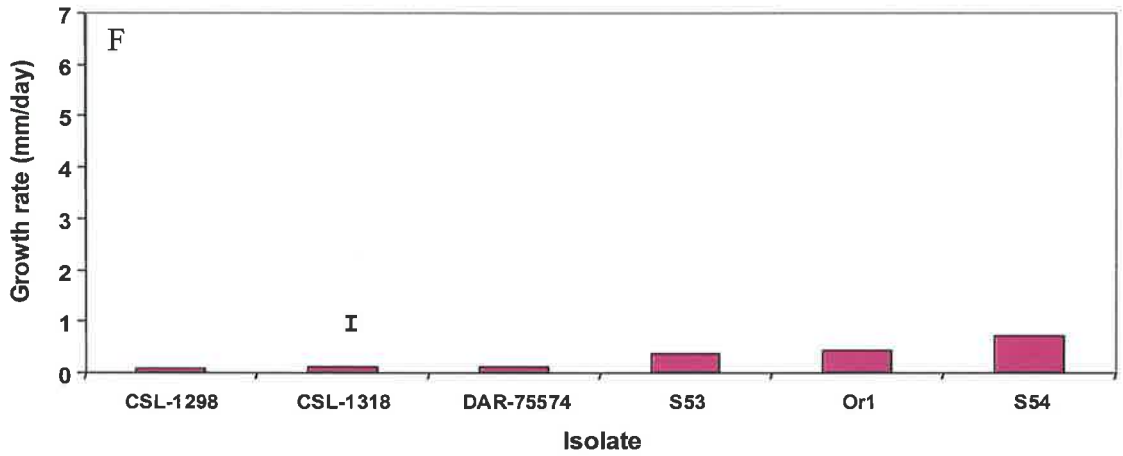
C. Mean mycelial growth rate at 20°C, bar represents Tukey's sd of 1.4.



D. Mean mycelial growth rate at 25°C, bar represents Tukey's sd of 1.1.



E. Mean mycelial growth rate at 30°C, bar represents Tukey's sd of 0.9.



F. Mean mycelial growth rate for isolates that grew at 35°C, bar represents Tukey's sd of 0.2.

Fig 4.7. The mean mycelial growth rate (mm/day) of representative isolates of *Colletotrichum* spp. after incubation for 7 days at 10-35°C. Error bars represent Tukey's significant difference (sd) at the 0.05% level. **A.** Mean mycelial growth rate at 10°C, **B.** Mean mycelial growth rate at 15°C, **C.** Mean mycelial growth rate at 20°C, **D.** Mean mycelial growth rate at 25°C, **E.** Mean mycelial growth rate at 30°C and **F.** Mean mycelial growth rate for those isolates at 35°C that grew.
Isolates of *C. acutatum* from almond in Australia are CSL-1687, CSL-1688, W153, CSL-1690, MPD1, S43, W16, CSL-1689, S44 and CSL-1318; *C. acutatum* from almond in California, US-1796 and US-1813; *Colletotrichum* sp. from almond in Israel, ALM-KSH-10; *C. acutatum* from strawberry, S53, from wine grape, DAR-75574, from pistachio, P1, from olive, OL3 and from avocado, CSL-1298; *C. gloeosporioides* from orange, S54 and Or1.

morphological characteristics examined and the analysis used, the isolates fitted the range of descriptors used for *C. acutatum* (Simmonds, 1965; Dyko and Mordue, 1979; Sutton, 1980; Gunnell and Gubler, 1992). Based on colony colour, three main subgroups or morphotypes were evident, in which isolates were either pink, orange or cream. The main morphological features of the majority of isolates of *C. acutatum* from almond in Australia comprised; bright pink colour of the culture from above and reverse, smooth and moderately dense mycelium, an abrupt and regular colony margin, maximum mycelial growth rate at 25°C, and conidia rounded at one end and pointed at the other. The range for length of conidia of *C. acutatum* from almond in Australia (4.24-21.2 μm) was larger than the descriptions of conidial size range for *C. acutatum* (Simmonds, 1965; Dyko and Mordue, 1979) (8-16 μm), however, Gunnell and Gubler (1992) reported that conidia of *C. acutatum* from strawberry were longer than 16 μm.

Several isolates differed from the above descriptors, in that the colour of the culture from above and reverse was bright orange. In general, the conidia of these isolates were more fusiform than those of other isolates of *C. acutatum* from almond in Australia; fusiform conidia are generally considered typical of *C. acutatum* (Simmonds, 1965; Gunnell and Gubler, 1992). Dyko and Mordue (1979) noted that colonies of *C. acutatum* can be pale orange and, recently, Denoyes-Rothan *et al.* (2003) and Jayasinghe and Fernando (2004) described isolates of *C. acutatum* from strawberry and ugurassa (a tropical fruit crop cultivated in Sri Lanka), respectively, as orange. Although cream or white colonies have been described for *C. acutatum* (Simmonds, 1965; Dyko and Mordue, 1979; Gunnell and Gubler, 1992), staling of isolates in culture and repeated subculturing can lead to changes in colony characters including colour and mycelial texture. This may be the case for several of the isolates placed in the cream group, although they had been passaged through strawberry (data not shown) and none had reverted to pink. However, several cream cultures exhibited pink

sectors and/or a concentric ring of pink pigment was observed from the underside of the culture plate, further demonstrating the morphological variability among cultures. In addition, several of these isolates were cream in culture even when derived directly from a single spore (Section 3.1) and had not been subjected to repeated subculturing.

Colony colour was the only distinguishing feature of these groups, as all of the other cultural and morphological characteristics examined, including mycelial growth rate, did not support clear differentiation into groups. Likewise, statistical analysis of morphological data using PCA, MDS or cluster analysis did not support these groups. Subgroups of *C. acutatum* and *C. gloeosporioides,* based on colour, have been described (Gunnell and Gubler, 1992; Liyange *et al.*, 1992; Förster and Adaskaveg, 1999; Abang *et al.*, 2002; Denoyes-Rothan *et al.*, 2003) and, in several cases, other features such as conidial morphology and growth rate supported the classification of these subgroups (Förster and Adaskaveg, 1999; Denoyes-Rothan *et al.*, 2003; Abang *et al.*, 2005).

The morphological and cultural characteristics of the isolates of *C. acutatum* from almond in Australia were more similar to those of the pink subpopulation of *C. acutatum* from almond in California, than to those of isolates from the grey subpopulation in California and Israel. This was particularly true for colony colour, mycelial characteristics, and spore shape and size. Although the predominant colony colour for Australian isolates of *C. acutatum* was bright pink, the isolates representative of the pink subpopulation in California were orange-pink. None of the isolates from almond in Australia was grey, however, it should not be discounted that genotypes of this colour exist.

The findings of this study with respect to the isolates of *C. acutatum* from almond in California were consistent with those of Förster and Adaskaveg (1999), in that the maximal mycelial growth for the pink and grey subpopulation of *C. acutatum* from almond in

California occurred at 25°C, the conidia of the grey isolates had less pointed ends than those of the pink isolates, the grey isolates grew significantly faster at 10°C and slower at 32°C than isolates from the pink subpopulation, and the isolates of the grey subpopulation sporulated less profusely on PDA than did those of the pink subpopulation. Here, the optimal temperature for mycelial growth for the representative isolate of *Colletotrichum* sp. from almond in Israel occurred at 20 and 25°C, which is similar to the findings of Freeman *et al.* (1998), who reported that the isolates of *Colletotrichum* sp. from almond in Israel grew fastest at 20-22°C. In the present study, the growth rates of the representative Israeli and Californian isolates were similar at their respective optimal temperatures (20 and 25°C). In contrast, Freeman *et al.* (1998) found that isolates of *Colletotrichum* sp. from Israel grew more slowly than isolates of *C. acutatum* from California at their respective optimal temperatures. However, it must be noted that, here, single isolates representative of each population were used in the mycelial growth rate experiments, whereas multiple isolates were used in the study of Förster and Adaskaveg (1999), whereas such details were not provided by Freeman *et al.* (1998). Although inferences can be made using small numbers of isolates, more isolates should be studied to assess the variability of mycelial growth rate within a population. In addition, care must be taken when making inferences about fungal growth in the field, as growth in culture may not necessarily reflect what occurs in nature.

The effect of culture medium and environmental conditions on morphological characteristics, particularly those considered useful for species delimitation, e.g., conidial shape, has been observed by several researchers (Smith and Black, 1990; Sutton, 1992; Gunnell and Gubler, 1992; Andersen and Thrane, 1996; Adaskaveg and Hartin, 1997; Kim *et al.* 2005). For example, Adaskaveg and Hartin (1997) found that conidia of *C. acutatum* were fusiform on pea straw agar and on almond fruit, whereas on PDA conidia were shorter and had forms intermediate between both ends rounded and both ends pointed. Such effects can lead to

taxonomic confusion (Sutton, 1992), and make it difficult to compare results from different laboratories. In addition, secondary conidia are produced directly from germinating primary conidia for many *Colletotrichum* spp. Secondary conidia are generally smaller and more variable in shape than primary conidia, making it difficult to differentiate among species of *Colletotrichum*, such as *C. acutatum*, *C. gloeosporioides* and *C. fragariae* (Buddie *et al.*, 1999). It was often difficult to categorise the conidial shape in this study, as many intermediate forms existed. Gunnell and Gubler (1992) reported that the conidial shape was uniform and consistent within *Colletotrichum* spp. only if a medium such as strawberry leaf agar (SLA) was used. The range of conidial forms found in this study confounded attempts to differentiate between *C. acutatum* and *C. gloeosporioides* or among groups of isolates of *C. acutatum*. Likewise, spore size did not provide a useful means to distinguish isolates at the species level or subspecies level as ranges overlapped, a finding also reported by Talhinhas *et al.* (2002).

Although the cultures used in this comparison were progeny from single spore-derived isolates (Section 3.1), variation with respect to colony colour and amount of sectoring had been observed among replicates of the parent single spore-derived isolates when first established (Section 3.1). Variation among cultures established from single spores and sectoring among cultures has been reported for isolates *Colletotrichum* spp. (Liyange *et al.*, 1992; Johnston and Jones, 1997; Kurame-Izioka *et al.*, 1997; Talhinhas *et al.*, 2002). The results of the present study support the findings of others, that delineation of species and subspecies should not rely on morphology alone (Sutton, 1992; Johnston and Jones, 1997; Martin and García-Figueres, 1999; Freeman *et al.*, 2000a). For examination of the morphology and cultural characteristics of fungi of the genus *Colletotrichum*, it would be useful to standardise protocols among laboratories, as recommended by Johnston and Jones (1997). In order that morphological and cultural characteristics be used to differentiate

groups reliably within the genus, Johnston and Jones (1997) recommend that cultures used for comparing isolates must be derived from a single conidium. This was not done here, due to time constraints. In addition, the use of a different culture media, e.g., SLA, may lead to different results. Such changes to protocols are recommended for future studies of the morphological variation of isolates of *C. acutatum* from almond in Australia. In addition, a larger sample of isolates from other plants is recommended to improve the understanding of the relationship among these isolates and those from almond.

The morphological features of isolates of *C. acutatum* from olive, pistachio and avocado were similar to those from almond in Australia and the pink subpopulation from California, and could not be distinguished from isolates of *C. acutatum* from almond in Australia based on the morphology and cultural characteristics examined in this study. Isolates from wine grape, blueberry, strawberry (from Australia and Israel) and one isolate from olive in Australia, differed from those isolates from almond in Australia, in that the colony colour was grey. However, apart from colony colour, there was overlap of the other morphological and cultural characteristics examined, making it difficult to distinguish isolates, although all of the isolates fitted the range of descriptors for *C. acutatum* (Simmonds, 1965; Dyko and Mordue, 1979; Sutton, 1992).

The isolates of *C. gloeosporioides* examined, from avocado in Israel and orange in Australia, could be distinguished from the isolates of *C. acutatum* from almond in Australia. The isolates of *C. gloeosporioides* were grey and, in general, the conidia were larger than conidia of *C. acutatum*. Conidial shape was consistent with that described for *C. gloeosporioides*, i.e., rounded at both ends (Simmonds, 1965; Mordue, 1971; Sutton, 1992). Results reported here for mycelial growth rate were consistent with the findings of others, such that *C. gloeosporioides* has a higher temperature for optimal mycelial growth (Simmonds, 1965) and

grows faster than *C. acutatum* (Smith and Black, 1990; Gunnell and Gubler, 1992; Sutton, 1992; Talhinhas *et al.*, 2002; Jayasinghe and Fernando, 2004). However, the morphology and cultural characteristics examined in this study, apart from optimal temperature and mycelial growth rate, were not sufficient to differentiate clearly the isolates of *C. gloeosporioides* and *C. acutatum*, which highlights the problems with species differentiation based on morphology alone. Testing the sensitivity of isolates to benomyl may have been a useful method to differentiate *C. acutatum* from *C. gloeosporioides*, as has been documented by a number of authors (Bernstein *et al.*, 1995; Adaskaveg and Hartin, 1997; Talhinhas *et al.*, 2002; Jayasinghe and Fernando, 2004), however, this was beyond the scope of this project.

There was no apparent link between geographical area, and morphological and cultural characteristics of the isolates of *C. acutatum* from almond in Australia. However, the majority of isolates were obtained from Willunga and the Northern Adelaide Plains region and several almond growing regions were represented by a single isolate, such as Nangiloc and Western Australia, and other important areas were not represented at all, such as the Riverland, Lindsay Point and Redcliffs. A survey of almond growing regions of South Australia, Victoria and New South Wales was carried out in October 2001 (Section 3.1) and plant material collected from these regions did not yield *C. acutatum*, nor was anthracnose observed on any trees in these areas at this time. To determine if isolates of *C. acutatum* from almond obtained from the almond growing regions of Australia differ with respect to morphology, a more comprehensive collection of isolates is required.

The results from PCA, MDS and cluster analysis of the max, mod and min data sets confirmed the general observations that there was considerable variation among the isolates of *Colletotrichum* spp. as well as among the isolates of *C. acutatum* from almond, with respect to morphological and cultural characteristics. Overall, it was considered that methods such as

PCA, MDS and clustering were not particularly useful, apart from confirming the variation noted during general observation of the cultures. This was due to: the subjective nature of the descriptors within each category; the fact that a change in the number of descriptors within a category led to different results; the difficulty of using a single adjective to describe a morphological character. For example, comparing the results from the analyses of the max and mod data sets highlights how small changes to the number of descriptors within one category, *viz.* mycelial texture, led to different results. The number of descriptors was different for half of the categories in the min *versus* the max data set, and the analyses revealed considerably different groups following MDS and cluster analyses. However, analyses of all three data sets did reveal, in general, that isolates of *Colletotrichum* sp. from almond in Israel differed considerably from *C. acutatum* from almond in Australia and California, but isolates of *C. gloeosporioides* from Australia and Israel were not clearly different on morphological grounds from isolates of *C. acutatum*. The aim of PCA is to obtain the minimum number of variables which account for the maximum variance in the data, and this usually involves a decrease in the number of variables, leading to simplification of a data set used in subsequent analyses. However, a decrease in the number of variables occurred for the mod data set only, suggesting that PCA, in general, did not simplify the analyses. However, further studies, using different numbers of adjectives in each category and other categories, e.g., appressorial shape, could be investigated in future.

In summary, there was considerable variation among isolates of *Colletotrichum* spp. and also among isolates of *C. acutatum* from almond with respect to morphological and cultural characteristics. Three main morphotypes were observed among isolates of *C. acutatum* from almond in Australia, namely pink, orange and cream colonies, the former being predominant. The majority of isolates of *C. acutatum* from almond in Australia were morphologically similar to those of the pink subpopulation of *C. acutatum* from almond in California and less

so to those from the grey subpopulation of *C. acutatum* from California and to the isolates of *Colletotrichum* sp. from Israel. Colour is a simple and natural grouping, especially for practical and applied plant pathology, even though the grouping of isolates into three morphotypes were not supported by analysis of other mycelial and reproductive characters. As stated by Sutton (1992), Johnston and Jones (1997) and Freeman *et al.* (2000a), morphology alone should not be relied upon to delineate species and subspecies of *Colletotrichum* spp., other approaches that may be taken include analysis of genetic variation using molecular biology techniques and pathogenicity tests. Such experiments are reported in Chapters 5 and 6, respectively.

Chapter 5 Genetic variation among *Colletotrichum* spp.

5.1 Introduction

Investigations into the taxonomy of the genus *Colletotrichum* are of interest to researchers because relationships among the species have not yet been resolved (Baxter *et al.*, 1985; Sutton, 1992; Guerber and Correll, 2001; Ureña-Padilla *et al.*, 2002; Guerber *et al.*, 2003). As discussed in Section 2.3, *C. acutatum* is considered a species complex, consisting of several subspecific groups (Johnston and Jones, 1997; Lardner *et al.*, 1999) including *C. acutatum sensu* Simmonds. Furthermore, Freeman *et al.* (2001) found evidence of subgroups within *C. acutatum sensu* Simmonds and that genetic variation existed within these subgroups.

Förster and Adaskaveg (1999) reported that the pink and grey subpopulations of *C. acutatum* from almond in California differed from one another genetically as well as morphologically but isolates within each subpopulation were genetically identical (see Section 2.5.3). Isolates representing each subgroup in California were also genetically distinct from isolates of *Colletotrichum* sp. from almond in Israel (Förster and Adaskaveg, 1999; Freeman *et al.*, 2001). Based on morphology, isolates of *Colletotrichum* sp. from almond in Israel had been identified as *C. gloeosporioides* (Freeman *et al.*, 1996) but, subsequently, using PCR with species-specific primers, they have been identified as *C. acutatum* (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a) (see Section 2.4.2). Isolates of *Colletotrichum* sp. from almond in Israel were shown to be genetically identical using a varicty of DNA-based molecular techniques suggesting a clonal population structure (see Section 2.5.3). Despite the uncertainty with respect to species delineation, Freeman *et al.* (2001) assigned isolates of *Colletotrichum* sp. from almond in Israel, as well as isolates of *C. acutatum* of the pink subpopulation from almond in California, to the *C. acutatum sensu* Simmonds group (see

Section 2.3). The genetic variability of isolates of *C. acutatum* from almond in Australia was not known prior to this study, nor was it known if the Australian isolates were genetically different from those in California and Israel. An understanding of the extent of genetic variation in the population of *C. acutatum* from almond in Australia is necessary for the selection and breeding of almond cultivars resistant to anthracnose, as resistance should be conferred to all variants of the pathogen (Bentley *et al.*, 1995).

As discussed in Section 2.3, the teleomorph of *C. acutatum, Glomerella acutata* (Guerber and Correll, 2001), has been described recently. However, the teleomorph has not been observed in cultures of *Colletotrichum* sp. from almond from California, Israel (Freeman *et al.*, 1998) or Australia. To date, the teleomorph of *C. acutatum* has not been found in nature, so it remains likely that *C. acutatum* from almond reproduces asexually and, this being the case, then little genetic diversity would be expected in *C. acutatum* from almond in Australia. Understanding genetic diversity, and the detection of genetic groups among populations of fungi, can provide information regarding the possible origin(s) of primary inoculum and host specificity (Ureña-Padilla *et al.*, 2002).

Genetic diversity can be investigated using PCR-based techniques, several of which are relatively simple, rapid and do not require prior knowledge of DNA sequences. ISSR-primed PCR (see Section 2.5.2.2) has been used widely to investigate intra-species diversity among populations of fungi, as reviewed by Bridge and Arora (1998), inlcuding *C. acutatum* from almond in California and Israel (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a). The aims of the experiments described in this chapter were to investigate genetic diversity among isolates of *C. acutatum* from almond in Australia and to compare these isolates to those from almond in California and Israel.

5.2 Materials and methods

5.2.1 PCR amplification

DNA was extracted and purified from the 132 isolates listed in Table 3.2, as described in Section 3.3.1. The isolates used were as described in 4.2.1.1, with the addition of an isolate of *Eutypa lata* from grapevine, M280, which was used as an outgroup. Isolates W42, W77 and W188 did not produce PCR products, therefore, were not included in the final analysis. The primers R1 (Weining and Langridge, 1991), M13 (Stenlid *et al.*, 1994) and ten ISSR primers were first screened with genomic DNA of isolates CSL-1690, ALM-NRB-3DK, CSL-1691, P1, DAR-72407, CSL-1689 and US-1776 (Table 3.2). The ISSR primers were $(CAG)_5$ (Rodriguez and Yoder, 1991), $(GACA)_4$ (Weising *et al.*, 1989), $(GTG)_5$, $(GACAC)_3$ (Freeman *et al.*, 1993), $(CAC)_5$, $(GATA)_4$, $(TGTC)_4$ (Freeman *et al.*, 2000a), $(GAA)_5$, $(GGAT)_4$ (Stummer *et al.*, 2000) and $(TCT)_5$. Based on production of clear and reproducible banding patterns, six primers, namely $(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(GACAC)_3$, $(CAC)_5$ and $(GGAT)_4$, were selected for use in further experiments. The sequences of these primers are given in Table 5.1.

Table 5.1. The nucleotide sequences of the inter-simple sequence repeat primers selected to assess genetic variation among 132 isolates of *Colletotrichum* spp. from Australia, California and Israel.

Primer	Nucleotide sequence (5'-3')
$(CAG)_5$	CAGCAGCAGCAGCAG
$(GACA)_4$	GACAGACAGACAGACA
$(GTG)_5$	GTGGTGGTGGTGGTG
$(GACAC)_3$	GACACGACACGACAC
$(CAC)_5$	CACCACCACCACCAC
$(GGAT)_4$	GGATGGATGGATGGAT

PCR amplification was performed in a total volume of 20 μL, comprising 5 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Promega, USA) and 1 μM primer. The DNA was amplified in a thermocycler (PTC-100, MJ Research, Watertown, MA), starting with 5 min of denaturation at 95°C, followed by 30 cycles consisting of 30 sec at 95°C, 30 sec at either 60°C [for $(CAG)_5$,$(GTG)_5$, and $(CAC)_5$] or 48°C [for $(GACA)_4$, $(GGAT)_4$, and $(GACAC)_3$] and 1.5 min at 72°C (Freeman *et al.*, 2000a). Amplification products were separated in agarose gels (1.5% w/v) in 0.5 x TBE buffer, subjected to electrophoresis at 80 V for 2.5 h. Gels were stained, viewed and photographed as described in Section 3.3.1. Amplification products were compared to a 100-bp ladder (Geneworks, Australia).

For each PCR, a negative control containing all reagents, and with sterile dd H_2O instead of DNA, was included. Two amplifications of each isolate were performed and bands compared. If the fingerprints were not identical, a third amplification was performed. In that case, only bands that were reproducible across the three repetitions were used for further analysis. PCR products with a molecular weight of approximately 180 to 1300 bp were included in the analysis.

Bands were scored as present (1) or absent (0) and a binary data matrix generated. Using the binary matrix, similarity among isolates was determined using the simple matching coefficient, resulting in a similarity matrix. The similarity matrix was clustered by UPGMA, using the SAHN program, generating a phenogram (see Section 4.2.1.2). A cophenetic coefficient was calculated to determine "goodness of fit" of the cluster analysis to the data as described in Section 4.2.1.2. Data were analysed using two- and three-dimensional nonmetric MDS and stress statistics were calculated (see Section 4.2.1.2). All analysis was carried out

using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System, version 2.02k, Exeter Software, Setauket, New York).

5.3 Results

5.3.1 Genetic variation of *C. acutatum*

A total of 71 clear and reproducible bands was produced following PCR with the six selected ISSR primers, and the primers amplified between 18 and 31 bands for all isolates, without monomorphic bands. Fig 5.1 shows representative banding patterns obtained following PCR amplification with primer $(GTG)_5$.

Cluster analysis, using UPGMA, revealed several groups among the collection as depicted in the phenogram (Fig 5.2). The cophenetic coefficient was 0.99, which suggested that the clusters formed by the phenogram were highly likely real, not random. There were four clusters of *Colletotrichum* sp. from almond, and each cluster contained isolates that shared 100% similarity; these are subsequently referred to as groups one, two, three and four. Group one contained all of the isolates from almond in Australia, except for CSL-1318. Isolate CSL-1318 from almond was most similar (99%) to an isolate from avocado in Australia, (CSL-1298), and, these two isolates shared 79% similarity with those from group one. Group one also included isolate P1 from pistachio in Australia as well as three isolates from olive in Australia, Se37, OL3 and OL4. Group two contained all isolates from the pink subpopulation from almond in California. This group was 79% similar to the isolates from Australia assigned to group one. Group three contained isolates from almond in Israel. Group four contained both isolates from the grey subpopulation of *C. acutatum* in California. Groups three and four were most similar to one another, and they were 77% similar to the isolates

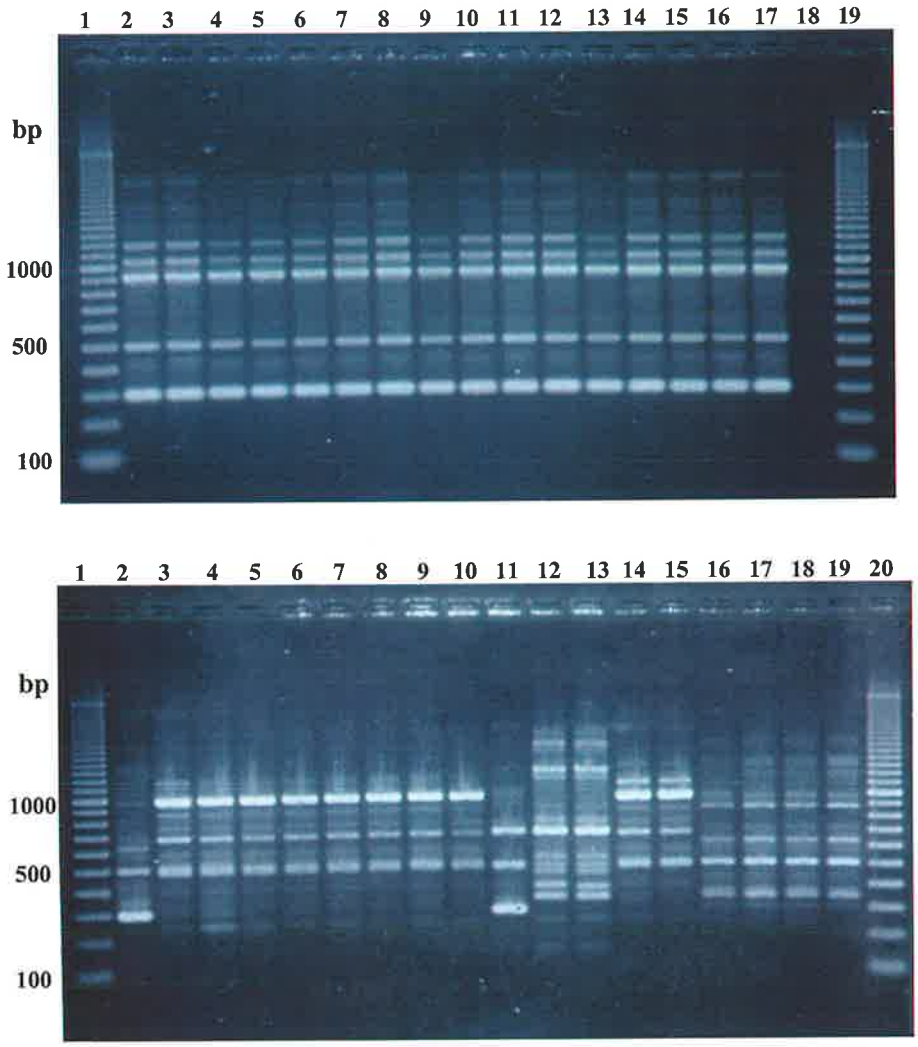
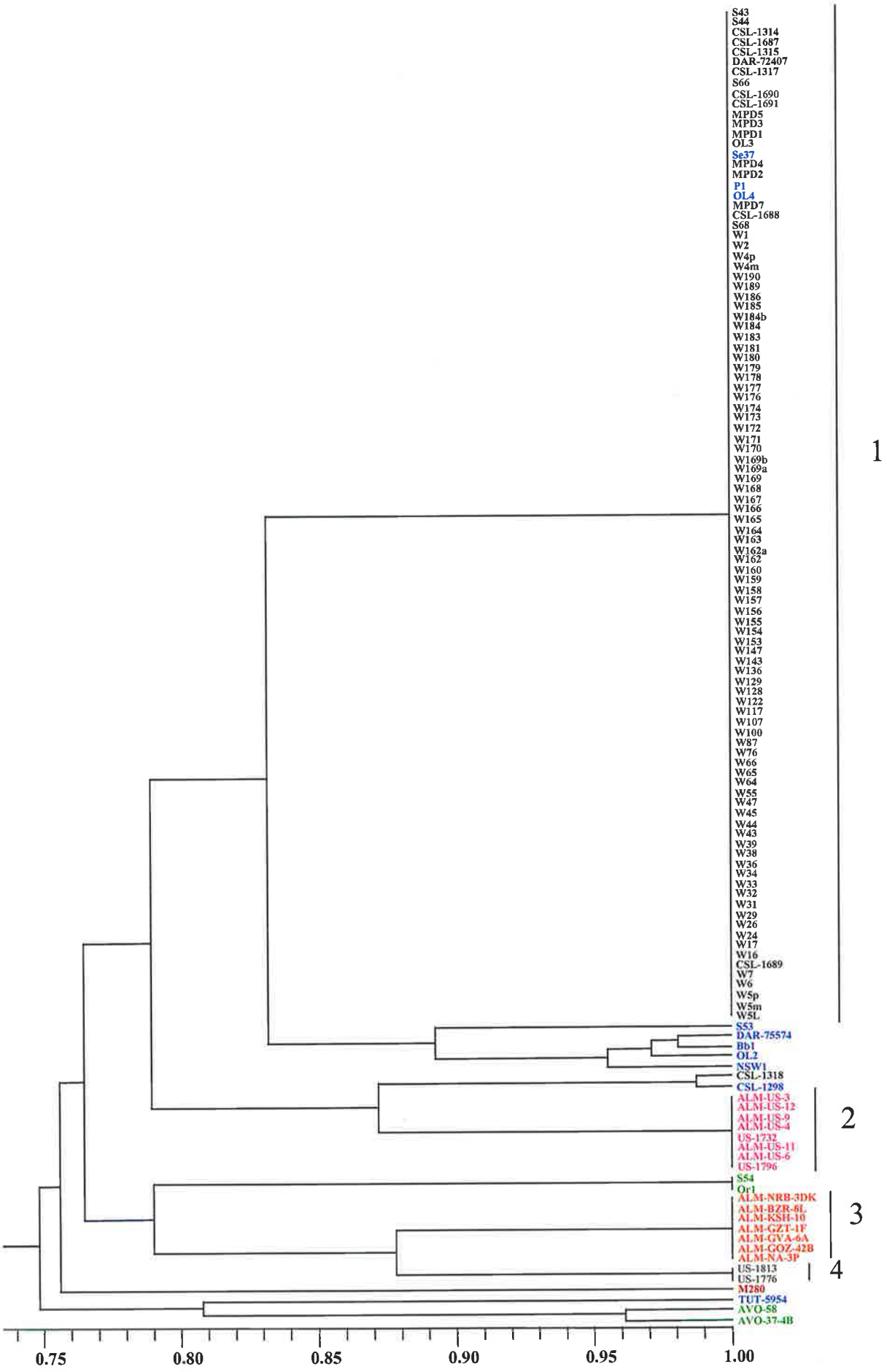


Fig 5.1. DNA banding patterns of 34 isolates of *Colletotrichum* spp. obtained following PCR with the inter-simple sequence repeat primer $(GTG)_5$.
A. Lanes 1 and 19, 100-bp ladder; lanes 2-17, *C. acutatum* from almond in Australia (CSL-1690, W1, W2, W4p, W4m, W5m, W5p, W5L, W6m, W7p, W8, W16, W17, W26, W29); lane 18, dd H_2O.
B. Lanes 1 and 20, 100-bp ladder; lane 2, *C. acutatum* from avocado in Australia (CSL-1298); lanes 3-10, *Colletotrichum* sp. from almond in Israel (ALM-NRB-3DK, ALM-BZR-8L, ALM-KSH-10, ALM-GZT-1F, ALM-GOZ-42B, ALM-GOZ-42B, ALM-NA-7Q); lane 11, *C. acutatum* from strawberry in Israel (TUT-5954); lane 12 and 13, *C. gloeosporioides* from avocado in Israel (AVO-58 and AVO-37-4B); lanes 14-19, *C. acutatum* from almond in California (US-1776 and US-1813, representative of the grey subpopulation, and ALM-US-3, ALM-US-4, ALM-US-6B and ALM-US-9, representative of the pink subpopulation).

