STUDIES OF CAMELLIA FLOWER BLIGHT (*CIBORINIA CAMELLIAE* KOHN)

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ABSTRACT

Camellias are popular ornamental plants and the most serious pathogen of this plant is camellia flower blight, caused by the fungal pathogen *Ciborinia camelliae* Kohn. Ascospores of this fungus attack the flowers, turning them brown, rendering infected flowers unattractive. Little is known about the pathogen and control measures are not particularly effective.

In this thesis, various aspects of the pathogen's basic and molecular biology and interaction with host species were studied.

Surveys of the distribution and spread of *C. camelliae* within New Zealand determined that the pathogen was present in most regions of the North Island, and north and east coasts of the South Island. Over the distances and time involved, it appeared that the disease was spreading mainly by windborne ascospores rather than human transfer.

Sclerotia were germinated out of season to increase the period during which ascospores were available for infection work. Greatest germination was achieved at low temperatures (5°C-10°C) in 24 h darkness.

Isolate-specific primers were designed to the ribosomal DNA Internal Transcribed Spacer region to detect the pathogen *in planta* and distinguish between New Zealand isolates of *C. camelliae* and other fungal pathogens. Phylogenetic analysis of the ITS region with other *Ciborinia*, *Sclerotinia* and *Botrytis* species showed that *C. camelliae* was more closely related to *S. sclerotiorum* than other *Ciborinia* species.

Two inoculation techniques for infecting *Camellia* petals with ascospores of *C. camelliae* were developed and tested. Inoculation using airborne ascospores in a settling chamber was a simple and quick method for testing large numbers of species for resistance. Inoculation of ascospores in suspension produced qualitative data, but was more time consuming.

Of the four mechanisms of resistance tested, levels of aluminium hyperaccumulation and the presence of phenolic compounds did not correlate with resistance in *Camellia* species. The large uptake of aluminium, however, did indicate that *Camellia* species would be good plants for phytoremediation of acid soils. Some resistant species were found to have cell wall modifications and/or lignification of cell walls in response to *C. camelliae* infection and chitinase activity was found in most resistant *Camellia* species tested. Further research into these latter two mechanisms is recommended and indicates that the development of resistant *Camellia* cultivars is possible.

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LIST OF ABBREVIATIONS

a.i.	active ingrediant
ANOVA	ANalysis Of VAriance
cc	cubic centimetre(s)
d	day(s)
df/DF	Degrees of Freedom
GA	Gibberellic Acid
GLB	Gel Loading Buffer
h	hour(s)
Hg	mercury
hpi	hours post inoculation
HR	Hypersensitive Response
IPTG	isopropylthio-β-D-thiogalactopyranoside
kPa	kilo Pascals
LB	Luria-Bertani broth or agar
L/D	Light/Dark
LS	Least Squares
min	minute(s)
mm	millimetre(s)
MS	Mean Square
nm	nanometres
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppm	parts per million
PR	Pathogenesis Related
p.s.i	pounds per square inch
RAPD	Randomly Amplified Polymorphic DNA
RH	Relative Humidity
RO	Reverse Osmosis
SS	Sums of Squares
μF	micro Farad
$\mu g\ g^{-1}$	micrograms per gram

V	volt
wk	week(s)
w/v	weight/volume
X-gal	5-bromo 4-chloro 2-indolyl-β-D-galactoside

CHAPTER ONE – GENERAL INTRODUCTION

1.1 INTRODUCTION

Camellias are popular ornamental plants, widely grown in parks and private gardens throughout the world. The International Camellia Register (Savige 1993) lists 267 species, all of which originated in Asia or south-east Asia, however, only a few are economically important. Tea is the most important economic product and is made from the leaves of *C. sinensis* L. var. *sinensis* Kuntze and *C. sinensis* var. *assamica* (Mast.) Kitamura. The crushed seeds of some species produce high quality oil for use in cooking (Sealy 1958; Chang & Bartholomew 1984; Guomei 1986; Xia *et al* 1993) and cosmetics (Nuccio 1975; Xia *et al* 1993) and the pharmaceutical and manufacturing industries also use camellia products (Chang & Bartholomew 1984; Xia *et al* 1993).

As ornamentals, the three most popular species are the autumn/winter flowering *C. sasanqua* Thunb., and the winter/spring flowering *C. japonica* L. and *C. reticulata* Lindl. The wide range of camellia cultivars and hybrids available are the result of many centuries of selection for desirable characteristics, first in China and Japan (Sealy 1958; Chang & Bartholomew 1984; Guomei 1986), then worldwide, as breeders continue to produce new cultivars. There is considerable variation in flower form (e.g. single, anemone, formal), colour and size of the flower; the evergreen glossy leaves are also an attraction for gardeners.

The most destructive pest or disease problem of camellias is camellia flower blight (Raabe *et al* 1978) caused by *Ciborinia camelliae* Kohn. The fungus is a specialist pathogen, attacking only the floral parts of *Camellia* species (Raabe *et al* 1978; Kohn & Nagasawa 1984) and the general health of the plant does not appear to be affected. Infected flowers turn brown, but since camellias are grown mainly for their flowers, the effects of the disease impact on people who grow camellias, or would otherwise choose to grow camellias, but do not because of the disease.

1.2 *CIBORINIA CAMELLIAE*

1.2.1 Distribution

The pathogen was first identified in Japan in 1919 (Hara 1919) and then in California, USA in 1938 (Hansen & Thomas 1940). At that time, plant nurseries had been importing camellias from Japan and it is likely that the disease was introduced on these imported plants (Hansen & Thomas 1940; Hansen & Thomas 1946; Thomas & Hansen 1946). Within 20 years of its arrival, the pathogen had spread to most regions of the United States where camellias are grown.

In 1993 the pathogen was found in a garden in Wellington, New Zealand (Stewart & Neilson 1993) and was found to be widespread in Wellington (C. F. Hill, 1993 unpubl. data, MAF). A survey in 1997 (Long & Taylor 1998; Taylor & Long 1998) showed that it was well established in the lower North Island and indicated that windborne dispersal was probably the principle method of spread. A second survey in 1998 (Taylor & Long 1999a; Taylor *et al* 1999) found the disease in the north of the South Island, with isolated outbreaks in Christchurch, Kaiapoi, and Auckland probably due to spread on planting material. Its current distribution is unknown.

The pathogen has been reported in southern and south-western England (Cook 1999; Scott 1999), Portugal and Spain (Mansilla *et al* 1999a,b), Italy (Garibaldi *et al* 2001), France, Switzerland and Germany (Peper 1999). Assuming that the disease is widespread in Asia, from which it presumably originates, the only two major camellia growing countries free of the pathogen are Australia and South Africa.

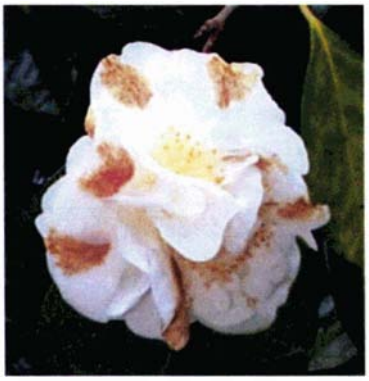
1.2.2 Taxonomy

C. camelliae is an inoperculate Discomycete of the Sclerotiniaceae. Whetzel (1945) created the genus *Ciborinia* to contain species in which either wholly or partly digested plant host tissue is incorporated into the sclerotial medulla, in contrast to the sclerotia of *Sclerotinia*, which do not contain host tissue. It was originally described as *Sclerotinia camelliae* Hara (Hara 1919). Kohn (1979) described the Californian specimens as *Ciborinia camelliae* Kohn.

Twenty *Ciborinia* species are known, all are plant pathogens and most are limited to one host species or to several species within a genus. The life cycle is annual, with sclerotia forming in infected host tissue over summer and autumn, then producing apothecia the following spring. Macroconidia have not been reported in any *Ciborinia* species, while microconidia have been reported for most (Groves & Bowerman 1955; Batra & Korf 1959; Batra 1960).

1.2.3 Life Cycle of *C. camelliae*

The life cycle is shown in Figure 1.1. In New Zealand, infection occurs in spring, when windborne ascospores land on camellia petals, germinate, and penetrate the petal tissue to set up infections. Each infection causes an irregular brown spot on the petal, and these infections enlarge and coalesce until the entire petal is brown. When the infection reaches the base of the petal, it spreads into the base of the other petals of the flower, and up these, so that a distinctive pattern of browning is often seen. At the base of the flower, a white or grey ring of mycelium can be seen when the calyx is removed. Most sclerotia form at the base of the flower, and microconidia may form on the petal surface. Sclerotia in fallen flowers lie dormant on the ground or in plant debris over summer, autumn and winter. Towards the end of winter the sclerotia germinate, producing apothecia from which the ascospores are released. In New Zealand, sclerotial germination occurs over 2-3 months in spring, but in California the fungus is mostly active in winter. One sclerotium can produce many apothecia over a season, and the same sclerotium may germinate in successive seasons (C. H. Taylor, unpubl. data). They may remain viable in the soil for at least four years (Baxter & Thomas 1995).



Ascospore infection of camellia petal leads to rapid spread of a brown rot.

Apothecia develop on sclerotia in spring. Ascospores are spread by wind.

Sclerotia form in petals after the blighted flowers fall to the ground.

Flowers rot and sclerotia overwinter in plant debris.

Figure 1.1
Illustrated disease cycle diagram of camellia (*Camellia* spp.) flower blight caused by the fungal pathogen *Ciborinia camelliae.*

1.2.4 Molecular Biology of *C. camelliae*

Little molecular work has been done on any member of the genus *Ciborinia*. Holst-Jensen *et al* (1997a,b) investigated the phylogeny of various members of the Sclerotiniaceae based on ribosomal DNA sequences. They showed that *C. erythronii* was more closely related to various other members of the family Sclerotiniaceae than it was to *C. foliicola* and *C. whetzelii*. In a study with *C. ciborium* and *C. erythronii*, Carbone & Kohn (1993) concluded that the genus *Ciborinia* was a heterogenous grouping of species and in 'need of monographic revision'.

Van Toor (2002) investigated the genetic diversity of *C. camelliae* isolates in New Zealand and concluded that there was a low level of genetic variation.

1.2.5 Control of C. camelliae

Control of *C. camelliae* has proved to be very difficult and measures have concentrated on disruption at two points in its life cycle. Various actions are recommended, but generally involve preventing the release of ascospores, either by collecting and destroying diseased flowers and sclerotia or by preventing the sclerotia from germinating (e.g. plastic matting laid under plants or application of fungicides). The second approach is to protect the camellia flowers through the application of fungicides to the plant. The second method may be more effective in urban areas where ascospores are blown in from surrounding gardens, but requires regular application of fungicides as new flowers open. The first method is not practical in many New Zealand garden situations. No fungicide has been found to give total control of the pathogen.

The disease can be avoided completely by growing autumn-flowering species or very early- or late-flowering species and cultivars. Taylor (1999) and Taylor & Long (1999b) found that there may be sources of resistance in the lesser known species, largely grown for botanical interest. Resistance mechanisms were studied by Vingnanasingam (2002) who found evidence of papillae, hypersensitive reaction and antifungal metabolites in some resistant species and possibly the effect of biocontrol activity on germinated ascospores. There is, therefore, a possibility of breeding resistant varieties.

1.2.6 Review of Literature on Camellia Flower Blight

Literature on camellia flower blight is contained in a comprehensive review paper (Taylor & Long 2000) (Appendix 1). Relevant details from this paper have been summarised for this introduction, with the addition of more recent publications.

1.3 PLANT DEFENCE MECHANISMS

For most plant/pathogen interactions, disease is the exception. In a compatible interaction, infection of the host plant by the pathogen depends on the interaction of three factors; a) environmental conditions that favour the pathogen, b) virulence of the pathogen, and c) the response of the plant to the pathogen.

Plant responses – or defence mechanisms – can be constitutive or induced by the pathogen. They may form a physical barrier to the pathogen or chemically inhibit their growth.

1.3.1 Constitutive Physical Defence Mechanisms

The external surface of the plant forms the first barrier to the pathogen. The waxy outer layer, cuticle, epidermis and stomata are potential sites of entry for a pathogen, with the amount, quality, morphology or behaviour influencing resistance. Internally, bundles or extended areas of sclerenchyma cells may stop further spread of a pathogen (Agrios 1988).

For example, the increased thickness of the epidermis in older leaves of barberry increased resistance to *Puccinia graminis* (Guest & Brown 1997) while the structure of stomata in *Citrus* determined resistance to *Pseudomonas citri* (McLean 1921). In resistant *C. nobalis*, the stomata are small and do not hold water, whereas the shape of the stomata holds water in the susceptible *C. grandis*, which *P. citri* requires for infection.

1.3.2 Constitutive Chemical Defence Mechanisms

Constitutive defence chemicals are usually secondary metabolites that are already present before infection; they may be exuded, or contained within the host.

Onion smudge, caused by the fungus *Colletotrichum circinans*, infects white onion varieties but not red onions, which have pigmented outer scales that contain the phenolic compounds protocatechuic acid and catechol (Link *et al* 1929; Link & Walker 1933). These compounds leach out into the soil and inhibit germination of *C. circinans* spores (Link & Walker 1933) providing external resistance for red onion varieties.

In tulips, *Botrytis tulipae* causes tulip fire disease, while *B. cinerea* fails to infect the tulip. Tulips were found to produce tulopisides, antifungal lactones (Beijersbergen & Lemmers 1972). *B. tulipae* was found to be less sensitive to tulopalin than *B. cinerea*, because it converted the tuliposides to non-toxic products, thus enabling it to bypass this defence mechanism (Schönbeck & Schroeder 1972).

1.3.3 Induced Physical Defence Mechanisms

Physical defence structures formed in response to infection by a pathogen may include strategies such as the deposition of substances to strengthen cell walls and/or isolation of the pathogen from healthy parts of the plant.

Strengthening of cell walls through the deposition of extra lignin and/or the deposition of extra materials, both at the site of pathogen penetration, have been documented for a number of host/pathogen interactions (e. g. Cohen *et al* 1990; Rey *et al* 1996; Picard *et al* 2000; Silva *et al* 2002).

Spread of the pathogen or substances secreted by the pathogen may be halted by the formation of barriers such as the cork layers developed by potato tubers infected by *Rhizoctonia* (Ramsey 1917), or the Hypersensitive Response (HR). HR involves the rapid death of plant cells at or near the site of infection, isolating the pathogen and preventing its spread into neighbouring healthy cells.

1.3.4 Induced Chemical Defence Mechanisms

Induced chemical defences are secondary resistance responses, requiring the *de novo* transcription or translation of genes that code for defensive proteins and enzymes such as phytoalexins and enzymes.

Pathogenesis Related (PR) proteins are low-molecular-weight polypeptides (10-40 kDa) which accumulate extra-cellularly, are relatively protease resistant, and often have extreme isoelectric points (van Loon 1985). They are generally assumed to be present in most plant species (Linthorst 1991), accumulate after infection by viroids, viruses, bacteria, and fungi (van Loon 1985) and may be induced by a variety of chemicals (van Loon 1983).

Five different 'families' of PR-proteins have been identified (van Loon *et al* 1987). Each family of proteins has similar features, such as molecular mass, serologic relationships, nucleotide and amino acid sequences, enzymatic activities, and acidic and basic isoforms (Prell & Day 2001).

Phytoalexins are antimicrobial compounds of low molecular weight that are both synthesised and accumulated in plants after exposure to pathogens or chemical or mechanical injury (Prell & Day 2001). In the carrot/*Candida albicans* interaction, the host was found to produce 6-methoxymellein, a phytoalexin that disrupted the membrane of *C. albicans* (Amin *et al* 1986).

Various enzymes, such as β-1,3-glucanase, chitinase, amylase and proteinase have also been implicated in host resistance. Details and evidence for the involvement of chitinase, phenolic phytoalexins and cell wall modifications in plant defence are discussed further in relevant chapters.

1.4 THESIS OBJECTIVES

There is little basic information on *C. camelliae* and most research has concentrated on chemical control methods. The overall goal of this thesis was to increase knowledge of the fungus through investigation of some basic and molecular biology, and provide data on resistant species for the development of resistant cultivars in the future.

The objectives of this study were to:

a) determine the distribution of *C. camelliae* within New Zealand and study its spread.
b) determine the conditions for inducing sclerotia to germinate out of season.

c) develop a species-specific primer for the accurate detection of *C. camelliae in planta.*

d) determine the relationship of *C. camelliae* to other *Ciborinia* species, and *Sclerotinia sclerotiorum.*

e) develop a robust and reliable method(s) for testing *Camellia* species for resistance and susceptibility.

f) develop a rating system for categorising levels of resistance.

g) investigate mechanisms of resistance found in *C. camelliae*/*Camellia* resistant reactions.

CHAPTER TWO – GENERAL MATERIALS & METHODS

2.1 FUNGAL CULTURES

2.1.1 *C. camelliae* Isolates used in this Study

C. camelliae isolates used in this study are listed in Table 2.1.

Table 2.1 *C. camelliae* Isolates used in this Study

Isolate	Region/Country of Origin	Year of Isolation	Source
NEWP	New Plymouth, New Zealand	2000	this study
PALM	Palmerston North, New Zealand	2000	this study
WELL	Wellington, New Zealand	2000	this study
HK	Hong Kong	2001	this study
LMK7	Japan	-	E. Nagasawa, Tottori Mycological Institute
2001835	West Sussex, England	1999	Roger Cook, Mycology Diagnosis, Central Science Laboratory, York
2001980	Hampshire, England	1999	Roger Cook, Mycology Diagnosis, Central Science Laboratory, York
12996	San Marino, California, USA	2000	this study
11160	San Marino, California, USA	2000	this study
793	Ticino, Switzerland	1999	Daniel Grindrat, Station fédérale de recherches agronomiques de Changins, Nyon
Spain 1	Spain	2000	J. Pedro Mansilla Vázquez, Ing. Jefe Servicio Fitopathologia
Spain 2	Spain	2000	J. Pedro Mansilla Vázquez, Ing. Jefe Servicio Fitopathologia
II	Italy	2001	A Garibaldi, Settore Patologia Vegetale, Università Degli Studi di Torino
MHC13	France	2001	Carole Saurat, Laboratoire National de la Protection des Végétaux, Unité de Mycologie Agricole et Forestière, Nancy
990038	France	2001	Carole Saurat, Laboratoire National de la Protection des Végétaux, Unité de Mycologie Agricole et Forestière, Nancy

2.1.2 Isolates of other Fungal Species used in this Study

Isolates of other fungal species used in this study are listed in Table 2.2.

Table 2.2 Isolates of other Fungal Species used in this Study

Isolate	Species	Region/Country of Origin	Year of Isolation	Host	Source
MONO	*Monochaetia* spp.	Palmerston North, New Zealand	2001	camellia flower	Peter Long
SSCL	*Sclerotinia sclerotiorum*	Te Kuiti, New Zealand	-	kiwifruit	Stephen Hoyte
GORE	*Sclerotinia sclerotiorum*	Gore, New Zealand	-	-	M. Braithwaite
WAIR	*Sclerotinia sclerotiorum*	Wairarapa, New Zealand	-	-	M. Braithwaite
BOTR	*Botrytis cinerea*	Palmerston North, New Zealand	2001	camellia flower	this study
PENE	*Penicillium* spp.	Palmerston North, New Zealand	2001	camellia flower	this study

2.1.3 Subculturing

All fungal isolates were maintained on Difco PDA in 12/12 L/D at 20°C. Cultures were sub-cultured monthly by removing a 5 mm diameter plug of mycelium and agar and placing mycelium-side down on fresh agar.

2.2 FLOWER SPECIES

2.2.1 *Camellia* Species, Hybrids and Cultivars used in this Study

The *Camellia* species, hybrids and cultivars used in this study, are listed in Table 2.3.

Table 2.3 List of *Camellia* Species, Hybrids and Cultivars used in this Study and their Flowering Season (F.S.)

Species	Cultivar	F.S.
C. cuspidate	-	S
C. cuspidata x *C. saluenensis*	'Cornish Snow'	S
C. cuspidata hybrid	'Spring Festival'	S
C. euryoides	-	S
C. forrestii	-	S
C. fraternal	-	S
C. granthamiana	-	S
C. grijsii	-	S
C. grijsii	'Juhuacha'[b], 'Zhenzhu Cha'	S
C. hiemalis	'Shishigashira', 'Shôwa-no-sakae', 'Shôwa Supreme'	A

[a] main flowering season in S=Spring or A=Autumn in New Zealand. Autumn flowering species overlapped with those of spring flowering species in Los Angeles and were available for resistance testing there only.

[b] cultivar not listed in the International Camellia Register (Savige 1993). Referenced to Camellia Nomenclature (1999), the person who supplied it, or declared unknown where appropriate.

Table 2.3 List of Camellia Species (Continued)

Species	Cultivar	F.S.
C. japonica	'Alba Plena', 'Aquarius', , 'Blood of China', 'Bob's Tinsie', 'Covina', Dahlohnega', 'Desire', 'Dixie Knight', 'Dixie Knight Supreme', 'Dolly Dyer', 'Easter Morn', 'Elegans Champagne', 'Elegans Supreme', 'Flame', 'Fleur Dipater', 'Grand Slam', 'Great Eastern', 'Guilio Nuccio', 'Hagoromo', 'Katherine Nuccio', 'Lady Loch', 'Laurie Bray', 'Lemon Drop', 'Leonora Novick', 'Lily Pons', 'Little Red Riding Hood', 'Little Slam', 'Mark Alan', 'Maroon and Gold', 'Mary Paige', Nuccio's Jewel', 'Otto Hopfer', 'Prince Frederick William', 'R. L. Wheeler', 'Roger Hall', 'Sarasa', 'Silver Chalice', 'Stella Sewell', 'Sugar Babe', 'The Czar', 'Twilight', Ville de Nantes', 'Zambo'	S
C. japonica x *C. fraterna*	'Tiny Princess'	S
C. japonica x *C. lutchuensis*	'Cinnamon Cindy', 'Scentuous'. 'Spring Mist', 'Sweet Emily Kate'	S
C. longicarpa	-	S
C. lutchuensis	-	S
C. lutchuensis x *C. japonica*	'Koto-no-kaori'[b], 'Minato-no-akebono', 'Minato-no-haru', 'Nymph'	S
C. lutchuensis hybrid	'Fairy Blush'[b]	S
C. maliflora	-	A
C. pitardii var. *pitardii*	-	S
C. pitardii var. *pitardii*	'Sprite'	S
C. pitardii var. *pitardii* x *C. japonica*	'Nicky Crisp'	S
C. polydonta	-	S
C. reticulata	'Simpatica', 'Zhangjia Cha'	S
C. reticulata x *C. japonica*	'Hulyn Smith'	S
C. reticulata hybrid	'Lovely Lady'	S
C. rosiflora	'Cascade'[b]	S
C. rusticana	'Botanyuki'	S
C. rusticana x C. lutchuensis	'Fragrant Joy'	S
C. saluenensis	-	S
C. saluenensis x *C. reticulata*	'Brian', 'Warwick Berg'	S
C. sasanqua	-	A
C. sasanqua	'Moon Moth', 'Setsugekka'	A
C. sinensis	-	A
C. transnokoensis	-	S
C. trichocarpa	-	S
C. x *vernalis*	'Ginryû'	A
C. x *williamsii*	'Donation', 'E. G. Waterhouse', 'Les Jury'[b], 'Mirage', 'Rendezvous', 'Rose Bouquet', 'Rose Holland', 'Softly'	S
C. x *williamsii* x *C. japonica*	'Night Rider'	S
C. yuhsienensis	-	S
C. yunnanensis	-	S
C. hybrid	'Alpen Glo', 'Gay Baby', 'Fairy Wand', 'Punkin'	S
Unknown (probably *C. japonica*)	'Alba Plena Imp'[b], 'Ameniflora'[b], 'Bella Rosa'[b], 'Bizarre'[b], 'Cox's Fancy'[b], 'Diorella'[b], 'Melody Time'[b], 'Ole Hybrid'[b]	S

[a] main flowering season in S=Spring or A=Autumn in New Zealand. Autumn flowering species overlapped with those of spring flowering species in Los Angeles and were available for resistance testing there only.

[b] cultivar not listed in the International Camellia Register (Savige 1993). Referenced to Camellia Nomenclature (1999), the person who supplied it, or declared unknown where appropriate.

2.2.2 Non-Camellia Species used in this Study

C. camelliae non-host flower species used in this study are listed in Table 2.4.

Table 2.4 Other Flower Species used in this Study

Genus	Species	Cultivar
Magnolia	*salicifolia*	-
	stellata	-
	soulangeaua	-
Rhododendron	-	'Chrysomanicum'
	-	'Medusa'
	unknown-	-
	tephropelum	-
	arboreum spp. zeylanicum	-
Tulip	-	'Monsella'

2.2.3 Classification System for *Camellia* Species

Camellia species and cultivars are referred to by the names and spellings given in the International Camellia Register (Savige 1993). Taxonomic revisions have been suggested, but I have maintained the classification and naming system as given in the Register to save confusion. Cultivars were often labelled with the Western common name, where the Chinese or Japanese name now takes precedence; for example, a plant labelled *C. reticulata* 'Chang's Temple' is referred to as 'Zhangjia Cha' in this thesis.

Plant names followed by a reference number, e. g. *C. fraterna* 15476-D are plants from the Huntington Botanical Gardens, the United States, identified by their reference number. At Descanso Gardens, where there were many plants of the same cultivar, plants were numbered, e. g. *C. japonica* 'Alba Plena' Plant 3, for individual identification.

2.2.4 Identification of Flower Species or Cultivars

Identification of species and cultivars was made by the label attached to the plant, by the name given by the plant owner, or identified by experts (e.g. Vonnie Cave, Wanganui; Dave Bull, Massey University). In some cases, plants were obviously mislabelled, e.g. *C. fraterna* was incorrectly labelled as *C. yunnanensis* in the Arboretum, Massey University, and the correct name was used.

2.2.5 Origin of Flowers

Flowers were collected from eleven sites in New Zealand and California, the United States. The sites are referred to by letter (Table 2.5) and indicated for each flower species or cultivar in each experiment.

Table 2.5 Location of Flower Collection Sites and Reference

Site: Address/Park/Farm	Reference
Massey University, Arboretum (Turitea Campus), New Zealand	A
Massey University, (Turitea Campus), New Zealand	M
VN, Palmerston North, New Zealand	V
MS, Palmerston North, New Zealand	MS
The Esplanade, Palmerston North, New Zealand	E
DP, Mangatainoka, New Zealand	DP
JV, Mangatainoka, New Zealand	JV
VC, Woodville, New Zealand	VC
Descanso Gardens, 1418 Descanso Drive, La Cañada Flintridge, Los Angeles, California, USA	D
The Huntington, 1151 Oxford Road, San Marino, Los Angeles, California, USA	H
Nuccio's Nurseries, 3555 Cheney Trail, Altadena, Los Angeles, California, USA	N

2.2.6 Origin of Apothecia

Apothecia were collected from two sites in New Zealand and one in California, the United States. The sites are referred to by letter (Table 2.6) and indicated for each experiment.

Table 2.6 Location of Apothecia Collection Sites and Reference

Site	Reference
Wellington Botanic Gardens	WBG
Glen Logie Garden, Wanganui	GL
Descanso Gardens, 1418 Descanso Drive, La Cañada Flintridge, Los Angeles, California, USA	DG

2.3 MEDIA

All media were prepared using RO or milli-Q purified water and sterilised by autoclaving at 103.4 kPa (15 p.s.i) at 121°C for 15 min. Liquid media were cooled to room temperature before the addition of supplements. Solid media were cooled to 50°C before the addition of supplements and pouring. Uninoculated plates were stored at 4°C.

2.3.1 Potato Dextrose Agar (PDA)

PDA contained 39 g/L of Difco PDA.

2.3.2 Luria-Bertani (LB) Broth and Agar

Luria-Bertani media contained (g/L): tryptone 10.0; NaCl 5.0; yeast extract 5.0. The ingredients were dissolved and pH adjusted to 7.4 (NaOH) prior to autoclaving. For LB agar, agar was added at the rate of 15 g/L. LB broth was supplemented after autoclaving with ampicillin to give a final concentration of 100 μg/ml. For blue/white selection, LB agar was supplemented after autoclaving to give final concentrations of: ampicillin 100 μg/ml; isopropylthio-β-D-thiogalactopyranoside (IPTG) 30 μg/ml; 5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal) 60 μg/ml.

2.3.3 Chitin Agar

Agar plates were prepared by adding 6 g of agar powder (Becton Dickinson) to 390 ml of McIlvaine buffer pH 5 containing 0.02% (w/v) sodium azide. The suspension was autoclaved then cooled before adding 4 ml of 1% (w/v) pH 4 glycol chitin (Sigma). The suspension was thoroughly mixed then poured into 90 mm diameter petri plates (20 ml/plate). After the agar had set, seven evenly spaced 5 mm diameter wells were punched into the agar using a cork borer.

2.4 BUFFERS AND SOLUTIONS

2.4.1 McIlvaine Buffer

McIlvain buffer pH 5 contained 48.50 ml citric acid (21.01 g/L citric acid monohydrate 0.1 M) and 51.50 ml Na_2HPO_4 (28.4 g/L Na_2HPO_4 0.2 M).

2.4.2 TE Buffer (Tris EDTA)

TE buffer contained 10 mM Tris-HCl and 1 mM Na_2EDTA (TE 10:1) and was prepared to the required concentration from 1 M Tris-HCl (pH 7.5) and 0.5 M Na_2EDTA (pH 8.5) stock solutions.

2.4.3 TAE Buffer (Tris Acetate EDTA)

TAE buffer contained final concentrations of Tris-HCl 40 mM, Na_2EDTA 2 mM and acetic acid 20 mM, pH 8.5 (NaOH).

2.4.4 TBE Buffer (Tris Borate EDTA)

TBE buffer contained final concentrations of Tris-HCl 89 mM, Na_2EDTA 2.5 mM and boric acid 89 mM, pH 8.2 (HCl).

2.4.5 10x Gel Loading Buffer (GLB)

GLB contained final concentrations of urea 2 M, 50% (v/v) glycerol, Tris acetate 50 mM, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol.

2.4.6 Lysis Buffer (Al-Samarrai & Schmid 2000)

Lysis buffer contained final concentrations of Tris acetate 40 mM, sodium acetate 20 mM, EDTA 1 mM and 1% (w/v) SDS, pH 7.8.

2.5 STAINS

2.5.1 Ethidium Bromide

The ethidium bromide solution used for staining agarose electrophoresis gels was prepared by adding 1 µl of a 10 mg/ml stock to 10 ml of milli-Q water to give a final concentration of 1 µg/ml.

2.5.2 Toludine Blue O

Toludine Blue O contained 0.01% (w/v) toludine blue in 0.1 M KH_2PO_4-NaOH buffer (pH 6).

2.6 STATISTICAL ANALYSIS

All data, except that of Chapter Seven, were analysed using SAS for Windows Version 6.12 or 8e under the guidance of Duncan Hedderley, Statistics Research and Consulting Centre, Massey University. The statistical tests used in this thesis were: Analysis Of Variance (ANOVA) (one and two factored), using Tukey's test for significant differences between means where appropriate; non-parametric ANOVA; repeated measures ANOVA; Chi square; Poisson; and logistic regression. Examples of SAS code for these tests are given in Appendix II. Data from each experiment met assumptions of normality of distribution and constant variance of residuals unless indicated otherwise, where the relevant transformation is noted. Relevant statistics are quoted for each experiment, with the entire result table given in Appendix IV. In Chapter Seven, data was analysed using MINITAB Release 12.1, with single factor ANOVAs and Fisher's individual comparisons to compare means under the guidance of Dr Brett Robinson, HortResearch, Palmerston North.

CHAPTER THREE - DISEASE SURVEY

3.1 INTRODUCTION

The disease was inadvertently introduced, from Japan, to California, the United States, and subsequent measures to prevent its spread within the United States and to other countries were ineffective. It is now present in all major camellia-growing regions with the exception of South Africa and Australia. In the United States, quarantine restrictions and cultural control methods failed to contain the disease and within 20 years it was widespread in the 'camellia belt'. In New Zealand, a survey (C. F. Hill, 1993 unpubl. data, MAF) determined that the disease was already widespread in Wellington and control or eradication measures were not undertaken.

3.1.1 Distribution within New Zealand

From its discovery in a private garden in Wellington, New Zealand, in 1993 (Stewart & Neilson 1993), to surveys in 1997 and 1998 which showed it to be widespread in the lower North Island and upper South Island (Taylor & Long 1998; Taylor 1999; Taylor *et al* 1999), *C. camelliae* has dispersed widely.

The objective of the surveys in 1997 and 1998 was to determine the distribution of the disease in New Zealand and to assess the method(s) and rate of spread. Figure 3.1 shows the distribution of the disease in the North Island prior to and during the 1997 and 1998 surveys and Figure 3.2, the distribution in the South Island in 1998.

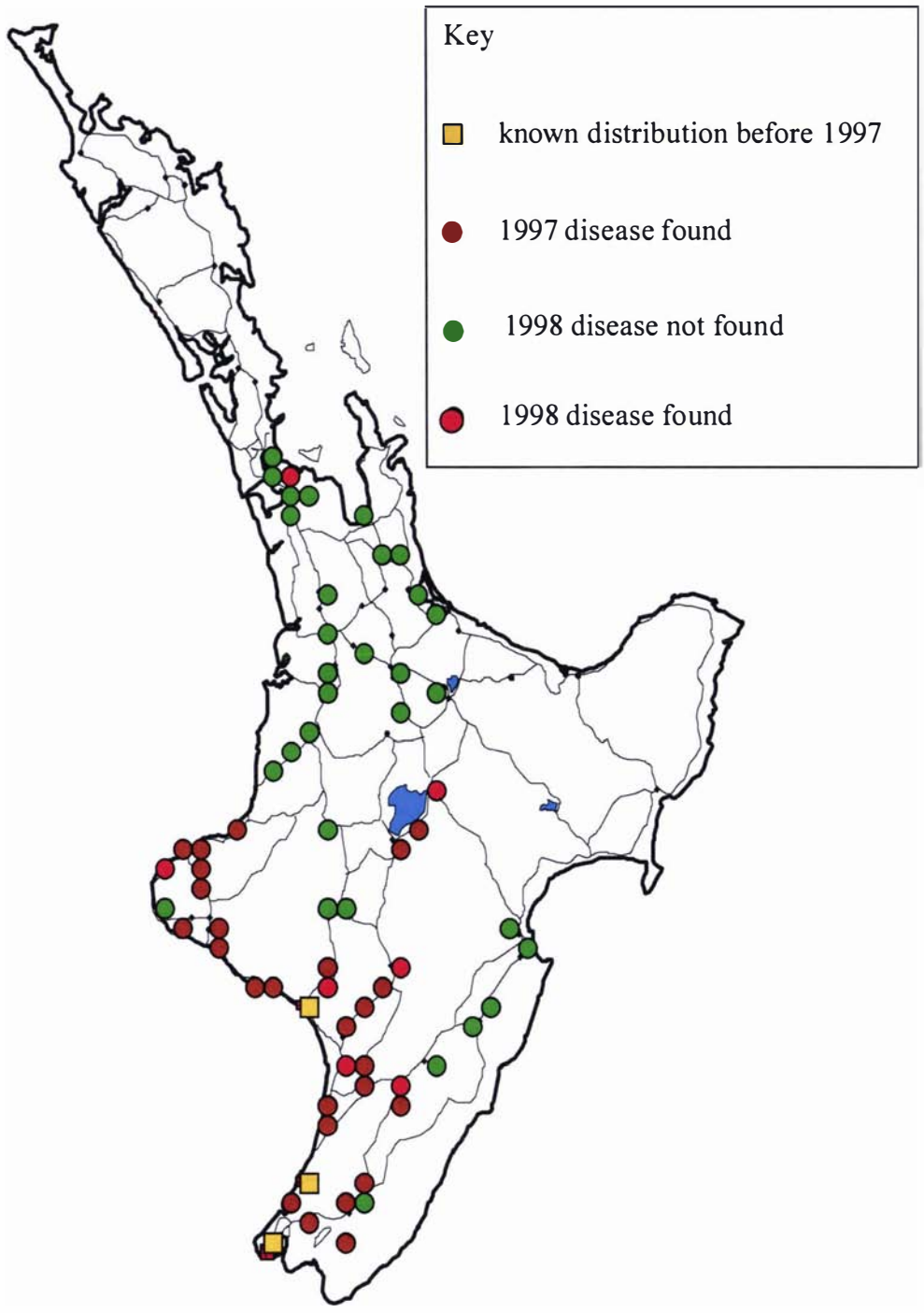


Figure 3.1
Distribution of *C. camelliae* in the North Island in 1998, indicating known distribution in previous years. For reasons of scale, points where the disease was found are not individually marked and points are indicative of site only.