Fig 5.2. Genetic similarity among 129 isolates of *Colletotrichum* spp. from almond and other plants following PCR with inter-simple sequence repeat primers. Similarity was determined using the simple matching coefficient and UPGMA cluster analysis. Four groups in which isolates were identical to one another are numbered 1-4.
Isolates of *C. acutatum* from almond in Australia are black text; *C. acutatum*, representative of the pink and grey subpopulations from almond in California, are pink and grey text, respectively; Colletotrichum sp. from almond in Israel are red text; *C. acutatum* from other plants are blue and *C. gloeosporioides*, green.


S43
S44
CSL-1314
CSL-1687
CSL-1315
DAR-72407
CSL-1317
S66
CSL-1690
CSL-1691
MPD5
MPD3
MPD1
OL3
Se37
MPD4
MPD2
P1
OL4
MPD7
CSL-1688
S68
W1
W2
W4p
W4m
W190
W189
W186
W185
W184b
W184
W183
W181
W180
W179
W178
W177
W176
W174
W173
W172
W171
W170
W169b
W169a
W169
W168
W167
W166
W165
W164
W163
W162a
W162
W160
W159
W158
W157
W156
W155
W154
W153
W147
W143
W136
W129
W128
W122
W117
W107
W100
W87
W76
W66
W65
W64
W55
W47
W45
W44
W43
W39
W38
W36
W34
W33
W32
W31
W29
W26
W24
W17
W16
CSL-1689
W7
W6
W5p
W5m
W5L
S53
DAR-75574
Bb1
OL2
NSW1
CSL-1318
CSL-1298
ALM-US-3
ALM-US-12
ALM-US-9
ALM-US-4
US-1732
ALM-US-11
ALM-US-6
US-1796
S54
Or1
ALM-NRB-3DK
ALM-BZR-6L
ALM-KSH-10
ALM-GZT-1F
ALM-GVA-6A
ALM-GOZ-42B
ALM-NA-3P
US-1813
US-1776
M280
TUT-5954
AVO-58
AVO-37-4B
1
2
3
4
0.75
0.80
0.85
0.90
0.95
1.00
Coefficient of similarity

from almond in Australia in group one. The two isolates of *C. gloeosporioides* from orange in Australia were identical, and were most similar to isolates in groups three and four.

Isolates of *C. acutatum* from several plants other than almond, including strawberry, olive, wine grape and blueberry from Australia, formed a cluster in which isolates were 89-98% similar to each other and, as a group, were most similar (83%) to *C. acutatum* isolates from almond in Australia (group one). Both isolates of *C. gloeosporioides* from avocado from Israel (AVO-58 and AVO-37-4B) were 96% similar to each other and most similar, 81%, to an isolate of *C. acutatum* from strawberry (TUT-5954) from Israel. These three isolates from Israel were the least similar, 75%, to all of the other isolates tested in this study, including *E. lata*, isolate M280.

Two-dimensional MDS analysis was carried out and the stress value was 0.24, indicating that the goodness of fit was fair to poor, therefore the analysis is not presented.

The results of the three-dimensional MDS (Fig 5.3) were similar to those following UPGMA clustering, and the four groups described previously were evident. Isolates from almond in Australia (group one) formed a distinct group, separate from the isolates of *C. acutatum* from plants other than almond in Australia. Groups three and four were shown as separate groups. The outliers were again evident. The stress value for the three-dimensional analysis was 0.11, which indicated that the plot of the distances among the clusters of the isolates was a good fit to the original similarity matrix, leading to the conclusion that the three-dimensional MDS analysis was a better representation of the data than the two-dimensional MDS analysis.

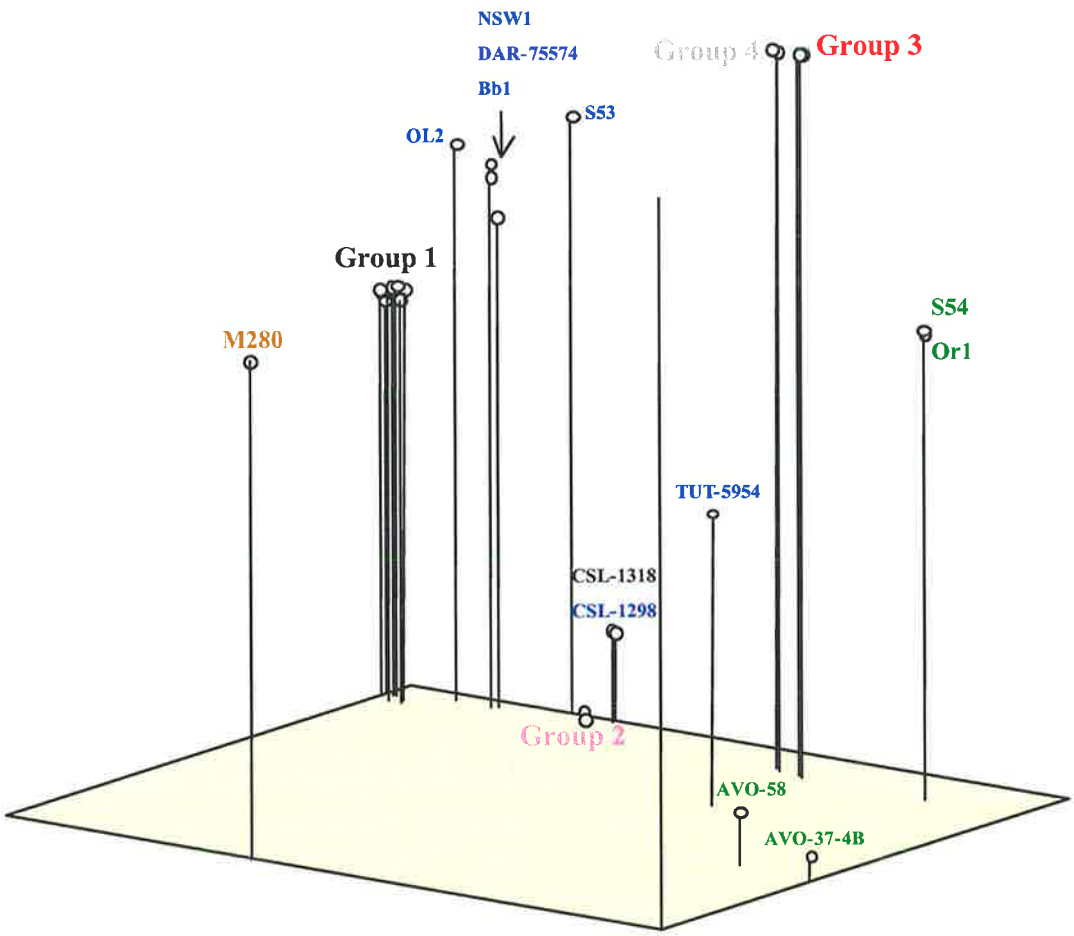


Fig 5.3. Three-dimensional nonmetric multi-dimensional scaling analysis of 129 isolates of *Colletotrichum* spp. from almond in Australia, California and Israel and, other plants. Data were derived following PCR with inter-simple sequence repeat primers and analysed with NTSYSpc (v. 2.02k).
Isolates of *C. acutatum* from almond in Australia are shown in black (Group 1 and CSL-1318); *C. acutatum* representative, of the pink and grey subpopulations from almond in California are pink and grey, respectively (Groups 2 and 4); *Colletotrichum* sp. from almond in Israel are red (Group 3); *C. acutatum* from other plants are blue; *C. gloeosporioides* are green; *E. lata*, M280, is brown.

5.4 Discussion

With the exception of isolate CSL-1318, there was no genetic diversity evident amongst the isolates of *C. acutatum* from almond in Australia. According to Lu *et al.* (2004), isolates that show more than 90% similarity to one another following ISSR- and RAPD-PCR share genomes that are "at least near-identical". Therefore, the population of *C. acutatum* from almond in Australia was most likely to be clonal. These results may also suggest that there may have been a single introduction of *C. acutatum* onto almond in Australia and that selection pressure for change has remained low, resulting in little genetic diversity (Hodson *et al.*, 1993). The use of UPGMA cluster analysis showed the isolates that shared 100% similarity with one another and is useful to depict groups among isolates. However, differences in band patterns do not necessarily indicate that significant genetic divergence has occurred (Cannon *et al.*, 2000), so care must be taken making if conclusions about genetic relationships and phylogeny from ISSR-PCR results. UPGMA analysis forces the data into two dimensions and connects isolates with lines, which makes isolates appear connected to one another, e.g., *E. lata*, isolate M280, is depicted as more similar to all of the isolates of *C. acutatum* than some of the isolates of the closely related species, *C. gloeosporioides*. The three-dimensional MDS analysis also clearly depicts the groups in which isolates are identical, but no connection is inferred among isolates, which makes it difficult to tell how similar they are to one another, yet it is easy to observe the outliers, such as M280. Such analyses were useful and easy to interpret for binary data produced by ISSR-PCR, in contrast to the UPGMA cluster analysis and the MDS ordination for the morphological data.

Isolate CSL-1318, obtained from the Angle Vale region of South Australia, differed from other isolates of *C. acutatum* obtained from almond in Australia, and was most similar to CSL-1298, an isolate of *C. acutatum* from avocado, originating from an unknown region in

South Australia. This result may suggest that the two isolates were closely related and may have diverged from a common ancestor. The slight variation in ISSR-PCR banding patterns could reflect exchange of genetic material following hyphal fusion and mitotic recombination, however, testing this hypothesis would require further research, using methods such as vegetative compatibility group (VCG) analysis. Alternatively, such variation may have arisen from mutation, or insertion and deletion events. It is possible that infected plant material from avocado in another region, e.g., the Riverland, was transferred to the Angle Vale region, and *C. acutatum* spread from the avocado to the almond. Orchard to orchard spread in the Angle Vale region was unlikely because avocados are not grown commercially there. However, given that isolates of *C. acutatum* from almond with the same DNA fingerprint as CSL-1318 have not been recovered during the course of this study, it could be that this phenotype failed to establish widely on almond or it was overlooked when recovery of *C. acutatum* from plant material was undertaken. The genetic diversity of *C. acutatum* on avocado in Australia is unknown, and such knowledge would be useful to elucidate the relationship between *C. acutatum* from almond and avocado.

It is possible that two introductions of *C. acutatum* on almond into Australia occurred. One phenotype may have spread to most of the major almond growing regions and become established, whereas the other was either introduced at a later date and has not yet spread to other almond growing regions or has failed to establish in any area apart from Angle Vale. Alternatively, the difference between DNA fingerprints of isolate CSL-1318 and the other isolates of *C. acutatum* from almond in Australia may be a result of mutation in the predominant phenotype of *C. acutatum* in Australia or *Colletotrichum* sp. on avocado.

Isolates from almond in Australia were genetically different from those in California and Israel. The pink and grey subpopulations of *C. acutatum* from almond in California were

different from one another, yet genetically homogeneous within each subpopulation, which is consistent with the findings of Förster and Adaskaveg (1999) and Freeman *et al.* (2000a). This study also showed that the isolates from the pink and grey subpopulations from almond in California differed from the isolates of *Colletotrichum* sp. from almond in Israel. Freeman *et al.* (2000a) found that the Israeli isolates differed from those of the pink subpopulation in California, however, they did not have access to the isolates from the grey subpopulation in California at the time of their study (S. Freeman, pers. comm., 2003). Förster and Adaskaveg (1999) reported that the grey isolates from almond in California had banding patterns 'almost identical' to those of the isolates from Israel based on RAPD-PCR. However, in the present study the isolates from Israel and those from the grey subpopulation in California were distinct, but isolates from each group were genetically more similar to each other than to isolates from either Australia or from the pink subpopulation in California.

Although homogeneity was found among the majority of isolates of *C. acutatum* from almond in Australia following ISSR-primed PCR, co-migrating bands of the same molecular weight do not necessarily share sequence homology (Ouellet and Seifert, 1993). It is possible that genetic diversity may be revealed using other molecular approaches, as was found by Arenal *et al.* (1999) who used several techniques which detected different levels of genetic similarity among isolates of *Eppicoccum nigrum*. Several groups, in which isolates were genetically homogeneous following ISSR-primed PCR or RAPD-PCR, have been identified among isolates of *C. acutatum* from almond (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a), strawberry (Freeman *et al.*, 2000b; Denoyes-Rothan *et al.*, 2003), lupin (Talhinhas *et al.*, 2002), anemone (Freeman *et al.*, 2000b) and tamarillo (Afanador-Kafuri *et al.*, 2003). Analysis of the isolates within these groups using other molecular approaches, including A + T-rich-DNA analysis (Freeman *et al.*, 2000a; Afanador-Kafuri *et al.*, 2003), restriction

enzyme digestion of rDNA (Förster and Adaskaveg, 1999; Freeman *et al.*, 2000a), analysis of patterns of nuclear hybridisation of restriction enzyme-digested nuclear DNA (Freeman *et al.*, 2000a), sequence analysis of the ITS region (Freeman *et al.*, 2000a; Talhinhas *et al.*, 2002; Denoyes-Rothan *et al.*, 2003; Afanador-Kafuri *et al.*, 2003), sequencing of ß-tubulin 2 and histone 4 genes (Talhinhas *et al.*, 2002) and AFLP analysis (Talhinhas *et al.*, 2002), supported the findings of genetic homogeneity within each group. In contrast, the genetically homogeneous group containing isolates of *C. acutatum* from strawberry and anemone was differentiated into two groups following VCG analysis (Freeman *et al.*, 2000b), showing that the VCG approach can elucidate differences amongst isolates both between and within populations that have apparent genotypic homogeneity (Freeman *et al.*, 2000b). Sequencing of the ITS regions of representative isolates of *C. acutatum* from almond in Australia is being carried out by Dr S. Freeman and colleagues at The Volcani Center, Bet Dagan, Israel, but results were not available at the time of writing.

Three isolates of *C. acutatum* from olive and one from pistachio had DNA fingerprints identical to isolates of *C. acutatum* from almond in Australia (excluding CSL-1318). Given that anthracnose, caused by *C. acutatum*, on pistachio and olive was reported in Australia as recently as 2000 (Ash and Lanoiselet, 2001) and 2001 (A. Watson, pers. comm., 2002) respectively, whereas anthracnose was first recorded on almond in Australia in 1996 (Hall *et al.*, 1998), it is more likely that *C. acutatum* spread to pistachio and olive from almond than *vice versa*. Single isolates from strawberry, wine grape and blueberry, and two isolates from olive yielded different DNA fingerprints, but were genetically more similar to one another than to other isolates of *C. acutatum*. However, the genetic diversity of populations of *C. acutatum* infecting strawberry, avocado, pistachio, wine grape and blueberry in Australia is not known. More isolates from these plants should be collected in the future, and experiments

conducted to investigate genetic diversity within and among these populations, further clarifying the relationships among isolates of *C. acutatum sensu* Simmonds.

The two isolates of *C. gloeosporioides* from orange in Australia were genetically identical in terms of DNA fingerprints following ISSR-primed PCR. Although, these two isolates formed a distinct group, they were genetically more similar to the isolates of *Colletotrichum* sp. from Israel and to the isolates of the grey subpopulation of *C. acutatum* in California than to the other isolates of *C. gloeosporioides* from avocado from Israel. Freeman *et al.* (1996) found that there were genetic differences among isolates of *C. gloeosporioides* from avocado, as was found here, but here the difference between the two isolates was small. Based on the findings of others (Hodson *et al.*, 1993; Hayden *et al.*, 1994; Freeman *et al.*, 1996; Buddie *et al.*, 1999; Ureña-Padilla *et al.*, 2002), isolates of *C. gloeosporioides* were expected to display genetic variation, and one explanation for this is that sexual recombination by the teleomorph, *Glomerella cingulata,* contributes to genetic diversity (Hayden *et al.*, 1994; Ureña-Padilla *et al.*, 2002). Conclusions regarding genetic diversity are not warranted here, as only two isolates of *C. gloeosporioides* from Australia were examined.

This study provides further evidence that subgroups exist within *C. acutatum sensu* Simmonds (Freeman *et al.*, 2001), however, genetic diversity was not evident among the subgroups of *C. acutatum* from almond in Australia, California and Israel. The existence of populations of asexually reproducing fungi displaying relatively similar genetic profiles suggests common ancestry and recent distribution (Mills *et al.*, 1992; Ureña-Padilla *et al.*, 2002). Here, the populations of *C. acutatum* from almond in Australia, California and Israel were genetically distinct, which suggested that they did not originate from a common ancestor. However, the degree of polymorphism among the groups from each country may indicate that sexual reproduction may have once played a role in the life cycle, generating

individuals of different genotypes, which have spread to different countries. Subsequently, each genotype has reproduced asexually, resulting in the clonal population structure observed within each group. Brasier (1987) suggested a hypothesis by which a "basal population" of a fungus, which is sexually reproducing and highly polymorphic, is subject to sudden stress, e.g., new geographical area, climate change, new host, which Brasier (1987) referred to as 'episodic selection'. This event distorts the basal population, in that sexual reproduction is suppressed and clonal populations result. Could the clonal populations of *C. acutatum* from almond in Australia, California and Israel be a result of episodic selection on a basal, once sexually reproductive population of *Glomerella acutata*? Alternatively, mutation rates, population size and age of a population (Aquadro, 1992; Bengtsson, 2003) may have contributed to the genetic diversity.

In summary, genetic diversity existed among the isolates of *C. acutatum* examined in this study, however, the diversity among isolates from almond in Australia detected using ISSR-primed PCR was extremely low. These results were consistent with what would be expected of a fungal population that reproduces asexually. The teleomorph of *C. acutatum* from almond has not been found in nature or *in vitro*, and it was concluded that the population from almond in Australia is most likely clonal. These results also support the findings of other researchers, in that the isolates of *Colletotrichum* sp. from almond in Israel were genetically identical, as were the isolates within each subpopulation of *C. acutatum* from almond in California. However, isolates of *C. acutatum* from almond in Australia were found to be genetically different from Californian and Israeli isolates. Although this does not necessarily mean that the isolates from each country are biologically different, it could indicate that the response of *C. acutatum* on almond in Australia to environmental and host factors, and to fungicides may differ from that of the pathogen in California and Israel.

Chapter 6 Pathogenicity of *Colletotrichum* spp.

6.1 Introduction

In addition to the morphological and genetic variation documented for *C. acutatum* (see Section 2.3.1 and 2.5.3), pathogenic variation has been demonstrated amongst isolates of *C. acutatum* from strawberry in France (Denoyes and Baudry, 1995) and Adaskaveg and Hartin (1997) found differences in lesion size between representative isolates of the pink and grey subpopulations of *C. acutatum* from almond in California. However, no studies have been published of the pathogenicity of Australian isolates of *C. acutatum* from almond, and pathogenic variation among the isolates has not been investigated.

It is not known if all commercial almond cultivars are susceptible to infection by *C. acutatum* nor if there are differences in susceptibility. There is limited evidence, primarily based on field observations, that Nonpareil is less susceptible to infection by *C. acutatum* than the other main commercial cultivars (Adaskaveg and Förster, 2000; McMichael, 2000; Diéguez-Uribeondo *et al.*, 2002), but this has not been thoroughly investigated. Also, it is not known if isolates of *C. acutatum* from other hosts are able to infect almond. Several fruit tree crops, e.g., olive, avocado, pistachio and orange, on which anthracnose has been reported in Australia, are grown in the same regions as almonds in Australia. It would be useful to know if almonds are potentially at risk from cross-infection by *C. acutatum* from other hosts. If this was the case, and anthracnose was found on other fruit crops adjacent to an almond orchard, the almond grower may need to be more vigilant with respect to hygiene between orchards and monitoring for anthracnose in the orchard than otherwise. In addition, a reliable and rapid method to assess pathogenicity and, in turn, cultivar susceptibility, is desired by the

Australian almond industry, in particular, the almond breeding program, conducted at the University of Adelaide, to assess potential genotypes for susceptibility to anthracnose.

The aims of the experiments described in this chapter were to determine if;

1 pathogenic variation exists amongst Australian isolates of *C. acutatum* from almond and, if so, how the isolates compare with those from other countries,

2 there are differences in susceptibility to infection by *C. acutatum* from almond among the main almond cultivars grown in Australia,

3 isolates of *C. acutatum* obtained from other tree crops in Australia are pathogenic on almond.

6.2 Materials and methods

6.2.1 General materials and methods

Conidial suspensions were prepared from 7-10-day-old cultures that had been grown in the dark at 25°C. Two mL of SDW was pipetted on the surface of the culture, the conidia gently released using a glass spreader, then the resultant suspension was pipetted into sterile Eppendorf tubes and vortexed for 5 sec. Serial dilutions were carried out and, using a haemocytometer, the concentration was adjusted to 1×10^6 conidia/mL. The spore suspensions were stored at 4°C for up to 48 h. At the commencement of each experiment, 5 μL of the suspension for each isolate was spread onto PDA to confirm viability of the conidia. The SDW used in the controls was spread onto PDA to check for contamination.

Plant material was collected, stored on ice and transferred to a cool room, 4°C, until required. Material was surface sterilised as described in Section 3.1 and air dried in a laminar flow cabinet unless otherwise stated.

Plant material was inoculated with a 5-μL droplet of either spore suspension (1 x 10^6 conidia/mL) or SDW, as a control, unless otherwise stated, then material was either wounded or remained intact. Leaves and fruit were wounded using the following protocol: A sterile needle was passed though the droplet of spore suspension or SDW and pierced the leaf cuticle or the fruit surface by approx 1 mm. Leaves or fruit were either wounded and inoculated with a spore suspension, wounded and sham-inoculated with SDW, intact and inoculated with a spore suspension, or intact and sham-inoculated with SDW. These treatments are hereafter referred to as wounded/inoculated, wounded/control, intact/inoculated or intact/control, respectively, unless otherwise stated.

Following inoculation, plant material was incubated in the dark at 25°C unless otherwise stated. At the completion of each experiment, mycelium from a representative sample of each treatment category was cultured onto PDA and the morphological characters of the fungal colony were examined, to confirm that the lesions were a result of inoculation by the isolate(s) of *C. acutatum* or *Colletotrichum* sp. used in that particular experiment. This procedure was carried out for each experiment unless otherwise stated.

Data for all experiments were subjected to analysis of variance (ANOVA) (Genstat® for Windows, 6th edn.) unless otherwise stated. A result was considered to be significant if $P<0.001$, and treatment means were separated using LSD of 5% unless otherwise stated.

6.2.2 Pathogenicity on detached leaves

6.2.2.1 Experiment L1 - preliminary

This experiment was intended to provide the basis for a protocol for inoculating detached leaves with *C. acutatum*. Leaves of the cv. Fritz were collected from the Waite Campus orchard in March 2002 and surface sterilised as described in Section 3.1. Leaves were placed, with adaxial surface upwards, on the surface of 0.5% TWA (Oxoid, No. 3) in 90-mm diameter Petri dishes. Spore suspension of *C. acutatum*, isolate DAR-72407, was placed at six spots on the surface of each leaf, with three drops placed on either side of the midrib approximately 1 cm apart and leaves were either wounded or remained intact. The treatments were wounded/inoculated, intact/inoculated, wounded/control and intact/control. Due to the growth stage of the almond trees, the leaves were too large to fit into the Petri dishes, so the leaf tip and base including the petiole were removed. The experiment was established in a completely randomised design with five replicates of each treatment. The Petri dishes were placed into a tray in a single layer, the tray sealed inside a clear plastic bag, and incubated on the laboratory bench at room temperature (approximately 22°C) in natural light. After 7 days, lesion diameter (Agostini *et al.,* 1992) was measured at right angles to the midrib, and the number of lesions and mean lesion diameter determined.

6.2.2.2 Experiment L2 - pathogenicity of *C. acutatum* from Australia and Israel

To compare the pathogenicity of isolates of *C. acutatum* representative of different almond growing regions of Australia and of *Colletotrichum* sp. from almond in Israel, detached leaves were inoculated with spore suspensions. Healthy leaves were collected from the cvs Price and Nonpareil on 26 September 2002 (approximately 6 weeks post-petal fall) from an orchard at Jubilee Almonds located at Waikerie, South Australia.

Spore suspensions were prepared for 13 isolates of *C. acutatum* and SDW was used for the control. The isolates used were as follows: S43 and CSL-1690 from two orchards in Angle Vale, South Australia; CSL-1687, CSL-1689, W16, W77, W117 and W153 from three orchards in Willunga, South Australia; MPD1 and CSL-1691 from one orchard in Munno Para Downs, South Australia; CSL-1688 from Nangiloc in Victoria; S44 from Western Australia and ALM-KSH-10 from Israel.

Leaves that were at a similar growth stage and size were selected and were surface sterilised as described in Section 3.1. Leaves with petioles attached were placed adaxial surface upwards onto three sterile toothpicks, which were on the surface of 0.5% TWA in 90-mm Petri dishes. A droplet of spore suspension was placed onto the right side of the midrib of each leaf and the leaves were wounded. Treatments were either wounded/inoculated or wounded/control. The Petri dishes were incubated for 7 days and lesion diameter was measured as previously described. The experiment was a completely randomised design with eight replicates of each treatment combination (isolate by cultivar).

6.2.2.3 Experiment L3 - pathogenicity of *C. acutatum* from Australia, Israel and California

This experiment with detached leaves was intended to compare the pathogenicity of isolates of *C. acutatum* representative of; different almond growing regions of Australia, *Colletotrichum* sp. from almond in Israel, and the pink and grey subpopulations of *C. acutatum* from almond in California. Healthy leaves were collected from the cvs Price and Nonpareil on 20 October 2003 (approximately 10 weeks post-petal fall) from the orchard at Jubilee Almonds. Spore suspensions were prepared for 13 isolates of *C. acutatum* and SDW was used for the control. The isolates that were used in experiment L2 (Section 6.2.2.2) were used in this experiment, except that isolates W117 and CSL-1691 were replaced by two

isolates from California, US-1776 and US-1796. The leaves were prepared and inoculated as described in Section 6.2.1 and lesion diameter measured after 7 days. The experiment was a completely randomised design with eight replicates of each treatment combination (isolate by cultivar).

6.2.2.4. Experiment L4 - susceptibility of Price and Nonpareil at different temperatures

Detached leaves were inoculated with *C. acutatum* and incubated at different temperatures to determine if a difference in susceptibility between Price and Nonpareil could be distinguished. Healthy plant material from Price and Nonpareil was collected and spore suspensions of isolates CSL-1687, CSL-1688 and W16 were prepared as described in Section 6.2.1, and SDW was used for the control. Isolates were selected on the basis of previous findings (Colmagro, unpublished) and the results of experiments L2 and L3 on detached leaves as described above. The preparation of the leaves and inoculation were as described in Section 6.2.1. Each cultivar/isolate combination was incubated at 15, 20 or 25°C in the dark for 7 days and the lesion diameter measured. The experiment was a split-plot design with eight replicates representing blocks, the temperatures were the whole-plots and isolate/cultivar combinations were the sub-plots.

6.2.3 Pathogenicity on detached fruit

6.2.3.1 Experiment F1 - preliminary

This experiment was designed to develop a protocol for the inoculation of detached almond fruit with *C. acutatum*. Almond fruit, cv. Fritz, were collected from the Waite Campus orchard in March 2002. Following surface sterilisation as described in Section 6.2.1, single fruits were placed on individual wire mesh racks, which were placed in plastic trays. Trays

were lined with two layers of sterile Chux® cloth above two layers of sterile paper towel, which were moistened with 200 mL of SDW per tray. Two 5-μL droplets of spore suspension of isolate DAR-72407 were applied approximately 1 cm apart on the surface of each fruit, with SDW for the control. Fruit was either wounded or remained intact, so the treatment categories were wounded/inoculated, wounded/control, intact/inoculated and intact/control. The trays were enclosed in plastic bags, incubated for 7 days in the dark at 25°C, and the lesion diameter and number of lesions were measured. The experiment was a randomised complete block design with five replicates.

6.2.3.2 Experiment F2 - pathogenicity of *C. acutatum* from Australia and Israel

Detached almond fruit were inoculated to compare the pathogenicity of isolates of *C. acutatum* representative of different almond growing regions of Australia and of *Colletotrichum* sp. from almond in Israel. Healthy almond fruit were collected from the cvs Price and Nonpareil on 26 September 2002 (approximately 6 weeks post-petal fall) from the orchard at Jubilee Almonds. Fruit was surface sterilised and spore suspensions were prepared for the same 13 isolates of *C. acutatum*, as were used for experiment L2 (Section 6.2.2.2). Almond fruit were placed onto wire mesh racks in plastic trays as described for experiment F1 (Section 6.2.3.1). One 5-μL droplet of spore suspension was pipetted onto the upper surface of each fruit and the fruit remained intact. The trays were enclosed in plastic bags and incubated for 7 days in the dark at 25°C. There were no lesions on the fruit after 7 days, and the fruit appeared dry despite the high humidity within the plastic bag, so a fine mist of SDW was sprayed over the fruit. Lesions subsequently developed on most of the fruit and lesion diameter (mm) was measured, following a total of 14 days of incubation. The experiment was a randomised complete block design with six replicates.

6.2.3.3 Experiment F3 - pathogenicity of *C. acutatum* from Australia, Israel and California

The aim of this experiment was to compare the pathogenicity, to detached fruit, of isolates of *C. acutatum* representative of; different almond growing regions of Australia, *Colletotrichum* sp. from almond in Israel, and the pink and grey subpopulations of *C. acutatum* from almond in California. Healthy almond fruit were collected from the cvs Price and Nonpareil on 20 October 2003 (approximately 10 weeks post-petal fall) from the orchard at Jubilee Almonds. Spore suspensions were prepared for the same isolates as were used in experiment L3 (Section 6.2.2.3).

This experiment was carried out as described in Section 6.2.3.2, with the following changes; the fruit were sprayed with a fine mist of SDW 3 days after inoculation, and there were eight replicates per isolate/cultivar combination. After 7 days of incubation, very few fruit bore lesions and several were infected with fungi other than *C. acutatum*, making it difficult to measure anthracnose lesions. Therefore, this experiment was repeated using a similar protocol, but the fruit were wounded. and this approach was used there after The fruit were sprayed with a fine mist of SDW 3 days after inoculation and lesion diameter (mm) measured after 7 days.

6.2.3.4 Experiments F4a and F4b - susceptibility of almond cultivars and hybrids to *C. acutatum*

Two experiments, F4a and F4b, were conducted using detached fruit to assess the relative susceptibility of several almond cultivars and hybrids to infection by *C. acutatum*.

Experiment F4a

Healthy almond fruit were collected on 30 September 2004 from an orchard belonging to Andrew Lacey at Lindsay Point, Victoria. Fruit was collected from the following cultivars

and hybrids; Nonpareil, Carmel, Price, Peerless, Lauranne, Carmel x Lauranne, Nonpareil x Lauranne, Price x Nonpareil, Nonpareil x Carmel, Nonpareil x Keane and Nonpareil x Peerless. These cultivars were selected following consultation with representatives of the almond industry and the almond breeding program based at the University of Adelaide. A spore suspension of 1 x 10^6 conidia/mL was prepared using equal parts of suspensions of the following isolates S43, DAR-72407, CSL-1318 and CSL-1689, all of which were 1 x 10^6 conidia/mL. This mixture will subsequently be referred to as 'bulk inoculum'.

Using a randomised complete block design with 10 replicates, each cultivar was wound-inoculated with 5 μL of either bulk inoculum or SDW. Fruit was incubated in the dark at 25°C, sprayed with a fine mist of SDW after 3 days, and lesion diameter measured after 7 days.

Experiment F4b

Fruit from Nonpareil, Price and Lauranne were inoculated with either bulk inoculum, isolate S43 or SDW, as described in experiment F4a, and fruit was either wounded or intact. The experiment was a randomised complete block design with eight replicates. Fruit was incubated for 7 days and lesion diameter measured as above. Inoculation of the almond fruit which were intact produced very few lesions, therefore the data were analysed using the mean lesion diameter from the wounded/inoculated treatment only.