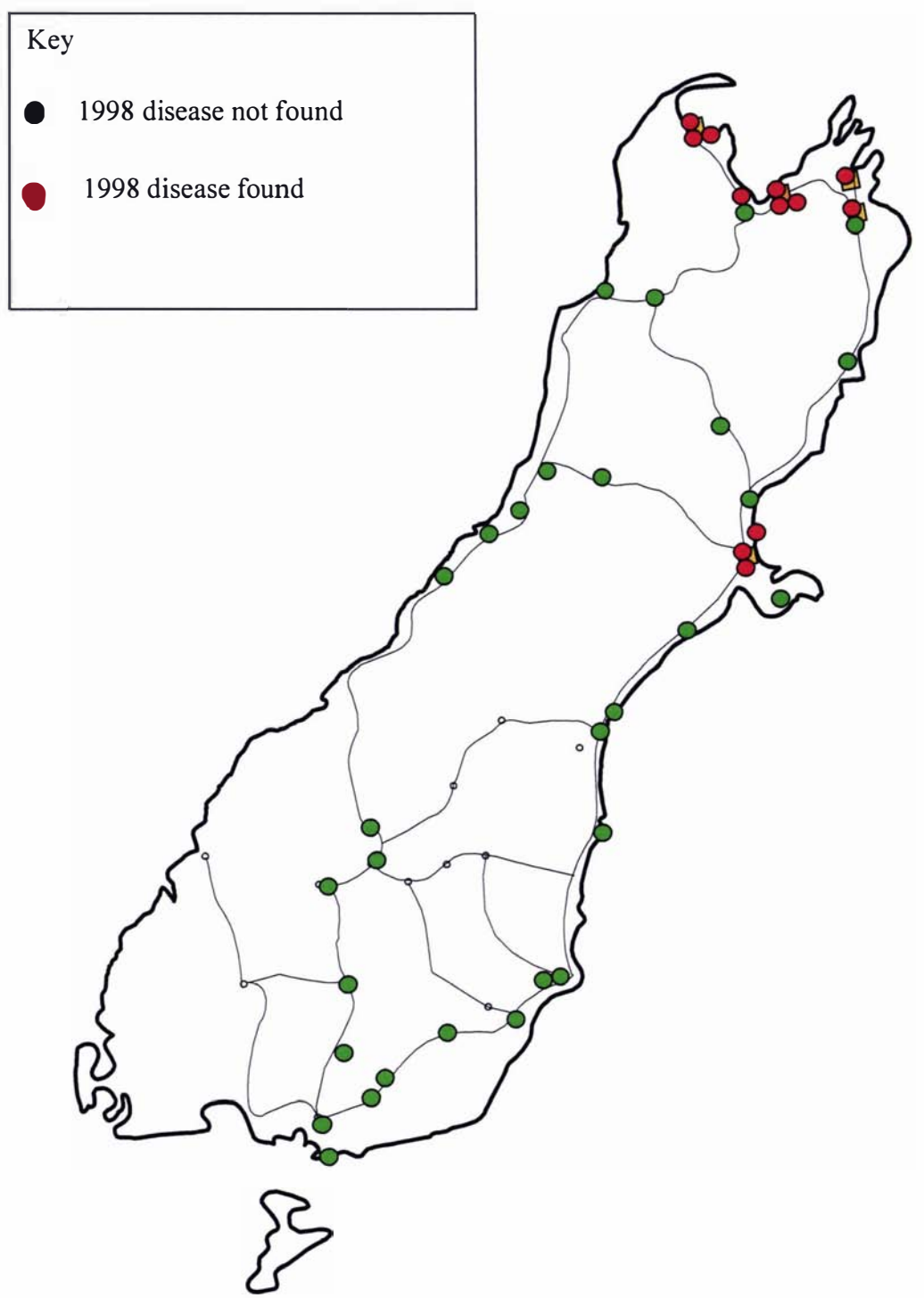


Figure 3.2
Distribution of *C. camelliae* in the South Island in 1998. For reasons of scale, points where the disease was found are not individually marked and points are indicative of site only.

Prior to these surveys, the disease was not known to be in the South Island nor had it been reported outside Wellington, Waikanae and Wanganui in the North Island. Discovery of its widespread occurrence, therefore, was a surprise to many in New Zealand but typical of disease reports from the United States. The large number of infected flowers seen in some centres (e.g. Nelson, Golden Bay, Christchurch, Masterton, New Plymouth) indicated the disease had been present at least two years, while at other sites (e.g. Paihiatua, Urenui, Kaiapoi, Taihape) low levels of infection indicated that the disease was newly established in the area.

3.1.2 Methods of Spread

The widespread occurrence of *C. camelliae* indicated that the main method of spread had been through windborne ascospores, particularly in the North Island, where the human population is greater and the land more intensively settled. Camellias are widespread in many small towns and farms, thus allowing the disease to continue its advance each spring.

Windborne ascospores have been estimated to travel from 500 m to several kilometres in a viable state, (Zummo 1960; Zummo & Plakidas 1959; Zummo & Plakidas 1961; Baxter & Epps 1981; Baxter & Seagars 1989; Stewart & Neilson 1993; Stewart 1994; Baxter & Thomas 1995; Bond 1996) but only Zummo (1960) and Zummo & Plakidas (1961) appear to have based their estimates on experimental evidence. Brown (1983) reported anecdotal evidence that ascospores might be blown up to 25 km and the results of the survey by Taylor (1999) and Taylor *et al* (1999) supported dispersal over these longer distances. Given that one apothecium may produce up to 12 million spores over 10 days (Bright & Long 2000), New Zealand's windy weather and the ubiquity of the camellia plant in New Zealand, such long-distance dispersal should be expected.

While wind dispersal accounted for much of its widespread distribution, human activity has also been implicated in its distribution. Four infected sites in the 1997 and 1998 surveys appeared to be the result of transfer/introduction of disease material (either through infected camellia plants or transfer of soil) by humans. In Christchurch and Auckland, no other infections were found within 280 km of the outbreaks, indicating that infected material had been transported by people into these

centres. Anecdotal evidence suggests that it is not uncommon for people to dig up garden plants when moving house, and this would be an ideal method for transferring the disease to new areas. In Mangaweka, a newly planted camellia, probably purchased in an infected area, appeared to be the origin of the disease. In Hatepe, the origin of the disease was less obvious, as the infected plants were mature camellias. It is, however, a bach-type settlement on the shores of Lake Taupo, and it may be that infected material was brought into a nearby property and spread to others.

3.1.3 Rate of Spread

From the 1997 and 1998 surveys (Taylor 1999) the rate of spread of the disease was calculated to average 32-35 km per year. This assumes that the disease was present in Wellington much longer than previously thought (circa 1987), suggested by its wide distribution in 1993 (Hill 1993) and observations of its arrival and proliferation in new areas during the course of this study. Small increments each season by windborne ascospores, combined with leap frogging on infected material, make such a dispersal rate possible.

3.1.4 Objective

The objective of this study was to:

a) re-survey New Zealand in 2001 to monitor disease distribution and spread.

3.2 MATERIALS & METHODS

The survey was designed to determine the distribution of the disease in both North and South Islands further to the surveys already completed. Survey time was limited by experimental work during the disease seasons, and financial considerations.

A small survey was conducted in October 2000, covering State Highway 2 from Dannevirke and Waipukurau to the Hastings and Napier region. The main survey, covering most of the North and South Islands, was conducted in November and December 2001.

The survey routes were the main State Highways in each Island and on some backroads. Stops were made at each town or city along the route to examine camellia plants. Some rural properties were also checked.

Camellia plants in public places such as road verges, churchyards, cemeteries, public parks and gardens were surveyed together with those in private gardens, where the plants could be examined without entering the property (e.g. adjacent to the footpath), as well as some private gardens, when invited.

The number of camellia plants examined in each city or town varied depending upon size of the locality and availability. Disease distribution is often patchy, even in areas where it has been present for several years (Hill 1993; Taylor 1999), so where possible, camellias in different areas of the same town were examined.

Disease identification was made on the basis of signs and symptoms exhibited by the flower. Blight-infected flowers were easily distinguished by colour from other flower rots caused by pathogens, saprophytes, tissue senescence and weather damage (Figure 3.3).

Figure 3.3
Blight-infected flowers have a characteristic grey-brown rot and the symptoms frequently spread upwards from the base of the flower. Browning symptoms caused by other factors are typically orange-brown, and the pattern of symptom spread differs. (A) Grey-brown colour of *C. camelliae*-infected flower and (B) orange-brown colour of a naturally ageing flower.

In diseased flowers, the fungus forms a characteristic ring of grey or white mycelium around the base of the flower that can be seen when the calyx is removed. This does not occur in petal-shattering cultivars and the ring is not usually evident in older flowers (circa 2 mo) unless they have been kept moist.

Positive identification of the disease was made where sclerotia were found in petals. Alford (1961) and Alford *et al* (1961) positively identified blight only if they found apothecia or sclerotia, but the sclerotia alone were considered sufficiently distinctive to be used for positive identification here. In all cases where the disease was identified, or suspected, samples were taken back to the laboratory and held under suitable conditions for sclerotial formation. One flower with definite signs and symptoms of *C. camelliae* infection counted as a positive find, but in all cases, more than one flower and more than one plant were found to be infected at a site.

3.3 RESULTS

The main survey took place after the camellia flowering and blight season, when most flowers had shrivelled and dried out. Fresh flower infections and infections less than ~3 weeks old were not seen. Combined with a drier than average spring, this made surveying for blight more difficult than in previous years. Where a plant and fallen flowers could be viewed for fresh blight symptoms in previous years, the November 2001 survey was limited, as this was not possible. The distinctive colour of blight-infected flowers had disappeared, and infected flowers were indistinguishable from non-blighted flowers. It was necessary for each dried out flower to be squeezed at the base to detect whether sclerotia were present and sclerotia tended to be smaller than usual (based on my observations) because of low rainfall. Overall, fewer flowers were checked because of the greater time required to check each flower individually. Fewer plants were surveyed because of the extra time required to check each plant, the need to get under the plant to check each flower (i.e. private property issues) and because many flowers had been raked up or mown over.

In the North Island in the 2000 mini-survey, blight was found at Dannevirke, Norsewood, Clive and Napier. It was not detected in Havelock North or Hastings.

In the 2001 North Island survey, the blight was found in many new areas. Travelling south from Auckland on State Highway 1, the disease was found in Huntly, Hamilton, Cambridge, Putaruru and Tokaroa. On State Highways 3 and 4 south of Hamilton it was found in Te Awamutu, Te Kuiti and Taumaranui. It was also found in Rotorua and on State Highway 2 in Paeroa, Waihi, Tauranga and Whakatane. It was not found in Ngaruawahia, Te Puke, Katikati and Thames.

Travelling north from Auckland, it was not found in Warkworth, but was present in Whangarei.

Within greater Auckland the disease was found in several eastern and southern suburbs (Mt Wellington, Pakuranga, Takanini and Manurewa) but a full survey was not conducted because of time constraints.

In the 2001 South Island survey, blight was found on State Highway 1 at Ashburton, Timaru and Oamaru. It was not found in Kaikoura, Hanmer Springs, Dunedin, Mosgiel, Balclutha, Gore, Invercargill, Queenstown or Geraldine. On the West Coast, it was not found in Murchison, Karamea, Westport, Greymouth or Ross.

The distribution of the disease as found in the recent surveys is shown in Figures 3.4 and 3.5.

Full details of survey sites are given in Appendix III.

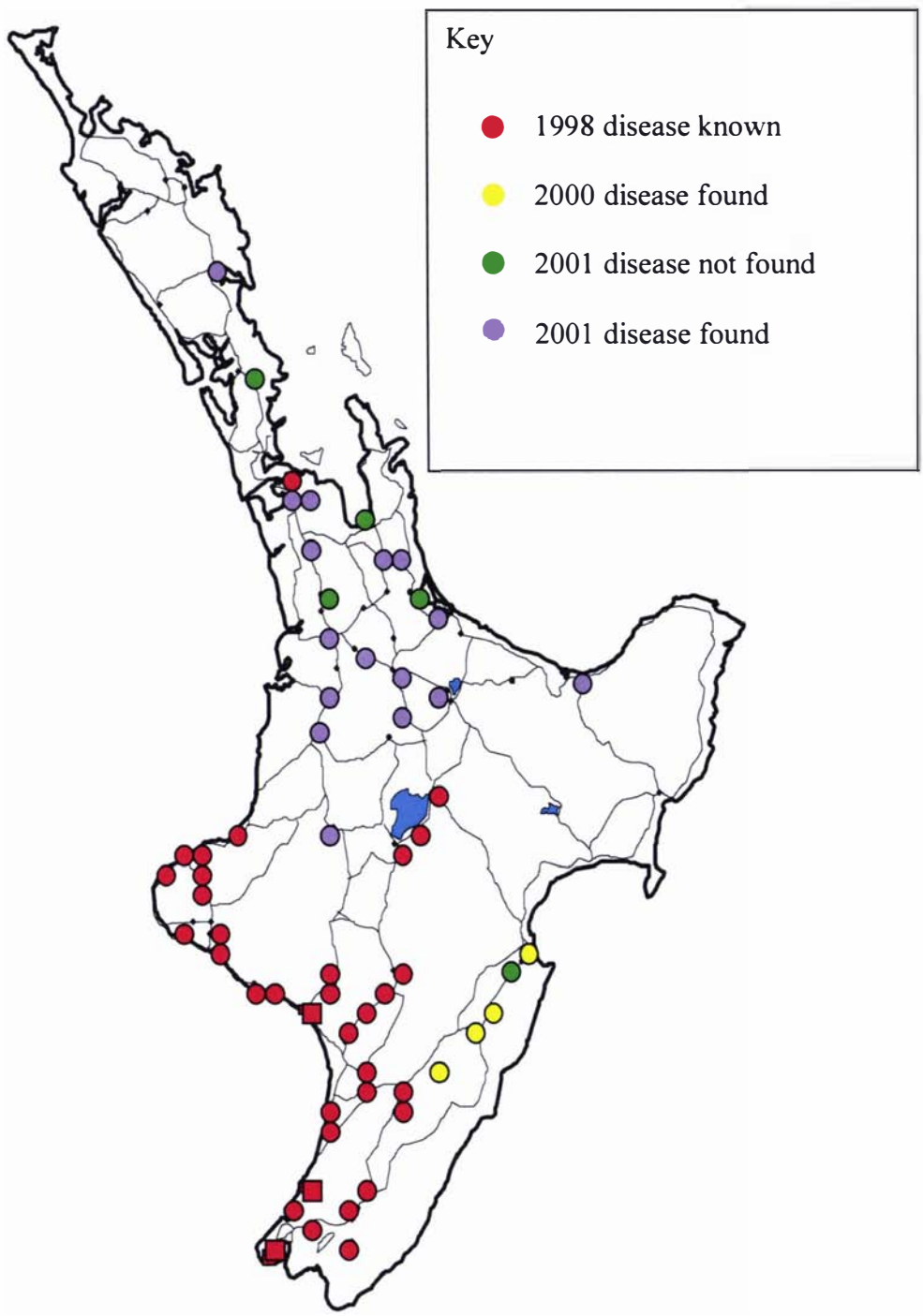


Figure 3.4
Distribution of *C. camelliae* in the North Island in 2001, indicating known distribution in previous years. For reasons of scale, points where the disease was found are not individually marked and points are indicative of site only. Areas north of Whangarei and the east coast around Gisborne were not surveyed.

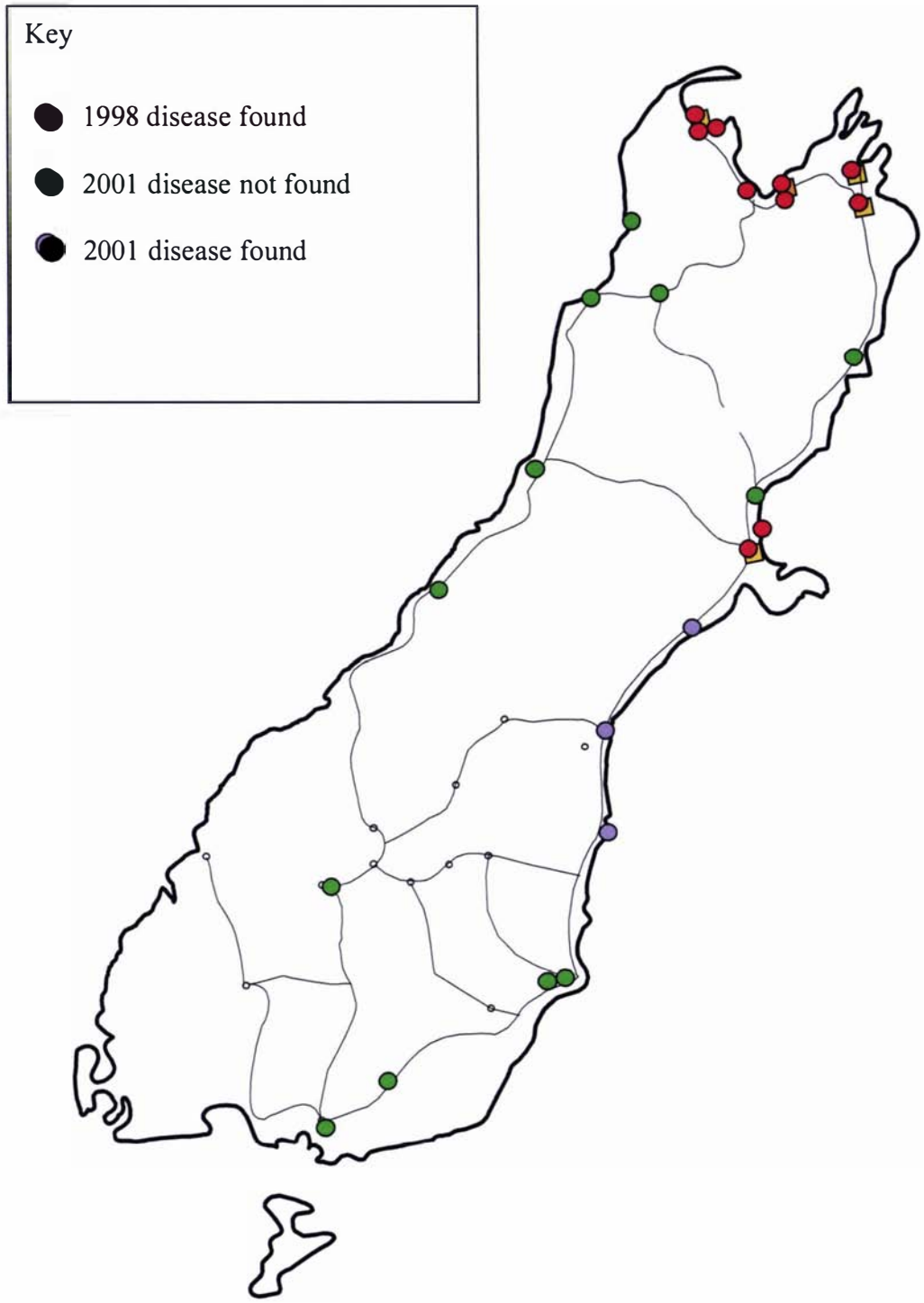


Figure 3.5
Distribution of *C. camelliae* in the South Island in 2001, indicating known distribution in previous years. For reasons of scale, points where the disease was found are not individually marked and points are indicative of site only.