6.2.3.5 Experiment F5 - pathogenicity to almond of *C. acutatum* from almond and other hosts

This experiment was conducted to determine if isolates of *Colletotrichum* sp. obtained from plants other than almond were pathogenic on almond fruit. Healthy almond fruit, cv. Price,

was collected on 30 September 2004 from an orchard belonging to Andrew Lacey at Lindsay Point, Victoria. Spore suspensions were prepared for the following isolates of *C. acutatum*; CSL-1298 from avocado, S53 from strawberry, NSW1 and Se37 from olive, P1 from pistachio, S43, DAR-72407, CSL-1318 and CSL-1689 from almond in Australia, US-1813 and US-1796 from almond in California, ALM-KSH-10 from almond in Israel and an isolate of *C. gloeosporioides* (S54) from orange in Australia. In addition, bulk inoculum was also prepared. Fruit were wound-inoculated with either a spore suspension or SDW for the control. Fruit were incubated for 7 days and lesion diameter measured. The experiment was a randomised complete block design with 10 replicates.

6.2.4 Pathogenicity to almond shoots - preliminary experiment

This experiment was conducted to develop a protocol for inoculating excised almond shoots to compare pathogenicity of isolates of *C. acutatum*. Shoots, approximately 15 cm long, were cut from the cv. Fritz in the Waite Campus orchard, March 2002. Older and damaged leaves were removed from each shoot until nine remained. There were four treatments, *viz*; 1. leaves wounded and sprayed with a spore suspension, 2. leaves wounded and sprayed with SDW, 3. leaves intact and sprayed with a spore suspension and 4. leaves intact and sprayed with SDW. Leaves were wounded as described in Section 6.2.1, with three wounds approximately 1 cm apart on each side of the midrib. Each shoot was placed into a single 250 mL conical flask containing 150 mL of SDW. Shoots were sprayed until runoff with a conidial suspension of *C. acutatum* isolate CSL-1317 (1 x 10^6 conidia/mL 0.2% Tween 80) or with 0.2% Tween 80 in SDW for the control. A clear plastic bag was placed over each shoot and sealed around the neck of the flask using a rubber band. Plant material in flasks was placed on the laboratory bench at room temperature and in diurnal light. The plastic bags were removed temporarily and a fine mist of SDW was sprayed over the shoots several times per day for 3 days to maintain humidity.

The plastic bags were removed after 3 days. The number of lesions and lesion diameter (mm) at right angles to the midrib were measured. The experiment was a completely randomised design with five replicates (shoots) per treatment. The data from the nine leaves per shoot were pooled and the number of lesions and area (mm^2) per lesion for each shoot were measured.

6.3 Results

6.3.1 Pathogenicity on detached leaves

6.3.1.1 Experiment L1 - preliminary

Lesions developed at the site of inoculation on all leaves that had been wounded and inoculated and an example of lesions is shown in Fig 6.1. The mean lesion diameter was 6.6 mm. There were no lesions on any leaves that received any of the other treatments. As a result of this finding, it was decided to use wound-inoculation for future experiments with detached leaves.

6.3.1.2 Experiment L2 - pathogenicity of *C. acutatum* from Australia and Israel

There were no lesions on any of the control leaves. All isolates, when applied to leaves, resulted in lesions on both Price and Nonpareil. There was significant variation in the mean lesion diameter among isolates (Fig 6.2). Isolate W16 caused the largest mean lesion diameter, 8.2 mm, and isolate W153 the smallest, 5.3 mm. The isolate from Israel (ALM-KSH-10) caused smaller lesions than most of the Australian isolates. The mean lesion diameter caused by ALM-KSH-10 was 5.6 mm. When data for all the isolates were

combined, there was no significant difference in lesion diameter between Price and Nonpareil, nor was there a significant isolate by cultivar interaction.

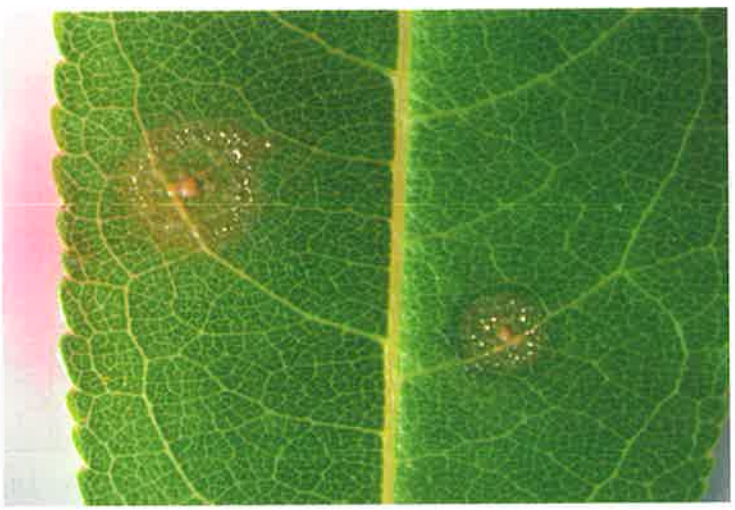


Fig 6.1. An example of anthracnose lesions on a detached almond leaf (cv. Fritz) 7 days after wounding and inoculation by an isolate (DAR-72407) of *C. acutatum*.

6.3.1.3 Experiment L3 - pathogenicity of *C. acutatum* from Australia, Israel and California

Sham inoculation with SDW did not result in lesions on any leaves. Inoculation by all isolates resulted in lesions on both Price and Nonpareil and there was significant variation of the mean lesion diameter among isolates (Fig 6.3). The lesion diameter for the isolates from Australia ranged from 11.1 mm for S44 to 6.7 mm for CSL-1690. The isolate from Israel, ALM-KSH-10, resulted in the smallest lesion diameter of 5.2 mm. The isolate representing the pink subpopulation from California (US-1796) caused a mean lesion diameter of 10.1 mm, and the isolate representing the grey subpopulation (US-1776), 9.1 mm. Price and

Nonpareil did not differ significantly in terms of mean lesion diameter, and there was not a significant isolate by cultivar interaction.

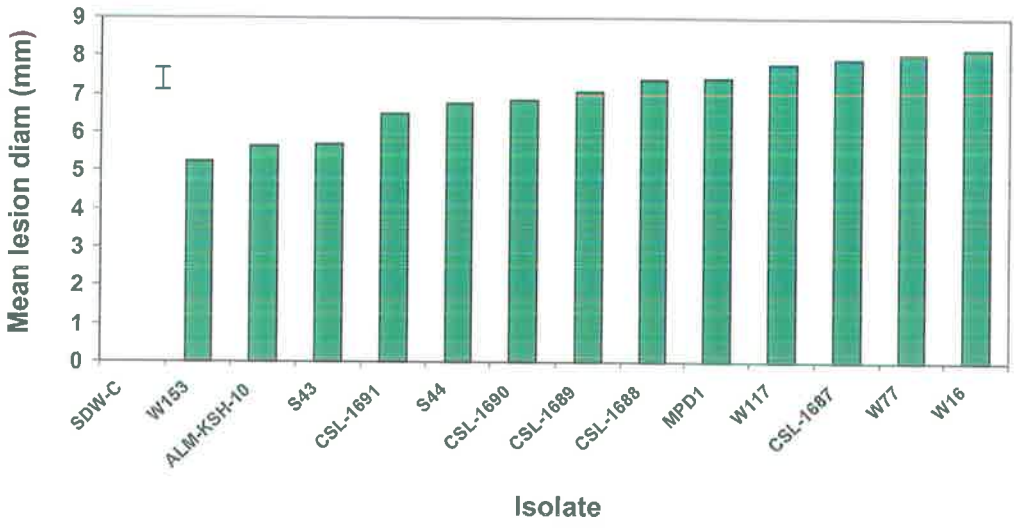


Fig 6.2. The mean lesion diam (mm) on detached almond leaves of cvs Price and Nonpareil combined, 7 days after wounding and inoculation with isolates of *C. acutatum* from almond obtained in Australia and Israel. Error bar represents LSD of 0.6.

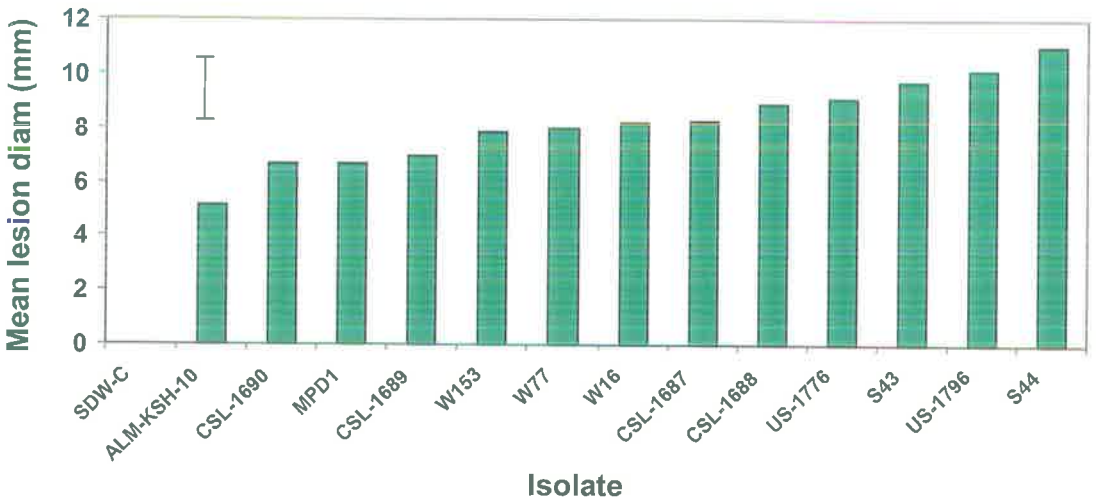


Fig 6.3. The mean lesion diam (mm) on detached almond leaves of cvs Price and Nonpareil combined, 7 days after wounding and inoculation with isolates of *C. acutatum* from almond obtained in Australia, California and Israel. Error bar represents LSD of 2.2.

6.3.1.4 Experiment L4 - susceptibility of Price and Nonpareil at different temperatures

There were no lesions on the control leaves. There was a significant effect of temperature on lesion diameter (Fig 6.4), with the smallest lesions for all isolates combined and on both cultivars at 15°C, and the largest at 25°C. There was a significant difference among isolates, in that isolate CSL-1687 produced the smallest lesions and CSL-1688 the largest (Fig 6.5). However, for all isolates combined, there were no significant difference between Price and Nonpareil at any temperature tested. In addition, there were no significant interactions between any combination of temperature, isolate and cultivar.

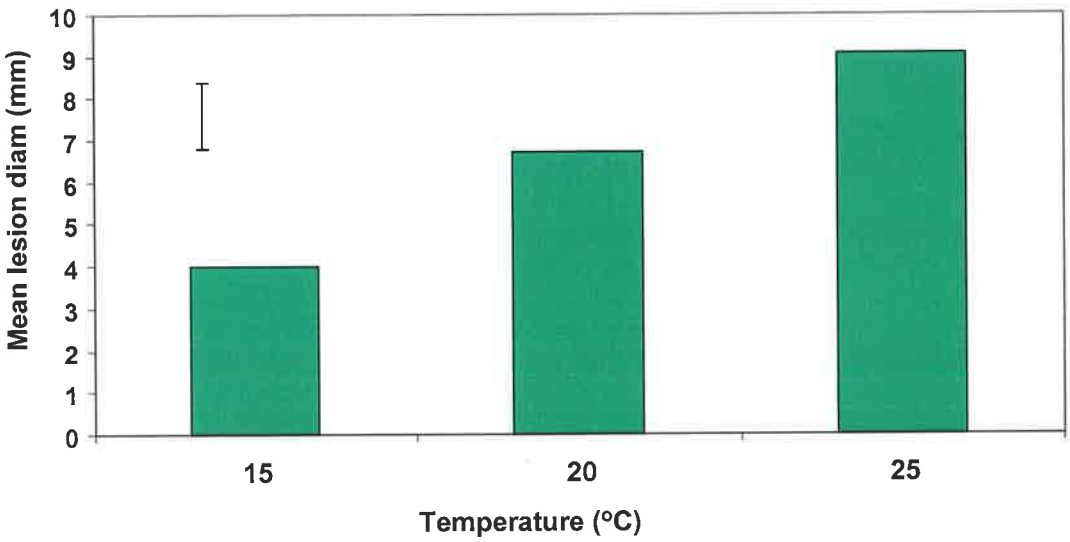


Fig 6.4. The mean lesion diam (mm) on detached almond leaves (cvs Price and Nonpareil combined). Leaves were wounded and inoculated with isolates of *C. acutatum* from almond obtained in Australia (CSL-1687, CSL-1688 and W16) and incubated at 15, 20 or 25°C for 7 days. Data represent the isolates combined and the error bar represents LSD of 1.6.

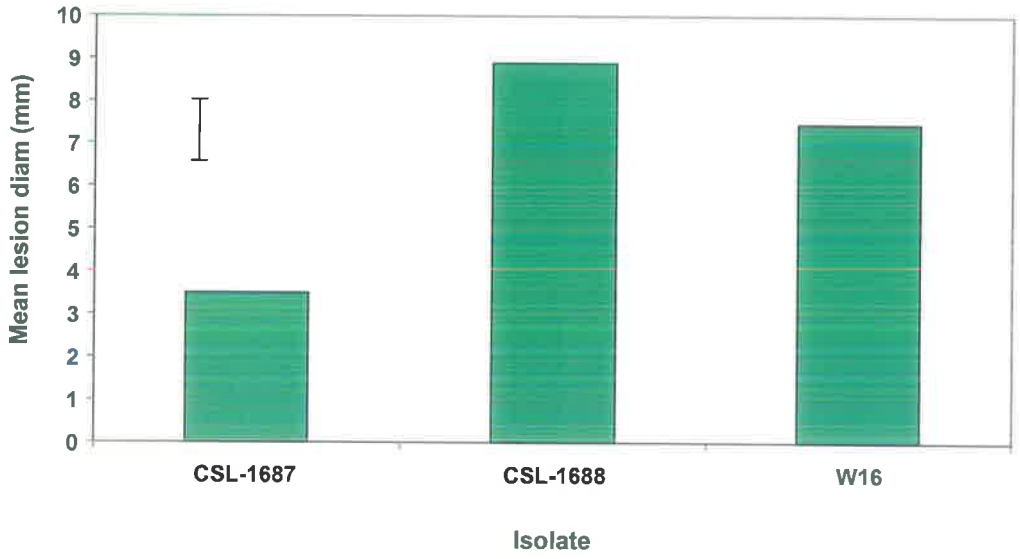


Fig 6.5. The mean lesion diam (mm) on detached almond leaves (cvs Price and Nonpareil combined). Leaves were wounded and inoculated with isolates of *C. acutatum* from almond obtained in Australia, and incubated at 15, 20 or 25°C for 7 days. Data represent all temperatures combined and the error bar represents LSD of 1.4.

6.3.2 Pathogenicity on detached fruit

6.3.2.1 Experiment F1 - preliminary

No lesions were observed on the fruit treated with SDW. Lesions were observed on both the wounded/inoculated and intact/inoculated fruit, and *C. acutatum* was re-isolated from representative lesions. On the basis of these results, it was decided that fruit would remain intact for subsequent pathogenicity experiments on detached fruit, as this is likely to resemble field conditions most closely.

6.3.2.2 Experiment F2 - pathogenicity of *C. acutatum* from Australia and Israel

There were no lesions observed on the control fruit, however, all isolates of *C. acutatum* produced lesions on fruit of Price and Nonpareil. There was a significant interaction between isolate and cultivar. Inoculation with isolate CSL-1689 resulted in the smallest mean lesion diameter on Nonpareil and the largest on Price (Fig 6.6). Several isolates produced lesions that were of a similar size on both Price and Nonpareil, for example, CSL-1687, CSL-1690, W16 and ALM-KSH-10. There was a general trend for lesions to be larger on Price than on Nonpareil, but this difference was not statistically significant.

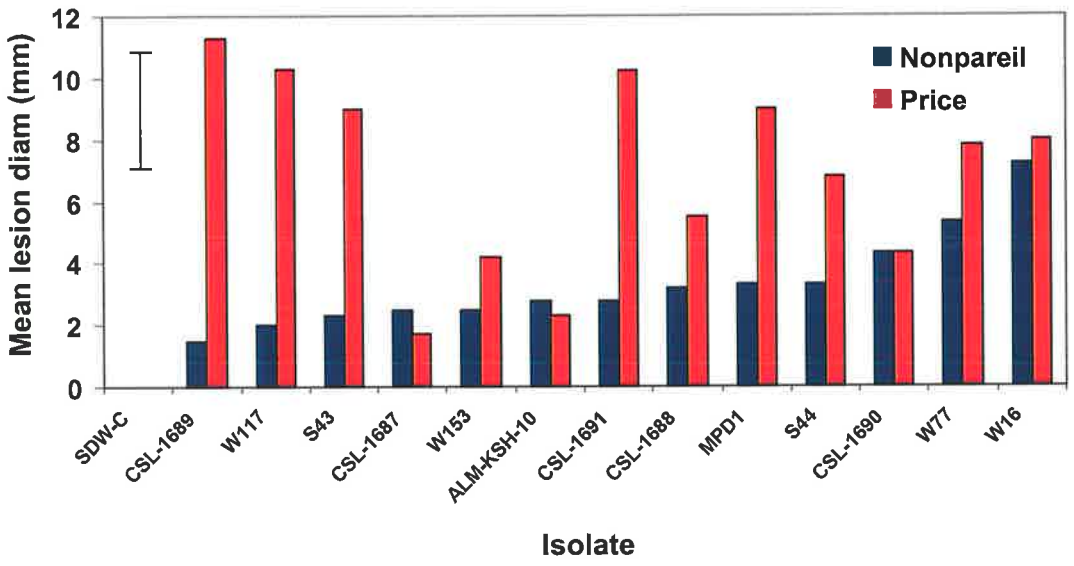


Fig 6.6. The mean lesion diam (mm) on detached almond fruit (intact) of cvs Price and Nonpareil, 14 days after inoculation with isolates of *C. acutatum* from almond obtained in Australia and Israel (Experiment F2). Error bar represents LSD of 4.0.

6.3.2.3 Experiment F3 - pathogenicity of *C. acutatum* from Australia, Israel and California

Sham inoculation by SDW did not result in lesions on any fruit. There was a significant difference in the mean lesion diameter between isolates (Fig 6.7) and between cultivars (Fig 6.8), however, there was no significant interaction between isolate and cultivar. The range of lesion diameter for Australian isolates was 5.8 mm (CSL-1687) to 12.7 mm (S43) (Fig 6.7). The mean lesion diameter for the Israeli isolate, ALM-KSH-10, and the isolate from the Californian grey subpopulation, US-1776, was below this range. ALM-KSH-10 caused the smallest lesions, 0.9 mm diameter, followed by US-1776 with 2.4 mm. US 1796 caused the largest lesion diameter, 12.8 mm, of all the isolates tested, but this isolate was within the range of several of the Australian isolates (LSD = 3.2). Lesions were smaller on Nonpareil, 5.7 mm, than on Price, 9.5 mm (Fig 6.8). Figure 6.9 shows representative lesions on detached fruit of Price and Nonpareil following inoculation by isolates of *C. acutatum*.

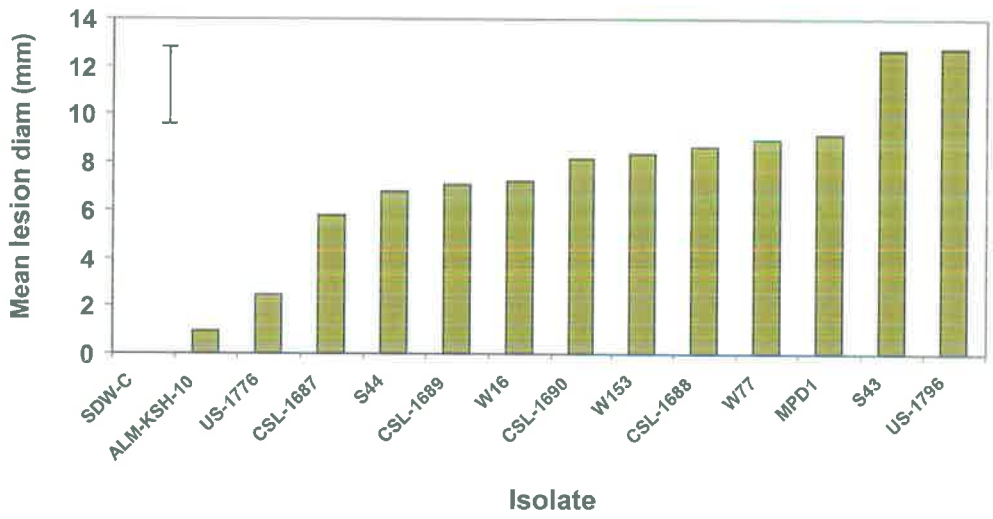


Fig 6.7. The mean lesion diam (mm) on detached almond fruit (cvs Price and Nonpareil combined) 7 days after wounding and inoculation with isolates of *C. acutatum* from almond in Australia, California and Israel (Experiment F3). Error bar represents LSD of 3.2.

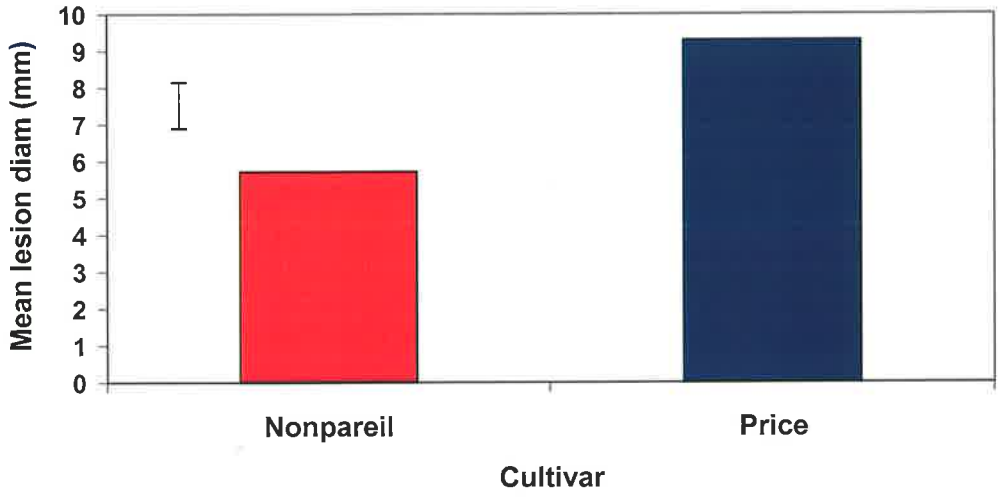


Fig 6.8. The mean lesion diam (mm) on detached almond fruit 7 days after wounding and inoculation with isolates of *C. acutatum* from almond in Australia, California and Israel (Experiment F3). Data are for all isolates combined for each cultivar. Error bar represents LSD of 1.3.

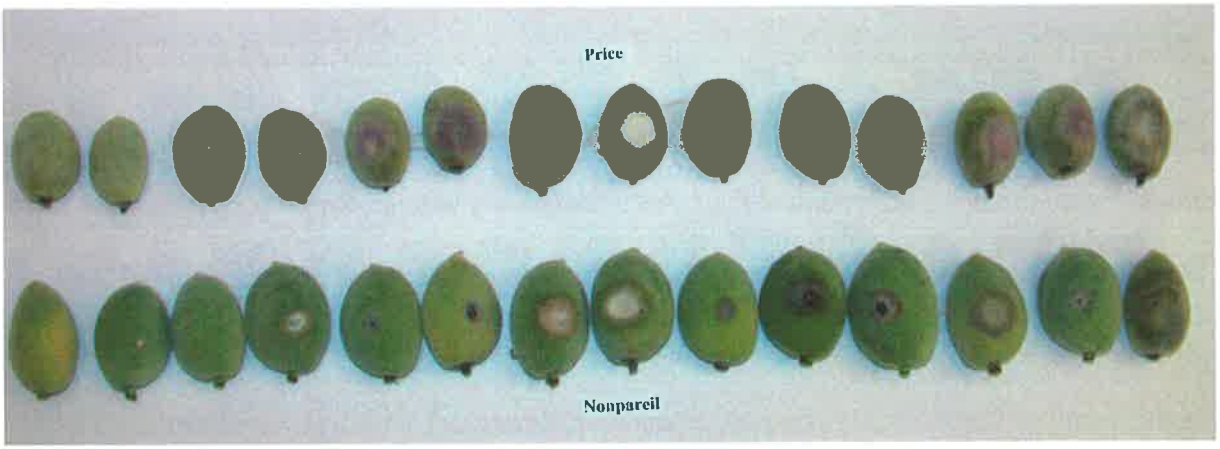


Fig 6.9. Representative anthracnose lesions on almond fruit 7 days after wounding and inoculation by spore suspensions of isolates of *C. acutatum* from almond from Australia, Israel and California, Nov 2003. Top row: fruit from cv. Price; bottom row: fruit from cv. Nonpareil. The isolates used to inoculate the fruit were (from left to right); SDW-C, ALM-KSH-10, US-1776, CSL-1687, S44, CSL-1689, W16, CSL-1690, W153, CSL-1688, W77, MPD1, S43 and US-1796.

6.3.2.4 Experiments F4a and F4b - susceptibility of almond cultivars and hybrids to *C. acutatum*

Experiment F4a

There were no lesions on the fruit treated with SDW. Inoculation resulted in lesions on all of the cultivars and hybrids, with significant variation among plant material (Fig 6.10). In general, lesions on Lauranne, Nonpareil x Keane, Nonpareil x Peerless, Carmel x Lauranne, Nonpareil x Lauranne and Nonpareil x Carmel were smaller than those on Carmel, Peerless, Price, Nonpareil and Price x Nonpareil. The smallest lesion diameter, 2.9 mm, was on the fruit of Lauranne, and largest lesion diameter was on Price x Nonpareil, 14.7 mm. There was no significant difference between Price and Nonpareil. Fig 6.11 shows representative lesions on the cultivars and hybrids tested.

Experiment F4b

There were no lesions on the control fruit. Lesions developed on wounded fruit following inoculation with isolate S43 and with bulk inoculum of *C. acutatum* from Australia. The mean lesion diameter on wounded fruit was significantly smaller for Lauranne than for either Price or Nonpareil, which were not significantly different from one another (Fig 6.12). The mean lesion diameters following inoculation by isolate S43 and the bulk inoculum were not significantly different from one another (data not shown). These data were consistent with those obtained in experiment F4a, as described above.

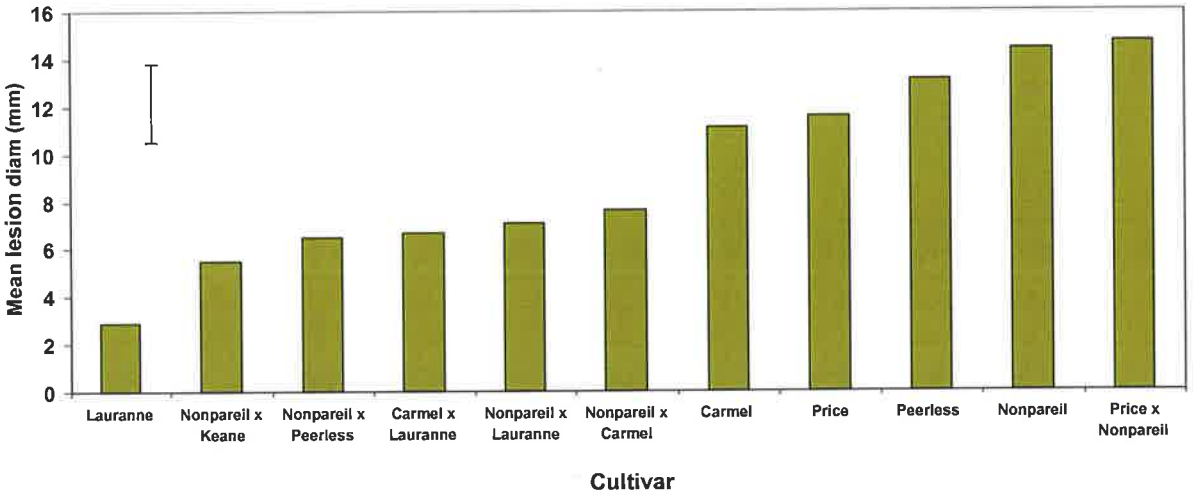


Fig 6.10. The mean lesion diam (mm) on detached fruit from five almond cultivars and six hybrids 7 days after wounding and inoculation with bulk inoculum of isolates (S43, DAR-72407, CSL-1318 and CSL-1689) of *C. acutatum* from almond in Australia (Experiment F4a). Error bar represents LSD of 3.2.

6.3.2.5 Experiment F5 - pathogenicity of *C. acutatum* from almond and other host plants on almond

Sham inoculation with SDW did not result in lesions on any fruit. Inoculation by all isolates of *C. acutatum* from almond and other hosts, and the isolate of *C. gloeosporioides* from orange resulted in lesions on the fruit of Price. There was significant variation in the mean lesion diameter among all of the isolates and among the Australian isolates (Fig 6.13). The mean lesion diameter for the Australian isolates ranged from 10.4 mm for CSL-1318 to 13.6 mm for CSL-1689. The isolate from California, US-1796, was close to that range, with a mean lesion diameter of 13.7 mm. ALM-KSH-10 and US-1813 produced significantly smaller lesions than did the Australian isolates from almond and US-1796, with mean lesion diameter of 6.8 mm and 8.5 mm, respectively. Isolates of *C. acutatum* from other host plants produced lesions of a diameter within the range of that for Australian isolates of *C. acutatum*

Fig 6.11. Anthracnose lesions on detached fruit from several cultivars and hybrids of almond 7 days after wounding and inoculation with a mixed spore suspension of *C. acutatum* comprising the following isolates; S43, DAR-72407, CSL-1318 and CSL-1689, from almond in Australia (Experiment F4a).

from almond. Inoculation by *C. gloeosporioides* (S54) resulted in the largest lesion diameter, of 14.6 mm, but this was not significantly different from US-1796, CSL-1689 and CSL-1298. Figure 6.14 shows representative lesions on detached almond fruit after inoculation.

6.3.3 Pathogenicity to almond shoots - preliminary experiment

No lesions developed on leaves that received the intact/control treatment. Lesions were evident on the wounded/control leaves, however, *C. acutatum* was not re-isolated from any of these lesions. Lesions occurred on all of the wound sites on every leaf, as well as on areas

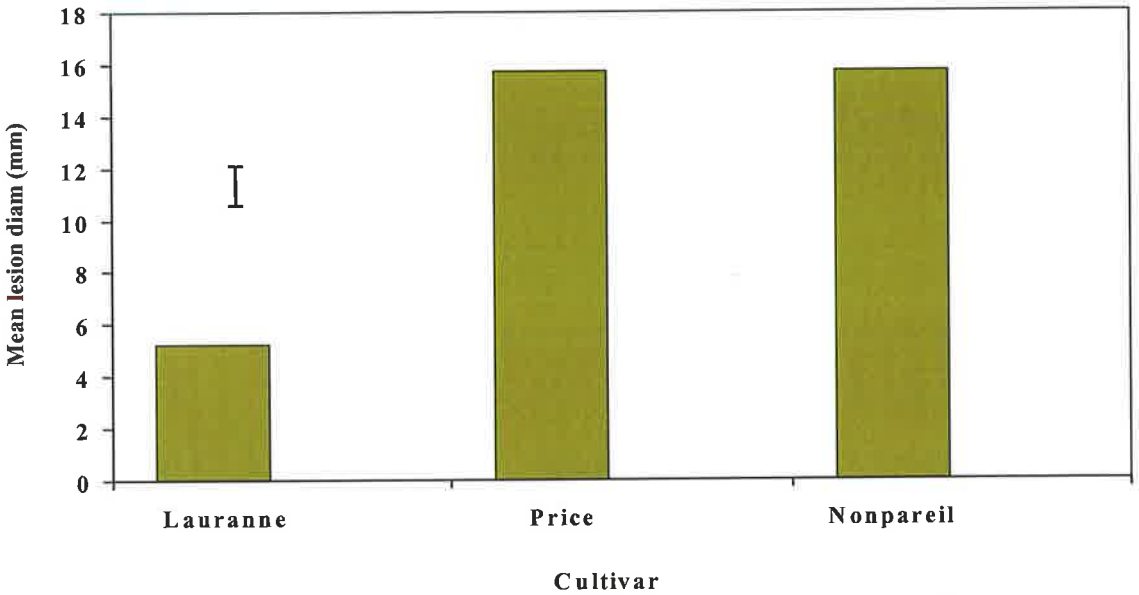


Fig 6.12. Mean lesion diam (mm) on detached almond fruit 7 days after wounding and inoculation with isolates of *C. acutatum* (bulk inoculum made of S43, DAR-72407, CSL-1318 and CSL-1689, and S43 alone) from almond in Australia (Experiment F4b). Error bar represents LSD of 1.8.

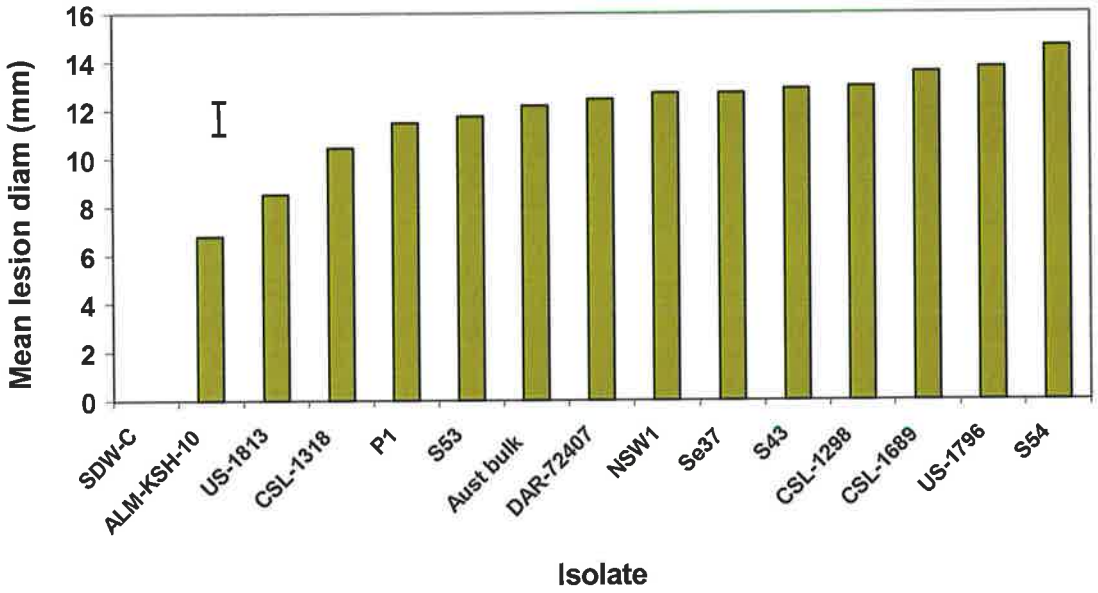


Fig 6.13. Mean lesion diam (mm) on detached almond fruit (cv. Price) 7 days after wounding and inoculation with isolates of *C. acutatum* from almond and other hosts, and an isolate of *C. gloeosporioides* (Experiment F5). Error bar represents LSD of 1.6.
Isolates: CSL-1318, DAR-72407, S43, CSL-1689 and bulk inoculum (mixture of CSL-1318, DSR-72407, S43 and CSL-1689) from almond in Australia; P1 from pistachio; S53 from strawberry; NSW1 from olive; Se37 from olive; CSL-1298 from avocado; S54 is *C. gloeosporioides* from orange; ALM-KSH-10 is *Colletotrichum* sp. from Israel; US-1813 and US-1796 are *C. acutatum* from California.

Fig 6.14. Anthracnose lesions on detached almond fruit (cv. Price) 7 days after inoculation with spore suspensions of isolates of *Colletotrichum* spp (Experiment F5). The isolates are as follows (left to right): Top row-US-1776, S54, NSW1, S53; Middle row-US-1776, Se37, CSL-1298, P1; Bottom row-DAR-72407, CSL-1318, ALM-KSH-10 and bulk inoculum (S43, DAR-72407, CSL-1318 and CSL-1689).

that were not artificially wounded, for the wounded/inoculated treatment. The mean number of lesions for the wounded/inoculated treatment was 7.2 per leaf. The mean number of lesions per leaf for the intact/inoculated treatment was 4.8. Lesions on leaves that had been wounded were distinct, whereas the lesions on the intact/inoculated treatment coalesced and often were an irregular shape, making it difficult to measure lesion area. The shoots and leaves wilted and began to senesce quickly after the plastic bag was removed, also contributing to the difficulty in measurement of lesions. Therefore, data for lesion area are not presented. This experiment demonstrated that intact and wounded leaves on excised shoots could develop anthracnose lesions following spray inoculation with *C. acutatum*.

6.4 Discussion

Pathogenicity experiments revealed pathogenic variation among Australian isolates of *C. acutatum* obtained from almond when tested on detached leaves and fruit of the cultivars Price and Nonpareil. However, no single isolate or group of isolates was consistently more pathogenic than any other. There was no evidence that isolates from certain almond growing areas were more pathogenic than others. In contrast, pathogenic variation has been reported among isolates of *C. acutatum* from strawberry in Europe (Denoyes and Baudry, 1995; Denoyes-Rothan *et al.*, 2003), between the two subpopulations of *C. acutatum* from almond in California (Förster and Adaskaveg, 1999) and among isolates of *C. gloeosporioides* from avocado in Israel (Freeman *et al.*, 1996). The present study confirmed, however, that all of the isolates tested were capable of infecting wounded almond leaves and young fruit of Price and Nonpareil, resulting in anthracnose lesions. However, pathogenicity of a range of isolates to other cultivars has not been demonstrated and it is possible that isolates may exhibit variability in pathogenicity on different cultivars, as was found by Denoyes and Baudry (1995), who reported that isolates of *C. acutatum* from strawberry differed in pathogenicity on several strawberry varieties. However, the variation amongst isolates may have little biological meaning *per se*, as minor differences in lesion diameter among individual isolates may have little significance in the orchard.

The experiments described in this chapter showed that the representative isolates from California and Israel were pathogenic on detached leaves and detached fruit of the cultivars Price and Nonpareil. Inoculation with these isolates resulted in lesions typical of anthracnose and the lesions were similar to those caused by Australian isolates. The lesions resulting from inoculation by the isolate from Israel were smaller than those caused by Australian isolates on detached fruit in experiments F3 and F5. However, the lesion size was within the range of

that caused by the Australian isolates on detached fruit in experiment F2 and on detached leaves, although the lesion size was consistently at the lower end of the range. Although, the Israeli isolate was pathogenic, it appeared to be generally less aggressive than the Australian isolates tested. The results of the detached fruit experiments were consistent with those of Förster and Adaskaveg (1999), who found that a representative isolate from the Californian pink subpopulation was significantly more aggressive than the isolate from the grey subpopulation, but they found that disease symptoms did not vary between the two isolates. In the current study, inoculation by the isolate from the pink subpopulation resulted in lesions that were within the range of the Australian isolates, but were consistently at the larger end of the range. However, the isolate from the grey subpopulation was less aggressive and produced smaller lesions on detached fruit than did isolates from Australia. This was not the case on detached leaves, where the lesion size for the grey isolate from California was well within the range of that caused by Australian isolates. It could be inferred from these pathogenicity tests that, if introduced, isolates from Israel and California from almond have the potential to cause anthracnose on almond in Australia.

Results from experiment F5 on detached fruit suggested that *C. acutatum* obtained from plants other than almond, and *C. gloeosporioides*, have the potential to infect almond. Of possible concern to the almond industry is that pistachio, avocado, olive and citrus orchards commonly occur close to almond orchards, particularly in the Riverland and Sunraysia regions of Australia. These results support the findings of others, that *Colletotrichum* isolates, including *C. acutatum* from host plants other than almond, were pathogenic to almond in detached fruit assays, e.g., *Colletotrichum* isolates from citrus and papaya (Adaskaveg and Hartin, 1997), *C. acutatum* from peach and strawberry (Adaskaveg and Hartin, 1997; Förster and Adaskaveg, 1999), and *C. gloeosporioides* from avocado (Freeman *et al.*, 1996).

Cross-infectivity from almond to other crops was not assessed in this study, but other researchers have found that *Colletotrichum* isolates from almond were pathogenic to detached fruit from other crops, e.g., peach (Adaskaveg and Hartin, 1997) and avocado (Freeman *et al.*, 1996). Future studies could assess the potential for *C. acutatum* from almond to infect other crops, and elucidate the host specificity of almond isolates of *C. acutatum*. This information would contribute to determining if anthracnose from almond has the potential to spread to other fruit tree crops in the temperate fruit growing regions of Australia and to understanding the relationship among *C. acutatum* taxa on different hosts. This was beyond the scope of the present study.

Overall, the results of these experiments suggested that there is no significant difference between Price and Nonpareil in susceptibility to anthracnose. A significant difference in susceptibility between these two cultivars was found only for experiment F3 on detached fruit. Evidence from field observations (Adaskaveg and Förster, 2000; McMichael, 2000) and from one study following inoculation of blossom and leaves of Nonpareil and Carmel *in vitro* (Diéguez-Uribeondo *et al.*, 2002), suggested Nonpareil is less susceptible than other cultivars, in particular Price and Carmel, however, overall, results obtained in the current study did not support these observations. There were differences in susceptibility among the almond cultivars and the hybrids tested, however, all were susceptible. The cultivars Carmel, Price, Peerless and Nonpareil were more susceptible than the hybrids and Lauranne, and Lauranne was the least susceptible cultivar. The concept of 'hybrid vigour' refers to the 'superiority of a hybrid over either inbred parent with respect to one or more traits' (Hartl, 1994). This may explain why four of the five hybrids were considerably less susceptible to infection than their parents. An exception was the hybrid of Price x Nonpareil, which was highly susceptible. This hybrid differed from the others in that Nonpareil was the pollen donor, whereas

Nonpareil was the female parent in the other hybrids. However, the influence of this factor is not known.

Although incubation at 15, 20 and 25°C did not assist in differentiating between Price and Nonpareil in terms of susceptibility in this study, Denoyes-Rothan *et al.* (2003) found that an incubation temperature of 18°C allowed better discrimination among strawberry varieties than 25°C, in detached fruit assays. Future experiments could test fruit of a range of almond cultivars at different temperatures, using bulk inoculum, to develop a screening test for anthracnose susceptibility.

The results of both the detached leaf and detached fruit experiments confirmed that isolates of *C. acutatum* from almond from Australia and other countries were pathogenic. However, there was inconsistency with respect to mean lesion diameter and the ranking of the isolates based on lesion diameter between the detached leaf and detached fruit experiments, and among the experiments carried out in different years. In addition, the results for differences in susceptibility between Price and Nonpareil were inconsistent among the detached fruit experiments. Several factors could account for these inconsistencies, e.g., the age of the trees from which the fruit was collected, the growth stage of the fruit may have been different each year and fruit was collected from an orchard in 2004 which was different from that in 2002 and 2003. Striem *et al.* (1989) found that the susceptibility of detached almond fruit to infection by *C. acutatum* declined with increasing fruit age. This may be important for interpreting the results of this current study on almond in Australia, as fruit age (days since anthesis) was not known, and would have varied among cultivars and from year to year. Another reason for inconsistency could be that management strategies varied between the two orchards from which fruit was collected and among the different years. Although the trees from which the fruit was collected had reportedly not been sprayed with fungicides in the 6

weeks prior to collection, this was difficult to confirm. Anecdotal evidence suggests that young fruit (<6-8 weeks post-petal fall) are most susceptible to infection, however, there was not a noticeable effect of age with respect to lesion development here. In order to investigate this, future experiments could involve the inoculation of fruit at different growth stages to determine if susceptibility changes over time.

Factors relating to the experimental protocol could also have contributed to the variability observed among the results. A critical factor for infection appeared to be a requirement for a moisture film on the fruit surface. However, in order to maintain the spore suspension droplet over the wound, the fruit must initially be dry, then lightly misted with water. Striem *et al.* (1989) found that almond fruit must initially be dry, but misting with water after inoculation was not necessary, and high humidity was sufficient for infection to occur. In this study on almond in Australia, high humidity was insufficient for consistent infection. Several amendments that could be evaluated with a view to improving consistency, e.g., using a larger droplet of spore suspension, making a larger and deeper wound, or making several wounds. Striem *et al.* (1989) found that such factors influenced the lesion size after inoculation of detached fruit. The effect of inoculation method on infection was also demonstrated by Adaskaveg and Hartin (1997), who found that disease incidence on detached fruit and fruit attached to trees in the field was more with the 'wounded drop' than the 'nonwounded spray' method.

In this study, pathogenicity was assessed on detached plant material in controlled conditions. In addition, for infection to occur reliably and repeatedly, it was necessary to wound the plant material at the time of inoculation. Although pathogenicity was demonstrated, as was variation amongst isolates and cultivars, these results may not necessarily reflect what might occur in the field. Ideally, it would be valuable to assess pathogenicity on fruit *in planta*,

however, this was not possible during the course of this study. In addition, the use of isolates of *C. acutatum* from other counties *in planta* would require strict quarantine conditions not available for this study. The advantage of using detached leaves over detached fruit is that leaves are available throughout most of the growing season, and not limited to a small window of 'susceptibility', as is the case with young almond fruit. Experiments in controlled conditions are not subject to the vagaries of the environment, and are more likely to yield consistent and repeatable results. For example, Biggs and Miller (2001) assessed the relative susceptibility of apple cultivars to infection by *C. acutatum*, and found that the results for field experiments were not repeatable, whereas the results obtained by inoculation of intact fruit were more repeatable. In addition, inoculation of plant material either in the glasshouse or in the field is not always possible, and risks dissemination of inoculum into commercial orchards.

It was hoped that a reliable and quick method suitable for assessing pathogenicity could be developed and, hence, provide a tool for assessing susceptibility to anthracnose. Following the preliminary experiment, in which pathogenicity was assessed on detached shoots (Section 6.2.4), the pathogenicity of three isolates of *C. acutatum* was assessed on potted plants of the cultivars, Price, Carmel and Nonpareil. On each tree, four branches, each with eight leaves, were inoculated with a spore suspension of a different isolate. A plastic bag was placed over each branch, sealed around the base, and the trees were placed in a shadehouse. Several problems were encountered with the protocol;

- there was variation of the quality of the leaves among cultivars, and it was difficult to obtain healthy and undamaged leaves for all cultivars, in particular, Nonpareil,
- leaves touched each other and the inside of the bag,

- the weight of the plastic bags, as well as high humidity inside the bag, led to the bags becoming heavy and the branch drooped, increasing the problem of leaves touching each other and the inside of the bags and

- some leaves fell off and rested in the moisture which had collected at the base of the bag, or stuck to the sides of the bags.

Such problems made it difficult to assess disease incidence and severity in a meaningful way. Therefore, results from this experiment are not presented. For future experiments, it is recommended that entire potted almond trees be inoculated, with one isolate used to inoculate each tree. Trees could be incubated in growth rooms, with misters to maintain high humidity to maximise the potential for infection. In addition, it is recommended that fruit be inoculated *in planta*, if bearing trees were available.

Further work is required to determine if susceptibility of almond fruit changes over time, as this factor may influence the results of a screening assay for cultivar susceptibility. Although the results of this study did not reveal a difference in susceptibility between Price and Nonpareil in terms of detached leaves, it is considered that the use of detached leaves in a screening assay warrants further investigation, using several hybrids and cultivars, as leaves are available throughout the growing season.

In summary, pathogenicity experiments on detached leaves and fruit demonstrated that there was variation of pathogenicity among isolates of *C. acutatum* from almond from Australia and other countries, however, all isolates were pathogenic. Isolates from California and Israel caused lesions that were generally of a similar size to that caused by Australian isolates. Isolates of *Colletotrichum* sp. from plants other than almond were pathogenic to detached almond fruit, cv. Price, using wound inoculation. The results of these experiments did not

reveal a consistent difference in susceptibility between Price and Nonpareil, however there were differences in susceptibility among several hybrids and other cultivars tested. The protocols used in this study show promise in the development of a rapid and reliable screening method to assess cultivar susceptibility.

Chapter 7 Epidemiology of anthracnose on almond

7.1 Introduction

The development of anthracnose on fruit tree crops is favoured by warm, wet conditions (Davis *et al.*, 1987; Waller, 1992; Adaskaveg and Förster, 2000; Timmer and Brown, 2000). In Israel, epidemics of anthracnose on almond developed following spring rains (Shabi and Katan, 1983) and in California epidemics followed extended rainfall and warm (20-25 °C) weather (Adaskaveg and Förster, 2000). Anecdotal evidence suggests that such conditions also are the most conducive for anthracnose on almond in Australia, however, McMichael (2000) found that the response of the anthracnose pathogen to fungicides in Australian almond orchards differed from that in California. It could be hypothesised that the response of the pathogen to environmental conditions in Australia may also differ from that in California, but the environmental conditions for maximum development of anthracnose on almonds in Australia have yet to be investigated and defined.

In California, symptoms of anthracnose occur on blossoms, fruit, leaves and woody tissue of almond at all stages of development (Adaskaveg and Förster, 2000), whereas blossom blight has not been observed in Australia (McMichael, 2000) nor in Israel (Shabi and Katan, 1983). McMichael (2000) and Adaskaveg and Förster (2000) reported that almond fruit are susceptible to infection by *C. acutatum* from early development until hull split, however, Shabi and Katan (1983) reported that infection occurred on fruit early in the growing season, from spring to early summer in Israel. A thorough investigation of the progression of both symptoms and epidemics of anthracnose on almond in Australia would assist growers to identify anthracnose and facilitate decisions about the timing of fungicide sprays.

Based on general observations in the field, there is some evidence which suggests that cv. Nonpareil is less susceptible to infection by *C. acutatum* than cv. Price (Adaskaveg and Förster, 2000; McMichael, 2000; Diéguez-Uribeondo *et al.*, 2002). Intensive monitoring of disease development on these cultivars could provide a deeper insight into the disease progress and the possible differences in relative susceptibility of these two cultivars.

C. acutatum has been recovered from mummified almond fruit (Shabi and Katan, 1983; Förster and Adaskaveg, 1999; McMichael, 2000), symptomatic blossom (Förster and Adaskaveg, 1999), asymptomatic blossom (McMichael, 2000), leaves, peduncles and woody tissue showing signs of die back (Shabi and Katan, 1983; Förster and Adaskaveg, 1999; McMichael, 2000). It is generally considered that mummified fruit are the main source of inoculum for anthracnose, however, this has not been thoroughly investigated in Australia, and it is not known whether other plant tissues could also be sources of primary inoculum. Shabi and Katan (1983) suggested that pruning out twigs and branches bearing mummified fruit and infected peduncles may decrease the amount of primary inoculum. However, removal of such inoculum sources is a labour intensive and time consuming process. A grower is unlikely to allocate much time and money to this practice unless there is strong evidence to show that disease and crop loss can be significantly decreased.

The aims of the research described in this chapter were to;

1 determine the environmental conditions that are most conducive for the development of anthracnose in a commercial almond orchard in South Australia,

2 monitor and document the progression of symptoms and disease on two cultivars, one reportedly susceptible and the other less susceptible to anthracnose, over several growing seasons,

3 determine which plant tissues are potential sources of primary inoculum of *C. acutatum*, and on which plant tissues the fungus survives throughout the year.

7.2 Materials and methods

7.2.1 Disease development in the field

7.2.1.1 Field site

The almond orchard used in this project belongs to Sue and Leath Hunt, and is located at Almond Grove Rd, Willunga, South Australia. Willunga is 110 km south of Adelaide and has a Mediterranean climate. The site was selected on the basis of several factors; proximity to the University of Adelaide, grower cooperation and a recent history of anthracnose (McMichael, 2000). The almond orchard was approximately 0.4 ha in size and the layout is shown in Fig 7.1. Rows of trees were orientated north-south, and consisted of the cultivar Nonpareil (odd rows) alternating with a polliniser variety, e.g., Carmel, Price, Fritz, Ne Plus, in the even rows. Trees were all grafted on Nemaguard® rootstocks and were approximately 12-15 years old. Many trees within rows and some trees in adjacent rows had overlapping canopies and, as the trees were not pruned extensively, significant amounts of dead wood remained on the trees throughout each season. There were established vineyards immediately east, a house and creek to the south beyond which was a vineyard, a road and housing to the north and an old, non-commercial almond orchard to the west. In July 2003, one half of the almond orchard (a section not used in this project) was removed and replaced by vines.

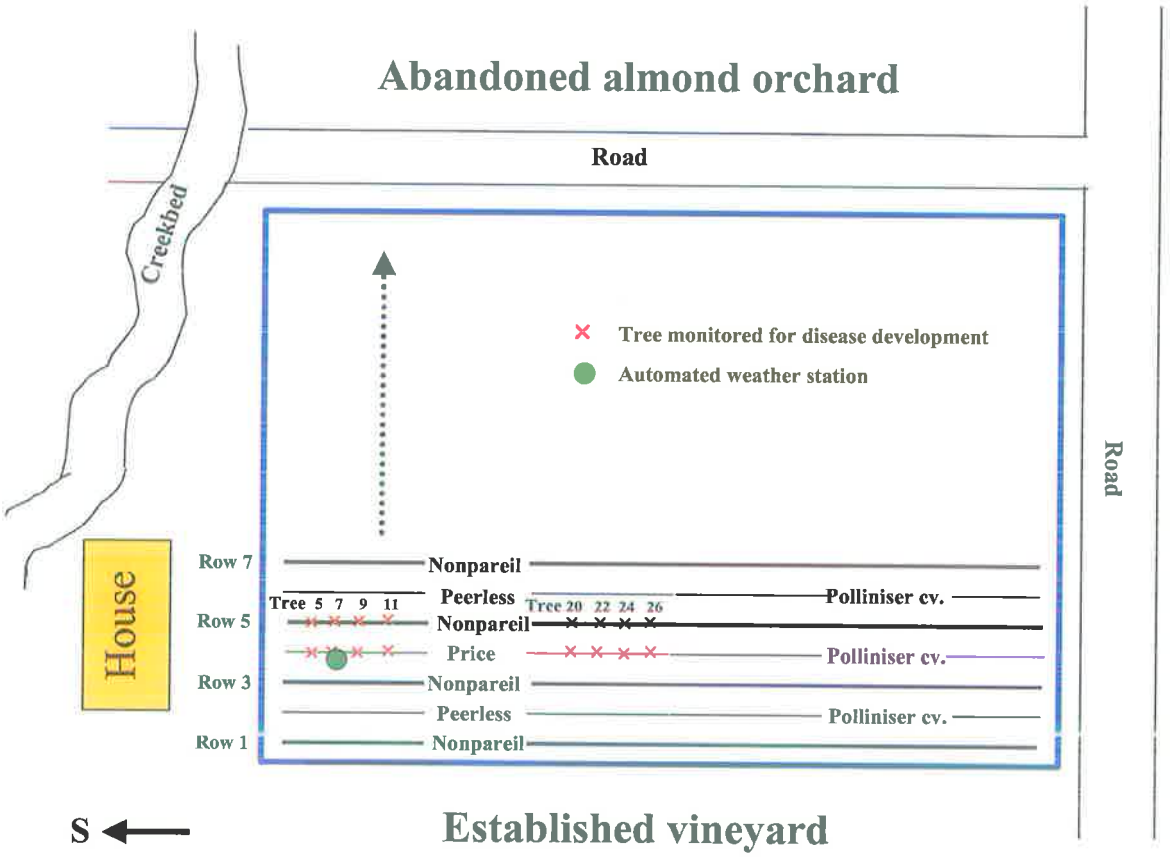


Fig 7.1. The layout of the almond orchard at Willunga, South Australia. Trees were numbered consecutively from the southern edge of the orchard.

7.2.1.2 Monitoring anthracnose disease

Trees numbered 5, 7, 9, 11, 20, 22, 24 and 26 in both row 4, cv. Price, and row 5, cv. Nonpareil (Fig 7.1), were selected for monitoring anthracnose throughout the seasons 2002, 2003 and 2004. Approximately six branches per tree were selected for monitoring. Branches were selected on the basis of ease of reach, had a minimum of 50 fruit per branch, and

represented northerly, southerly, easterly, westerly aspects and the centre of the tree as far as possible. Branches were tagged with flagging tape and, at regular intervals during the almond growing season, the number of asymptomatic fruit and the number of fruit affected by anthracnose were counted on each branch. A fruit was deemed to be infected if one or more anthracnose lesions were visible. The fruit, leaves and woody tissue were observed for the development of disease symptoms as described in Section 2.6.2 over the course of each season. Disease incidence for each cultivar was calculated by dividing the total number of fruit for all eight trees combined by the number of diseased fruit and multiplying by 100, giving the disease incidence as a percentage.

In addition to monitoring and counting asymptomatic and diseased fruit, the following parameters were measured

- Total count of mummified fruit on each tree (counted in June)
- Number of mummified fruit on each branch (counted in July)
- Number of mummified fruit within a 1 m radius of each tagged branch (counted in July)

For the season 2002, due to unforseen circumstances, it was not possible to obtain disease incidence data between 7 October and 20 October, and after 5 November.

There was 10-fold decrease in the amount of blossom in season 2003 compared to 2002, therefore it was not possible to select six branches each with a minimum of 50 fruit per branch. Up to 12 branches per tree were selected and as many fruit as possible, up to 300 per tree, were monitored and counted through the season.

Representatives samples of fruit and leaves with anthracnose symptoms were removed at regular intervals from trees adjacent to those used for monitoring. These samples were returned to the laboratory, incubated, and *C. acutatum* isolated and identified, as previously described (Section 3.1), to confirm the presence of anthracnose.

7.2.1.3 Weather data collection

Weather data were collected using an automated weather station (Measurement Engineering Australia Pty Ltd), which was located adjacent to tree 7 in row 4 (Fig 7.1). The following data were collected; air temperature at 30 cm above the ground, rainfall below the tree canopy and leaf wetness on a branch 120 cm from the ground. Measurements were recorded every 15 minutes, stored in the data logger of the weather station and downloaded using Magpie for Windows® software onto a laptop computer. Data were transferred to Microsoft Excel® for manipulation and further analysis.

7.2.1.4 Data analysis

Data were analysed separately for each growing season using repeated measures ANOVA (Genstat® for Windows, 6th edn.), which takes into account that each time period is not an independent treatment factor, to determine if there were statistically significant differences between the cultivars with respect to disease incidence over time.

Disease incidence data from the logistic portion of the disease progress curve were logit-transformed using the formula, Ln=(y/(1-y), then simple linear regression was performed on the transformed data using Microsoft Excel®. From the linear regression model, the apparent infection rate of the epidemic for each cultivar for each season was determined (Van der Plank, 1963).

The area under the disease progress curve (AUDPC) was calculated using the formula

$$\text{AUDPC} = \sum \ [(y_i + y_{i+1})/2] \ (t_{i+1} - t_i)$$

where y_i = the disease incidence at the ith observation, t_i = time (days) at the ith observation, and n = the total number of observations (Shaner and Finney, 1977). The AUDPC was standardised, by dividing the AUDPC value by the total duration of the epidemic in days (Fry, 1978), giving the relative area under the disease progress curve (RAUDPC) for each cultivar. For this analysis, the total duration of the epidemic was defined as from the first day that symptoms were evident until the day of maximum disease incidence. The use of AUDPC takes into account many important biological factors relating to each epidemic, e.g., disease onset, rate of increase, duration and final disease incidence (Campbell and Madden, 1990), and calculating the RAUDPC is useful when the epidemics vary in duration. RAUDPC is useful to compare and describe epidemics using one value. The RAUDPC is a proportion, between 0 and 1, with 1 being equivalent to 100% disease. For this study, RAUDPC was multiplied by 100 and is therefore expressed as percent (D. Shtienberg, pers. comm., 2004).

The relationship between the number of mummified fruit per tree and maximum disease incidence for Price and Nonpareil combined for each season was analysed by simple linear regression. In addition, the relationship between the maximum disease incidence and number of mummified fruit for Price and Nonpareil combined and for each season combined was analysed by simple linear regression (Genstat® for Windows, 6th edn.). The relationships between the environmental factors of rainfall, temperature and leaf wetness, and the apparent infection rate were analysed using simple linear and polynomial regression (Genstat® for Windows, 6th edn.).

7.2.2 Isolation of *C. acutatum* from plant material

Plant material was collected from almond trees located in the Willunga orchard. The following samples were collected, at random, from Price and Nonpareil trees in the first week of every month from September 2003 to August 2004 inclusive:

- Thirty mummified fruit (remaining on the tree from the previous season);
- Thirty peduncles, many of which had mummified fruit attached;
- Thirty bark samples, each comprising four pieces of bark approximately 2 mm x 5 mm (each). These were removed from the area within 10 mm of the base of a peduncle or spur. Several of the peduncles or spurs had mummified fruit attached.

The samples were surface sterilised as described in Section 3.1, placed onto LAPDA and incubated in the dark at 25°C for up to 21 days. All of the peduncles sampled were desiccated, as was most of the bark.

In addition, the following plant tissues were collected from Price and Nonpareil at various times throughout the year:

- Thirty asymptomatic leaves were collected at monthly intervals from September 2003 to July 2004;
- Thirty asymptomatic fruit were collected at monthly intervals from September 2003 to February 2004;
- Thirty asymptomatic buds and blossom were collected on 19 and 30 July 2003.

Fruit, leaves, buds and blossom were surface sterilised and incubated in moist conditions as described in Section 3.1 for up to 14 days. Leaves were placed with the adaxial surface uppermost, and blossoms with the receptacle uppermost.

C. acutatum was identified using morphological characteristics in culture and microscopic features. Representative isolates of *C. acutatum* were subcultured onto PDA and identification was confirmed using PCR with *C. acutatum*-specific primers as described in Section 3.3.2.1.

7.3 Results

7.3.1 Disease development in the field

7.3.1.1 Season 2002

Disease symptoms on fruit were first observed on 25 August on Price and 2 October on Nonpareil. Sunken, orange lesions developed on fruit (Fig 7.2A, 7.2C), and colonisation usually progressed until the fruits were misshapened, shrivelled and subsequently died (see Fig 2.3, Section 2.6.2). Fruit was generally < 20 mm long during this time and the majority of these fruits remained attached to the tree. Fruit that was first observed to be infected during late October and early November developed sunken, orange lesions, but did not necessarily shrivel and die (Fig 7.2D). Fruit was approximately 15-25 mm long at this time of the year and, in these cases, the fruit often continued to grow, but the lesions would not increase in size. Commonly, a tan-coloured gum would exude from the lesion (Fig 7.2D). Fruit larger than *ca* 30 mm did not develop anthracnose symptoms. Leaf symptoms were first evident on 25 September on both Price and Nonpareil. Typically, water-soaked lesions developed along the leaf margins, the lesions became pale orange, then became necrotic (Fig 7.2B, 7.2C). The

leaves wilted, shrivelled and died. Symptoms of blossom blight were not observed and blossom remained healthy throughout.

There was a significant interaction ($P< 0.001$) between cultivar and time with respect to disease incidence (%) on fruit. Following the first appearance of symptoms on Price on 25 August, disease incidence increased throughout September and October to reach 21.6% on 5 November when assessment ceased. Disease incidence in Nonpareil, first observed on 2 October, increased during October to reach 8.1% on 5 November (Fig 7.3A). The period during which increase in disease incidence was observed was 68 days for Price, whereas it was 34 days for Nonpareil. Disease incidence for this time was used to calculate the RAUDPC, which was twice as much for Price, 6.1%, as for Nonpareil, 3.2% (Table 7.1).

A linear regression model using logit-transformed disease incidence data describes the relationship between disease incidence and time for Price ($P< 0.001$, $R^2=0.96$) and Nonpareil ($P<0.05$, $R^2=0.98$) with the equations $y = 0.0615x - 5.3777$ for Price and $y = 0.0917x - 8.5241$ for Nonpareil (Fig 7.3B). The apparent rate of infection was faster for Nonpareil, 0.09, than Price, 0.06 (Table 7.1), however using 95% confidence intervals, there was no significant difference between these.

In terms of variation among trees of each cultivar, the incidence of disease on fruit increased for all Price and Nonpareil trees, with the exception of Nonpareil tree 22, for which disease appeared to decrease from 21 October until 5 November. However, this was due to a cluster of infected fruit falling from the tree. Price tree 5 showed a lower disease incidence at the end of the monitoring period than did the other trees of this cultivar. Price tree 11 showed a higher disease incidence, as did Nonpareil tree 24, than did the other trees. This could reflect

differences in susceptibility between individual trees of a cultivar, or differences in environmental or growing conditions within the orchard.