3.4 DISCUSSION

The lateness of the main survey reduced the number of flowers and plants sampled and perhaps reduced the chances of detecting flower blight in areas where the incidence was low. The absence of blight in an area did not prove that blight was not present in the area, merely that at the sites sampled, blight was not present. In Dunedin, for example, relatively few plants were sampled for the city size, and I would not definitively state that blight was not present in the city. Locating and accessing camellia plants with dead flowers underneath was difficult and the result is based mostly on plants examined at one site; the Botanic Gardens.

Many sites that were surveyed in previous years were revisited in the 2001 survey, but in some cases (e.g. Temuka, Clinton, Bluff, Wanaka, Cromwell) the flowers had been cleared away and could not be assessed. In others, sites that were previously surveyed (e.g. Otorohanga, Owhango, Seddon) were not visited in 2001 because of lack of time.

In the two disease seasons since the last major survey, blight has become widespread in the North Island. Apart from an isolated outbreak in Mt Eden, Auckland, the most northerly occurrence of the disease was in Taupo; it has now spread throughout the central North Island, Bay of Plenty, Waikato, Auckland and is now in Whangarei. Although the survey did not detect the disease in towns such as Katikati, Te Puke and Thames, because of its generally widespread distribution, this could be due to the small sample size and detection problems rather than its absence from these areas. The disease was not detected between Auckland and Whangarei (169 km AA Road Map) but whether this is due to absence of the disease at intermediate points (indicating human transfer to Whangarei) or lack of sampling opportunities (coastal settlements tend to have fewer camellia plants) is not known. Access to more plants and a greater time to conduct the survey would define this point. It is also not known if the disease is present north of Whangarei.

In the South Island, blight had spread to three new centres (Ashburton, Timaru and Oamaru) since the 1998 survey. Low levels of blight were found in Ashburton and Oamaru, but blight appeared to be quite common in Timaru. The distance from Christchurch to Oamaru is 247 km (AA Road Map). A wind dispersal rate of 32-35

km per year, as estimated from the 1997 and 1998 surveys (Taylor & Long 1998; Taylor 1999, Taylor *et al* 1999) would not account for this distribution. There is not sufficient evidence to determine whether wind dispersal – at greater distances than previously calculated – or human activity is responsible. If human activity is responsible for the majority of outbreaks at these sites, then it is likely that the disease is present, but currently undetected, in Dunedin.

Overall, the blight is now widespread in the North Island, and is widespread in the populous areas of the South Island, except Dunedin and Invercargill. The disease will continue to spread to new areas, either by wind or human activity, limited only by environmental factors. The colder winters of the far south and interior of both islands, hotter summers in the South Island's interior, and the drier climates of the east coasts may limit the disease somewhat, either preventing establishment, or reducing sclerotial survival/longevity. In two visits to Los Angeles, California, I noticed that the disease was less prevalent in this desert climate compared to New Zealand. Many gardens in this region would not survive without the benefit of irrigation, but the irrigation permits the fungus to exist. Drier climate areas in New Zealand may also limit the disease.

Any future surveys would be better carried out about three quarters of the way through the blight season (perhaps early-mid October in New Zealand) in order to facilitate detection of the disease and increase the number of plants that can be found at the best time for disease identification.

CHAPTER FOUR – SCLEROTIAL GERMINATION

4.1 INTRODUCTION

Camellia flower blight research is currently limited by the availability of *C. camelliae* ascospores, which are produced over 3-4 months in late winter and spring in New Zealand. In order to increase the period during which research into the biology of infection and resistance can be conducted, it is essential to discover the conditions that stimulate sclerotial germination and apothecial development so that ascospores can be obtained out of season.

Information from diverse fungal groups regarding details of sclerotial structure, growth, composition and types of germination is given, while factors involved in sclerotial germination refer solely to literature on members of the Sclerotiniaceae. A description of *C. camelliae* sclerotia and apothecia from Kohn & Nagasawa's (1984) taxonomic revision is given along with a summary of previous (unsuccessful) germination experiments.

4.1.1 Fungal Sclerotia

A general definition of the sclerotium describes a range of "morphologically variable, nutrient-rich, multi-hyphal structures which can remain dormant or quiescent when their environment is adverse and then, when conditions improve, germinate to reproduce the fungus" (Willetts & Bullock 1992). Ainsworth (1961) gives the definition as "a firm, frequently rounded, mass of hyphae with or without the addition of host tissue or soil, normally having no spores in or on it". It is suggested that sclerotia from different species are functionally, and in many instances anatomically, alike as a result of convergent evolution, evolving from different aborted structures, such as sexual fruiting bodies, asexual sporogenous tissue and vegetative mycelia (Willetts 1972; Willetts 1978; Willetts & Bullock 1992).

Sclerotial formation starts when hyphae aggregate to form discrete small initials, a process that is the least understood of the growth stages. Initiation of the sclerotial primordium is followed by branching, growth and compaction of hyphae (Willetts

1978) to form one of three types of sclerotial development: a) loose sclerotial development where hyphae branch irregularly and there is no definite pattern of hyphal organisation (e.g. *Rhizoctonia solani*); b) terminal development where branching of one or several hyphal tips form the initial, and there is often fusion between branches (Willetts 1978) (e.g. *Botrytis cinerea* and *Sclerotinia sclerotiorum*); and c) lateral or strand development, where the branch or branches of hyphal strand(s) become interwoven (Willetts 1978) as in *Sclerotium rolfsii*.

Within 7-10 d, sclerotia are often fully mature (Willetts 1978). In the case of *S. sclerotiorum*, 88% of the sclerotial dry weight is attained within 3 d of initiation (Cooke 1971) and glycogen appears to be the major insoluble carbohydrate reserve (Willetts 1978). The mature sclerotium usually forms a rind and becomes independent of its parent colony and surroundings.

The rind forms a barrier that reduces the movement of water and nutrients into and out of the mature sclerotium (Young & Ashford 1992). It is dark coloured from accumulation of melanin (Chet *et al* 1967; Chet & Henis 1968). When complexed with chitin in fungal walls, melanins are inhibitors of polysaccharases (Bull 1970) thus protecting against antagonistic microorganisms and enzymes of the fungus itself (Willetts & Bullock 1992). The cortex, where present, consists of close-fitting, rounded cells which merge into the rind and medulla (Willetts & Bullock 1992).

The majority of the sclerotium consists of the medulla, which is formed by the interweaving of hyphae with few septa and branches (Willetts & Bullock 1992). Often the interhyphal spaces contain a gelatinous matrix, predominantly of ß-1,3 linked glucans with ß-1,6 linked side branches (Saito 1974; 1977; Gómez-Miranda & Leal 1979; Bullock *et al* 1980; Backhouse & Willetts 1984). Its most important function is probably to provide a large reserve of carbohydrate, both for use during resting periods and during germination (Willetts & Bullock 1992).

The number and size of sclerotia produced are directly determined by the nutrient status of the substrate (Willetts & Wong 1971) and most studies indicate that organic nitrogen sources are more suitable for growth than inorganic sources (Willetts 1978).

The main cytoplasmic reserves that have been detected in sclerotia are glycogen, protein, polyphosphate and lipids.

Exudates, small liquid droplets on the surface of the sclerotium, have been reported from many sclerotia. Analysis of these exudates from *S. sclerotiorum* have shown the presence of salts, carbohydrates, lipids, amino acids, soluble proteins and enzymes (Cooke 1969; Colotelo *et al* 1971; Colotelo 1974). The function of exudates is unclear but some suggest they maintain the internal physiological balance of the sclerotium (Cooke 1969; Colotelo 1978; Willetts 1978).

4.1.1.1 Survival and Longevity

The structure and physiology of the sclerotium enables it to survive and persist in conditions that would kill vegetative or sexual fungal structures. In general, survival is greater at cooler than at warmer temperatures, especially when combined with dry conditions (Willetts 1971). Extreme desiccation, however, is unfavourable, whilst alternate wetting and drying (often a factor of burial depth) favours biological degradation (Smith 1972). Sclerotia of *Sclerotium cepivorum* and *Stromatinia gladioli* survived field burial for 20 years in the absence of host plants (Coley-Smith *et al* 1990) and *Verticillium dahliae* for 14 years (Wilhelm 1955).

Soil texture and pH can also affect sclerotial survival, with sclerotia of *S. sclerotiorum* surviving better in sandy clay loam (pH 6.0) than in sandy loam (pH 8.7) (Merriman 1976), although Singh *et al* (1991) reported that pH had no effect.

4.1.1.2 Dormancy: Exogenous vs. Endogenous

Two types of dormancy are recognised – endogenous (or constitutive) and exogenous (or quiescence). Endogenous dormancy depends on the internal development of the sclerotium. It is a period of maturation during which the internal physiological state of the sclerotium prevents germination. There is some evidence that the sugar-mannitol pathway is involved in this inhibition (Cooke & Mitchell 1970). Sclerotia may be activated to germinate during this period by specific environmental stimuli (Coley-Smith & Cooke 1971). Exogenous dormancy occurs when there are unfavourable external conditions. When these conditions change in favour of the fungus, sclerotial germination can occur. Sclerotial germination, therefore, may be a

two step process, as both types of dormancy must be broken separately and the conditions required for each may vary.

4.1.2 Sclerotial Germination

Three types of sclerotial germination are known. In myceliogenic germination, vegetative hyphae are produced by the sclerotium and these are capable of infecting the host plant. This is normally a feature of small sclerotia such as those produced by some root-infecting fungi (Willetts & Bullock 1992). Sporogenic (producing asexual spores) germination and carpogenic (producing sexual spores) germination are characteristic of larger sclerotia, which tend to be produced by some foliage-infecting fungi (Garrett 1970).

The sclerotia of some fungi are able to germinate by more than one method under certain conditions. For example, carpogenic germination of *Sclerotinia* spp. was favoured at intermediate RH and completely inhibited at 100% RH (Achbani *et al* 1995), whilst after exposure to –10°C or –20°C for 4 wk, the germination of a Canadian isolate of *S. sclerotiorum* changed from carpogenic to myceliogenic (Huang 1991). Huang & Kozub (1994) found that immature sclerotia (6-8 d) of *S. sclerotiorum* germinated myceliogenically, but mature sclerotia (>14 d) germinated carpogenically.

Some of the environmental conditions known to affect sclerotial germination are summarised here. There is a large amount of literature on the subject, but this is sometimes confusing and frequently conflicting, even for work on the same species. The following summaries refer mainly to work on genera related to *Ciborinia*, particularly *Sclerotinia* spp., which could be more relevant to the project.

4.1.2.1 Effect of Sclerotial Formation Conditions on Subsequent Germination

The temperature and substrate in which sclerotia are formed prior to any conditioning treatments may influence germination. With *S. sclerotiorum*, Bedi (1962) found that 25°C was the optimum temperature for sclerotial growth to obtain apothecia, and that sclerotia formed below 15°C did not germinate. In contrast Keay (1939) reported that no apothecia were produced on sclerotia grown at 25°C, but did

so on those produced at 5°C, 10°C, 15°C and 20°C, while Purdy (1956) found that apothecia were produced on sclerotia formed at most temperatures (4-27°C).

More recent work indicates that variation between isolates of *S. sclerotiorum* may account for some of this contradictory information (Huang & Kozub 1991; Huang & Kozub 1993). Huang & Kozub (1991) also demonstrated that a conditioning treatment might break dormancy of a previously non-germinating sclerotium.

Budge & Whipps (1991) and Ferraz & Café Filho (1998) demonstrated that although culture media high in carbohydrates increased the number and weight of *S. sclerotiorum* sclerotia, apothecial production was greatest for sclerotia grown on media low in carbohydrate.

4.1.2.2 Effect of Incubation Temperature on Subsequent Germination

Low temperatures or temperature cycling have been found to be effective for germination of sclerotia. A chilling period of 0-10°C was required by *Claviceps purpurea* for activation and the longer the chilling period, the higher the proportion of sclerotia that germinated (Mitchell & Cooke 1968). Too long a chilling period, however, might reduce germination and chilling was not required if the sclerotia were placed on a damp substrate instead (Mitchell & Cooke 1968).

For *S. sclerotiorum*, Bedi (1962) and Saito (1977) found that pretreating the sclerotia at 0°C and 4°C stimulated apothecial formation, but low temperature treatment was not essential. In contrast, thermocycling – without chilling – stimulated germination of *S. trifoliorum* (Sproston & Pease 1957) and *S. minor* (Hawthorne 1973).

Thus, the temperature(s) required to stimulate germination of sclerotia vary, both between and within species. This variation may be due to differences in the life cycle of pathogens or may reflect ecotypic variation, where isolates from different climatic regions behave dissimilarly.

4.1.2.3 Effect of Substrate on Subsequent Germination

Various soil types and substrates ranging from soils to artificial substrates (e.g. moist filter papers, vermiculite and perlite) have been used successfully as a medium in which sclerotia can germinate. While Mitchell & Wheeler (1990) found that soil type did not affect apothecial production of *S. sclerotiorum*, other studies showed that sand was better than clay (Singh & Singh 1983; Singh *et al* 1991; Singh & Tripathi 1996). Singh *et al* (1991) found that germination was reduced in soils with higher organic carbon content while Ferraz & Café Filho (1998) found that sand, organic, water agar and compost/sand substrates were all equal for apothecial formation. Tores & Moreno (1986), however, found strong interactions between different isolates and different substrates (earth, peat, sand and filter paper). Sclerotia also germinated well in distilled water (Singh & Singh 1984), on moist filter papers (Tores & Moreno 1986; Budge & Whipps 1991) and on moist perlite (Sansford & Coley-Smith 1992).

4.1.2.4 Effect of Light on Subsequent Germination

Light is not thought to affect the early stages of apothecial initiation and germination. Sclerotia of *S. sclerotiorum* produced stipes in the dark (Kosasih & Willetts 1975a; Smith & Boland 1989) and Achbani *et al* (1995) found that light was not required for induction of apothecial initials of *S. sclerotiorum, S. trifoliorum* and *S. minor*. However, Letham (1975) and Bedi (1958) showed that it might play a part in *S. sclerotiorum*. Germination experiments have frequently used a combination of light and temperature so that it was not possible to determine the relative importance of each (e. g. Sproston & Pease 1957; Fuentes *et al* 1964; Sansford & Coley-Smith 1992).

Light does appear to be an essential factor for subsequent development of the stipe into a mature apothecium. The stipe tips of *Sclerotinia* spp. are photosensitive and initials bend towards a light source (Willetts & Wong 1980). For *S. trifoliorum* white light was most effective, with continuous darkness inhibiting stipe evolution (Raynal 1987). With *S. sclerotiorum*, Singh & Singh (1987) found larger apothecia were produced under white light; apothecia were formed most rapidly under blue light (440-480 nm) but were reversibly inhibited by red light (630-700 nm), whilst

Thaning (2000) found light between 298-319 nm (ultraviolet) was essential for apothecium formation. Ikegami (1959) found that light intensity was important in apothecial formation of *S. trifoliorum*, with no formation below 100 Lux and 78-100% germination at 600-1400 Lux.

4.1.2.5 Effect of Burial Depth on Subsequent Germination

In general, germination decreases with an increase in burial depth of the sclerotium but the depth at which germination decreases varies between studies: for *S. sclerotiorum*, Mitchell & Wheeler (1990) cite burial at >2 cm depth as the point at which germination fails and Krüger (1975) >1 cm, but burial at greater depths does not preclude successful germination since stipe lengths of 6 cm have been reported (Cook *et al* 1975).

4.1.2.6 Effect of Moisture on Subsequent Germination

Free water or water potentials of around 0 kPa were required for germination of *S. sclerotiorum* (Grogan & Abawi 1974; Morrall 1977; Phillips 1987), possibly indicating a water-soluble inhibitor (Casale & Hart 1983). Grogan & Abawi (1974) concluded that even slight osmotic stress inhibited apothecial production and that water saturation was essential, although Morrall (1977) did obtain apothecia at ~-750 kPa.

Casale & Hart (1983) found that germination was quicker if sclerotia were leached, and that the germination rate was higher if the water was changed regularly. Batra & Korf (1959) found leaching by water was a prerequisite to germination of cultured sclerotia of *C. erythronii*. Leaching was not necessary for the germination of *C. wisconsinensis* (Batra 1960) and *C. pseudobifrons* (Groves & Bowerman 1955).

4.1.2.7 Effect of Other Factors on Subsequent Germination

Other factors have been shown to influence the germination of sclerotia. Exudates from roots were shown to stimulate germination in several species, but this may be more important in myceliogenic germination for infection of host roots. LeTourneau (1979) states that the sclerotia of *Sclerotinia* spp. do not require an external source of nutrients in order to germinate.

Coley-Smith (1960) showed that gentle abrasion of the rind broke dormancy of *S. cepivorum* and Harvey *et al* (1995) that germination of *S. sclerotiorum* was greater in exposed, rather than sheltered, locations.

Known inhibitors that suppress or reduce sclerotial germination or apothecial production include ammonia (Huang & Janzen 1991), high concentrations of Na^{+}, Ca^{++}, Cl^{-}, SO_4^{2-} (Singh *et al* 1996), allyl alcohol (Huang *et al* 1997), some herbicides (Teo *et al* 1992; Singh & Kapoor 1996) and mycoparasites (Thaning 2000; Huang & Erickson 2000). In addition, some herbicides cause abnormal apothecial development (Huang & Blackshaw 1995).

4.1.3 Apothecia

The Family Sclerotiniaceae, containing both the genera *Sclerotinia* and *Ciborinia*, belongs to the Order Helotiales, in the Class Discomycetes of the Subdivision Ascomycota. Whetzel (1945) created the family to accommodate fungi that produced 'true' sclerotia on which apothecia developed and the inoperculate asci lining the apothecium contained ellipsoid ascospores.

Under suitable conditions, the tip of the stipe develops a small pore, with the inner portion going on to form the hymenium and related structures and the outside forming the underside of the mature apothecial disc. Mature ascospores are ejected from the upper side of the disc. The maximum lifespan recorded for apothecia of *S. sclerotiorum* was up to 33 d (Kopmans 1993; Twengstrom *et al* 1998).

4.1.4 Sclerotia and Apothecia of *C. camelliae*

Kohn & Nagasawa (1984) describe the sclerotia as being up to 12 x 10 x 2 mm in size, determinate, discoid and developing on senescent petals of *C. japonica* flowers. Remnants of the petal tissue remain embedded in the cortex and medulla, the distinguishing feature of the Genus *Ciborinia* (Whetzel 1945).

From infection, formation of sclerotia takes 2-3 wk, depending on environmental conditions. Most sclerotia form in the petals of fallen flowers, although sclerotia may be found in flowers that have been caught in branches or not properly abscised

from the stalk. Nunomura (1980) reported that sclerotia formed in sepals, but neither I, nor Kohn & Nagasawa (1984) have observed this.

Four to eight apothecia per compound sclerotium (i. e. several fused together) is common (Johnson 1971) although one with 45 apothecia was recorded (Haasis & Winstead 1954). Sclerotia may form in a single petal, or an aggregate sclerotium may form from the fusion of many petals, and including more than one fungal genotype. The length of the stipe is variable, depending upon the depth at which the sclerotium is buried in the soil (Kohn & Nagasawa 1984). The disc is 5-18 mm in diameter, capulate at first, but becoming flattened and then plano-convex as the disc matures (Kohn & Nagasawa 1984). Yokohama (1980) showed that the appearance of apothecia in spring is linked to low air and soil temperatures coupled with high RH and soil moisture. Bright & Long (2000) found that a single apothecium released between 3.7×10^6 to 11.9×10^6 ascospores over a lifetime that lasted around 17 d (in laboratory conditions), with most released in the first 8-10 d.

Microconidia are produced on the senescent flowers and in culture (Kohn & Nagasawa 1984). Microconidia are produced abundantly in culture (for most isolates) and where wet or damp conditions predominate in the field. It is not known whether microconidia are required to fertilise the sclerotium prior to germination. Macroconidia have not been observed (Kohn & Nagasawa 1984) and have not been reported for any *Ciborinia* species. (Kohn 1979).

4.1.5 Previous Sclerotial Germination Trials

Attempts to germinate both natural and cultured sclerotia of *C. camelliae* have been made (Taylor 1999). The methods of Hawthorne (1973) (*S. minor*), Sansford & Coley-Smith (1992) (*S. sclerotiorum*), Batra & Korf (1959) (*C. erythronii*), Groves & Bowerman (1955) (*C. pseudobifrons*) and Faretra & Antonacci (1987) (*B. fuckeliana*) were all unsuccessful. A range of novel treatments that simulated winter chilling and spring conditions, leaching and camellia root exudates were also unsuccessful (Taylor 1999). One treatment, where natural sclerotia were held at 10°C in constant darkness produced sclerotia with stipes (14/40 sclerotia). None of these stipes was visible above the surface of the perlite medium. Considering how

profusely the sclerotia appear to germinate under field conditions, this lack of success was surprising.

4.1.6 Objectives

The objectives of this study were to:

a) investigate factors influencing sclerotial germination and apothecial development of *C. camelliae*.
b) develop a reliable technique for producing apothecia under laboratory conditions.

4.2 MATERIALS & METHODS

All sclerotia were formed naturally in infected flowers and cultured sclerotia were not used. They were collected from the plant debris under camellia plants. The age of each sclerotium was not known, but presumably the large number collected for each experiment represented a range of ages. Sclerotia were collected for each experiment in a single day, stored outside the Agricultural Engineering Building, Massey University overnight, and processed (washed, weighed, sorted etc.) the next day. The garden site outside the Agricultural Engineering Building consisted of two large camellias, hedging and annuals, with several m^2 of accumulated plant debris where bags of sclerotia could be placed on the ground and covered lightly with debris, thus replicating 'normal' conditions for sclerotia in plant debris. Apothecia formed on sclerotia in plant debris that were present from the two camellia plants, indicating that temperature, moisture, shade conditions etc. were suitable for sclerotia.

Most experiments examined multiple factors, and the factors were repeated in more than one experiment (Table 4.1). Washing or leaching of sclerotia to imitate several seasons of rainfall in natural conditions was trialled through submerging sclerotia in RO water in the laboratory or in a stream in case there were any germination stimulants in run-off water that would not be present in RO water. Cold-conditioning of sclerotia, followed by incubation at more moderate temperatures attempted to imitate a winter chilling period followed by an artificial 'spring'. The chemical, medium and light treatments attempted to compare germination of *C. camelliae* sclerotia with other species as discussed in Section 4.1.2.

Table 4.1 Summary of Factors Investigated in Sclerotial Germination Experiments

Experiment No.	Factors Investigated (+/-)						
	Conditioning Treatments			Incubation Treatments			Other
	Washing	Temperature	Chemical	Temperature	Medium	Light[a]	
4.1	+	+	-	+	-	+	water quality
4.2	-	-	+	+	-	+	-
4.3	+	-	+	-	-	-	wounding
4.4	-	-	-	+	-	+	size
4.5	-	-	-	-	+	-	site

[a] the light source consisted of two Phillips TLD 15W/33 fluorescent tubes.

Experiments were monitored for stipe formation and watered regularly (with RO water unless otherwise indicated) to keep the medium moist. Priority of analysis was given to the number of stipes produced, rather than number of germinated sclerotia, as in Experiments 4.2 and 4.5 it was not possible to determine the number of sclerotia that germinated, only the number of stipes or apothecia (Experiment 4.5) as the sclerotia were submerged in the medium.

Data were analysed using SAS for Windows Version 6.12. Examples of SAS code are given in Appendix II. The data from each experiment met assumptions of normality of distribution and constant variance of residuals unless indicated otherwise. The symbol '*' indicates an interaction between factors, as in Temperature*Concentration.

4.2.1 Experiment 4.1

Aims:

a) to determine whether a conditioning wash is required for sclerotial germination.
b) to determine whether incubation temperature and light conditions influence sclerotial germination.

Sclerotia (WBG) from two seasons, either ~18 or ~6 months old, were prepared for treatment. At each age, sclerotia were sorted into three treatments of 120 sclerotia each and each treatment contained a range of different sized sclerotia. In the first treatment, sclerotia were sewn into 120 mm^2 mesh bags and nailed to a submerged log in the Turitea Stream, Palmerston North. In the second treatment, sclerotia were placed in a 2 L conical flask filled with 1600 ml of RO water, aerated by an

aquarium pump (Elite 802, air output 2500 mm^3/min) and the water was changed twice daily. For the control treatment, unwashed sclerotia were placed in a humid chamber at room temperature.

After 7 d of wash conditioning, the sclerotia were transferred to 200 x 90 x 250 mm containers containing ~1 cm of compacted wet sphagnum moss (Eden Agencies Ltd., Auckland) and covered with more loose wet sphagnum. These were covered with aluminium foil and incubated for 4 wk at 5°C in darkness. At the completion of this incubation period, all 18 month old sclerotia were discarded due to microbial infections and rotting. Of the 6 month old sclerotia, 2-5 sclerotia were removed from each treatment due to similar infections.

Three 200 x 90 x 250 mm containers containing ~1 cm of wet compacted sphagnum were divided into three sections with plastic dividers. After temperature conditioning, each treatment of 120 sclerotia was subdivided into three batches of ~40 sclerotia and each batch placed in a section of each of the containers, so that each container contained sclerotia from each of the three treatments. Containers were incubated at 10°C and 15°C (both in darkness) and 10°C/15°C 12/12 L/D.

ANOVA could not be used to analyse the data because the error variance for checking size effects could not be estimated since no degrees of freedom remained (meaning variation could be sampling variation). Chi square was used instead.

4.2.2 Experiment 4.2

Aims:

a) to evaluate the method of Anzalone (1959) for germination of sclerotia of *C. camelliae* using gibberellic acid (GA).
b) to determine the necessity of light for germination.

Anzalone (1959) soaked three month old sclerotia in three concentrations of 8% GA (1, 10 and 100 $\mu l\ m^{-1}$) for 1, 2, 4 or 8 h before incubation. No other details were given.

Seven month old sclerotia (WBG) that had been stored outside were rinsed in RO water to remove petal tissue and debris. Pots containing five dilutions of GA (Gibco BHL - mainly GA_3) and one water control were prepared. Sclerotia were placed in the appropriate dilution for 1 h or 8 h, then placed on ~20 mm wet peat moss (Hauraki Gold) in 200 ml squat plastic pots (No.6074, LilyPak Industries Ltd., Auckland, New Zealand) (Table 4.2). Pots were covered with the lid of a 90 mm diameter plastic petri dish that had had a small hole burnt into the centre for ventilation. Pots were placed in larger (500 x 140 x 300 mm) containers that were covered with either aluminium foil or gladwrap, and incubated in either 24 h darkness or 12/12 L/D at the temperatures shown in Table 4.2.

Table 4.2 Treatment Factors for Sclerotia in Experiment 4.2

GA Concentration ($\mu l\ ml^{-1}$ 100% a.i.)	No. Sclerotia	Soak Duration (h)	Light/Dark	Incubation Temperature (°C)
RO water control	10	8	24 darkness	15
	10	8	12/12 L/D	15
1 $\mu l\ ml^{-1}$	10	1	24 darkness	15
	10	1	12/12 L/D	15
	10	8	24 darkness	15
	10	8	12/12 L/D	15
10 $\mu l\ ml^{-1}$	10	1	24 darkness	15
	10	1	12/12 L/D	15
	10	8	24 darkness	15
	10	8	12/12 L/D	15
40 $\mu l\ ml^{-1}$	10	1	24 darkness	15
	10	1	12/12 L/D	15
	10	8	24 darkness	15
	10	8	12/12 L/D	15
70 $\mu l\ ml^{-1}$	10	1	24 darkness	15
	10	1	12/12 L/D	15
	10	8	24 darkness	15
	10	8	12/12 L/D	15
100 $\mu l\ ml^{-1}$	10	1	24 darkness	15
	10	1	12/12 L/D	15
	10	8	24 darkness	15
	10	8	12/12 L/D	15
	10	8	24 darkness	5
	10	8	24 darkness	10
	10	8	24 darkness	20

A three-factor ANOVA was performed on the log transformed data. The experiment was analysed in two parts: the first analysis was performed on all treatments incubated at 15°C which comprised the main part of Experiment 4.2 and the second on all treatments including those incubated at 5°C, 10°C and 20°C. Since there was little information on the effect of incubation temperatures, this experiment was not a balanced design as a wide range of treatments were trialled for an indication of the

most promising conditions for further experiments. The Temperature*Concentration, Temperature*Soak Duration and Temperature*Light/Dark interactions were removed from the analysis in order to generate LS means.

4.2.3 Experiment 4.3

Aims:

a) to evaluate the method of Anzalone (1959) for germination of sclerotia of *C. camelliae* using GA.

b) to test a variation of this method using washing and wounding treatments.

Eight month old sclerotia (MS) were rinsed to remove debris. For each treatment (Table 4.3), 50 sclerotia were held for 7 d in either a humid chamber at room temperature or in water with constant aeration (1L RO water in 1L conical flask, aeration by aquarium Elite 802, air output 2500 cc/min, water changed twice daily). Prior to soaking in 20 $\mu l\ g^{-1}$ GA for 8 h, the rinds of sclerotia in Treatments 1 and 3 were wounded with a scalpel (Figure 4.1).

Table 4.3 Treatment Factors for Sclerotia in Experiment 4.3

Treatments	7 d in:	Wounding (+/-)	20 $\mu l\ ml^{-1}$ GA (+/-)
1. Wound + GA	humid chamber	+	+
2. GA only	constant aeration	-	+
3. Wound + GA	constant aeration	+	+
4. Control – no water	humid chamber	-	-
5. Control – water only	humid chamber	-	RO water only

Figure 4.1
Three wound-sites in a large sclerotium for Experiment 4.3. Smaller sclerotia were wounded once or twice only.

Sclerotia were placed on wet peat moss in covered pots as described in Experiment 4.2. Pots were placed in larger containers (420 x 280 x 100 mm) that were covered in aluminium foil and incubated at 15°C in 24 h darkness.

ANOVA was not used because the error variance for checking size of effects could not be estimated since no degrees of freedom remained (meaning any variation could be sampling variation). The Poisson was used instead, as variance=mean.

4.2.4 Experiment 4.4

Aims:

a) to test germination of sclerotia left on the ground to condition naturally.
b) to test % germination of sclerotia from the previous season.
c) to test if % germination and number of stipes was dependent on sclerotial size.
d) to test if temperature affected stipe and/or apothecial formation.

As soon as the first stipes were observed in naturally conditioned sclerotia (MS), non-germinated sclerotia were washed to remove plant and soil debris and sorted by size (large, medium and small). The average weight of sclerotia in each size category was calculated by weighing 50 sclerotia. The average weight of large sclerotia was 1.31 g, medium sclerotia 0.24 g and small sclerotia 0.05 g.

One litre plastic food trays (BS-1000B, Bonson Industrial Company Ltd., Auckland, New Zealand) with wet sphagnum moss were prepared for each of the three sclerotial categories in five treatments (total 15). Twenty grams of large sclerotia (~15 sclerotia), 30 g of medium sclerotia (~125 sclerotia) and 6 g of small sclerotia (~127 sclerotia) were placed in separate food trays. Each tray was wrapped in aluminium foil to ensure complete darkness and incubated at 5°C, 10°C, 15°C, 20°C or 25°C.

A non-parametric two-factor ANOVA was performed on the ranked data (Conover & Iman 1981). A non-parametric test was used in order to accommodate the large number of zero values.

4.2.5 Experiment 4.5

Aims:

a) to determine the effect of medium on sclerotial germination.

The media were: four regional soil types (Tokomaru silt loam, Eltham organic, Hawera volcanic and Foxton dark-grey sand), three camellia-growing soils (WBG, A, M), two commercial media (peat and sphagnum moss) and one inorganic medium (perlite) (Table 4.4).

Table 4.4 Source and pH of Media for Sclerotia in Experiment 4.5

Medium	Collection Site	pH
Tokomaru silt loam	Sheep Farm Rd, Massey University	4.2
Eltham organic	Mangawhero Rd	4.5
Hawera volcanic	State Highway 1, rail overbridge, Hawera	6.9
Foxton dark-grey sand	Himatangi	5.1
Wellington Botanic Gardens	WBG	5.2
Massey Arboretum	A	4.7
Agricultural Engineering Building, Massey University	M	5.3
Peat moss	Hauraki Gold	-
Sphagnum	Eden Agencies Ltd, Auckland	-
Perlite	-	-

A terracotta plant pot (13", Vaberie Trevigiane, Italy) was filled to within ~20 mm of the rim for each medium. Three hundred sclerotia (WBG) were gently rinsed in RO water and sorted into three size categories. The average weight of sclerotia in each size category was calculated by weighing 50 sclerotia. The average weight of the

large sclerotia was 0.96 g, of the medium sclerotia 0.40 g and 0.13 g for the small sclerotia. Thirty sclerotia (10 from each size category) were placed in each pot and covered by ~5 mm of medium. The pots were placed in the garden plot at the Agricultural Engineering building, at Massey University. The pots were recessed into the ground so that only the lip projected above the soil. The porous nature of terracotta allowed the temperature and moisture conditions of the surrounding soil to equilibrate with the sclerotia in the medium.

The pots were monitored for production of apothecia throughout the 2000 and 2001 blight seasons.

Originally, this experiment was replicated over four sites in Palmerston North, but various calamities befell three of the sites. Because of the large number of sclerotia involved, replication of media had been planned by site (pseudo-replication) rather than within site. Thus, the results could not be statistically analysed, as only data from one site was available, and variability in germination within a medium could not be determined. The difference in apothecial production between media at the remaining site was marked however, and these results are presented.

4.3 RESULTS

4.3.1 Experiment 4.1

Stipes were first observed after 35 d of incubation (Figure 4.2). A double layer of shade cloth was added in an attempt to prevent stipes drying out and dying before reaching maturity, but many stipes continued to die.

Figure 4.2
Three germinated sclerotia from Experiment 4.1. Five stipes were produced by the sclerotium on the left.

The total number of stipes produced during the 14 months of Experiment 4.1 are shown in Table 4.5.

Table 4.5 Sclerotial Germination by Treatment for Experiment 4.1

Incubation Treatments	No. Germinated Sclerotia (No. Stipes)/Total No. Sclerotia		
	Turitea Stream	RO Water	Control
10°C 24 h darkness	1(1)/38	1(2)/35	9(16)/35
15°C 24 h darkness	5(11)/38	2(3)/37	5(8)/38
10°C/15°C 12/12 L/D	0/38	0/38	5(7)/38

There was a significant difference between wash treatments (F_2, $\chi^2$26.75, p=0.0001) with the largest number of germinated sclerotia and stipes in the unwashed control treatment. There was also a significant difference between the incubation treatments (F_2, $\chi^2$10.17, p=0.0062) with few stipes in the 10°C/15°C 12/12 L/D treatment compared to those incubated at either 10°C or 15°C in 24 h darkness. There was a significant interaction between the incubation and wash treatments (F_4, $\chi^2$21.24, p=0.0003) as a result of most stipes occurring in the unwashed control treatment at 10°C and in the Turitea-washed treatment at 15°C.

Overall, very few sclerotia germinated in this experiment (28/335 – 8%). Over the three wash treatments, the unwashed control treatments produced the greatest number of stipes (31) while over the three incubation treatments, incubation at 10°C in 24 h darkness produced 19 stipes while 15°C in 24 h darkness produced 22 stipes.

4.3.2 Experiment 4.2

Sclerotia germinated in the water control and GA-soaked treatments (Table 4.6).