There was no relationship between the number of mummified fruit per tree and disease incidence per tree for either Price (P>0.1) or Nonpareil (P>0.05). The mean number of mummified fruit and the range for Price were 759 and 373-1112, respectively, whereas those for Nonpareil were 78 and 35-131, respectively (Appendix B). There was no apparent relationship between the number of mummified fruit on an individual branch, or within 1 m of each tagged branch, and disease incidence on that branch.

7.3.1.2 Season 2003

Symptoms on young fruit were first observed on 10 September and on leaves on 17 September for Price, and on 17 September for fruit and leaves of Nonpareil. Symptoms on fruit and leaves were as described in Section 7.3.1.1. Mummified fruit generally remained attached to the trees for the entire season. Leaves with anthracnose also remained attached to the tree on both Price and Nonpareil for longer than leaves which were either senescent or showed symptoms of other diseases. Blossom blight was not observed.

In terms of disease incidence (%) on fruit, there was a significant interaction (P<0.001) between cultivar and time. Disease incidence for Price increased steadily throughout September to 80% on 8 October, from which time there was no further increase for the rest of the season (Fig 7.4A). Disease incidence for Nonpareil also increased during September and, in contrast to Price, disease incidence continued to increase slightly throughout October, until a maximum of 25% was reached on 5 November. The period during which increase in

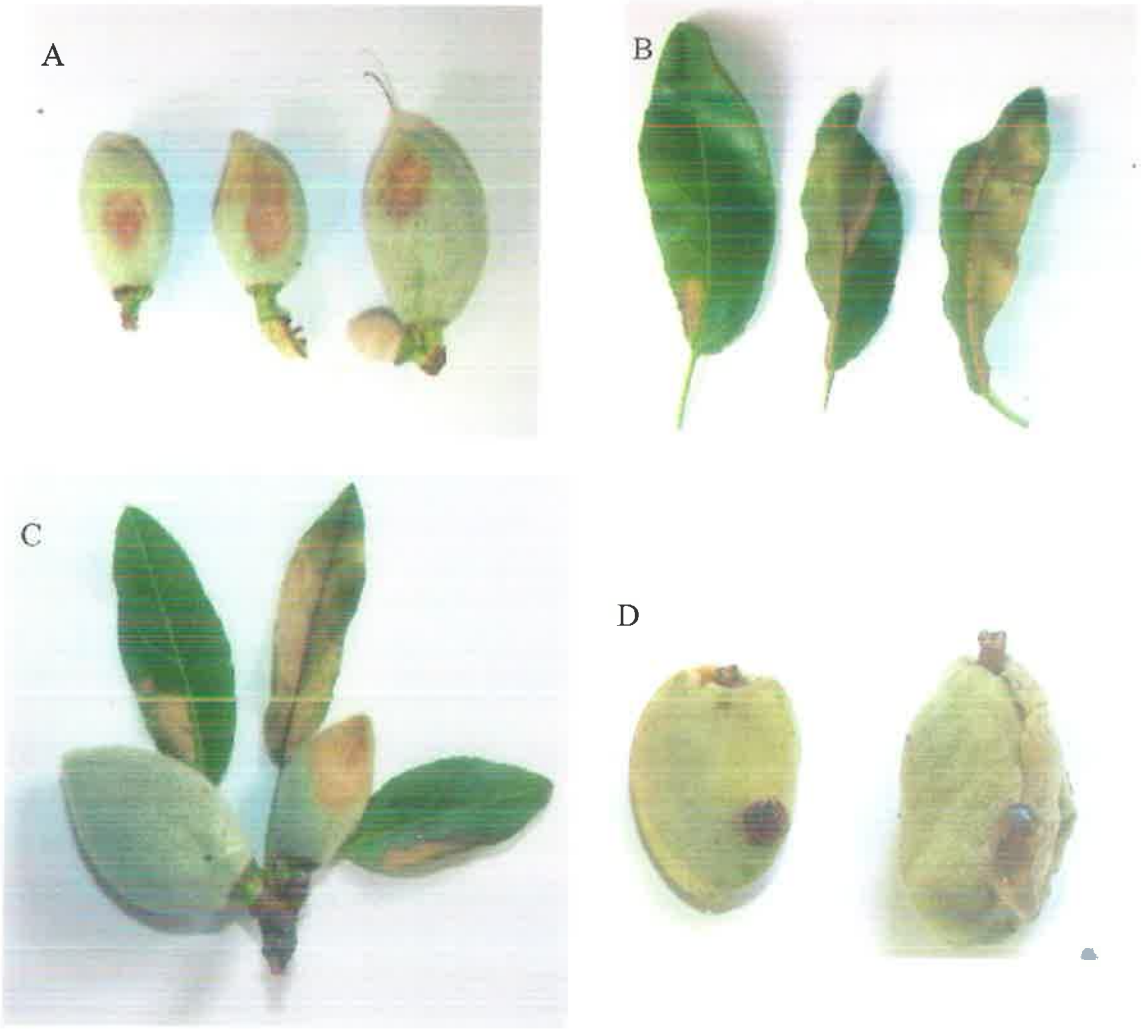


Fig 7.2. Symptoms of *C. acutatum* infection on almond. **A.** Lesions on young fruit, cv. Price. **B.** Lesions on leaves, cv. Price. **C.** Lesions on leaves and fruit (right) and an asymptomatic fruit (left), cv. Price. **D.** Gum exuding from a lesion on mature fruit, cv. Nonpareil (left) and cv. Price (right).

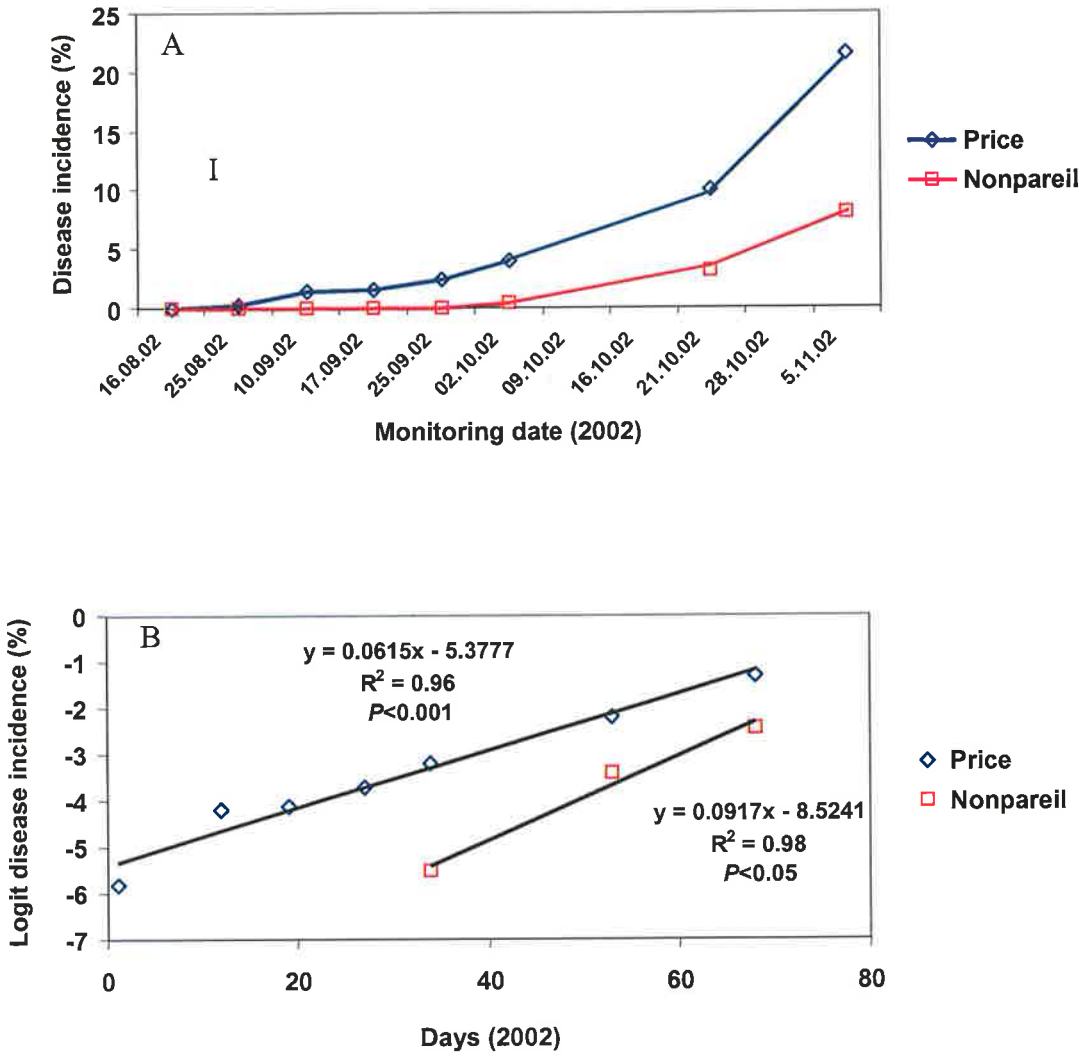


Fig 7.3. Incidence of anthracnose on almond fruit caused by *C. acutatum*, on the cultivars Price and Nonpareil, at Willunga, South Australia, 2002. **A.** Disease progress curves ($P<0.001$). Error bar represents LSD of 2.8. **B.** The relationship between logit transformed disease incidence data and time is described by the equations $y = 0.0615x - 5.3777$ ($P<0.001$, $R^2 = 0.96$) for Price and $y = 0.0917x - 8.5241$ ($P<0.05$, $R^2 = 0.98$) for Nonpareil.

Table 7.1. The variables describing the anthracnose 'epidemic' on the almond cultivars Price and Nonpareil at Willunga, South Australia, to November 2002.

Cultivar	Time of disease onset	Initial amount of disease (%)	Apparent infection rate[a]	Maximum amount of disease (%)[b]	Overall duration of epidemic (days) [bc]	RAUDPC (%)[d]
Price	25.08.02	0.3	0.06	21.6	68	6.1
Nonpareil	02.10.02	0.4	0.09	8.1	34	3.2

[a] Apparent rates of infection were obtained by regressing logit-transformed disease incidence percentages against time.
[b] The final date of monitoring was 05.11.02
[c] Duration of epidemic was calculated from day 1 until the day that the maximum level of disease was reached
[d] Relative area under the disease progress curve (RAUDPC) was obtained by dividing the AUDPC by the duration (days) of the epidemic and multiplying by 100.

disease incidence was observed was 36 days for Price, compared to 50 days for Nonpareil. The RAUDPC was approximately three times greater for Price, 45.7%, than for Nonpareil, 16.4% (Table 7.2).

A linear regression model using the logit of disease incidence describes the relationship between disease incidence and time for Price ($P<0.05$, $R^2=0.99$) and Nonpareil ($P<0.05$, $R^2=0.99$) with the equations $y = 0.2235x - 3.9947$ for Price and $y = 0.2199x - 6.3952$ for Nonpareil (Fig 7.4B). The apparent rate of infection was 0.23 for Price and 0.22 for Nonpareil (Table 7.2). Using 95% confidence intervals, there is no significant difference between the apparent infection rate for the two cultivars.

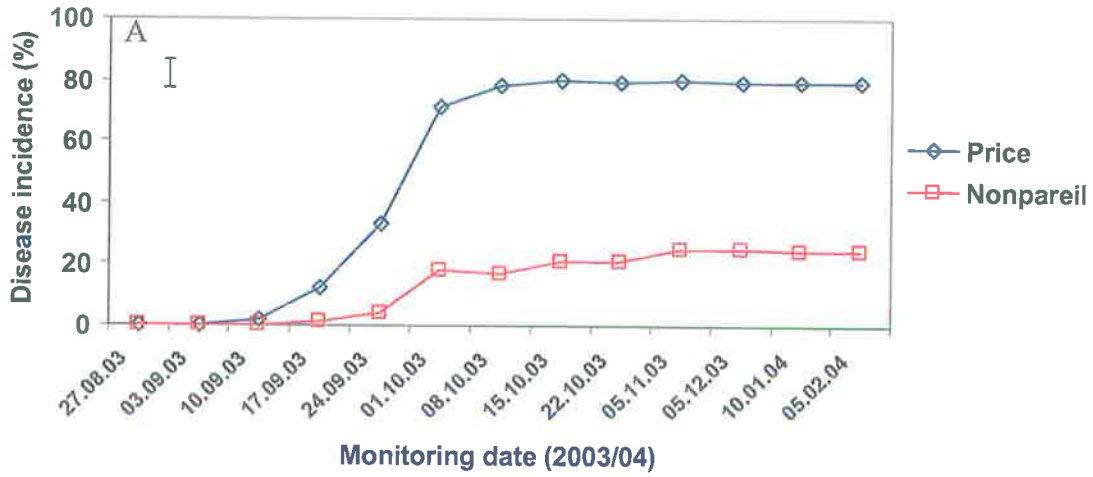
In terms of individual trees, there was minimal apparent variation with respect to disease incidence (%) of fruit throughout the season, with the exception of Price trees 22 and 24, which were less diseased than the others. Fifty-six percent and 62% of fruit showed anthracnose symptoms on Price trees 22 and 24 respectively, whereas 80 to 90% of fruit showed symptoms of anthracnose on the other Price trees. There was slightly more

variability among Nonpareil trees than Price, with the range of disease incidence (%) of fruit between 14% for tree 24 and 33% for tree 11.

There was no relationship between the number of mummified fruit per tree and disease incidence per tree for either Price (P >0.1) or Nonpareil (P >0.05). The mean number of mummified fruit and the range for Price were 106 and 76-161, respectively, whereas those for Nonpareil were 13 and 6-18, respectively (Appendix B). There was no apparent relationship between the number of mummified fruit on an individual branch, or within 1 m of each tagged branch, and disease incidence on that branch.

7.3.1.3 Season 2004

Symptoms on fruit were first evident on 6 September for Price and 13 September for Nonpareil. Leaf symptoms on both cultivars were first evident on 13 September and symptoms were seen from early September to mid-October. The symptoms observed were as reported in Section 7.3.1.1. However, only one tree of Nonpareil, tree 7, showed signs of disease, bearing one diseased fruit for the entire season and showing some leaf symptoms. There was an increase in disease in early November for both Price and Nonpareil (data not shown), and this was due to infection of fruit that was unpollinated and had aborted. This fruit had not been counted in previous monitoring, therefore, was excluded from further analysis.



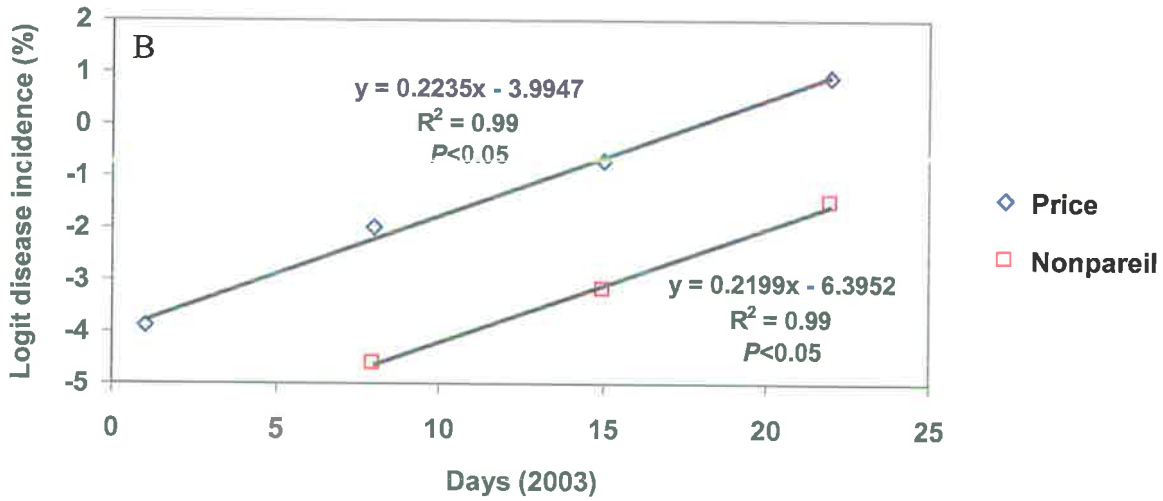


Fig 7.4. Incidence of anthracnose on almond fruit caused by *C. acutatum*, on the cultivars Price and Nonpareil, at Willunga, South Australia, 2003/04. **A.** Disease progress curves ($P<0.001$). Error bar represents LSD of 9.7. **B.** The relationship between logit transformed disease incidence data and time is described by the equations $y = 0.2235x - 3.9947$ for Price ($P<0.05$, $R^2 = 0.99$) and $y = 0.2199x - 6.3952$ for Nonpareil ($P<0.05$, $R^2 = 0.99$).

Table 7.2. The variables describing the anthracnose 'epidemic' on almond fruit of the cultivars Price and Nonpareil at Willunga, South Australia, 2003.

Cultivar	Date of disease onset	Initial disease incidence (%)	Apparent infection rate[a]	Maximum disease incidence (%)	Overall duration of epidemic (days) [b]	RAUDPC (%)[c]
Price	10.09.03	2	0.22	80	36	45.7
Nonpareil	17.09.03	1	0.21	25	50	16.4

[a] Apparent rates of infection were obtained by regressing logit-transformed disease incidence percentages against time
[b] Duration of epidemic was calculated from day 1 until the day that the maximum disease incidence was reached
[c] Relative area under the disease progress curve (RAUDPC) was obtained by dividing the AUDPC by the duration (days) of the epidemic and multiplying by 100.

There was a significant interaction ($P<0.001$) between cultivar and time on disease incidence (%) of fruit (Fig 7.5A). Disease incidence increased steadily from mid-September to reach 25.2% on 9 November for Price, whereas disease incidence for Nonpareil was below 0.1% until 11 October, and it remained at 0.1% for the rest of the season. The small but apparent increase in disease incidence for Nonpareil prior to 11 October was due to the number of healthy fruit decreasing (falling from the tree) and not an increase in fruit with anthracnose symptoms. The period during which increase in disease incidence was observed was 50 days for Price, compared to 35 days for the apparent increase in disease incidence for Nonpareil. The RAUDPC was 12.7% for Price compared to 0.05% for Nonpareil (Table 7.3).

A regression model using the logit of disease incidence describes the relationship between disease incidence and time for Price ($P<0.05$, $R^2=0.89$) and Nonpareil ($P<0.05$, $R^2=0.94$) with the equations $y = 0.048x - 3.2437$ for Price and $y = 0.0197x - 7.8345$ for Nonpareil (Fig 7.5B). The apparent rate of infection was 0.05 for Price and 0.02 for Nonpareil (Table 7.3) and, using 95% confidence intervals, there is no significant difference between the apparent infection rate of the two cultivars.

With respect to variation among trees, disease incidence (%) on fruit of Price increased over the season. However, trees 5, 11, 22 and 26 showed lower disease incidence, between 13 and 16 %, than the other trees, which were between 30 and 39 % at the end of the monitoring period. As disease was evident only on Nonpareil tree 7 for the entire monitoring period, it is not possible to assess apparent variation among trees for Nonpareil.

There was no relationship between the number of mummified fruit per tree and disease incidence per tree for either Price ($P>0.1$) or Nonpareil ($P>0.1$). The mean number of mummified fruit and the range for Price were 65 and 45-100, respectively, whereas those for Nonpareil were 19 and 2-56, respectively (Appendix B). There was no apparent relationship between the number of mummified fruit on an individual branch, or within one metre of each tagged branch, and disease incidence on that branch.

7.3.1.4 Mummified fruit and disease incidence for all seasons

The maximum disease incidence was significantly greater for Price than for Nonpareil for seasons 2002, 2003 and 2004 and, overall, there was considerably more mummified fruit on the trees of Price than Nonpareil for each season. When the data for maximum disease incidence and the mean number of mummified fruit for all trees of each cultivar and each season were combined and analysed by linear regression, there was no significant linear relationship ($P>0.1$) between maximum disease incidence and the total number of mummified fruit.

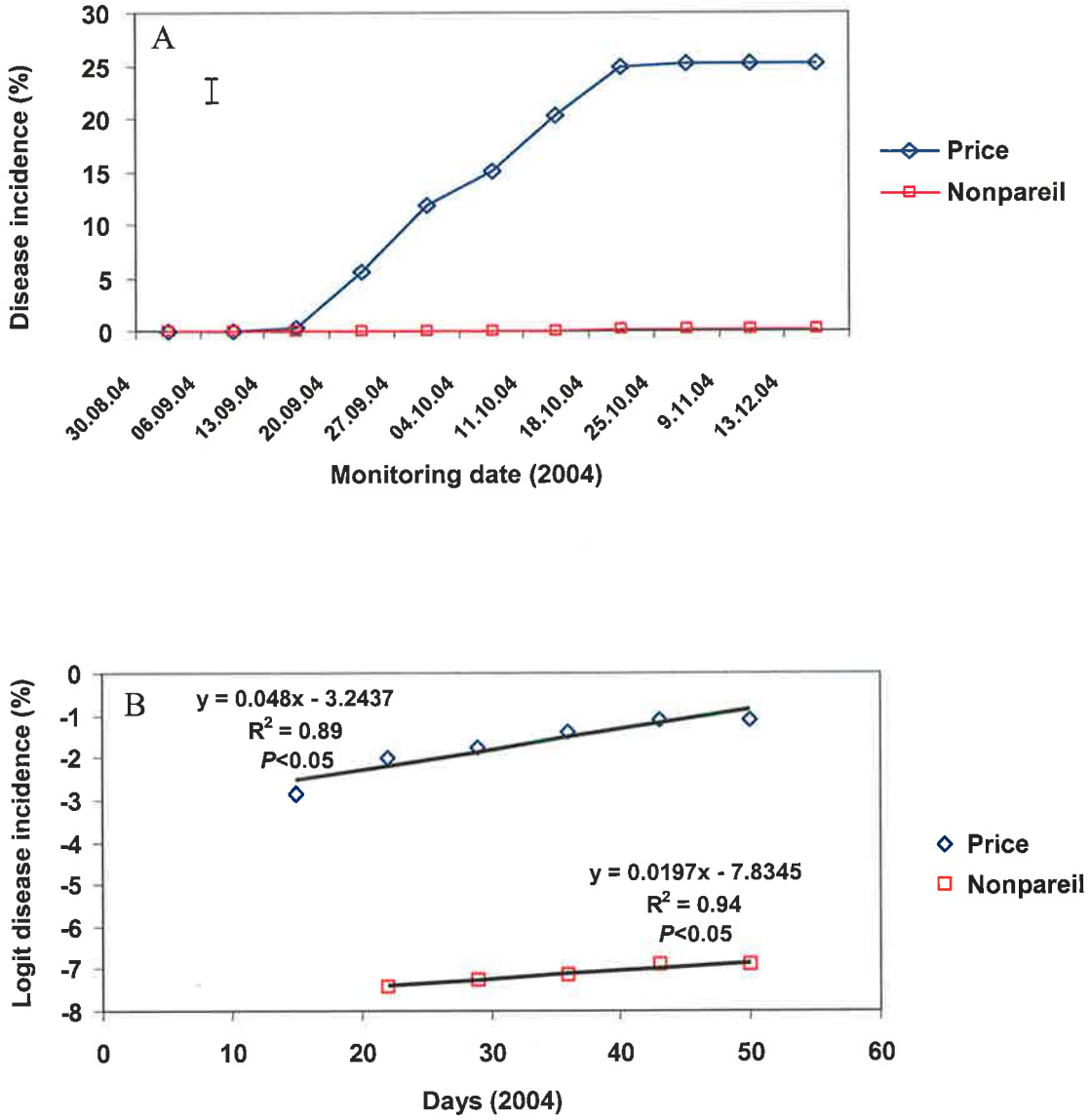


Fig 7.5. Incidence of anthracnose on almond fruit caused by *C. acutatum*, on the cultivars Price and Nonpareil, at Willunga, South Australia, 2004. **A**. Disease progress curves ($P<0.001$). Error bar represents LSD of 5.2. **B**. The relationship between logit transformed disease incidence data and time is described by the equations $y = 0.048x-3.2437$ ($P<0.05$, $R^2 =0.87$) for Price, and $y = 0.0197x -7.8345$ ($P<0.05$, $R^2 =0.94$) for Nonpareil.

Table 7.3. The variables describing the anthracnose 'epidemic' on almond fruit of the cultivars Price and Nonpareil at Willunga, South Australia, 2004.

Cultivar	Date of disease onset	Initial disease incidence (%)	Apparent infection rate[a]	Maximum disease incidence (%)	Overall duration of epidemic (days) [b]	RAUDPC (%)[c]
Price	06.09.04	0.05	0.05	25.2	50	12.7
Nonpareil	13.09.04	0.05	0.02	0.1	35	0.05

[a] Apparent rates of infection were obtained by regressing logit-transformed disease incidence percentages against time
[b] Duration of epidemic was calculated from day 1 until the day that the maximum disease incidence was reached
[c] Relative area under the disease progress curve (RAUDPC) was obtained by dividing the AUDPC by the duration (days) of the epidemic and multiplying by 100.

7.3.1.5 Environmental variables and disease development

The average daily rainfall (mm), temperature and leaf wetness were determined for the duration of the logistic portion of the disease progress curve for each cultivar and compared to the apparent rates of infection (Table 7.4). Overall, the apparent infection rates were highest in 2003 for both Price and Nonpareil, likewise, rainfall and leaf wetness for the duration of the logistic part of disease progress curves were higher in 2003 than 2002 and 2004, whereas the temperature was generally lower.

The relationship between the apparent rates of infection for the two cultivars and the measurements of the environmental parameters taken for the duration of the logistic portion of the disease progress curve for each cultivar was explored by simple linear regression. There was a positive linear relationship ($P<0.001$) between the apparent rates of infection for Price and for Nonpareil and the average daily rainfall (mm) for each season (Table 7.4)

Table 7.4. The apparent rate of infection of anthracnose on the cultivars Price and Nonpareil compared to the average daily rainfall, leaf wetness and temperature for the years 2002, 2003 and 2004.

Year	Cultivar	Apparent rate of infection[a]	Average daily rainfall (mm)[b]	Average daily temperature (°C)[b]	Average daily leaf wetness (%)[b]
2002	Price	0.06	1.2	13.4	3.3
	Nonpareil	0.09	0.9	14.2	3.6
2003	Price	0.23	2.7	11.7	4.2
	Nonpareil	0.22	2.6	11.8	4.1
2004	Price	0.05	0.4	14.1	0.3
	Nonpareil	0.02	0.5	14.7	0.3

[a] Apparent rates of infection were obtained by regressing logit-transformed disease incidence percentages (for the logistic portion of the disease progress curve) against time
[b] Rainfall, temperature and leaf wetness were measured for the duration of the logistic portion of the disease progress curve for each cultivar

which is described by the equation $y = 0.0843x - 0.0065$ ($R^2 = 0.93$) (Fig 7.6). This finding suggests that there was more disease when there was more rain. There was no significant relationship ($P > 0.05$) between apparent infection rate and mean leaf wetness for Price and Nonpareil. There was a significant linear relationship ($P<0.05$) between mean daily temperature and apparent infection rate, described by the equation $y = -0.0660x + 0.989$ ($R^2 = 0.88$) (Fig 7.7). This result suggests that there was less disease when mean daily temperature was around 12°C then when it was 13-14°C.

Rainfall was considered as discrete events, which were measured as either >0.05 mm, >1 mm or >2 mm. The relationship between apparent infection rates and the number of rain events, either >0.05 mm, >1 mm or >2 mm, was explored by simple linear regression. There was no significant relationship ($P>0.05$) between apparent infection rate and the number of rain events of either >0.05 mm, >1 mm or >2 mm.

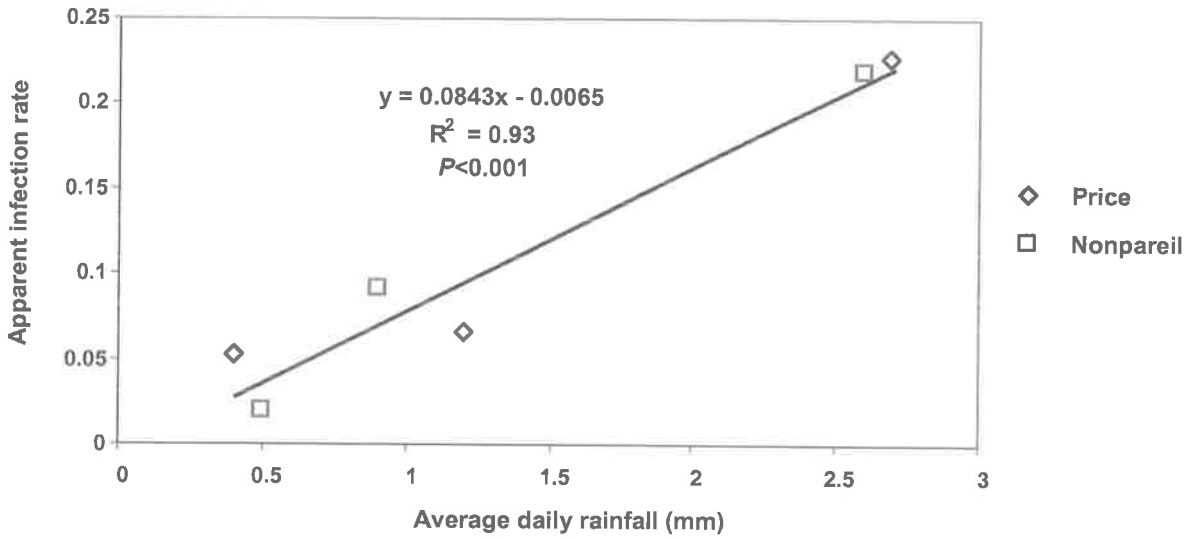


Fig 7.6. The relationship between the apparent infection rate of each anthracnose 'epidemic' on almond fruit and the average daily rainfall (mm) for Price and Nonpareil for the years 2002, 2003 and 2004.

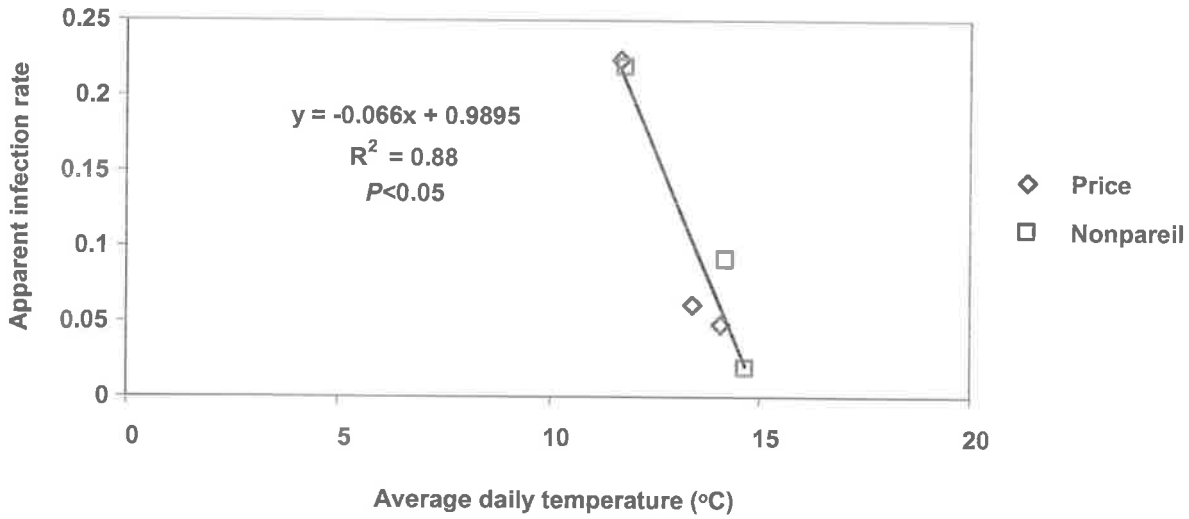


Fig 7.7. The relationship between the apparent infection rate of each anthracnose 'epidemic' on almond fruit and the average daily temperature (°C) for Price and Nonpareil for the years 2002, 2003 and 2004.

7.3.2 Isolation of *C. acutatum* from plant material

C. acutatum was recovered from each type of the plant tissues sampled in 2003/04, most commonly from mummified fruit, peduncles, bark and healthy fruit, and rarely from healthy buds, blossom and leaves.

C. acutatum was recovered from mummified fruit from Price and Nonpareil every month of the sampling period (Fig 7.8). However, there does not appear to be any obvious relationship between the recovery of *C. acutatum* from mummified fruit and any particular time period. Recovery of *C. acutatum* from mummified fruit of Price ranged from 73% in April and May to 23% in September. Recovery of *C. acutatum* from mummified fruit of Nonpareil ranged from 80% in October to 17% in November. When the results for the 12-month sampling period were combined, 47% and 44% of mummified fruit of Price and Nonpareil yielded *C. acutatum*, respectively. Other fungi that were frequently recovered included *Fusarium*, *Botrysphaeria*, *Penicillium* and *Aspergillus* spp.

C. acutatum was recovered from peduncles (Fig 7.9) and bark (Fig 7.10) from Price and Nonpareil every month of the sampling period. As for mummified fruit, there did not appear to be any obvious relationship between the recovery of *C. acutatum* from peduncles and the month, for either cultivar, throughout the 12-month sampling period. However, with the exception of the recovery from peduncles from Price in February, there appeared to be a slight trend towards a decrease in recovery from both peduncles and bark in late summer and early autumn, February and March, respectively. Recovery from peduncles of Price was greatest in April, 83%, and least in March, 10%. *C. acutatum* was recovered most frequently from peduncles from Nonpareil in September, 70%, and least in the winter months of June and July, 6%.

When the results for the 12-month sampling period were combined, 53% and 29% of peduncles of Price and Nonpareil yielded *C. acutatum*, respectively. Recovery of *C. acutatum* from bark of Price ranged from 67% in September to 6% in March, and for Nonpareil, the range was 40% in June to 3% in February. The mean recovery from bark of Price over the 12-month sampling period was 43%, which was almost twice that for Nonpareil, 23%.

C. acutatum was first observed on mummified fruit, peduncles and bark as pink mycelium growing on the plant tissue and onto the culture medium. Frequently, in particular for bark samples and, to a lesser degree for peduncles, other fungi predominated, often making it difficult to establish pure cultures of *C. acutatum* and growth of *C. acutatum* was slow. However, for mummified fruit and with peduncles to which mummified fruit were previously attached, pure cultures could be easily established because *C. acutatum* was the predominant fungus.

C. acutatum was recovered rarely from asymptomatic leaves (Fig 7.11). The fungus was isolated from 16% of leaves from Price in September, 6% in March and 3% in each of December, January and June. *C. acutatum* was recovered from 3% of leaves from Nonpareil in December only. Orange slimy spore masses (sporodochia) were the predominant sign of infection on the detached leaves during incubation. Sporodochia occurred frequently along the petioles, leaf margins and main vein and less frequently on the leaf blade. Very sparse, whitish mycelium was occasionally visible to the naked eye after 2-3 weeks of incubation.

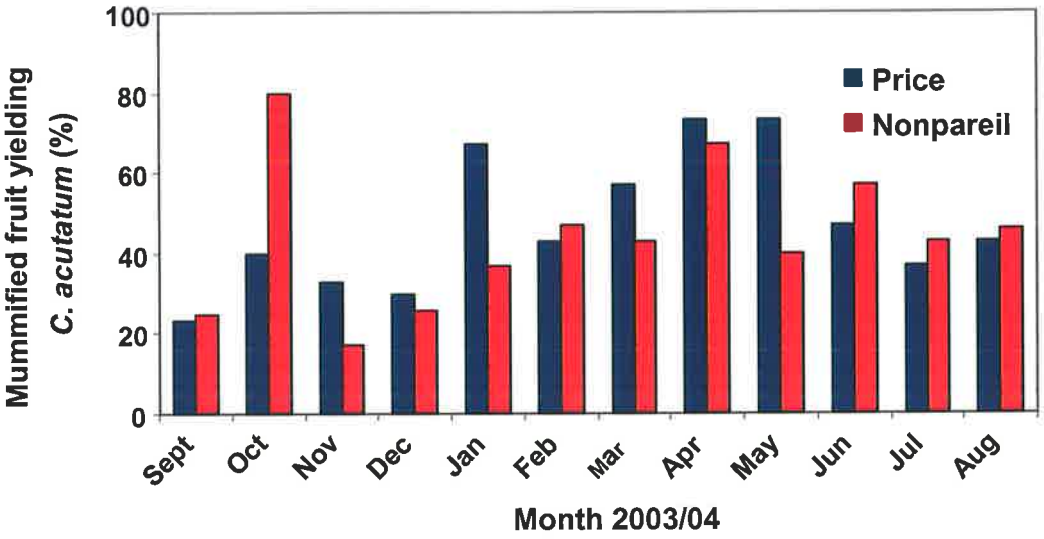


Fig 7.8. The percentage of mummified fruit of the cultivars Price and Nonpareil yielding *C. acutatum*, from Sept to Aug 2004. Mummified fruit were surface sterilised with bleach and incubated on LAPDA.

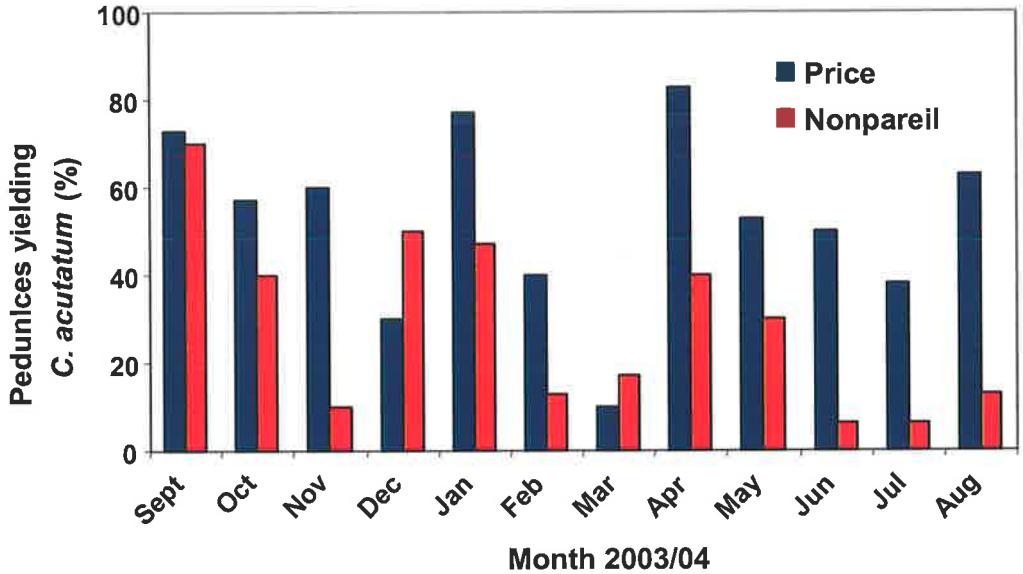


Fig 7.9. The percentage of fruit peduncles of the cultivars Price and Nonpareil yielding *C. acutatum*, from Sept to Aug 2004. Peduncles were surface sterilised in bleach and incubated on LAPDA.

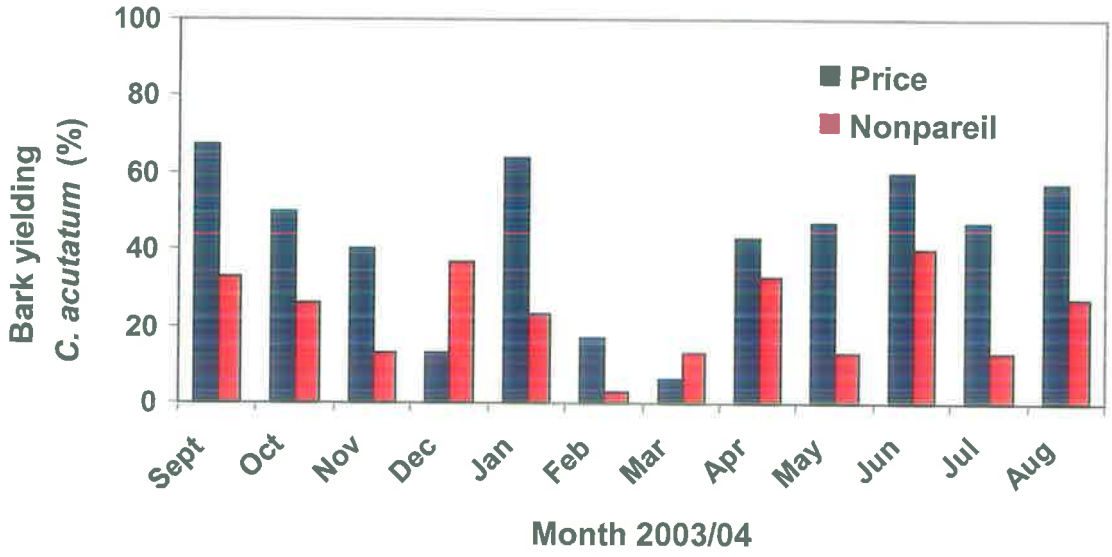


Fig 7.10. The percentage of bark samples of the cultivars Price and Nonpareil yielding *C. acutatum*, from Sept to Aug 2004. Bark was surface sterilised in bleach and incubated on LAPDA.

C. acutatum was recovered from asymptomatic fruit of Price and Nonpareil each month of the sampling period (September to February) (Fig 7.12). Recovery of *C. acutatum* from fruit of Price was greatest in November, December and February, 90%, and least in September, 27%. Recovery of *C. acutatum* from Nonpareil ranged from 17% in September to 53% in October. On average, 75% of asymptomatic fruit from Price yielded *C. acutatum*, compared to 37% of Nonpareil fruit. Infection on both cultivars was evident by the appearance of abundant pink mycelium, which covered the fruit and, once the mesocarp had split, the mycelium covered the interior and exterior of the mesocarp and the endocarp. Generally, mycelium was first observed on the proximal end of the fruit, especially around the point of attachment to the peduncle. Sporodochia developed on the fruit surface after moist incubation for 7-10 days onwards. Other micro-organisms were isolated infrequently from asymptomatic fruit.

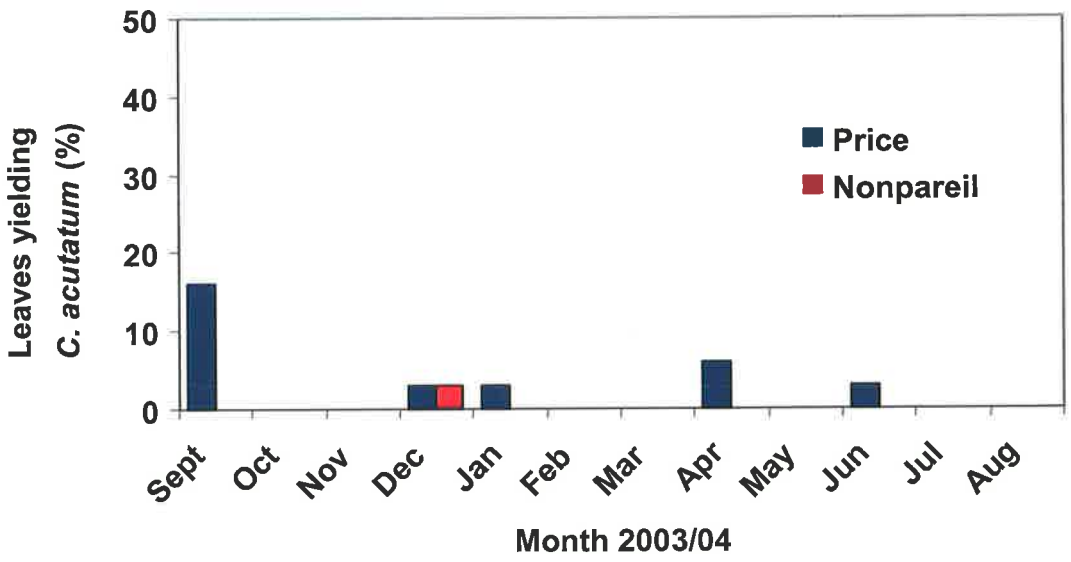


Fig 7.11. The percentage of asymptomatic leaves of the cultivars Price and Nonpareil yielding *C. acutatum*, from Sept to Aug 2004. Leaves were surface sterilised and incubated in humid conditions.

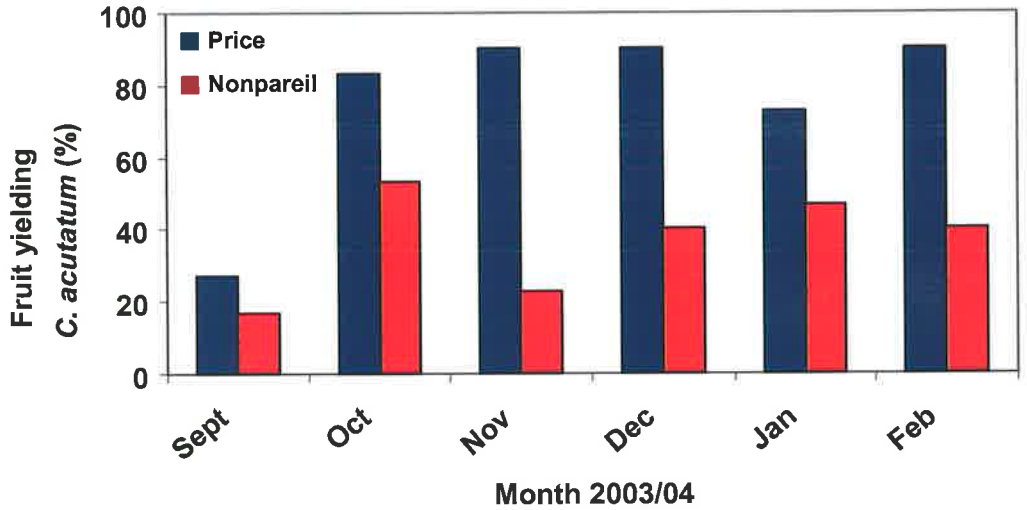


Fig 7.12. The percentage of asymptomatic fruit of the cultivars Price and Nonpareil yielding *C. acutatum*, from Sept to Aug 2004. Fruit were surface sterilised in bleach and incubated in humid conditions.

C. acutatum was recovered from asymptomatic buds and blossom of both Price and Nonpareil for the two sampling dates of 19 July and 30 July 2004. Seventeen percent of buds of Price yielded *C. acutatum* on 19 July, as did 57% of blossom on 30 July. Only 3.5% of buds and blossom of Nonpareil yielded *C. acutatum* on both sampling dates. Typically, signs of infection were sporodochia on the calyx of the buds and blossoms. There was no evidence of infection by *C. acutatum* on the petals of either cultivar. *Botrytis cinerea* was frequently isolated from blossom, and sometimes made it difficult to establish a pure culture of *C. acutatum*.

C. gloeosporioides was not isolated from any plant samples collected during this study. Occasionally, mycelium of *C. acutatum* appeared grey/greyish-pink, especially on the asymptomatic fruit. However, once isolated onto LAPDA or PDA, the colour of such mycelium changed to pink and remained pink.

Perithecia were not observed on any plant tissue, nor on the fungal colonies in culture.

7.4 Discussion

7.4.1 Disease development in the field

The anthracnose symptoms observed on fruit and leaves in each year of this study were generally consistent with those reported in the literature. However, blossom blight was not observed on the trees examined in this study, which is consistent with the observations of McMichael (2000) and Shabi and Katan (1983), although *C. acutatum* was recovered from asymptomatic blossom here.

The main damage observed was to the young, developing fruit. Young fruit, <20 mm long, appeared to be the most susceptible to infection and the majority of fruit that was infected at this size shrivelled, died and generally remained attached to the tree as mummies. On young infected fruit, sometimes more than one lesion was observed. Fruit, *ca* 20-30 mm long and still less than about 8-10 weeks old could still be infected, but this was much less common than infection of younger, smaller fruit. Infection on fruit of *ca* 20-30 mm long resulted in distortion of shape and the lesions produced a tan-coloured gum. Frequently, such fruit that was infected progressed to maturity and harvest. No new infections were observed on fruit *ca* >30 mm long or on fruit greater than about 12 weeks old (early November). Shabi and Katan (1983) observed lesions only on young fruit and early in the growing season, whereas Adaskaveg and Förster (2000) and McMichael (2000) reported that fruit are susceptible to infection throughout the growing season. Leaf symptoms were observed on Price and Nonpareil early in the growing season, but symptoms on leaves were less common than symptoms on fruit. Anthracnose lesions on leaves have been observed in California (Adaskaveg and Förster, 2000) but not in Israel (S. Freeman, pers. comm., 2004), although Shabi and Katan (1983) reported wilting of leaves in association with anthracnose, but they were unable to isolate *Colletotrichum* sp. from such leaves. Die back of twigs and spurs, often with mummified fruit attached, was common and is consistent with signs of anthracnose reported in California (Förster and Adaskaveg, 1999).

In season 2002, the initial incidence of infection was very similar for Price and Nonpareil, as was the apparent rate of infection, however, the final incidence of disease was significantly different. This can be explained by the fact that the duration of the epidemic for Price was almost twice that for Nonpareil, and this is reflected in the RAUDPC, which for Price was

double that of Nonpareil. The delay of onset of disease on Nonpareil in this season led to an 'epidemic' significantly less severe than that for Price.

The initial incidence of infection and apparent infection rate were similar for Price and Nonpareil in season 2003. However, the RAUDPC was almost two and a half times greater for Price than for Nonpareil and the final incidence of disease was over three times more. In contrast to the results for 2002, the 'epidemic' was shorter for Price than for Nonpareil. Infection of Price increased rapidly very early in the season when fruit was small and this is reflected by the fact that 80% of fruit on Price was infected 6 weeks post-petal fall.

In season 2004, the initial incidence of infection was similar for Price and Nonpareil, yet the final amount of disease was significantly different. Symptoms were first observed one week earlier on Price than Nonpareil and, statistically, disease progressed at an apparent rate that was similar for both cultivars. However, disease on Nonpareil was almost non-existent, so the apparent rate of infection and RAUDPC had little biological and practical meaning for Nonpareil in this season.

As discussed in Chapters 2 and 5, Nonpareil has been perceived as less susceptible to anthracnose than Price (Adaskaveg and Förster, 2000; McMichael, 2000). In this study, the onset of disease for Price was consistently earlier than for Nonpareil. Price generally reached full bloom about one week before Nonpareil, so this factor could account for the difference in the date of disease onset between the two cultivars. The maximal disease incidence was significantly greater for Price than for Nonpareil for each season, which may suggest that Price was more susceptible to anthracnose than Nonpareil. Maximum disease incidence was reached for Price and Nonpareil on similar dates for each season, but due to the delay of onset of disease on Nonpareil, the duration of the epidemic was generally shorter and the RAUDPC

less on Nonpareil than Price. This could be interpreted as meaning that Nonpareil was less susceptible than Price, but the delay of onset of disease may be the reason that the duration was shorter and the RAUDPC less on Nonpareil than Price, rather than differences in genetic susceptibility.

The apparent rate of infection was not significantly different for the two cultivars for each season, which suggests that Price and Nonpareil were equally susceptible, as, once disease commenced, it progressed at the same rate for both cultivars. Calculation of apparent rate of infection only takes into account the logistic portion of the disease progress curve, whereas the disease incidence over time takes into account the progress of the epidemic from the first day that symptoms are observed until disease reaches a maximum incidence. A limitation in using the apparent rate of infection alone to compare susceptibility between cultivars is highlighted by examining the rates of infection for Price and Nonpareil for 2004. Disease was practically non-existent on Nonpareil in 2004, but the apparent rate of infection suggests that disease increased on Nonpareil at a statistically similar rate to Price, whereas biologically, this was not the case. However, the disease progress curves showed significant differences between Price and Nonpareil for each season, which suggests that Nonpareil was less affected by anthracnose than Price. Overall, these results do not provide a clear answer about the differences in the susceptibility between Price and Nonpareil. More study is required to investigate the differences in cultivar susceptibility. In this study, only two cultivars were examined, and only one area of one orchard was used. If possible, several cultivars, including Nonpareil, should be examined in more than one area of an orchard and, preferably, studies should include several orchards representing different climatic zones, e.g., Northern Adelaide Plains, Lindsay Point, Sunraysia.

With regard to variation among trees, there did not appear to be a pattern for particular trees from either cultivar or an area of the orchard to be more or less diseased than others over the three seasons.

Mummified fruit are generally considered to be the main source of primary inoculum. In this study, there was no correlation between the number of mummified fruit per tree and the incidence of disease on each tree, nor between maximum disease incidence and total mummified fruit for each cultivar and each season combined. In addition, there was no apparent correlation between the number of mummified fruit per branch or within close proximity to each branch and the amount of disease observed on that branch. Across all trees, there were seven times more mummified fruit on the trees in 2002 than in the 2003 season, yet the disease incidence in 2002 was less than in 2003. These results suggested that mummified fruit may not be the only significant source of primary inoculum, as epidemics of anthracnose occurred irrespective of the number of mummified fruit on the trees. However, it may be that even a small number of mummified fruit is sufficient to provide enough inoculum for an anthracnose epidemic when environmental conditions are suitable.

Rainfall and temperature appeared to be significant factors in the development of anthracnose on almond at this field site, with the former apparently more significant. Several factors must be taken into account when interpreting these results. The equations describing the relationships between apparent infection rate and rain, and apparent infection rate and temperature, need to be interpreted in a biological context. It does not necessarily follow that increased rainfall will increase apparent infection rate. For example, rainfall beyond a certain amount could wash the inoculum off the plant tissue such that infection may not occur, and intensity of rain affects splash dispersal of the inoculum. There is likely to be a threshold for

In particular, rainfall early in the season (August - November) was associated with an increase in disease.

environmental factors beyond which disease development will be affected, and the interaction among various environmental factors will also affect disease development. Other environmental factors, such as sunlight, wind and relative humidity may also influence disease development. Such investigations were beyond the scope of this project, however, data collection over more seasons, and studies of thresholds and interactions of environmental factors and disease development are required to produce a more definitive model to predict disease development. The effect of rainfall, temperature, dew, leaf wetness and relative humidity, and the interactions among some of these factors have been investigated by others (Wastie, 1972b; Wilson *et al.*, 1990; Dodd *et al.*, 1991; Timmer and Zitko, 1993; Timmer and Zitko, 1996). Wilson *et al.* (1990) found that different combinations of temperature and wetness periods significantly affected infection of strawberry by *C. acutatum*. Wastie (1972b) studied secondary leaf fall of rubber, caused by *C. gloeosporioides*, and found that the presence of free water, from rainfall or dew, resulting in periods of 100% relative humidity or leaf wetness, was required for lesion formation *in planta* and temperature affected lesion development *in vitro*. However, Wastie (1972b) concluded that it was not possible to develop a system of forecasting disease outbreaks in sufficient time for control measures to be applied, primarily because of the close link in time between atmospheric humidity and disease development. Timmer and Zitko (1993) found that total rainfall was the most important environmental factor in determining post-bloom fruit drop of citrus caused by *C. acutatum*, and average temperature and relative humidity were not major factors in disease development. The results of this current study on anthracnose on almond suggest that rainfall was the most important of the environmental factors investigated.

The limitation of using mean daily temperature is that it does not describe the temperature course during an epidemic. Different temperature courses can lead to identical means, and

Furthermore, one sensor in one tree could not capture the range of leaf wetness in the experimental plot.

mean temperature is not the most frequent temperature of the day (Friesland and Schröedter, 1988). The limitation of measuring leaf wetness is that the sensors do not have the same physical properties as a leaf, and even less the surface of an almond fruit, so they do not adequately describe the wetting and drying of the plant tissue. This may explain the lack of a significant relationship between leaf wetness and disease development in this study. General observations in the field and in the laboratory suggest that almond fruit appeared to retain moisture for longer than leaves, possibly because the furry exocarp of the fruit retains moisture, whereas moisture is not retained as easily on the waxy cuticle of a leaf. A leaf wetness sensor does not replicate these physical properties. Furthermore, as only one sensor was used in this project, it could not represent the variation that can occur in the canopy of an almond tree.

The results of this study suggested that almond fruit were not susceptible to infection from *ca* 12 weeks post-petal fall, which contrasts with the findings of Adaskaveg and Förster (2000) and McMichael (2000). Biggs and Northover (1988) found that peaches, a climacteric fruit, are susceptible to infection by *Monilinia fructicola* early in their development, then become resistant until later in the season when the fruit start to ripen. Although almonds are not a climacteric fruit, it would be useful to know if the fruit become resistant to infection at a certain size or growth stage, as this may reduce unnecessary fungicide sprays. Further investigations are needed to determine if there is variation in the susceptibility of almond fruit to infection, and if this relates to fruit size, growth stage or other factors.

7.4.2 Isolation of *C. acutatum* from plant material

Overall, more than 40% of mummified fruit, peduncles and bark from Price, and over 20% of these plant tissues from Nonpareil, yielded *C. acutatum*, and it was possible to recover *C.*

acutatum from these tissues every month of the year. All of these tissues have the potential to provide a source of primary and secondary inoculum. It is generally considered that mummified fruit are the main source of primary inoculum, yet these findings showed that dead woody tissue is also likely to be a source of primary inoculum. Thus *C. acutatum* was able to survive over summer months in mummified fruit and woody tissue. The thicker tissue of mummified fruit and peduncles to which mummified fruit were attached may protect the mycelium or spores from heat and desiccation, as recovery was less from bark over summer than from mummified fruit or peduncles. In contrast, Förster and Adaskaveg (1999) found the rate of recovery of *C. acutatum* from woody tissue to be low, even when the woody tissue was associated with diseased fruit. In this study, peduncles and bark were frequently associated with mummified fruit, however, samples were selected at random and mummified fruit frequently became detached during surface sterilisation, so the proximity of mummified fruit to the woody tissue was generally not known. However, it was noted that if mummified fruit was closely associated with woody tissue, then it was likely that *C. acutatum* would be recovered. Also, considering that the growth of *C. acutatum* from bark was often obscured by other fungi in culture, the incidence of *C. acutatum* surviving in woody tissue may be higher than was found in this study. The results of this study also showed that *C. acutatum* was able to survive in leaves and apparently healthy bark. The difference in findings between this study and those of Förster and Adaskaveg (1999) may be that the inoculum load was greater in the orchard used in this study than was found in California, however, there are no data to support this idea.

Mummified fruit of sweet cherry and fruit spurs that had been inoculated with *Monilinia laxa* were able to produce conidia for the entire growing season, and that mummified fruit produced more conidia than fruit spurs (Stensvand *et al.,* 2001). Kable (1965) and Sutton and

Clayton (1972) showed that peduncles were an important source of viable inoculum of *Monilinia fructicola* on peach, in particular early in the growing season, and Kable (1965) recommended that tissue with peduncles be pruned out before the growing season to reduce inoculum. Shabi and Katan (1983) were able to isolate *Colletotrichum* sp. from peduncles of almond and, although the rate of recovery was not quantified, they recommended the removal of overwintering mummified fruit and associated peduncles to reduce inoculum. Based on the findings of the current study, removal of mummified fruit, associated peduncles and woody tissue has the potential to decrease primary inoculum and subsequent disease in South Australia.

C. acutatum was isolated most frequently from healthy leaves in September, coinciding with the time that anthracnose symptoms on leaves were most common. This may suggest that young leaves are more susceptible to infection by *C. acutatum* than older leaves, which are likely to have a thicker cuticle that may impede penetration. Overall, in the field, anthracnose was observed more commonly on the leaves of Price than Nonpareil, and this was reflected by greater recovery of *C. acutatum* from leaves from Price than Nonpareil. It appears that leaves are less susceptible to infection by *C. acutatum* than other plant organs, and the proportion of infected leaves observed in this study would have minimal effect on the potential area available for photosynthesis, therefore, have little direct effect on yield or fruit quality. However, infected leaves could provide secondary inoculum for other tissues, in particular, young fruits early in the season. The results of this study are consistent with those of Förster and Adaskaveg (1999), who recovered *C. acutatum* from 13.6% of leaves, however, they sampled leaves that had foliar symptoms of anthracnose, including chlorosis, whereas asymptomatic leaves were sampled here. However, Förster and Adaskaveg (1999) noted that chlorosis of leaves may have been due to toxic secondary metabolite production by the

fungus, not directly to infection of the tissue. Damaged or unhealthy leaves were not incubated and assessed for inoculum in this study, so the proportion of leaves on which *C. acutatum* was surviving is likely to be greater than reported here. Also, it is likely that recovery would be more frequent if leaves showing signs of anthracnose infection had been sampled.

Although healthy fruit frequently yielded *C. acutatum*, there was no evidence of new lesions on fruit from early November onwards (Section 6.3). The furry exocarp of almond fruit may provide a physical environment that protects the conidia or mycelium of *C. acutatum*, and enables them to survive over summer. Thus, although *C. acutatum* was not able to cause infection on healthy almond fruit after a certain age or size, it was able to survive on the surface. Damage to processed, unblanched almonds due to anthracnose has been reported in California (Adaskaveg *et al.* 1998), however, but not in Australia. Förster and Adaskaveg (1999) demonstrated that kernel infection is likely to occur pre-harvest, but also post-harvest infection of kernels can occur if post-harvest moisture levels were greater than 13.1%. This study found that *C. acutatum* was able to survive on a high proportion of mature fruit, and one could speculate that if environmental conditions were suitable, post-harvest infection may occur in Australia.

C. acutatum was recovered from a high proportion of apparently healthy buds and blossom, in particular from Price, yet no symptoms of petal blight or anthracnose lesions were evident. Two weeks after bud and blossom samples were collected, symptoms on young fruit in the field were evident. Leandro *et al.* (2001) found that *C. acutatum* survived and produced abundant secondary conidia on symptomless strawberry leaves, without evidence of either host penetration or latent infection. They concluded that the production of secondary conidia may increase the amount of inoculum in the absence of suitable host tissue, and that spraying

with fungicides to reduce initial inoculum could reduce the amount of subsequent disease. It may be that buds and blossom are not infected by *C. acutatum*, but produce secondary conidia which could be spread by rain splash onto the surface of suitable host tissue, e.g., developing fruit. Thus, spraying almond trees with fungicides may be warranted, even in the absence of symptoms on buds or blossom, to reduce primary inoculum.

Although cultural methods for anthracnose control were not investigated in this study, removal of mummified fruit and woody tissue may decrease the inoculum potential. The extensive amount of dead wood that remained on the trees at Willunga may have contributed to an increase in inoculum potential compared to other orchards, which appear to use a rigorous pruning strategy. Pruning of mummified fruit and woody tissue as a strategy for anthracnose control is an area for future research. Such research would require considerable grower cooperation and either the availability of orchards infected with anthracnose or growers who would allow trees to be inoculated with *C. acutatum*.

The investigations described in this chapter were carried out at one field site, and it is recommended that similar research be carried out at other almond growing regions of Australia, in particular, those with different climate profiles, e.g., the Riverland, the Sunraysia region.

7.5 Summary

Rainfall early in the growing season (August until early November) appeared to be important in the development of anthracnose, and infection of almond tissues occurred from when fruit was young and small until they reached about 30 mm and/or early November. Assessing buds and blossom for blight symptoms is probably not necessary or useful for monitoring for

anthracnose in Australia, because such symptoms have not been observed by McMichael (2000) or here. Although blossom blight has not been observed, *C. acutatum* was recovered from asymptomatic buds and blossom, and infection can commence at the time of first fruit set. Therefore, the recommendation of McMichael (2000) that fungicide sprays commence early in the season, at pink bud, is endorsed. However, McMichael (2000) recommended that fungicide sprays continue throughout the season, whereas results from the current study indicated that sprays may not be needed after early/mid November, as there was no increase in disease incidence beyond this time. Young fruit and leaves should be monitored for signs of disease from fruit set until early November, to assess the efficacy of fungicide sprays.

With regard to cultivar susceptibility, these results did not answer conclusively the question of relative susceptibility of Price and Nonpareil. Further studies, to investigate the relative susceptibility of the main cultivars and those in the almond breeding program of the University of Adelaide, are recommended. If possible, field studies should be carried out in the different almond growing regions of Australia.

Although it is generally considered that mummified fruit are the main source of primary inoculum, other plant tissues, such as peduncles and woody tissue, are potentially significant sources of primary inoculum. These findings support the suggestion that removal of mummified fruit as well as associated peduncles and woody tissue may reduce inoculum potential and, therefore, subsequent disease. Further studies to compare pruning strategies and the impact on inoculum production and disease are recommended.

Chapter 8 General Discussion

This study has characterised a collection of isolates of *C. acutatum* from almond in Australia with respect to morphological and cultural characteristics, mycelial growth rate at a range of temperatures, genetic variation and pathogenicity to detached plant tissue. Information about disease progression in the field and the plant tissues that have the potential to be sources of primary inoculum will contribute toward understanding optimal conditions for disease development and improved management strategies for anthracnose on almond.