Table 4.6 Number of Stipes produced in Experiment 4.2

GA Concentration	Treatment			
	1 h Soak		8 h Soak	
	12/12 L/D	24 h darkness	12/12 L/D	24 h darkness
0 (control)	-	-	12	2
1 µl ml^{-1}	6	2	13	2
10 µl ml^{-1}	1	4	6	4
40 µl ml^{-1}	8	5	8	4
70 µl ml^{-1}	7	7	12	2
100 µl ml^{-1}	12	7	14	3

There was evidence for significant differences in the number of stipes produced in the two light incubation conditions ($F_{1,4}$=41.92, p=0.0029) with greater numbers produced in 12/12 L/D than in 24 h darkness (Figure 4.3). There was no evidence that GA concentration ($F_{5,4}$=3.89, p=0.1064) or length of soak ($F_{1,4}$=0.41, p=0.5567) influenced the number of stipes produced (ANOVA table Appendix IV) and there was no interaction between GA concentration and length of soak ($F_{4,4}$=2.51, p=0.1970).

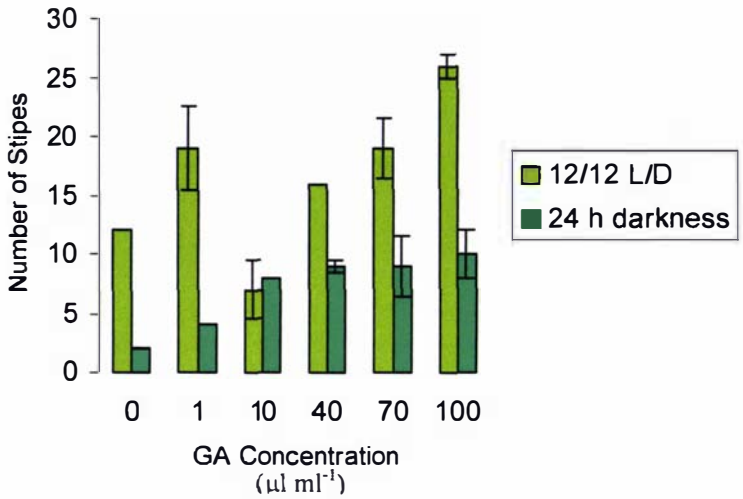


Figure 4.3
Number of stipes produced by sclerotia soaked in GA for 1 and 8 h (averaged) for Experiment 4.2 (RO water control data for 8 h only). Significantly more stipes were produced on sclerotia incubated in 12/12 L/D than in 24 h darkness, except at 10 µl ml^{-1} GA concentration where there was no difference between the incubation treatments (raw data).

There was some evidence for a significant interaction between GA concentration and light incubation conditions ($F_{5,4}$=5.28, p=0.0686). With the exception of the 10 µl ml^{-1} GA concentration (which caused the significant interaction), the greatest number of stipes for each GA concentration and the control, were produced in the 12/12 L/D incubation conditions, with fewer stipes produced in 24 h darkness (Figure 4.4).

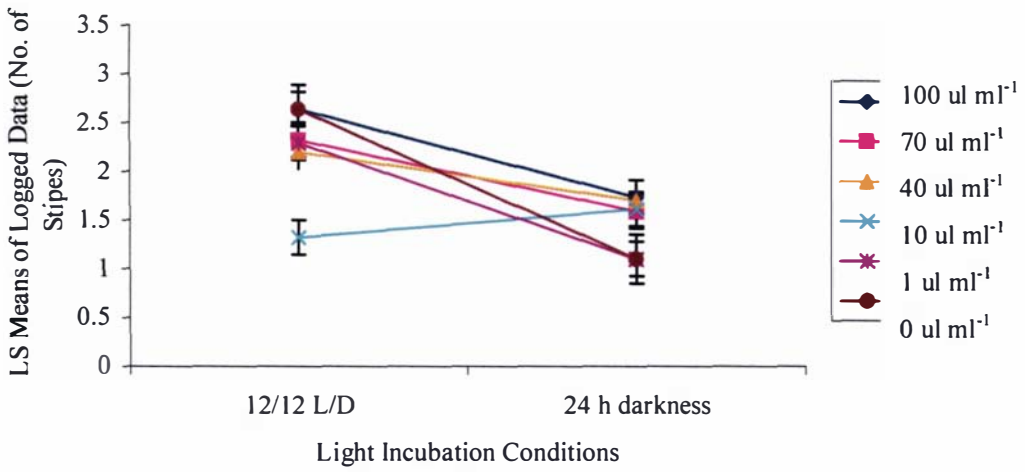


Figure 4.4
Interaction of GA concentration and light incubation conditions on stipe production for Experiment 4.2 (calculated from LS Means). Corresponding data points have been joined to show the interaction effect although biologically incorrect (Duncan Hedderley, pers. comm.). LS Means and SE for 0 µl ml^{-1} (water control) are calculated from one data point, all others from two data points.

There was evidence for a significant interaction between length of soak in GA and light incubation conditions ($F_{1,4}$=15.61, p=0.0168) (Figure 4.5). For sclerotia soaked in GA for 8 h, the greatest number of stipes (55) were produced by sclerotia incubated in 12/12 L/D. Fewer stipes (17) were produced by sclerotia incubated in 24 h darkness. For sclerotia soaked in GA for 1 h prior to incubation, the difference in stipe production was much less between the two light incubation regimes (34 and 25).

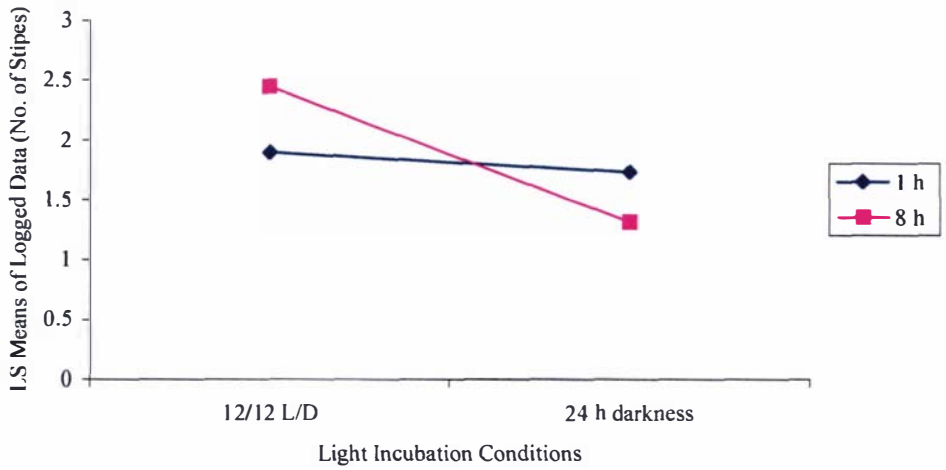


Figure 4.5
Interaction of length of soak period with light incubation conditions on stipe production for Experiment 4.2 (calculated from LS Means). Corresponding data points have been joined to show the interaction effect although biologically incorrect (Duncan Hedderley, pers. comm.). n=5 (1 h) and n=6 (8 h).

There was also evidence for a significant difference between incubation temperatures ($F_{3,\ 4}$=33.36, p=0.0027). Sclerotia incubated at 5°C and 20°C failed to germinate, while the treatment at 10°C produced 4 stipes and the treatment at 15°C produced 3 stipes.

None of the stipes held in laboratory conditions developed into mature apothecia. Those sclerotia with stipes that were placed outside in natural light did form mature apothecia.

4.3.3 Experiment 4.3

There was no evidence that sclerotial wounding (F_1, p=0.9661), wash treatments (F_1, 0.4962) or GA (F_1, p=0.6520) affected stipe production and there was no interaction between sclerotial wounding and wash treatments (F_1, p=0.2448) (Table 4.7). The sclerotial germination rate was 35%.

Table 4.7 Number of Stipes Produced by Sclerotia in Experiment 4.3

Treatments	Treatment Factors			No. Germinated Sclerotia(No. Stipes)/Total No. Sclerotia
	7 d in:	Wounding (+/-)	20 µl ml^{-1} GA (+/-)	
1. Wound + GA	humid chamber	+	+	15(15)/50
2. GA only	constant aeration	-	+	18(18)/50
3. Wound + GA	constant aeration	+	+	21(22)/50
4. Control – no water	humid chamber	-	-	17(21)/50
5. Control – water only	humid chamber	-	RO water only	16(16)/50

4.3.4 Experiment 4.4

Sclerotia in Treatments 1 (5°C), 2 (10°C) and 3 (15°C) produced stipes over a three month period (Table 4.8). Sclerotia did not germinate in Treatments 4 (20°C) or 5 (25°C).

Table 4.8 Number of Stipes Produced by Sclerotia in Experiment 4.4

Treatment	No. Germinated Sclerotia (No. Stipes)/Total No. Sclerotia		
	Large Sclerotia	Medium Sclerotia	Small Sclerotia
1 (5°C, 24 h darkness)	14(24)/15	47(65)/125	20(22)/125
2 (10°C, 24 h darkness)	3(5)/15	18(19)/125	0/125
3 (15°C, 24 h darkness)	5(10)/15	7(9)/125	0/125

There was evidence for a significant difference between incubation temperatures ($F_{4,8}$=6.06, p=0.0152) (ANOVA table Appendix IV). The greatest number of stipes was produced by sclerotia (all sizes) incubated at 5°C (Figure 4.6). Fewer stipes were produced at 10°C and at 15°C and none at 20°C and 25°C.

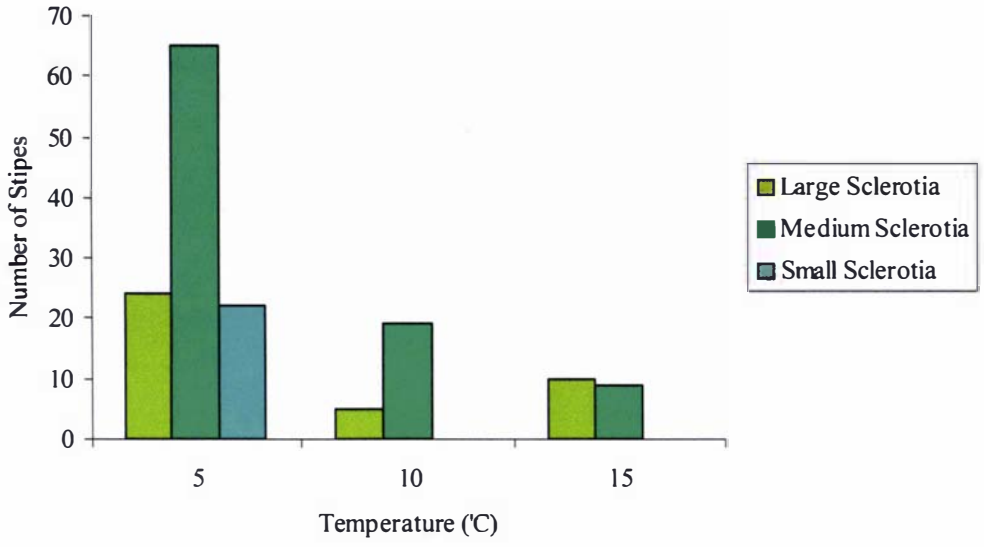


Figure 4.6
Stipe production by sclerotial size at three incubation temperatures in Experiment 4.4. Incubation of sclerotia at 5°C in 24 darkness was the most successful treatment. In all three sclerotial size categories, more stipes were produced at this temperature than at 10°C and 15°C.

There was no evidence that sclerotial size affected germination ($F_{2,8}$=2.24, p=0.1693) but there was some evidence that large sclerotia produced more stipes than small sclerotia (p=0.0677).

In total, 8.53 % of the sclerotia germinated to form stipes (Table 4.9).

Table 4.9 Percent Sclerotial Germination by Incubation Temperature and Sclerotial Size in Experiment 4.4

Treatment Temperature	Sclerotial Size Category			% Germination by Temperature
	Large	Medium	Small	
5°C	93.3	37.6	15.7	30.3
10°C	20.0	14.4	0.0	7.9
15°C	33.3	5.6	0.0	4.5
% Germination by Sclerotial Size	29.3	11.5	3.1	-

4.3.5 Experiment 4.5

No apothecia were observed in the first blight season. In the second season, the greatest number of apothecia was observed in the WBG (32) medium, followed by sand-soil from Foxton (12) and sphagnum moss (12) (Figure 4.7). Apothecia were not produced by sclerotia on two media: Agricultural Engineering soil and Hawera volcanic soil. The pH of the medium did not appear to be a factor as the soil from the Agricultural Engineering site was pH 5.3, compared to pH 5.2 for the most successful medium, soil from the WBG.

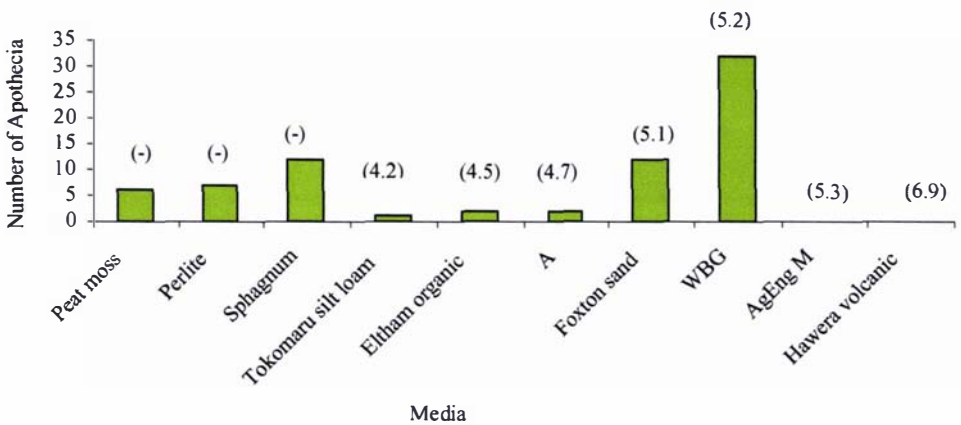


Figure 4.7
Apothecial production by media (ranked from low to high pH) at the Agricultural Engineering building site for Experiment 4.6.

4.3.7 General Observations on Sclerotia and Germination in *C. camelliae*

There is wide variation in the size and shape of sclerotia. In general, a large fleshy flower (e. g. *C. reticulata* or *C. japonica* 'Elegans Champagne') produces a large, multiple sclerotium. The entire petal base may be replaced by sclerotium, forming a ring, with sclerotial 'teeth' replacing each petal (Figure 4.8). Fleshy petals, which fuse together at the base of the flower and/or with many petals, more often form larger sclerotia. Smaller flowers, and those with thinner and/or fewer petals (e. g. *C. saluenensis* x *C. cuspidata* 'Cornish Snow' and *C. saluenensis* x *C. japonica* 'Fairy Wand') rarely form sclerotia. Even *C. cuspidata* seedling 'Spring Festival', which is a double rose form, rarely forms sclerotia, presumably because the petals are very thin. When a sclerotium is formed, it is always very small in comparison to those sclerotia formed in medium-large *C. japonica* and *C. reticulata.* From my

observations, I believe the difference is largely due to the amount of water (and available nutrients) contained in the petals and the quicker rate at which thin petals dehydrate. For sclerotia to form in the thin-petalled varieties, contact with moist/wet soil or plant debris is required after the flower has fallen from the plant. Petal-shattering varieties (e. g. *C. saluenensis* x *C. japonica* 'Water Lily') also frequently fail to form sclerotia, presumably because scattered single petals dehydrate rapidly. If a flower of 'Water Lily' remained intact on the ground, however, a single small 'pip' sclerotium formed towards the centre of each petal, rather than one or two larger sclerotia at the base. I have rarely seen this phenomenon in other flowers and assume it is a cultivar difference.

Figure 4.8
Sclerotium with fimbriated edges. This sculpting was occasionally seen on sclerotia formed by *C. japonica* 'Elegans Champagne', a cultivar with fimbriated petals.

Whatever the flower type, a prolonged period of dry weather reduces the size of the sclerotium, or prevents its development altogether, while wet weather appears to encourage development. While working in Los Angeles, I observed that even the large fleshy flowers failed to form sclerotia of the size seen in the same cultivar in New Zealand. Their average yearly rainfall is 380 mm (www.losangelesalmanac.com), most of it falling between January-March, and artificial irrigation is necessary. The soil never appeared saturated to the extent that is frequently seen in the Manawatu and moisture-retaining plant debris or undergrowth was minimal. In comparison the two main centres where sclerotia were collected for these experiments, Palmerston North and Wellington, the average yearly rainfall is 968 mm and 1249 mm, respectively (www.metservice.co.nz).

From my observations of sclerotial germination and apothecial formation in Los Angeles, it would appear that sclerotia may germinate before a significant rain event,

but the stipes remain undifferentiated within the plant debris on the ground. Once the rain occurred, the stipes rapidly matured into apothecia. While searching for apothecia for experiments in Los Angeles, many germinated sclerotia with undifferentiated stipes were found in the plant debris under camellia bushes (Figure 4.9). Prior to rain, apothecia were few and sclerotia with stipes many; after rain, apothecia appeared within hours. This behaviour would be advantageous in drier areas, allowing ascospores to be produced rapidly after rain.

Figure 4.9
Stipe in plant debris. The debris has been shifted to reveal the stipe, whose tip was just below the surface of the debris.

In the 2000 New Zealand blight season, a large number of abnormal apothecia were noted in Wellington, Palmerston North and Wanganui (Figure 4.10). These apothecia developed convoluted discs, as described by Kosasih & Willetts (1975b) for *S. sclerotiorum*. The reason for this abnormal development is unknown.

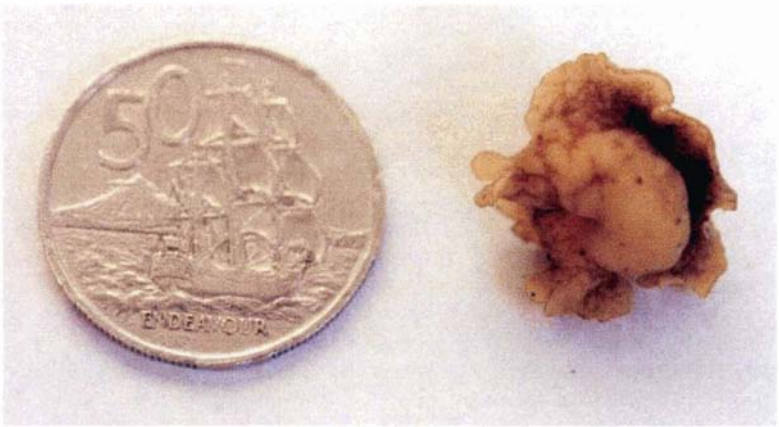


Figure 4.10
Example of abnormally-formed disc of a mature apothecium. This apothecium was collected at WBG.

4.4 DISCUSSION

This study did not investigate the percent germination of sclerotia under natural conditions, therefore it is not known how the percent germination rate reported in these experiments compares with natural germination rates. Overall, the germination rates in these experiments appears to be low. Although many apothecia may be found in plant debris under camellia plants that have had camellia flower blight for a number of years, the actual number of sclerotia that germinate is not known. A sclerotium may produce more than one apothecium at a time, many apothecia over a season, and germinate in more than one season, and since it is usually slightly buried in the plant debris (if not the soil), determining whether apothecia are produced by one sclerotium or several is impossible without destroying the natural conditions and terminating the experiment. At the beginning of this study, no suitable site for investigating the percentage germination of sclerotia in natural conditions was available, but this may now be possible at several sites around Massey, particularly the garden plot at the end of the Agricultural Engineering building where sclerotia have been brought in and spread under the camellias. There is still the difficulty of determining how many apothecia a sclerotium produces, and over what time period, without disturbing the sclerotium. From my observations, I suspect that quite a large number of the sclerotia present at a site germinate during a season.

The sclerotial germination experiments reported here consist of those in which sclerotia were successfully triggered to germinate out of season and/or the maturation of apothecia. A range of other experiments (not reported) investigated the same

factors as those reported, in addition to others that were designed to determine whether sclerotia formed early in the season germinated earlier than those formed late in the season and whether handling of sclerotia influenced subsequent germination. The latter experiment was instigated because of the poor (or no) germination gained in previous experiments. Sclerotia in the field germinate profusely during the blight season and it was expected that sclerotia in the laboratory would be similarly productive. Preliminary results from handled vs. non-handled sclerotia indicates that collecting, washing, removing debris, size sorting etc. does inhibit sclerotial germination for at least one season, and thus, future experiments should either use sclerotia which have been handled as little as possible, or wait for the second season after the experiment is set up, before results are gathered. The application of GA or incubation at low temperatures does, however, appear to overcome part of this problem.

Of the successful sclerotial germination experiments reported here, several factors were shown to be important for germination.

4.4.1 Effect of Washing on Germination

A conditioning wash of the sclerotia prior to incubation was not required for germination of sclerotia of *C. camelliae* as shown by Experiments 4.1 and 4.3. Washing was not required for germination of *C. wisconsinensis* (Batra 1960) or *C. pseudobifrons* (Groves & Bowerman 1955), but was essential for *C. erythronii* (Batra & Korf 1959).

4.4.2 Effect of Media on Germination

The three commercial media (perlite, sphagnum and peat moss) that were used in these and other experiments were suitable media for sclerotial germination. Until Experiment 4.5 investigated different media, these three had been used for all germination experiments and it was necessary to determine whether the choice of media was inhibiting germination, or other factors.

The most successful medium (32 apothecia) was soil collected at WBG (pH 5.2), and the least successful (no apothecia) soil from Agricultural Engineering (pH 5.3) and Hawera volcanic soil (pH 6.9) with only one apothecium each from Arboretum soil (pH 4.7) and Tokamaru silt loam (pH 4.2). Since the pH of the most and least

successful media were similar (pH 5.2 and 5.3) it is more likely that soil constituents, rather than pH, affected germination. Further replication is required to determine how much of the results are due to natural variation, but the three commercial media are suitable for future experiments.

4.4.3 Effect of Temperature on Germination

None of the sclerotia incubated at 20°C or higher produced stipes. Sclerotia incubated at lower temperatures (between 5°C - 15°C) germinated to produce stipes, the most successful temperatures being 10°C and 15°C in Experiments 4.1 and 4.2, and 5°C and 10°C in Experiment 4.4. Where sclerotia had been naturally stimulated to germinate (Experiment 4.4) the greatest number of stipes was produced at 5°C, and fewer at 10°C and 15°C. Where sclerotia were artificially stimulated to germinate using GA (Experiment 4.2) the greatest number of stipes was produced at 10°C and 15°C and none at 5°C. In Experiment 4.1, the least successful incubation treatment was 10°C/15 °C L/D, indicating that either temperature cycling and/or photoperiod inhibited germination; further replication is required to determine this point.

Experiments in which sclerotia were cold-conditioned at 4°C for 4-8 wk before incubation at higher temperatures (not reported) showed that cold-conditioning was not required for germination, and in fact inhibited germination.

Overall, these results indicate that low temperatures are important for sclerotial germination and that an incubation temperature of ~10°C would be optimum for further germination experiments. More research is required to determine whether the temperature difference between naturally and artificially stimulated sclerotia is consistent.

4.4.4 Effect of Light on Germination

There were conflicting results between experiments on the effect of 24 h darkness and 12/12 L/D on sclerotial germination. In Experiment 4.1, sclerotia incubated in 24 h darkness produced more stipes than those incubated in 12/12 L/D (though this was confounded by the 10°C/15°C thermocycle period). In Experiment 4.2, sclerotia incubated at 12/12 L/D produced more stipes than those in 24 h darkness, so either the thermocycling inhibited germination in Experiment 4.1 or there is an interaction

effect of GA with light cycling. Further experiments are required to determine which light conditions are most effective under different germination regimes and also to determine if light intensity or wavelength is important.

4.4.5 Effect of Chemicals on Germination

The method of Anzalone (1959) using the plant growth hormone GA did not induce stipe production in the two experiments reported here. The method was followed as closely as possible, but as it was not fully explained, there could be some variation in reproduction of his experiments or in the formulation of GA. Additional variables include the different environmental conditions under which the sclerotia formed in New Zealand, the different camellia cultivars grown here, and genetic variability of the isolates of *C. camelliae* in New Zealand. Although statistical analysis did not show that GA induced sclerotial germination any more than the water controls, the experiments were unusually successful (compared to the unreported failed experiments) in producing stipes, and an experiment with the control sclerotia incubated in a separate incubator, along with controls incubated with GA-treated sclerotia, would eliminate the possibility that volatile compounds were produced permeated into the control sclerotia.

Anzalone (1959) observed germination of sclerotia after ~one month of incubation and germination continued for a further five months. He used three month old sclerotia, GA acid (8% a.i.) at three concentrations (1, 10 and 100 $\mu l\ ml^{-1}$) and four soak durations (1, 2, 4 and 8 h). He may have used a commercial preparation of GA that had contaminants/additional chemicals that influenced germination. He specified that the peat moss must be kept "saturated". The GA used in my experiments was almost 100% a.i., so the 10 and 100 $\mu l\ ml^{-1}$ of his experiment should be equivalent to the 1 and 10 $\mu l\ ml^{-1}$ treatments here. Higher concentrations of GA increased germination of sclerotia, but the duration of the soak in GA was found to have little effect (Anzalone 1959).

The method of Radke (1986), who used various herbicides to stimulate germination of sclerotia of *S. sclerotiorum*, was trialled, with the herbicides Treflan® and Sencor® failing to stimulate *C. camelliae* sclerotia to germinate.

4.4.6 Maturation of Apothecia

The greatest problem was finding conditions that prevented stipes from dying. While mature apothecia were produced in both laboratory and the garden site, the apothecia of those produced at the garden site were larger (diameter >19 mm) than those produced in the laboratory (diameter <19 mm).

Sclerotia that produced stipes in any laboratory treatment were transferred to different containers and incubated in 12/12 L/D as light was shown to be an important factor in apothecial formation of *S. trifoliorum* (Ikegami 1959). Stipes did not progress to produce mature apothecia, but eventually withered and died, although two apothecia were found on stipes in an abandoned experiment (not reported here), indicating that the white light produced by the fluorescent tubes was adequate. I have also produced mature apothecia of *Botryotinia fuckeliana* under the same lights. Some sclerotia bearing stipes were left in both 12/12 L/D and 24 h darkness treatments in case handling the sclerotia during transfer was affecting apothecial maturation but these stipes also withered and died, possibly because of low RH. A number of sclerotia with stipes that were transferred to containers outside went on to produce mature apothecia, indicating that quality of light may be an important factor in maturation of the apothecial disc. Maturation of the stipes of *S. sclerotiorum* is also most easily achieved using natural light (Stephen Hoyte, pers. comm). An experiment to determine which wavelengths of light were required for maturation was considered, but was not done due to lack of equipment.

4.4.8 Conclusion

Sclerotial germination of *C. camelliae* out of season was most successfully achieved at low temperatures in 24 h darkness unless GA was used, in which case germination was greater in 12/12 L/D. Commercial media appear to be suitable materials on which to incubate sclerotia. A wash treatment was not required for germination.

Sustaining germinated sclerotia was more difficult and requires further research with artificial light sources. Currently, the only conditions in which the stipe developed into a mature apothecium were achieved in the garden plot in natural light. This would be unsuitable in winter months as the temperatures would fall too low.

CHAPTER FIVE – MOLECULAR STUDIES

5.1 INTRODUCTION

Little is known about the genus *Ciborinia* and few of its species have been studied at the molecular level. Such studies can reveal a range of information for use in a variety of ways. In this chapter, three aspects of the molecular biology of *C. camelliae* were investigated: a) genetic variation between isolates of *C. camelliae*, b) phylogenetic comparison with other closely related species and c) detection and identification of the pathogen *in planta.*

The degree of genetic variation within New Zealand isolates of *C. camelliae* was studied using Universally Primed PCR (UP-PCR) (van Toor 2002), which produce more complex banding patterns and with greater reproducibility than Randomly Amplified Polymorphic DNA (RAPD) techniques (Lübeck *et al* 1998; Tyson *et al* 2002). Van Toor found that the New Zealand isolates were distinctly different from the USA isolates with the majority of the genetic variation (87%) associated with the difference between the two countries, and only 9% associated with variation within the countries. There was also less genetic variability within isolates collected in New Zealand (9%) compared to those from the USA (17%). The lower variation seen in the New Zealand isolates may be explained because the initial introduction of *C. camelliae* to New Zealand was more likely to have been from a single source, while the USA made multiple imports of camellias from Japan before the pathogen was recognised. In addition, these differences could be accounted for by differential selection pressures in each country, and the greater length of time for which *C. camelliae* has been present in the United States (van Toor 2002).

Three *Ciborinia* species were used as part of phylogenetic studies of the family Sclerotiniaceae (Holst-Jensen *et al* 1997a; 1997b) and in a comparison of rDNA sequences of *C. ciborium* and *C. erythronii* (Carbone & Kohn 1993). The latter authors concluded that the genus *Ciborinia* was a heterogeneous grouping of species "in need of monographic revision".

There is currently no quick and accurate method for detecting and identifying *C. camelliae* from diseased plant material. Disease identification is made by symptoms and signs exhibited by the flower and its colony characteristics when grown on PDA. Establishing a pure culture of the pathogen from diseased material can be difficult (Watson 1950; Winstead *et al* 1954; Taylor 1999) and colony growth is relatively slow, taking from 7-14 d to attain the felty mycelium and form sclerotia characteristic of the fungus in culture (Kohn & Nagasawa 1984). Similarly, the characteristic symptoms and signs of *C. camelliae* flower infection may be confused with infections caused by other pathogens, such as *Botrytis, Pestalotia, Monochaetia, Penicillium* and *Sclerotinia.* Correct identification is also hindered when flowers display atypical symptoms, as well as by petal shattering varieties, where the petals desiccate before the pathogen is well established. For researchers and quarantine staff, the quality and accuracy of research and control management options at export/import points is limited by the lengthy and sometimes unreliable isolation and identification methods.

5.1.1 Molecular Detection and Identification of Fungal Plant Pathogens using rDNA

Molecular identification using the ribosomal gene unit has been used to compare and relate organisms at many levels, from Kingdoms (Gray *et al* 1984), to genera (Berbee & Taylor 1992; Holst-Jensen *et al* 1997a), to species (Lee & Taylor 1992; Kageyama *et al* 1997) and even strains of species (Gardes *et al* 1991). It contains highly conserved regions that allow comparisons to be made between distantly related organisms and faster evolving regions, for comparisons between more closely related organisms.

Ribosomes are the site of translation of mRNA codons into the amino acid sequence of a polypeptide chain. They are found in large numbers in all organisms and the genes coding for the production of ribosomal RNA are found in high copy number in tandem repeats, each ribosomal gene unit separated from the next repeat unit by the

InterGenic Spacer (IGS) region. The ribosomal gene unit consists (in Eukaryotes) of the large subunit (28S) of about 4,800 bp, the 5.8S (~160 bp), and the small subunit (18S) of around 1900 bp (Figure 5.1). The sequences of these subunits are highly conserved. Between the 18S and 5.8S is Internal Transcribed Spacer (ITS) 1 and between the 5.8S and 28S is ITS2. ITS1 (~200 bp) is shorter than ITS2 (~400 bp) and tends to be more polymorphic (Cooke & Duncan 1997).

Figure 5.1
Schematic of the ribosomal RNA gene unit showing the location of the ITS regions in relation to the subunits and approximate location of the universal primers ITS1 and ITS4.

The ITS regions are transcribed, but they do not form part of the functional ribosome. This lack of a functional product (Nues *et al* 1994) is thought to explain the high sequence variation that is found among species or populations (White *et al* 1990), as the ITS regions are not under pressure to conserve their sequence. Some sequences within the ITS regions are more highly conserved than other sequences, suggesting that some areas are subject to stronger selective pressures than others (Michot *et al* 1993; Torres *et al* 1990). This may indicate that parts of the ITS region are conserved to enable correct processing of the primary ribosomal transcripts and subsequent correct folding (Goldman *et al* 1983; Kasuga & Michelson 1983).

5.1.1.1 Species-Specific Primers for Identification

The combination of conserved subunits and variable ITS regions permit species-specific identification to be made. Using universal primers that bind to the (known) conserved sequences of the subunits, the unknown variable sequence of the ITS regions can be copied and sequenced. Species-specific primers can then be designed to the unique sequence variation of that species. This strategy has been successfully

developed for a number of plant pathogens (Trout *et al* 1997; Zhang *et al* 1997; Bulman & Marshall 1998; Lindqvist *et al* 1998; Kim *et al* 1999; Le Cam *et al* 2001; Mazzaglia *et al* 2001; Freeman *et al* 2002).

5.1.1.2 Advantages of the ITS region for Molecular Identification

There are several features that make the ITS region a commonly used molecular marker. The entire region is small, only 600-800 bp, and universal primers allow the region to be readily amplified (White *et al* 1990). Multiple copies of the ribosomal gene repeat unit makes the ITS region easy to amplify even if the DNA sample is small, dilute or partially degraded. Several studies have shown that the region is often highly variable among morphologically distinct fungal species (Gardes *et al* 1991; Gardes & Bruns 1991; Lee & Taylor 1992) but variation within a species is low (Gardes *et al* 1991; Lee & Taylor 1992). One known exception is *Fusarium sambucinum* (O'Donnell 1992).

5.1.2 Molecular Identification of Fungal Plant Pathogens using β-tubulin

Beta-tubulin is part of the tubulin gene family that make up microtubules. These are major components of the cytoskeleton, mitotic spindles and flagella of eukaryotic cells. As a protein-encoding gene, β-tubulin is highly conserved due to its structural function and has been used to distinguish between fungi at all levels (Baldauf & Palmer 1993; Baldauf & Doolittle 1997; Edlind *et al* 1996; O'Donnell *et al* 1998; Thon & Royse 1999).

In an analysis of partial (-tubulin sequences of basidiomycetes, Thon & Royse (1999) showed there were two lineages of ascomycete β-tubulin genes, and that gene duplication occurred before the divergence of basidiomycetes. Two copies of the β-tubulin gene have also been reported in *Colletotrichum graminicola* (Panaccione & Hanau 1990) and *Aspergillus nidulans* (May et al 1987) and five in *Epichloë* spp. (Tsai *et al* 1994). Mutations within the *β*-tubulin gene can also confer resistance to fungicides in the benzimidazole group (Koenraadt *et al* 1992; Koenraadt & Jones 1993; Yarden & Katan 1993; Albertini *et al* 1999; Matsuo *et al* 1999).

Like the ribosomal gene unit, β-tubulin contains highly conserved protein-coding region. It also contains less conserved introns that do not form part of the processed mRNA. Working with *Neurospora crassa* and *Aspergillus nidulans*, Glass & Donaldson (1995) created two pairs of primers that spanned one or more introns in β-tubulin. The partial structure of the β-tubulin gene and the location of one of the primer pairs (Bt2a and Bt2b) are shown in Figure 5.2.

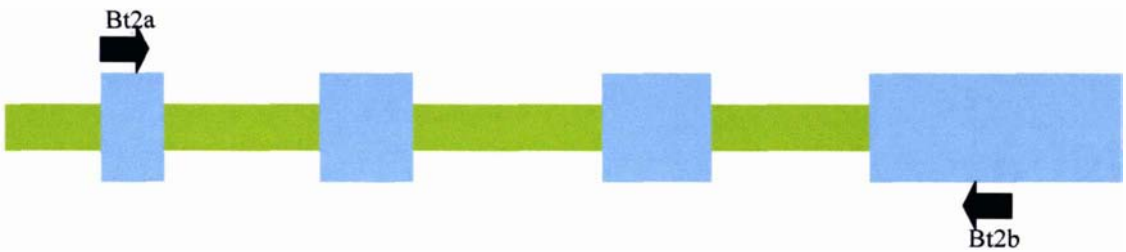


Figure 5.2
Schematic of the *N. crassa* partial β-tubulin gene and location of the primer pair (from Glass & Donaldson 1995). Blue boxes indicate protein-coding sequences and green boxes introns.

5.1.2.1 Species Identification using β-tubulin

The β-tubulin region has proved valuable for distinguishing between species where ribosomal genes (and/or other genes) have failed. Barnes *et al* (2001) used β-tubulin and histone genes to show that there were three species of *Seiridium* responsible for cypress canker, where sequences from the ITS regions could not distinguish between them. In the *Cylindrocladium candelabrum* spp. complex, Schoch *et al* (1999) found there was a low number of informative characters from ITS sequence data. In a later study, Schoch *et al* (2000) found that only β-tubulin sequences could distinguish between *Cylindrocladium scoparium* and *C. insulare*. Wyand & Brown (2003) and Crous *et al* (1999) also found the β-tubulin gene had greater divergence between species than ITS regions.

5.1.3 rDNA and β-tubulin in Phylogenetic Studies

The field of systematics includes phylogenetics, the study of the evolutionary development of organisms by tracing them to common ancestors and taxonomy, which names and classifies the diversity of organisms with ranks approximate to the

evolutionary distances between organisms. A range of properties have been used to classify organisms, such as morphology, physiology, biochemistry and increasingly, molecular data.

The ideal gene for phylogenetic studies would evolve between species, but have little intraspecific variation compared to interspecific variation (Hillis & Dixon 1991). For most single-copy genes, high levels of variation between species are typically correlated with great variation within the species, so that extensive sampling of the species population is necessary (Hillis & Dixon 1991). Since the rDNA and β-tubulin genes both contain highly conserved and more variable regions they have been used for phylogenetic analysis of closely and more distantly related organisms, as well as for species identification.