Considerable variation of morphological and cultural characteristics was found among isolates of *C. acutatum* from almond in Australia, however, three main morphotypes were evident, namely pink, orange and cream. Representative isolates also varied with respect to mycelial growth rate at a range of temperatures and pathogenicity to detached almond leaves and fruit. However, overall, there was no trend for isolates representative of the morphotypes to be more or less pathogenic than one another on either detached leaves or fruit, suggesting that morphology is not a good indicator of pathogenicity, as was found by Sanders and Korsten (2003). In contrast, two morphologically distinct populations of *C. gloeosporioides* from yam were found to differ significantly in virulence (Abang *et al.*, 2005). One isolate, CSL-1318, that belonged to the orange morphotype group, consistently grew faster than other isolates of *C. acutatum* from almond in Australia at all of the temperatures tested and was genetically different from all the other isolates of *C. acutatum* from almond in Australia.

In general, based on morphological and cultural characteristics and the results of pathogenicity experiments, the separation of isolates of *C. acutatum* from almond in Australia into distinct groups cannot be supported by more than colony colour alone. This differs from

the findings of Förster and Adaskaveg (1999), who reported that the two subpopulations of *C. acutatum* from almond in California can be differentiated on the basis of colony colour, conidial morphology, virulence in laboratory inoculation experiments, and mycelial growth rates at 10 and 32°C. However, there were several differences between this study and that of Förster and Adaskaveg (1999), in that the mean of seven isolates from each subpopulation was used by Förster and Adaskaveg (1999) to determine mycelial growth rate in culture, conidial morphology was differentiated by general observation only and conidia were harvested from colonies grown on wheat straw agar. Here, single isolates were used for mycelial growth rate experiments and variation determined among isolates, and conidia, harvested from colonies grown on PDA, were measured quantitatively. As such, it is difficult to draw definitive conclusions as to differences in the populations between California and Australia. Although differences were observed among isolates with respect to conidial size and shape, there was considerable overlap amongst characters, making it difficult to differentiate distinct groups among isolates from Australia and California based on conidial morphology alone.

In contrast to the variation observed for morphological characteristics, all isolates of *C. acutatum* from almond in Australia, except for CSL-1318, were the same using ISSR-primed PCR. The DNA fingerprints generated from isolate CSL-1318 by ISSR-primed PCR were similar to those of CSL-1298, an isolate from avocado, obtained from an unknown region of South Australia. After all experimental work and analysis was completed, DNA sequence data for the ITS2 region of selected isolates of *C. acutatum* from almond in Australia was provided by S. Freeman, the Volcani Center, Bet Dagan, Israel. Freeman and collegues (pers. comm., 2005) showed that the sequence of the ITS2 region of CSL-1318 to be different from that of other isolates of *C. acutatum* from almond in Australia, which supported the results of

mycelial growth rate experiments and of the ISSR-primed PCR reported here. Although it would be advisable to assess genetic variation using other DNA-based approaches (see Chapter 5), based on the results to date, it is hypothesised that the population of *C. acutatum* from almond in Australia is clonal. This supports the long-held opinion that *C. acutatum* reproduces asexually, as there has been no evidence that sexual reproduction occurs in nature. Förster and Adaskaveg (1999), likewise, inferred that the pink and grey subpopulations from almond in California were each clonal, based on results of ISSR-primed PCR and RAPD-PCR, and they also concluded that isolates of *Colletotrichum* sp. from almond in Israel were genetically homogeneous.

The findings that groupings based on morphology and pathogenicity were not supported by ISSR-primed PCR agrees with those of Ansari *et al.* (2004) and Sanders and Korsten (2003), who reported a lack of agreement between morphological and cultural characteristics, pathogenicity and genetic variation results, for isolates of *C. lindemuthianum* from bean and *C. gloeosporioides* from mango and avocado, respectively. In contrast, Abang *et al.* (2005) found that two morphotypes of *C. gloeosporioides* from yam were distinguishable based on colony and conidial morphology, growth rate, virulence, and VCG analysis as well results from ISSR-primed PCR and denaturing gradient gel electrophoresis. However, each of these studies used different molecular approaches and experimental techniques, which makes direct comparison difficult.

A few isolates from pistachio and olive in Australia were genetically identical to isolates of *C. acutatum* from almond in Australia using ISSR-primed PCR, which may suggest that the population of *C. acutatum* is not specific to almond. Denoyes-Rothan *et al.* (2003) found that a clonal population of isolates of *C. acutatum* from strawberry was more virulent on

strawberry than a genetically variable population from this host, leading to the hypothesis that the clonal population was more specialised on strawberry. Further study of cross-infectivity of almond isolates on other hosts and *C. acutatum* from other hosts on almond should be conducted to investigate host-specificity.

An isolate of *C. gloeosporioides* from orange in Australia caused lesions on detached almond fruit *in vitro*, which suggested that *C. gloeosporioides* has the potential to infect almond in the field. The optimal temperature for mycelial growth of *C. gloeosporioides* was 30°C and mycelial growth continued at 35°C, suggesting that *C. gloeosporioides* may grow and survive in hot climates. Orange and almond are cultivated in close proximity in the Riverland and Sunraysia regions of Australia which are warmer than the Southern Vales and Northern Adelaide Plains regions of South Australia. If anthracnose caused by *C. gloeosporioides* was to occur on orange in these hotter regions and spread to almond orchards nearby, *C. gloeosporioides* may pose a threat to the almond industry.

Field studies showed that anthracnose has the potential to cause severe crop loss in Australia, which concurs with the findings of McMichael (2000). Considerable disease was observed on both cvs Price and Nonpareil, with consistently greater incidence on Price than Nonpareil. Although there is some evidence to suggest that Nonpareil is less susceptible to anthracnose than Price and other cultivars, the results of this study do not fully support such an observation. Nonpareil is perceived as less susceptible than other cultivars because disease is less common in the field, but analysis of field monitoring data, isolation of *C. acutatum* from plant tissue and *in vitro* pathogenicity assays suggested that there is little difference in the susceptibility of Price and Nonpareil. Biggs and Miller (2001) suggested that ranking of susceptibility of fruit tree cultivars be based on results of systematic field and laboratory studies rather than observations in the field alone. If there were differences in resistance to

anthracnose among the cultivars commonly grown in Australia at present, then it is hypothesised that variation in pathogenicity among isolates be expected, as the pathogen and host co-evolve (Chakraborty *et al.*, 2002). Although this study found variation among representative isolates of *C. acutatum* from almond in Australia, there was no trend for isolates to be more or less pathogenic than one another, inferring that differences in cultivar susceptibility were too small to drive the selection of more or less pathogenic strains of *C. acutatum* in Australia. Furthermore, low genetic diversity may also indicate a lack of pathogenic diversity and cultivar specificity, as was shown by Freeman *et al.* (1996) who reported that pathogenic diversity was 'coupled with' genetic diversity among isolates of *C. gloeosporioides* from avocado in Israel. Further work is needed to substantiate the apparent lack of difference in susceptibility between Price and Nonpareil, and it is highly recommended that *in vitro* and *in planta* assays be carried out on progeny from the almond breeding program at the University of Adelaide, in conjunction with detailed field monitoring of a range of cultivars including Price and Nonpareil.

Data from field monitoring and environmental variables showed that there were significant relationships between apparent infection rates and rainfall and temperature, respectively. The equations resulting from the linear regression analysis form the basis for the development of a model to predict an increase in disease when particular environmental parameters are met. These relationships need to be validated on data from other cultivars and almond growing regions over several growing seasons. Results from field monitoring suggested that other factors, such as amount of potential inoculum and blossom time, appeared to influence disease incidence, so these factors would need to be taken into account for the development of a predictive model. A simple method for the estimation of inoculum potential is considered important in the development of 'grower friendly' disease risk and predictive models (Dodd

et al., 1991; Luo and Michailides, 2001). Estimation of the number of mummified fruit on representative almond trees, as used in this project, may be the simplest method for a grower to use, compared to spore traps, as are used in other experimental models (Dodd *et al.*, 1991). Using the linear regression equation for apparent infection rate *versus* rainfall as a simple model, i.e., increased rainfall in spring is positively correlated with a potential increase in anthracnose, then it is advised that a protectant fungicide be sprayed just prior to rain, or an eradicant or antisporulant fungicide be sprayed immediately after rainfall, however, both scenarios are not always possible due to weather and other factors which may restrict access to the orchard.

At this time, Captan is the only fungicide registered for control of anthracnose on almonds in Australia, but other fungicides can be and are used for control of other fungal diseases at the same time, of which some, e.g., Bravo® (chlorothalonil), have been reported to show efficacy against anthracnose (McMichael, 2000). Current recommendations are to spray with either Bravo® or Captan at intervals not exceeding 12 days from pink bud throughout the entire growing season (McMichael, 2000). However, it appears that control has not been consistent across locations, season-long protection has not been achieved and, in general, 'product efficacy trials have produced highly variable results' (McMichael, 2000). In general, good control of anthracnose has been achieved in California (Adaskaveg, 1999), but eradication has not been possible (Adaskaveg and Förster, 2000). The population of *C. acutatum* from almond in Australia was shown to be genetically distinct from both the pink and grey subpopulations of *C. acutatum* from almond in California, which may infer that these populations are biologically distinct, thus could respond differently to fungicides. Although this hypothesis may be plausible, there were no significant differences in pathogencity *in vitro* between the representative pink isolate from California and the Australian isolates of *C.*

acutatum, yet the representative grey isolate from California was less pathogenic than the Australian isolates. This result suggested that the isolates of *C. acutatum* from Australia may be biologically distinct from the Californian grey subpopulation, which could help explain, in part, the different fungicide responses. However, Förster and Adaskaveg (1999) reported that the pink subpopulation was generally more abundant than the grey subpopulation, but that this varied between orchards and counties that had different climate profiles. It is not known if isolates from the grey and pink subpopulations of *C. acutatum* from almond in California show similar responses to fungicides. To begin to understand fungicide responses, it is recommended that representative isolates of *C. acutatum* from almond in Australia, Israel and California be assessed *in vitro* and *in planta* for response to a range of fungicides that are used for anthracnose control, e.g., Bravo®, Captan, and others which have been effective in California (Adaskaveg and Förster, 2000).

Sprays at or around blossom time may not be warranted as no symptoms of anthracnose were seen on these plant tissues. Rather, fungicide application should commence from about 4 weeks post-petal fall. Fungicide efficacy trials suggested that fruit can be infected until hull split (McMichael, 2000), however, there was no evidence of new infection on fruit after early November in the present study, which suggests that fungicide application after this time may not be warranted. Thus the critical period for fungicide application appears to be the first 3 months of the growing season. These findings are consistent with those of Shabi and Katan (1983) and Shabi (1997) who reported that anthracnose affects only young fruit during spring. There would seem little value in applying fungicides if there are no signs of disease and if there is minimal rainfall in spring, but if symptoms of anthracnose are evident and rain occurs in spring then regular fungicide sprays seem warranted. If growers can be persuaded to spray only following confirmation of anthracnose in their orchard and in response to rain in spring,

then this may result in cost savings. Given the acknowledged limitations of McMichael's, (2000) study, it seems reasonable to recommend that systematic fungicide efficacy trials be conducted.

Orchard hygiene has been recommended as an important tool for disease management for almond in Israel by Shabi and Katan (1983) and for other crops (Kable, 1970). Mummified fruit and associated woody tissue, including peduncles and small branches, provided considerable potential inoculum sources, so it is suggested that woody tissue with attached mummified fruit be removed at the end of each growing season. Also, pruning dead and diseased wood may open the canopy, thus improve air circulation and decrease humidity within the canopy (Jeffries *et al.*, 1990) leading to decrease in disease.

During the 'survey' of almond growing regions in 2001 (Section 3.1), typical anthracnose symptoms were observed only on trees in the Willunga and Northern Adelaide Plains, whereas symptoms atypical of anthracnose were observed in the other regions. During the survey and throughout this study, many growers reported that they had fruit affected by anthracnose, yet observations and failed attempts to isolate *C. acutatum* from this plant material suggested strongly that these growers had confused anthracnose symptoms with other, unidentified diseases, disorders or naturally aborted fruit. Thus, there is a need to educate growers to identify anthracnose. The trees at Willunga were lightly pruned compared to the majority of trees at other locations which may result in increased humidity within the canopy. Also, Willunga receives more rainfall than the Riverland and Sunraysia regions. Both of these factors may provide conditions more conducive for the development of anthracnose in Willunga than in other regions. Anthracnose has been confirmed in all of the major growing regions (McMichael, 2000), however, *C. acutatum* was not recovered from plant samples collected during this study from these regions, thus, it is possible that

environmental conditions have not been suitable for the development of anthracnose over the last few years.

For future research, it is suggested that several other methods be explored to characterise isolates of *C. acutatum* from almond and other plants in more detail. It is recommended that additional DNA-based methods be used, e.g., AFLP analysis, sequencing of ITS regions and conserved genes, as such approaches may detect variation in the population. To investigate population genetics and aspects of anthracnose on almond, it is recommended that molecular markers be used, because phenotypic markers, e.g., morphological traits, are subject to variability, as gene expression governing morphological characteristics is subject to environmental conditions (Rodriguez and Redman, 1992). Molecular markers could be used to investigate aspects of the disease cycle, such as following the spread of an isolate in the field. In addition to DNA-based approaches, VCG analysis should be performed to determine if isolates of *C. acutatum* from almond have the potential to mate with one another, and if the possibility exists for genetic exchange among *C. acutatum* isolates from almond and other plants. This would have implications for genetic diversity and for the breeding and selection of almond cultivars with durable resistance to infection by all variants of the pathogen (Bentley *et al.*, 1995). It appeared as though there was one genotype of *C. acutatum* from almond in Australia and, thus, breeding and selection of almond cultivars in Australia for resistance to *C. acutatum* should be based on this one genotype.

This study has contributed to knowledge of *C. acutatum* from almond in Australia and the relationship to isolates of *Colletotrichum* from almond in California and Israel, as well as from other plants. Australian isolates appear to be genetically unique, yet they fit within the morphological descriptors for fungi of the *C. acutatum* species complex. The data obtained

from pathogenicity assays, monitoring anthracnose in the field and isolating *C. acutatum* from various plant tissues have enhanced our understanding of the disease, which will contribute to the development of improved management strategies.

Appendix A (media and reagents)

Lactic acid acidified PDA (LAPDA) (per litre)

PDA (Difco)	39 g
RO water	1000 mL

Sterilised by autoclaving, then the following was added:

Lactic acid	1 mL

Lysis buffer (Rodriguez and Yoder 1991; Freeman *et al.* 2001)

EDTA	50 mM
Tris-HCL	50 mM pH 8.0
N-Lauroyl sarkosine	1 %

Sterilised by autoclaving. On the day of use, the following was added:

Pronase E	500 µg/mL

Mycelial extraction buffer (Hamelin *et al.* 2000)

Tris-HCl	0.5 M pH 9.0
Triton® X-100	1%

Sterilised by autoclaving.

PEG/NaCl (Rodriguez and Yoder 1991)

PEG 6000	20%
NaCl	2.5 M

Sterilised by autoclaving.

Potato dextrose agar (PDA) (per litre)

PDA (Difco)	39 g
RO water	1000 mL

Sterilised by autoclaving.

Potato dextrose broth (PDB) (per litre)

PDB (Difco)	24 g
RO water	1000 mL

Sterilised by autoclaving.

Tap water agar (TWA) (per litre)

Agar (Oxoid, No. 3)	7.5 g
RO water	1000 mL

Sterilised by autoclaving.

Tris-Acetate-EDTA (TAE) buffer

Tris-HCl	40 mM
Sodium acetate	20 mM
EDTA	1 mM

Prepared as a 50 x solution, pH adjusted to 7.8 with glacial acetic acid, sterilised by autoclaving.

Tris-borate-EDTA (TBE) buffer

Tris-borate	45 mM
EDTA	1 mM

Prepared as a 5 x solution, sterilised by autoclaving.

Tris-EDTA (TE) buffer

Tris-HCl	10 mM
EDTA	1 mM

Sterilised by autoclaving.

Appendix B

Table 1. The number of mummified almond fruit and disease incidence (%) on individual Price and Nonpareil trees. A. 2002, B. 2003 and C. 2004.

A.	Price		Nonpareil	
Tree number	Number of mummified fruit	Disease incidence (%)	Number of mummified fruit	Disease incidence (%)
5	1110	10	70	4
7	1112	23	131	5
9	710	20	54	11
11	531	32	52	8
20	740	20	82	6
22	373	23	92	0.7
24	910	25	109	20
26	585	23	35	11

B.	Price		Nonpareil	
Tree number	Number of mummified fruit	Disease incidence (%)	Number of mummified fruit	Disease incidence (%)
5	106	90	6	26
7	76	81	10	30
9	105	90	11	16
11	118	83	11	33
20	161	86	17	28
22	72	56	16	20
24	120	82	18	14
26	90	62	15	18

C.	Price		Nonpareil	
Tree number	Number of mummified fruit	Disease incidence (%)	Number of mummified fruit	Disease incidence (%)
5	100	14.1	12	0
7	57	35.2	32	0.8
9	50	39.1	12	0
11	54	12.5	56	0
20	70	31.7	14	0
22	45	15.5	12	0
24	52	30.4	2	0
26	90	16.1	10	0

References

Abang, M.M., Fagbola, O., Smalla, K. and Winter, S. (2005) Two genetically distinct populations of *Colletotrichum gloeosporioides* Penz. causing anthracnose disease of yam (*Dioscorea* spp.). *Phytopathology,* **153**, 137-142.

Abang, M.M., Winter, S., Green, J.R., Hoffman, P., Mignouna, H.D. and Wolf, G.A. (2002) Molecular identification of *Colletotrichum gloeosporioides* causing yam anthracnose in Nigeria. *Plant Pathology,* **51**, 63-71.

Adaskaveg, J.E. (1999) Management of almond anthracnose in California: Detection and identification of the causal pathogen, epidemiology and new management practices. *Nut Grower Magazine,* **March**, 39-48.

Adaskaveg, J.E. and Förster, H. (2000) Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California. In *Colletotrichum: Host Specificity, Pathology and Host-pathogen Interaction* (Eds, Prusky, D., Freeman, S. and Dickman, M.B.) The American Phytopathological Society Press, St. Paul, Minnesota, USA, pp. 317-336.

Adaskaveg, J.E., Förster, H., Hartin, R.J., Connell, J.H., Teviotdale, B. and Duncan, R. (1998) Almond anthracnose in California- A new pre- and postharvest fungal disease outbreak. *Acta Horticulturae,* **470**, 553-561.

Adaskaveg, J.E. and Hartin, R.J. (1997) Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology,* **87**, 979-987.

Afanador-Kafuri, L., Minz, D., Maymon, M. and Freeman, S. (2003) Characterization of *Colletotrichum* isolates from tamarillo, passiflora, and mango in Columbia and identification of a unique species from the genus. *Phytopathology,* **93**, 579-587.

Agostini, J.P., Timmer, L.W. and Mitchell, D.J. (1992) Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. *Phytopathology,* **82**, 1377-1382.

Agrios, G.N. (1997) *Plant Pathology,* Academic Press, San Diego, California, USA.

Andersen, B. and Thrane, U. (1996) Differentiation of *Alternaria infectoria* and *Alternaria alternata* based on morphology, metabolite profiles, and cultural characteristics. *Canadian Journal of Microbiology*, **42**, 685-689.

Annamalai, P., Ishii, H., Lalithakumari, D. and Revathi, R. (1995) Polymerase chain reaction and its application in fungal disease diagnosis. *Journal of Plant Diseases and Protection,* **102**, 91-104.

Ansari, K.I., Palacios, N., Araya, C., Langin, T., Egan, D. and Doohan, F.M. (2004) Pathogenic and genetic variability among *Colletotrichum lindemuthianum* isolates of different geographic origins. *Plant Pathology,* **53,** 635-642.

Aquadro, C.F. (1992) Why is the genome variable? Insights from *Drosophila*. *Trends in Genetics,* **8,** 355-362.

Arenal, F., Platas, G., Martin, J., Salazar, O. and Peláez, F. (1999) Evaluation of different PCR-based DNA fingerprinting techniques for assessing the genetic variability of isolates of the fungus *Eppicoccum nigrum*. *Journal of Applied Microbiology*, **87**, 898-906.

Arx, J.A. v. (1957) Die Arten der Gattung *Colletotrichum*. *Phytopathology Zeitschrift,* **29**, 413-468.

Ash, G.J. and Lanoiselet, V.M. (2001) First record of *Colletotrichum acutatum* causing a leaf spot and hull rot of pistachio. *Australasian Plant Pathology,* **30**, 365-366.

Ayala, F.J. (1983) Genetic variation and evolution. *Carolina Biology Readers,* **126**, 1-16.

Bailey, J.A., O'Connell, R.J., Pring, R.J. and Nash, C. (1992) Infection strategies of *Colletotrichum* species. In *Colletotrichum: Biology, Pathology and Control* (Eds, Bailey, J.A. and Jeger, M.J.) CAB International, Wallingford, Oxon, UK, pp. 88-120.

Baker, K. (1957) *The U.C. System for Producing Healthy Container-grown Plants,* University of California, Division of Agricultural Sciences, USA.

Barker, I., Brewer, G., Cook, R.T.A., Crossley, S. and Freeman, S. (1994) Strawberry blackspot disease (*Colletotrichum acutatum*). In *Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification* (Eds, Schots, A., Dewey, F.M. and Oliver, R.) CAB International, Oxon, UK, pp. 179-182.

Baxter, A.P., van der Westhuizen, G.C.A. and Eicker, A. (1985) A review of literature on the taxomony, morphology and biology of the fungal genus *Colletotrichum*. *Phytophylactica,* **17**, 15-18.

Bengtsson, B.O. (2003) Genetic variation in organisms with sexual and asexual reproduction. *Journal of Evolutionary Biology,* **16**, 189-199.

Bennett, C. (2004). Almond Industry Annual Report, 2004.

Bentley, S. and Bassam, B.J. (1996) A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f. sp. *cubense*. *Journal of Phytopathology,* **144**, 207-213.

Bentley, S., Pegg, K.G. and Dale, J.L. (1995) Genetic variation among a world-wide collection of isolates of *Fusarium oxysporum* f. sp. *cubense* analysed by RAPD-PCR fingerprinting. *Mycological Research,* **99**, 1378-1384.

Bernstein, B., Zehr, E.I. and Dean, R.A. (1995) Characteristics of *Colletotrichum* from peach, apple, pecan and other hosts. *Plant Disease,* **79**, 478-482.

Biggs, A.R. and Miller, S.A. (2001) Relative susceptibility of selected apple cultivars to *Colletotrichum acutatum*. *Plant Disease*, **98**, 657-650.

Biggs, A.R. and Northover, J. (1988) Early and late-season susceptibility of peach fruits to *Monilinia fructicola*. *Plant Disease,* **72**, 1070-1074.

Bourke, P.M.A. (1970) Use of weather information in the prediction of plant disease epiphytotics. *Annual Review of Phytopathology,* **8**, 345-370.

Brasier, C.M. (1987) The dynamics of fungal speciation. In *Evolutionary Biology of the Fungi* (Eds, Rayner, A.D.M., Brasier, C.M. and Moore, D.) Cambridge University Press, Cambridge, UK, pp. 231-260.

Bridge, P.D. and Arora, D.K. (1998) Interpretation of PCR methods for species definition. In *Applications of PCR in Mycology* (Eds, Bridge, P.D., Arora, D.K., Reddy, C.A. and Elander, R.P.) CAB International, Wallingford, Oxon, UK, pp. 63-84.

Brizi, U. (1896) Eine neue krankheit (Anthracosis) des mandelbaumes. *Zeitschrift fur Pflanzenkrankheiten,* **6**, 63-72.

Brookman, J. and Denning, D.W. (2000) Molecular genetics in *Aspergillus fumigatus*. *Current Opinion in Microbiology,* **3**, 468-474.

Brown, J.K.M. (1996) The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytologist,* **133**, 183-195.

Bryson,R.J., Caten, C.E., Hollomon, D.W. and Baily, J.A. (1992) Sexuality and genetics of *Colletotrichum.* In *Colletotrichum: Biology, Pathology and Control* (Eds, Bailey, J.A. and Jeger, M.J.) C.A.B. International, Wallingford, Oxon, UK, pp. 27-46.

Buddie, A.G., Martínez-Culebras, P., Bridge, P.D., García, M.D., Querol, A., Cannon, P.F. and Monte, E. (1999) Molecular characterization of *Colletotrichum* strains derived from strawberry. *Mycological Research,* **103**, 385-394.

Buhler, M., Gessler, C. and Boos, J. (1993) An improved apple scab warning system: consideration of the biological parameters, ascospore presence and leaf growth, in addition to microclimatic factors. *Acta Horticulturae*, **347**, 115-125.

Butt, D.J. and Xu, X.M. (1994) VentemTM- a computerised apple scab warning system for use on farms. Norwe*gian Journal of Agricultural Sciences,* **17**, 247-251.

Campbell, C.L. and Madden, L.V. (1990) *Introduction to Plant Disease Epidemiology,* John Wiley and Sons, New York, USA.

Cannon, P.F. (1998) International Course on the Identification of Fungi of Agricultural and Environmental Significance. *Colletotrichum*, CABI Bioscience, UK.

Cannon, P.F., Bridge, P.D. and Monte, E. (2000) Linking the past, present, and future of *Colletotrichum* systematics. In *Colletotrichum Host Specificity, Pathology and Host-pathogen Interaction* (Eds, Prusky, D., Freeman, S. and Dickman, M.B.) The American Phytopathological Society Press, St. Paul, Minnesota, USA, pp. 1-20.

Chakraborty, S., Fernandes, C.D., Charchar, M.J.d' A. and Thomas, M.R. (2002) Pathogenic variation in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. in a center of diversity in Brazil. *Phytopathology,* **92**, 553-562.

Chakraborty, S., Ratcliff, D. and McKay, F.J. (1990) Anthracnose of *Stylosanthes scabra*: Effect of leaf surface wetness on disease severity. *Plant Disease,* **74**, 379-384.

Chandra Mohanan, R., Kaueriappa, K.M. and Nambiar, K.K.N. (1989) Epidemiological studies of *Colletotrichum gloeosporioides* disease of cocao. *Annals of Applied Biology,* **114**, 15-22.

Channuntapipat, C., Wirthensohn, M., Ramesh, S. A., Batlle, I., Arús, P., Sedgley, M. and Collins, G. (2003) Identification of incompatibility genotypes in almond (*Prunus dulcis* Mill.) using specific primers based on the introns of the *S*-alleles. *Plant Breeding,* **122**, 164-168.

Chiocchetti, A., Ghignone, S., Minuto, A., Lodovica Gullino, M., Garibaldi, A. and Migheli, Q. (1999) Identification of *Fusarium oxysporum* f. sp. *basilici* isolated from soil, basil seed, and plants by RAPD analysis. *Plant Disease,* **83**, 576-581.

Colhoun, J. (1973) Effects of environmental factors on plant disease. *Annual Review of Phytopathology,* **11**, 343-364.

Cook, R.T.A. and Hughes, K.J.D. (2004) *Glomerella acutata*. Diagnostic protocols for regulated pests. OEPP/EPPO Bulletin, **34**, 193-199.

Correll, J.C. (1992) Genetic, biochemical, and molecular techniques for the identification and detection of soilborne plant-pathogenic fungi. In *Methods for Research on Soilborne Phytopathogenic Fungi* (Eds, Singleton, L.L., Mihail, J.D. and Rush, C.M.) The American Phytopathological Society, St. Paul, Minnesota, USA, pp. 7-16.

Correll, J.C., Morelock, T.E. and Guerber, J.C. (1993a) Vegetative compatibility and virulence of the spinach anthracnose pathogen, *Colletotrichum dematium. Plant Disease*, **77**, 688-691.

Correll, J.C., Rhoads, D.D. and Guerber, J.C. (1993b) Examination of mitochondrial DNA restriction fragment length polymorphisms, DNA fingerprints and randomly amplified polymorphic DNA of *Colletotrichum orbiculare. Phytopathology,* **83**, 1199-1204.

Crowhurst, R.N., Hawthorne, B.C., Rikkerink, E.H.A. and Templeton, M.D. (1991) Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. *Current Genetics,* **20**, 391-396.

Curry, K.J., Abril, M., Avant, J.B. and Smith, B. (2002) Strawberry anthracnose: Histopathology of *Colletotrichum acutatum* and *C. fragariae. Phytopathology,* **92**, 1055-1063.

Czarnecki, H.L. (1916) A *Gloeosporium* disease of the almond probably new to America. *Phytopathology,* **6**, 310.

Davis, R.D., Irwin, J.A.G., Cameron, D.F. and Shepherd, R.K. (1987) Epidemiological studies on the anthracnose diseases of *Stylosanthes* spp. caused by *Colletotrichum*

gloeosporioides in North Queensland and pathogenic specialisation within the natural fungal populations. *Australian Journal of Agricultural Research,* **38**, 1019-1032.

Debets, A.J.M. (1998) Parasexuality in fungi: Mechanisms and significance in wild populations. In *Molecular Variability of Fungal Pathogens* (Eds, Bridge, P.D., Couteaudier, Y. and Clarkson, J.M.) CAB International, Wallingford, Oxon, UK, pp. 41-52.

Denham, T.G. and Waller, J.M. (1981) Some epidemiological aspects of post-bloom fruit drop disease (*Colletotrichum gloeosporioides*) in citrus. *Annals of Applied Biology,* **98**, 65-77.

Denoyes, B. and Baudry, A. (1995) Species identification and pathogenicity study of French *Colletotrichum* strains isolated from strawberry using morphological and cultural characteristics. *Phytopathology,* **85**, 53-57.

Denoyes-Rothan, B., Guérin, G., Délye, C., Smith, B., Minz, D., Maymon, M. and Freeman, S. (2003) Genetic diversity and pathogenic variability among isolates of *Colletotrichum* species from strawberry. *Phytopathology,* **93**, 219-228.

Diéguez-Uribeondo, J., Förster, H. and Adaskaveg, J.E. (2002) Temperature and wetness requirements for almond anthracnose development on leaves and blossoms. *Phytopathology,* **92**:S19.

Diéguez-Uribeondo, J., Förster, H. and Adaskaveg, J.E. (2003) Subcuticular, intramural and intracellual colonization of almond by *Colletotrichum acutatum. Phytopathology*, **93**:S21.

Dodd, J.C., Estrada, A. and Jeger, M.J. (1992) Epidemiology of *Colletotrichum gloeosporioides* in the tropics. In *Colletotrichum; Biology, Pathology and Control* (Eds, Bailey, J.A. and Jeger, M.J.) CAB International, Wallingford, Oxon, UK, pp. 308-325.

Dyko, B.J. and Mordue, J.E.M. (1979) *Colletotrichum acutatum*. Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria, **630**. Commonwealth Mycological Institute, Kew, Surrey, UK.

Dodd, J.C., Estrada, A.B., Matcham, J., Jeffries, P. and Jeger, M.J. (1991) The effect of climatic factors on *Colletotrichum gloeosporioides*, causal agent of mango anthracnose, in the Philippines. *Plant Pathology,* **40**, 568-575.

Edel, V., Steinberg, C., Avelange, I., Laguerre, G. and Alabouvette, C. (1995) Comparison of three molecular methods for the characterisation of *Fusarium oxysporum* strains. *Phytopathology,* **85**, 579-585.

Elena, K. (1999) The vascular wilt fungi of tomato in Greece: vegetative compatibility groups of *Verticillium dahliae* isolates. *Phytopathologia Mediterranea*, **38**, 13-143.

Ellis, M.A. and Miller, S.A. (1994) Evaluation of commercially available serological test kits for diagnosis of apple crown and root rot caused by *Phytophthora* spp. in Ohio. *Research Circular, Ohio Agricultural Research and Development Center,* **No. 298**, 68-72.

Fabre, J.V., Julien, J., Parisot, D. and Dron, M. (1995) Analysis of diverse isolates of *Colletotrichum lindemuthianum* infecting common bean using molecular markers. *Mycological Research,* **99**, 429-435.

Fernando, T.H.P.S., Jayasinghe, C.K. and Wijesundera, R.L.C. (2000) Factors affecting spore production, germination and viability of *Colletotrichum acutatum* isolates from *Hevea brasiliensis*. *Mycological Research,* **104**, 681-685.

Fidanza, M.A. and Dernoeden, P.H. (1995) Evaluation of an enzyme-linked immunosorbent assay method for predicting brown patch infection in turf. *HortScience,* **30**, 1263-1265.

Fitzell, R.D. and Peak, C.M. (1984) The epidemiology of anthracnose disease of mango: inoculum sources, spore production and dispersal. *Annals of Applied Biology,* **104**, 53-59.

Fitzell, R.D., Peak, C.M. and Darnell, R.E. (1984) A model for estimating infection levels of anthracnose disease of mango. *Annals of Applied Biology,* **104**, 451-458.

Förster, H. and Adaskaveg, J.E. (1999) Identification of subpopulations of *Colletotrichum acutatum* and epidemiology of almond anthracnose in California. *Phytopathology,* **89**, 1056-1065.

Freeman, S. and Katan, T. (1997) Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology,* **87**, 516-521.

Freeman, S., Katan, T. and Shabi, E. (1996) Characterization of *Colletotrichum gloeosporioides* isolates from avocado and almond fruits with molecular and pathogenicity tests. *Applied and Environmental Microbiology,* **62**, 1014-1020.

Freeman, S., Katan, T. and Shabi, E. (1998) Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease,* **82**, 596-605.

Freeman, S., Minz, D., Jurkevitch, E., Maymon, M. and Shabi, E. (2000a) Molecular analysis of *Colletotrichum* species from almond and other fruit. *Phytopathology,* **90**, 608-614.

Freeman, S., Minz, D., Maymon, M. and Zveibil, A. (2001) Genetic diversity within *Colletotrichum acutatum sensu* Simmonds. *Phytopathology,* **91**, 586-592.

Freeman, S., Pham, M. and Rodriguez, R.J. (1993) Molecular genotyping of *Colletotrichum* species based on arbitrarily primed PCR, A + T-rich DNA, and nuclear DNA analysis. *Experimental Mycology,* **17**, 309-322.

Freeman, S. and Rodriguez, R.J. (1995) Differentiation of *Colletotrichum* species responsible for anthracnose of strawberry by arbitrarily primed PCR. *Mycological Research*, **99**, 501-504.

Freeman, S., Shabi, E. and Katan, T. (2000b) Characterization of *Colletotrichum acutatum* causing anthracnose of anemone (*Anemone coronaria* L.). *Applied and Environmental Microbiology,* **66**, 5267-5272.

Friesland, H. and Schröedter, H. (1988) The analysis of weather factors in epidemiology. In *Experimental Techniques in Plant Disease Epidemiology* (Eds, Kranz, J. and Rotem, J.) Springer-Verlag, Heidelberg, Germany, pp. 115-134.

Frost, R.R. (1964) Seta formation in *Colletotrichum* spp. *Nature*, **201**, 730-731.

Fry, W.E. (1978) Quantification of general resistance of potato cultivars and fungicide effects for integrated control of potato late blight. *Phytopathology,* **68**, 1650-1655.

García- Muñoz, J., Belén Suárez, M., Grondona, I., Monte, E., Buddie, A.G., Bridge, P.D. and Cannon, P.F. (2000) A physiological and biochemical approach to the systematics of *Colletotrichum* species pathogenic to strawberry. *Mycologia*, **92**, 488-498.

Godwin, I.D., Aitken, E.A.B. and Smith, L.W. (1997) Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis,* **18**, 1524-1528.

Glass, N.L. and Kuldau, G.A. (1992) Mating type and vegetative incompatibility in filamentous ascomycetes. *Annual Review of Phytopathology,* **30**, 201-224.

Grove, G.G. and Boal, R.J. (1991) Influence of temperature and wetness duration on infection of immature apple and pear fruit by *Phytophthora cactorum*. *Phytopathology,* **81**, 1465-1471.

Gu, Y.H. and Ko, W.H. (2000) Occurrence of parasexual cycle in *Phytophthora parasitica* following protoplast fusion. *Botanical Bulletin of Academia Sinica,* **41**, 225-230.

Guerber, J.C. and Correll, J.C. (1997) The first report of the teleomorph for *Colletotrichum acutatum* in the United States. *Plant Disease,* **81**, 1334.

Guerber, J.C. and Correll, J.C. (2001) Characterization of *Glomerella acutata*, the teleomorph of *Colletotrichum acutatum*. *Mycologia,* **93**, 216-229.

Guerber, J.C., Lui, B., Correll, J.C. and Johnston, P. R. (2003) Characterization of diversity in *Colletotrichum acutatum sensu lato* by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia,* **95**, 872-895.

Gunnell, P.S. and Gubler, W.D. (1992) Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. *Mycologia,* **84**, 157-165.

Hall, B.H., Jones, M.K. and Wicks, T.J. (1998) First report of anthracnose of almond in South Australia. *Australasian Plant Pathology,* **27**, 127.

Hamelin, R.C., Bourassa, M., Rail, J., Dusabenyagasani, M., Jacobi, V. and Laflamme, G. (2000) PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderris canker of pine. *Mycological Research,* **104**, 527-532.

Hantula, J., Dusabenyagasani, M. and Hamelin, R.C. (1996) Random amplified microsatellites (RAMS) -A novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology*, **26**, 159-166.

Hartl, D. L. (1994) *Genetics,* Jones and Bartlett Publishers International, London, UK.

Hartung, J.S., Burton, C.C. and Ramsdell, D.C. (1981) Epidemiological studies of blueberry anthracnose disease caused by *Colletotrichum gloeosporioides*. *Phtyopathology*, **71**, 449-453.

Hayden, H.L., Pegg, K.G., Aitken, E.A.B. and Irwin, J.A.G. (1994) Genetic relationships as assessed by molecular markers and cross-infection among strains of *Colletotrichum gloeoporioides*. *Australian Journal of Botany,* **42**, 9-18.

Heintz, C. (1997) Board gears up to tackle anthracnose disease problem. *Australian Nut Grower*, **June-August**, 26.

Hendricks, L. (1998) What can we do with anthracnose in almonds in 1998? *Australian Nut Grower,* **March-May**, 12.

Henson, J.M. and French, R. (1993) The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology,* **31**, 81-109.

Hodson, A., Mills, P.R. and Brown, A.E. (1993) Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum gloeosporioides* isolated from tropical fruits. *Mycological Research,* **97**, 329-335.

Horejsi, T., Box, J.M. and Staub, J.E. (1999) Efficiency of randomly amplified polymorphic DNA to sequence characterised amplified region marker conversion and their comparative polymerase chain reaction sensitivity in cucumber. *Journal of the American Society of Horticultural Science,* **124**, 128-135.

Hovmøller, M. S. (2001) Disease severity and pathotype dynamics of *Puccinia striiformis* f. sp. *tritici* in Denmark. *Plant Pathology,* **50**, 181-189.

Hughes, K.J.D., Lane, C.R. and Cook, R.T.A. (1997) Development of a rapid method for the detection and identification of *Colletotrichum acutatum*. In *Diagnosis and Identification of Plant Pathogens* (Eds, Dehne, H.W., Adam, G., Diekmann, M., Frahm, J., Mauler-Machnik, A. and van Halteren, P.) Kluwer, Dordrecht, the Netherlands, pp.52-65.

Jailloux, F., Willocquet, L., Chapuis, L. and Froidefond, G. (1999) Effect of weather factors on the release of ascospores of *Uncinula necator*, the cause of grape powdery mildew, in the Bordeaux region. *Canadian Journal of Botany,* **77**, 1044-1051.

Jayasinghe, C.K. and Fernando, T.H.P.S. (2004) Re-identification and characterization of pathogens causing ugurassa (*Flacourtia inermis*) fruit anthracnose. *Mycopathologia,* **157**, 81-85.

Jeffries, P., Dodd, J.C., Jeger, M.J. and Plumbley, R.A. (1990) The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathology,* **39**, 343-366.

Jennings, D.H. and Lysek, G. (1996) *Fungal Biology: Understanding the Fungal Lifestyle,* Bios Scientific Publishers Ltd, Oxford, UK.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettscheider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Vockaert, G., Rueda, J., Linacero, R., Vazquez, A. and Karp, A. (1997) Reprducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding*, **3**, 381-390.

Johnston, P.R. and Jones, D.K. (1997) Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia,* **89**, 420-430.

Joseph, M.E. and Hering, T.F. (1997) Effects of environment on spore germination and infection by broad bean rust (*Uromyces viciae-fabae*). *Journal of Agricultural Science,* **128**, 73-78.

Kable, P.F. (1965) The fruit peduncle as an important overwintering site of *Monilinia fructicola* in the Murrumbidgee Irrigation Areas. *Australian Journal of Experimental Agriculture and Animal Husbandry,* **5**, 172-175.

Kable, P.F. (1970) Eradicant action of fungicides applied to dormant peach trees for control of brown rot (*Monilinia fructicola*). *Journal of Horticultural Science,* **45**, 143-152.

Katan, T. and Jimenez-Diaz, R.M. (2001) Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. *European Journal of Plant Pathology*, **107**, 443-456.

Katan, T., and Shabi, E. (1996) Vegetative compatibility among isolates of *Colletotrichum gloeosporioides* from almond in Israel. *European Journal of Plant Pathology,* **102**, 597-600.

Keiper, F.J. and McConchie, R. (2001) Morphological variation among *Sticherus flabellatus* (Gleicheniaceae) populations of the Sydney region. *Australian Journal of Botany,* **49**, 89-105.

Kester, D.E. and Gradziel, T.M. (1996) Almonds. In *Fruit Breeding, volume III. Nuts* (Eds, Janick, J. and Moore, J.N.) John Wiley and Sons, Inc., New York, USA, pp. 1-98.

Kester, D.E., Gradziel, T.M. and Grassely, C. (1991) Almonds (*Prunus)*. In *Genetic Resources of Temperate Fruit and Nut Crops 2* (Eds, Moore, J.N. and Ballington, J.R.) International Society for Horticultural Science, Wageningen, the Netherlands, pp. 701-758.

King, W.T., Madden, L.V., Ellis, M.A. and Wilson, L.L. (1997) Effects of temperature on sporulation and latent period of *Colletotrichum* spp. infecting strawberry fruit. *Plant Disease,* **81**, 77-84.

Kim, Y.K., Xiao, C.L. and Rogers, J.D. (2005) Influence of culture media and environmental factors on mycelial growth and pycnidial production of *Sphaeropsis pyriputrescens*. *Mycologia,* **97**, 25-32.

Kistler, H.C. (1997) Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathology*, **87**, 474-479.

Kistler, H.C., Bosland, P.W., Benny, V., Leong, S. and Williams, P.H. (1987) Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. *Phytopathology*, 77, 1289-1293.

Koball, D.C., Wilcox, W.F. and Seem, R.C. (1997) Influence of incubation-period humidity on the development of brown rot blossom blight of sour cherry. *Phytopathology*, **87**, 42-49.

Kohn, L.M. (2004) Applying comparative genomics to plant disease epidemiology. *Phytoprotection,* **85**, 45-48.

Koonjul, P.K., Brandt, W.F., Farrant, J.M. and Lindsey, G.G. (1999) Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Research,* **27**, 915-916.

Korolev, N., Perez-Artes, E., Bejarano-Alcazar, J., Rodriguez-Jurado, D., Katan, J. (2001) Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. *European Journal of Plant Pathology,* **107**, 443-456.

Krause, R.A., Massie, L.B. and Hyre, R.A. (1975) Blitecast: a computerized forecast of potato late blight. *Plant Disease Reporter*, **2**, 95-98.

Kruskal, J.B. (1964a) Multidimensional scaling optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika,* **29**, 1-27.

Kruskal, J.B. (1964b) Nonmetric multidimensional scaling: a numerical method. *Psychometrika,* **29**, 28-42.

Kurame-Izioka, E.E., Lopes, C.R., Souza, N.L. and Machado, M.A. (1997) Morphological and molecular characterization of *Colletotrichum* spp. from citrus orchards affected by postbloom fruit drop in Brazil. *European Journal of Plant Pathology,* **103**, 323-329.

Lardner, R., Johnston, P.R., Plummer, K.M. and Pearson, M.N. (1999) Morphological and molecular analysis of *Colletotrichum acutatum sensu lato*. *Mycological Research,* **103**, 275-285.

Lardner, R., Stummer, B.E., Sosnowski, M.R. and Scott, E.S. (2005) Molecular identification and detection of *Eutypa lata* in grapevine. *Mycological Research,* **109**, 1-10.

Leandro, L.F.S., Gleason, M.L., Nutter, F.W., Wegulo, S.N. and Dixon, P.M. (2001) Germination and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. *Phytopathology*, **91**, 659-664.

Leslie, J.F. (1993) Fungal vegetative compatibility. *Annual Review of Phytopathology*, **31**, 127-153.

Liyange, H.D., McMillan, R.T. and Corby Kistler, H. (1992) Two genetically distinct populations of *Colletotrichum gloeosporioides* from citrus. *Phytopathology,* **82**, 1371-1376.

Llorente, I., Vilardell, P., Bugiani, R., Gherardi, I. and Montesinos, E. (2000). Evaluation of BSPcast disease warning system in reduced fungicide use programs for management of brown spot of pear. *Plant Disease*, **84**, 631-637.

Lu, G., Cannon, P.F., Reid, A. and Simmons, C.M. (2004) Diversity and molecular relationsips of endophytic *Colletotrichum* isolates from the Iwokrama Forest Reserve, Guyana. *Mycological Research,* **108**, 53-63.

Luo, Y. and Michailides, T.J. (2001) Risk analysis for latent infection of prune by *Monilinia fructicola* in California. *Phytopathology,* **91**, 1197-1208.

Madden, L.V., Ellis, M.A., Lalancette, N., Hughes, G. and Wilson, L.L. (2000) Evaluation of a disease warning system for downy mildew of grapes. *Plant Disease*, **84**, 549-554.

Magarey, P. and Western, M. (1997) The Model T MetStation prototype - a useful low cost weather station/disease predictor. *Australian and New Zealand Wine Industry Journal*, **12**, 273-276.

Majer, D., Mithen, R., Lewis, B., Vos, P. and Oliver, R. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research*, **100**, 1107-1111.

Mantel, N.A. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research,* **27**, 209-220.

Martin, M.P. and García-Figueres, F. (1999) *Colletotrichum acutatum* and *C. gloeosporioides* cause anthracnose on olives. *European Journal of Plant Pathology,* **105**, 733-741.

Martínez-Culebras, P., Querol, A., Suarez-Fernandez, M.B., García-Lopez, M.D. and Barrio, E. (2003) Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. *Journal of Phytopathology*, **151**, 135-143.

McDonald, B.A. (1997) The population genetics of fungi: Tools and techniques. *Phytopathology,* **87**, 448-453.

McMichael, P.A. (2000) HRDC Project NT 98004. The control and management of 'anthracnose' of almonds. Scholefield Robinson Horticultural Services Pty Ltd, Netherby, South Australia.

Meng, X. and Chen, W. (2001) Applications of AFLP and ISSR techniques in detecting genetic diversity in the soybean brown stem rot pathogen *Phialophora gregata*. *Mycological Research,* **105**, 936-940.

Menzies, J.G., Bakkeren, G., Matheson, F., Procunier, J.D. and Woods, S. (2003) Use of inter-simple sequence repeats and amplified fragment length polymorphisms to analyze genetic relationships among small grain-infecting species of *Ustilago*. *Phytopathology*, **93**, 167-175.

Michelmore, R.W. and Hulbert, S.H. (1987) Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology,* **25**, 383-404.

Miehle, B.R. and Lukezic, F.L. (1972) Studies on conidial germination and appressorium formation by *Colletotrichum trifolii* Bain & Essary. *Canadian Journal of Microbiology,* **18**, 1263-1269.

Miller, S.A. and Martin, R.R. (1988) Molecular diagnosis of plant disease. *Annual Review of Phytopathology*, **26**, 409-432.

Millgroom, M.G. (1996) Recombination and the mutlilocus structure of fungal populations. *Annual Review of Phytopathology*, **34**, 457-477.

Mills, P.R., Hodson, A. and Brown, A.E. (1992) Molecular differentiation of *Colletotrichum gloeosporioides*. In *Colletotrichum: Biology, Pathology and Control* (Eds, Bailey, J. A. and Jeger, M. J.) C.A.B. International, Wallingford, Oxon, UK, pp. 269-288.

Mills, P.R., Sreenivasaprasad, S. and Brown, A.E. (1994) Detection of the anthracnose pathogen *Colletotrichum*. In *Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification* (Eds, Schots, A., Oliver, R. and Dewey, F.M.) CAB International, Oxon, UK, pp. 183-190.

Mills, P.R., Sreenivasaprasad, S. and Muthumeenakshi, S. (1998) Assessing diversity in *Colletotrichum* and *Trichoderma* species using molecular markers. In *Molecular Variability of Fungal Pathogens* (Eds, Bridge, P. D., Couteaudier, Y. and Clarkson, J. M.) CAB International, Oxon, UK, pp. 105-120.

Montesinos, E. and Vilardell, P. (1992) Evaluation of FAST as a forecasting system for scheduling fungicide sprays for control of *Stemphylium vesicarium* on pear. *Plant Disease,* **76**, 1221-1226.

Mordue, J.E.M. (1971) *Glomerella cingulata*. Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria, **315**. Commonwealth Mycological Institute, Kew, Surrey, UK.

Moricca, S., Ragazzi, A., Kasuga, T. and Mitchelson, K.R. (1998) Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant Pathology,* **47**, 486-494.

Newton, C.R. and Graham, A. (1994) *PCR: An Introduction to Biotechniques*. BIOS Scientific Publishers Pty Ltd., Oxford, UK.

Nicholson, R.L. and Moraes, W.B.C. (1980) Survival of *Colletotrichum graminicola*: the importance of the spore matrix. *Phytopathology,* **70**, 255-261.

Ouellet, T. and Seifert, K.A. (1993) Genetic characterization of *Fusarium graminearum* strains using RAPD and PCR amplification. *Phytopathology,* **83**, 1003-1007.

Pejic, I., Ajmone-Marsan, P., Morgante, M., Kozumplick, V., Castiglioni, P., Taramino, G. and Motto, M. (1998) Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. *Theoretical and Applied Genetics,* **97**, 1248-1255.

Penrose, L.J. and Nicol, H.I. (1996) Aspects of microclimate variation within apple tree canopies and between sites in relation to potential *Venturia inaequalis* infection. *New Zealand Journal of Crop and Horticultural Science,* **24**, 259-266.

Peres, N.A.R., Kuramae, E.E., Dias, M.S.C. and de Souza, N.L. (2002) Identification and characterisation of *Colletotrichum* spp. affecting fruit after harvest. *Journal of Phytopathology,* **150**, 128-134.

Pfender, W.F. (2001) A temperature-based model for latent-period duration in stem rust of perennial ryegrass and tall fescue. *Phytopathology*, **91**, 111-116.

Pocock, D. and Bennett, C. (1999) Investment Opportunity- Almonds. Department of Primary Industries and Resources South Australia, Adelaide, South Australia.

Pontecorvo, G. (1956) The parasexual cycle in fungi. *Annual Reviews in Microbiology,* **10**, 393-400.

Pryor, B.M. and Michailides, T.J. (2002) Morphological, pathogenic, and molecular characterisation of *Alternaria* isolates associated with Alternaria late blight of pistachio. *Phytopathology*, **92**, 406-416.

Quinn, G. (1928) The almond in South Australia. Department of Agriculture, Adelaide, South Australia, bulletin 219, 1-19.

Rafalski, J.A. and Tingey, S.V. (1993) Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. *Trends in Genetics,* **9**, 275-280.

Ramsdell, D.C. and Ogawa, J.M. (1973) Reduction of *Monilinia laxa* inoculum potential in almond orchards resulting from dormant benomyl sprays. *Phytopathology*, **63**, 830-836.

Raynor, R.W. (1970) *A Mycological Colour Chart.* CAB International, Surrey, UK.

Rizwana, R. and Powell, W.A. (1995) Ultraviolet light-induced heterokaryon formation and parasexuality in *Cryphonectria parasitica*. *Experimental Mycology,* **19**, 48-60.

Rodriguez, R.J. and Redman, R.S. (1992) Molecular transformation and genome analysis of *Colletotrichum*. In *Colletotrichum: Biology, Pathology and Control* (Eds, Bailey, J.A. and Jeger, M.J.) C.A.B. International, Wallingford, Oxon, UK, pp. 47-66.

Rodriguez, R.J. and Yoder, O.C. (1991) A family of conserved repetative DNA elements from the fungal plant pathogen *Glomerella cingulata* (*Colletotrichum lindemuthianum*). *Experimental Mycology,* **15**, 232-242.

Rohlf, F.J. (1997) NTSYS-pc: numerical taxonomy and multivariate analysis system. Version 2.00. Exeter Software, Setauket, New York, USA.

Saha, T., Kumar, A., Ravindran, M., Jacob, C.K., Roy, B. and Nazeer, M.A. (2002) Identification of *Colletotrichum acutatum* from rubber using random amplified polymorphic DNAs and ribosomal DNA polymorphisms. *Mycological Research*, **106**, 215-221.

Salazar, O., Julian, M.C. and Rubio, V. (2000) Primers based on specific rDNA-ITS sequences for PCR detection of *Rhizoctonia solani*, *R. solani* AG 2 subgroups and ecological types, and binucleate *Rhizoctonia*. *Mycological Research,* **104**, 281-285.

Sanders, G.M. and Korsten, L. (2003) A comparative morphological study of South African avocado and mango isolates of *Colletotrichum gloeosporioides*. *Canadian Journal of Microbiology,* **81**, 877-885.

Scherm, H., Kioke, S.T., Laemmlen, F.F. and van Bruggen, A.H.C. (1995) Field evaluation of fungicide spray advisories against lettuce downy mildew (*Bremia lactucae*) based on measured or forecast morning leaf wetness. *Plant Disease,* **79**, 511-516.

Schilling, A.G., Moller, E.V. and Geiger, H.H. (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum, F. graminearum,* and *F. avenaceum. Phytopathology,* **86**, 515-522.

Scholefield, P.B. (1995) Almonds. In *Horticulture Australia. The Complete Reference of the Australian Horticultural Industry* (Ed, Coombs, B.) Morescope Publishing Pty Ltd, Victoria, Australia, pp. 477-481.

Shabi, E. (1997) Disease management of almond pathogens *Glomerella cingulata*, *Polystigma ochraceum* and *Tranzschelia pruni-spinosae*. Bulletin OEPP/EPPO Bulletin, **27**, 479-480.

Shabi, E. and Katan, T. (1983) Occurrence and control of anthracnose of almond in Israel. *Plant Disease,* **67**, 1364-1366.

Shabi, E., Katan, T., Gera, H. and Elisha, S. (1994) Taxonomic determination of pathogenic *Colletotrichum gloeosporioides* of almond, anemone and avocado according to fungicide sensitivity. *Phytoparasitica,* **21**, 130-131.

Shaner, G. and Finney, R.E. (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology,* **67**, 1051-1056.

Simmonds, J.H. (1965) A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Queensland Journal of Agricultural and Animal Science,* **22**, 437-459.

Smith, B.J. and Black, L.L. (1990) Morphological, cultural and pathogenic variation among *Colletotrichum* species isolated from strawberry. *Plant Disease,* **74**, 69-76.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology,* **98**, 503-517.

Sreenivasaprasad, S., Brown, A.E. and Mills, P.R. (1992) DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. *Physiological and Molecular Plant Pathology*, **41**, 265-281.

Sreenivasaprasad, S. and Mills, P.R. (1998) Future directions for PCR in mycology. In *Applications of PCR in Mycology* (Eds, Bridge, P.D., Arora, D.K., Reddy, C.A. and Elander, R.P.) CAB International, Wallingford, Oxon, UK, pp. 325-345.

Sreenivasaprasad, S., Mills, P.R. and Brown, A.E. (1994) Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycological Research,* **98**, 186-188.

Sreenivasaprasad, S., Sharada, K., Brown, A.E. and Mills, P.C. (1996) PCR-based detection of *Colletotrichum acutatum* on strawberry. *Plant Pathology,* **45**, 650-655.

Stenlid, J., Karlsson, J. and Högberg, N. (1994) Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. *Mycological Research,* **98**, 57-63.

Stensvand, A., Talgø, V. and Børve, J. (2001) Seasonal production of conidia of *Monilinia laxa* from mummified fruits, blighted spurs and flowers of sweet cherry. *Gartenbauwissenschaft,* **66**, 273-281.

Stockwin, W. (1998) Almond growers out of weapons. Californian Farm Bureau Federation. [http://www.cfbf.com/archive/aa-0617c.htm]

Striem, M.J., Spiegel-Roy, P. and Shabi, E. (1989) Evaluating susceptibility of almonds to anthracnose disease caused by *Colletotrichum gloeosporioides*. *Phytoparasitica,* **17**, 107-122.

Stummer, B.E., Zanker, T., Scott, E.S. and Whisson, D.L. (2000) Genetic diversity in populations of *Uncinula necator*: comparison of RFLP- and PCR-based approaches. *Mycological Research,* **104**, 44-52.

Sutton, B.C. (1980) *The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata,* Commonwealth Mycological Institute, Kew, Surrey, England.

Sutton, B.C. (1992) The genus *Glomerella* and its anamorph *Colletotrichum.* In *Colletotrichum: Biology, Pathology and Control* (Eds, Bailey, J.A. and Jeger, M.J.) CAB International, Wallingford, Oxon, UK, pp. 1-26.

Sutton, T.B. and Clayton, C.N. (1972) Role and survival of *Monilinia fructicola* in blighted peach branches. *Phytopathology,* **62**, 1369-1373.

Swanson, T.A. and van Gundy, S.D. (1985) Influences of temperature and plant age on differentiation of races of *Fusarium oxysporum* f. sp. *tracheiphilum* on cowpea. *Plant Disease,* **69**, 779-781.

Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J. and Oliveira, H. (2002) Genetic and morphological characterization of *Colletotrichum acutatum* causing anthracnose of lupins. *Phytopathology,* **92**, 986-996.

Tautz, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research,* **17**, 6463-6471.

Thornton, C.R., O'Neill, T.M., Hilton, G. and Gilligan, C.A. (1999) Detection and recovery of *Rhizoctonia solani* in naturally infested glasshouse soils using a combined baiting, double monoclonal antibody ELISA. *Plant Pathology,* **48**, 627-634.

Timmer, L.W. and Brown, G.E. (2000) Biology and control of anthracnose diseases of citrus. In *Colletotrichum; Host Specificity, Pathology and Host-Pathogen Interaction* (Eds, Prusky, D., Freeman, S. and Dickman, M.B.) The American Phytopathological Society Press, St. Paul, Minnesota, USA, pp. 300-316.

Timmer, L.W. and Zitko, S.E. (1993) Relationships of environmental factors and inoculum levels to the incidence of postbloom fruit drop of citrus. *Plant Disease,* **77**, 501-504.

Timmer, L.W. and Zitko, S.E. (1996) Evaluation of a model for prediction of postbloom fruit drop of citrus. *Plant Disease,* **80**, 380-383.

Tinline, R.D. and MacNeill, B.H. (1969) Parasexuality in plant pathogenic fungi. *Annual Review of Phytopathology,* **7**, 147-170.

Ureña-Padilla, A.R., MacKenzie, S.J., Bowen, B.W. and Legard, D.E. (2002) Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. *Phytopathology,* **92**, 1245-1252.

Vaillancourt, L.J. and Hanau, R.M. (1992) Genetic and morphological comparisions of *Glomerella* (*Colletotrichum*) isolates from maize and from sorghum. *Experimental Mycology,* **16**, 219-229.

van der Aa, H.A., Noordeloos, M.E. and de Gruyter, J. (1990) Species concepts in some larger genera of the Coelomycetes. *Studies in Mycology*, **32**, 3-19.

Van der Plank, J.E. (1963) *Plant Diseases: Epidemics and Control,* Academic Press, New York.

Van der Zwet, T., Biggs, A.R., Heflebower, R. and Lightner, G.W. (1994). Evaluation of the MARYBLYT computer model for predicting blossom blight on apple in West Virginia and Maryland. *Plant Disease*, **78**, 225-230.

Vernière, C.J., Gottwald, T.R. and Pruvost, O. (2003) Disease development and symptom expression of *Xanthomonas axonopodis* pv. *citri* in various citrus plant tissues. *Phytopathology,* **93**, 832-843.

Vincente, M.J., Cifuentes, D., Cenis, J.L. and Abad, P. (1999) RAPD-PCR polymorphism and vegative compatibility group variation in Spanish isolates of *Acremonium cucurbitacearum*. *Mycological Research,* **103**, 1173-1178.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Homes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new concept for DNA fingerprinting, *Nucleic Acids Research*, **23**, 4407-4414.

Waller, J.M. (1972) Water-borne spore dispersal in coffee berry disease and its relation to control. *Annals of Applied Biology,* **71**, 1-18.

Waller, J.M. (1992) *Colletotrichum* diseases of perennial and other cash crops. In *Colletotrichum; Biology, Pathology and Control* (Eds, Bailey, J. A. and Jeger, M. J.) CAB International, Wallingford, Oxon, UK, pp. 167-185.

Wasilwa, L.A., Correll, J.C., Morelock, T.E. and McNew, R.E. (1993) Reexamination of races of the cucurbit anthracnose pathogen *Colletotrichum orbiculare*. *Phytopathology*, **83**, 1190-1198.

Wastie, R.L. (1972a) Secondary leaf fall of *Hevea brasiliensis*: factors affecting the production, germination and viability of spores of *Colletotrichum gloeosporioides*. *Annals of Applied Biology,* **72**, 273-282.

Wastie, R.L. (1972b) Secondary leaf fall of *Hevea brasiliensis*: meteorological and other factors affecting infection by *Colletotrichum gloeosporioides*. *Annals of Applied Biology,* **72**, 283-293.

Weeds, P.L., Manners, J.M. and Chakraborty, S. (2000) Intron sequence polymorphisms for genetic diversity and phylogenetics in *Colletotrichum. Phytopathology*, **90**:S83.

Weining, S. and Langridge, P. (1991) Identification and mapping of polymorphisms in cereals based on polymerase chain reaction. *Theoretical and Applied Genetics,* **82**, 209-216.

Weising, K., Weigand, F., Driesel, A. J., Kahl, A.J., Zischer, H. and Epplen, J.T. (1989) Polymorphic simple GATA/GACA repeats in plant genomes. *Nucleic Acids Research*, **17**, 10128.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research,* **18**, 7213-7218.

Wharton, P.S. and Diéguez-Uribeondo, J. (2004) The biology of *Colletotrichum acutatum. Anales del Jardin Botanico de Madrid,* **61**, 3-22.

Wharton, P.S. and Schilder, A.M.C. (2003) Infection and colonization of blueberry fruit by *Colletotrichum acutatum. Phytopathology,* **93**:S90.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A Guide to Methods and Applications* (Eds, Innes, M., Gelfand, D., Sninsky, J. and White, T.) Academic Press, San Diego, California, USA, pp. 315-322.

White, J.G., Lyons, N.F., Wakeham, A.J., Mead, A. and Green, J.R. (1994) Serological profiling of the fungal genus *Pythium*. *Physiological and Molecular Plant Pathology,* **44**, 349-361.

Wicks, T.J. (1981) Suppression of *Monilinia laxa* spore production by fungicides applied to infected apricot twigs during dormancy. *Plant Disease*, **65**, 911-912.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* **18**, 6531-6535.

Wilson, L.L., Madden, L.V. and Ellis, M.A. (1990) Influence of temperature and wetness duration on infection of immature and mature strawberry fruit by *Colletotrichum acutatum*. *Phytopathology,* **80**, 111-116.

Wyss, P. and Bonfante, P. (1993) Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycological Research,* **97**, 1351-1357.

Yang, H.A. and Sweetingham, M.W. (1998) The taxonomy of *Colletotrichum* isolates associated with lupin anthracnose. *Australian Journal of Agricultural Research,* **49**, 1213-1223.

Yuen, G.Y., Xia, J.Q. and Sutala, C.L. (1998) A sensitive ELISA for *Pythium ultimum* using polyclonal and species-specific monoclonal antibodies. *Plant Disease,* **82**, 1029-1032.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genome*, **20**, 176-183.

Zohary, D. and Hopf, M. (1988) *Domestication of Plants in the Old World*. Oxford University Press, New York, USA.

Zulfiqar, M., Brlansky, R.H. and Timmer, L.W. (1996) Infection of flower and vegetative tissues of citrus by *Colletotrichum acutatum* and *C. gloeosporioides*. *Mycologia*, **88**, 121-128.