5.1.4 Objectives

The objectives of this study were to:

a) determine genetic variation within the ITS regions of *C. camelliae* isolates.

b) develop *C. camelliae*-specific primers to the ITS region for detection of the pathogen *in planta*

c) examine the relationship of *C. camelliae* with other *Ciborinia* species and to members of the Sclerotiniaceae using ITS and β-tubulin data.

5.2 MATERIALS & METHODS

5.2.1 Preparation of Cultures for DNA Extraction

5.2.1.1 Fungal DNA

To harvest mycelium of fungal species for DNA extraction, the isolates were grown for three to eight days on PDA and cellophane (Waugh Rubber Bands Ltd, Wellington, New Zealand). Mycelia was scraped into microcentrifuge tubes (1.5 ml), frozen for >2 h, then freeze-dried. The dried cultures were stored at -20°C.

5.2.1.2 *Camellia* Petals

Six petals each of C. japonica 'Elegans Champagne' (A) and *C. reticulata* x *C.*

saluenensis 'Brian' (M) were inoculated with agar plugs of *S. sclerotiorum* (SSCL), *Botrytis cinerea* (BOTR), *C. camelliae* (PALM), *Penicillium* spp. (PENI) or agar-only control inoculations. The petals were placed in a humid chamber and incubated at 20°C in 12/12 L/D for 5 d.

Uninfected petals and petals on which infections had developed were placed in microcentrifuge tubes (1.5 ml), frozen for >2 h, then freeze-dried. Dried material was stored at -20°C.

5.2.1.3 *Camellia* Stems

The flowers and stems of *C. reticulata* x *C. saluenensis* 'Brian' and *C. saluenensis* x *C. japonica* 'Donation' naturally infected with *C. camelliae* were collected (M). The diseased flower was removed and the dead tissue around the abscission site cut away and placed in a microcentrifuge tube (1.5 ml) (Figure 5.3). A section of the stem approximately 10 mm below the abscission site was cut and placed in a microcentrifuge tube (1.5 ml). Corresponding sections from multiple flowers of the same hybrid were pooled in order to collect a sufficient amount of material for DNA extraction. The tubes were frozen for >2 h, then freeze-dried and the dried material stored at -20°C.

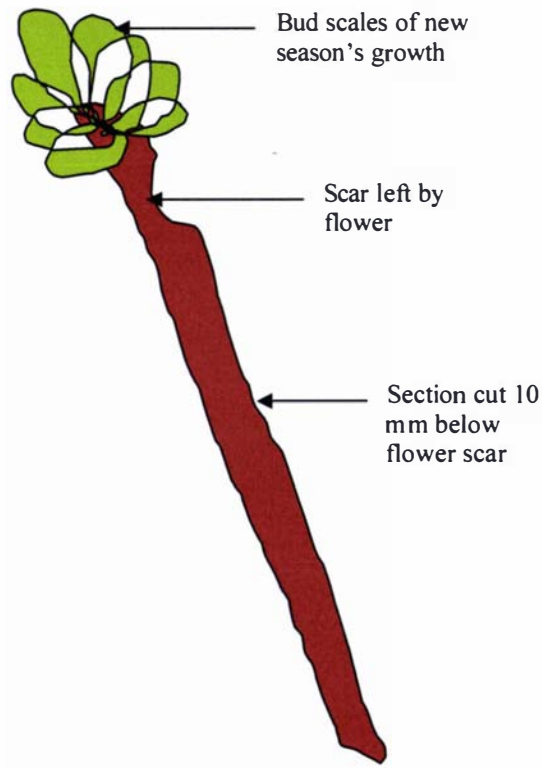


Figure 5.3
Diagram of camellia stem, showing site of scar left by abhisced flower and section further down the stem from which tissue was cut for analysis.

5.2.2 DNA Extraction

All DNA was extracted using the method of Al--Samarrai & Schmid (2000). Thirty mg of freeze-dried material was ground to a fine powder in a 1.5 ml microcentrifuge tube in liquid nitrogen using a pre-cooled pestle. Ground material was resuspended and lysed in 500 µl of lysis buffer (Section 2.4.6) and pipetted vigorously until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. Two microlitres of 10 mg/ml RNAse A (Sigma) was added and the mixture incubated at 37°C for 5 min. Precipitation of polysaccharides, protein and cell debris was facilitated by addition of 165 µl of a 5 mol/L NaCl solution and the components mixed by inverting the tube several times. The suspension was centrifuged at 13 000 rpm/min for 20 min at 4°C, the supernatant was immediately transferred to a fresh tube and 400 µl of chloroform and 400 µl of phenol were added. The solution was mixed by gently inverting the tube until the solution became milky. After centrifugation for 20 min, the aqueous phase was removed and extracted with an equal volume of chloroform. The DNA in the aqueous supernatant was precipitated with two volumes of 95% ethanol. To free the DNA from polysaccharide the precipitate was resuspended in 500 µl of lysis buffer and mixed by gentle pipetting. Then 165 µl of 5 mol/L NaCl was added and the tube gently inverted several times. The suspension was then chloroform-extracted as described above. After centrifugation for 10 min, the aqueous phase was clear and the DNA was precipitated with 95% ethanol. The precipitated DNA was washed thrice with 70% ice-cold ethanol, dried and dissolved in 50 µl TE buffer (Section 2.4.2).

Initially DNA extraction was undertaken using a Nucleon Phytopure kit (Amersham Life Sciences) designed for fungal DNA, however, it failed to produce usable DNA from *C. camelliae* isolates. The mycelium of *C. camelliae* isolates in culture was significantly thicker and more jelly-like than that of other fungi used in this study, and presumably a large amount of complexed polysaccharides prevented clean extraction. The method of Al-Samarrai & Schmid (2000) overcame this problem through the salt precipitation of polysaccharides and protein.

5.2.3 PCR of Genomic DNA

5.2.3.1 ITS Primers and Fungal DNA

A subset of the isolates available (Section 2.1) was used in this study. These were *C. camelliae* isolates 11160, 12996, 793, 990038, MHC13, II, Spain1, Spain2, 2001835, 2001980, NEW, PALM, WELL, LMK7, HK; plus *B. cinerea* (BOTR), *Penicillium* sp. (PENI), *Monochaetia* sp. (MONO) and *S. sclerotiorum* (GORE, SSCL and WAIR).

The ITS1, 5.8S and ITS2 regions of fungal DNA were amplified using the universal primer pair ITS1/ITS4 (White *et al* 1990) (Table 5.1). The PCR was carried out in a final volume of 50 (l, consisting of 1x PCR buffer (100 mM Tris-HCl pH 8.85, 250 mM KCl, 50 mM (NH4)2SO4; Roche) containing 2.0 mM $MgSO^4$ (Roche), 100 (M dNTPs, 2 (M of ITS1 and ITS4 primers, 2.5 U *Pwo Taq* DNA polymerase (Roche) and 20-50 ng of template DNA. Two separate PCR reactions were carried out on *C. camelliae* isolates HK and LMK7.

A negative control (no DNA) was included in every PCR to test for contamination, as well as a positive control (*C. camelliae* DNA).

Amplification conditions were an initial 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final elongation of 72°C for 5 min.

Five μl of the reaction mixture was loaded onto a 1.0% agarose gel in 1x TBE, separated by electrophoresis, stained with ethidium bromide and viewed and photographed under UV light.

5.2.3.2 β-tubulin Primers and Fungal DNA

A subset of the isolates available (Section 2.1) was used in this study. These were *C. camelliae* isolates 12996, Spain2, 2001980, NEW and PALM, and *S.*

sclerotiorum (isolates GORE and SSCL).

A partial segment of the β-tubulin gene was amplified using the primer pair Bt2a/Bt2b (Glass & Donaldson 1995) (Table 5.1). The PCR was carried out in a final volume of 50 µl, consisting of 1x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl; (Gibco, BRL) containing 1.5 mM $MgCl^2$ (Gibco BRL), 100 (M dNTPs, 2 µM of Bt2a and Bt2b primers, 1.0 U *Taq* DNA polymerase (Gibco BRL) and 20-50 ng of template DNA. Two separate PCR reactions were carried out on each of the isolates.

Controls, amplification conditions and analysis of PCR products were as described in Section 5.2.3.1.

Table 5.1 DNA Sequence of Primer Pairs

Target Gene	Primer Name	Primer DNA Sequence
ITS	ITS1	5'TCCGTAGGTGAACCTGCGG 3'
	ITS4	5' TCCTCCGCTTATTGATATGC 3'
β-tubulin	Bt2a	5' GGTAACCAAATCGGTGCTGCTTTC 3'
	Bt2b	5' ACCCTCAGTGTAGTGACCCTTGGC 3'

5.2.4 Purification and Ligation of PCR Products

PCR products were initially purified using the QIAquick PCR Purification Kit Protocol (Qiagen). A second round of purification was achieved by electrophoresis of the PCR products on a 1% Sea Plaque agarose gel (1x TAE) followed by gel extraction using the Concert Gel Extraction System (Life Technologies, Gibco BRL).

5.2.4.1 ITS PCR Products

Purified PCR products were ligated in to the plasmid pUC118 (Vieira & Messing 1982) linearised with *Sma*I. The ligation reaction was carried out in a final volume of 15 µl consisting of 1x ligation buffer and 1 U T4 DNA ligase (Roche), 150 ng linearised pUC118 and 40-100 ng of insert.

5.2.4.2 β-tubulin PCR Products

Purified PCR products were ligated using the pGEM(-T Easy Vector System (Promega). The ligation reaction was carried out in a final volume of 10 µl consisting of 1x Rapid Ligation Buffer and 3 U T4 DNA ligase, using an insert:vector ratio of 1:1.

For both transformation systems, a positive control using control insert DNA (Promega), and background control (no insert) were included in each ligation reaction to check ligation efficiency and the number of background colonies.

Five µl of the ligation product was loaded onto a 1% agarose gel in 1x TBE, separated by electrophoresis, stained with ethidium bromide and viewed under UV light to determine whether ligation had occurred.

5.2.5 Transformation and Plasmid Preparation

The ligation products were transformed by electroporation at 25 (F, 2.5 V and 200-600 Ω (Biorad Gene Pulsar and Pulse Controller). For each ligation reaction 2 µl of the ligation product was added to 40 µl competent cells (*E. coli* XL1-BLUE (genotype *Sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, *lac*-, F'[*pro*AB+. *lacZ*(M15, Tn10(tet^r)] Bullock *et al* 1987) and pipette mixed. The suspension was transferred to a cold dry cuvette and placed in the electroporation chamber. After electroporation, 220 µl of LB broth was added to the cuvette and mixed.

Efficiency of the transformation was checked by transforming XL1 cells with uncut plasmid pUC118 (*Amp*r, *lacZ*' (3.2 Kb) Messing 1987) and ampicillin sensitivity of the XL1 cells was checked by transforming them with no plasmid DNA.

After transformation, 100 µl of each transformation mixture was spread over either LB agar or LB agar amended with 100 (g/ml Amp, 50 mg/ml Xgal, 24 mg/ml IPTG. Cultures were incubated at 37°C overnight.

White colonies were inoculated into 3 ml LB broth containing 100 (g/ml Amp and incubated in shake culture at 37°C overnight. Plasmids were purified using the QIAprep Spin Miniprep Kit Protocol (Qiagen).

The presence of an insert in the plasmid was determined by digesting 3 µl of the purified plasmid in a total volume of 25 µl consisting of 1x H buffer (Roche) and either 2.5 U *Eco*RI (P-GEM(-T Easy system) or 2.5 U *Eco*RI and 2.5 U *Pst*I (pUC118) in a 37°C water bath for 2-3 h. Digests were analysed by gel electrophoresis as described in Section 5.2.4.2.

5.2.6 DNA Sequencing

Automated sequencing reactions were performed using the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an ABI 377 automated sequencer, with 20-100 ng of DNA template, and primers T7 and Sp6 (pGEM(-T Easy Vector System) or M13 forward and reverse (pUC118). Each insert was sequenced on both strands.

The NCBI Basic Local Alignment Search Tool (BLASTn) was used to confirm the ITS identify of each sequence and identify other similar sequences (www.ncbi.nlm.nih.gov/blast).

5.2.7 Sequence Alignments

DNA sequences were aligned using the CLUSTAL W algorithm (Thompson *et al* 1994) which is available in the Gene-Jockey II® program. For the ITS sequences, the 18S and 28S ribosomal DNA sequences which flank the ITS regions were deleted prior to alignment, while for the partial β-tubulin sequences, the primer sequences (embedded in the exons) flanking the regions of interest were deleted prior to alignment. A *B. cinerea* isolate (Z69263, The Netherlands) was included in the β-tubulin comparison.

In order to design *C. camelliae*-specific primers, one forward primer of 20 bp was

designed to a section of ITS1 which contained the greatest number of variations compared to the most similar sequences from NCBI, and used in conjunction with the reverse primer ITS4.

5.2.8 Phylogenetic Analysis of ITS Sequences

All ITS sequences of *Ciborinia, Sclerotinia* and *Botrytis cinerea* species contained on the NCBI database and the isolates of *C. camelliae, S. sclerotiorum* and *B. cinerea* sequenced in this study were used to examine the relationship of *C. camelliae* with other *Ciborinia* species, with *S. sclerotiorum* isolates and in relation to other *Sclerotinia* species and some *Botrytis* species (Table 5.2).

Sequences were aligned using CLUSTAL X (1.81). A Neighbour-Joining tree was constructed based on 1000 bootstrap replications of the data set using PHYLIP, branches with less than 50% support were collapsed. The tree was rooted using *Chlorociboria aeruginosa* as an outgroup.

5.3 RESULTS

5.3.1 PCR of Genomic DNA

Amplification of fungal DNA with universal primers ITS1/ITS4 resulted in PCR products approximately 550 bp in length, whilst with β-tubulin primers Bt2a/Bt2b products approximately 480 bp in length were obtained.

5.3.2 ITS Sequence Alignment of *C. camelliae* Isolates

A total of 15 *C. camelliae* isolates from 9 countries were sequenced. There was no sequence variation between the 13 isolates from the United States, New Zealand or Europe (United Kingdom, France, Spain, Italy and Switzerland). The two isolates from east Asia, LMK7 from Japan and HK from Hong Kong, differed by five and two substitutions respectively (Figure 5.4).

Table 5.2 Summary of ITS Sequences of Ciborinia, Sclerotinia and Botrytis spp. obtained from NCBI and this study.

Species	NCBI Accession Number/Source[a]	Origin of Isolate
Botryotinia fuckeliana	AF246943	France
Botrytis calthae	Z99667	Norway
Botrytis cinerea	Z73764 (Z99663)	Norway
Botrytis cinerea	Z99665	Norway
Botrytis cinerea	Z99662 (Z73765, Z99664, AF246942, AF246944)	Norway
Chlorociboria aeruginosa	Z81426	Norway
Ciborinia allii	AB026167 (AB26166)	Japan
Ciborinia camelliae	this study	New Zealand
Ciborinia camelliae	this study	Japan
Ciborinia camelliae	this study	Hong Kong
Ciborinia candolleana	Schumacher & Holst-Jensen (1997)	Norway
Ciborinia candolleana	Schumacher & Holst-Jensen (1997)	Norway
Ciborinia erythronii	Z73767	Canada
Ciborinia foliicola	Z80892	Canada
Ciborinia whetzelii	Z73768	Canada
Monolinia oxycocci	Z73789	Norway
Sclerotinia borealis	Z99675	Norway
Sclerotinia borealis	Z99674	Norway
Sclerotinia borealis	AF067644	Canada
Sclerotinia glacialis	Z99669	Norway
Sclerotinia homeocarpa	AF067640	United States
Sclerotinia minor	Z99673	Norway
Sclerotinia pirolae	Z73798	Norway
Sclerotinia sclerotiorum	AF455526	Austria
Sclerotinia sclerotiorum	AF455523	Austria
Sclerotinia sclerotiorum	AF455456	Austria
Sclerotinia sclerotiorum	AF455439	Austria
Sclerotinia sclerotiorum	AF455439	Austria
Sclerotinia sclerotiorum	Z73799	Norway
Sclerotinia sclerotiorum	Z73800	Norway
Sclerotinia sclerotiorum	this study	New Zealand
Sclerotinia tetraspora	Z99672	Norway
Sclerotinia tetraspora	Z99670 (Z99671)	Norway
Sclerotinia trifoliorum	Z99676	Norway

[a] references in brackets indicate isolates of identical sequence and were not used in the phylogenetic analysis.

5.3.3 ITS Sequence Alignment of *C. camelliae*, *S. sclerotiorum* and *Botrytis cinerea* Isolates and Location of *C. camelliae*-specific Primer

ITS sequences of the New Zealand isolates of *Penicillium* sp. and *Monochaetia* sp. were very different from those of *C. camelliae* and were not used in sequence

alignments. The sequences of the three New Zealand isolates of *S. sclerotiorum* were identical to each other and similar to that of *C. camelliae* (Figure 5.5), except for four substitutions, two additions and one deletion (compared with the majority of *C. camelliae* isolates). The *Botrytis cinerea* isolate was similar to both the *C. camelliae* and *S. sclerotiorum* sequences, having two deletions, two insertions and four substitutions difference when compared with the majority of *C. camelliae* isolates (Figure 5.5).

The *C. camelliae* isolates from New Zealand, the United States and Europe were sequenced and the specific primer designed before the two Asian isolates were available for sequencing. The *C. camelliae*-specific primer (CIB1) was designed to the section of the ITS1 where variation was found between *C. camelliae* isolates and *S. sclerotiorum* and *B. cinerea* isolates. Subsequently, the two Asian *C. camelliae* isolates were obtained and sequenced and found to differ within the CIB1 primer. Because sequence variation was relatively low both within *C. camelliae* isolates and between the closely related species *S. sclerotiorum* and *B. cinerea*, the design of the primer was not changed. It became an isolate-specific primer.

The similarity of the ITS sequences of *C. camelliae* and *S. sclerotiorum* was unexpected and several tests were carried out to ensure that a) there was no DNA contamination or mislabelling and b) that both fungi were identified correctly. Inoculation of carrots and *Camellia* petals with *C. camelliae* and *S. sclerotiorum* confirmed that the identification was correct (Figure 5.6). In addition, the three *S. sclerotiorum* isolates were collected by two different organisations, in three different locations (Te Kuiti, Wairarapa and Gore).

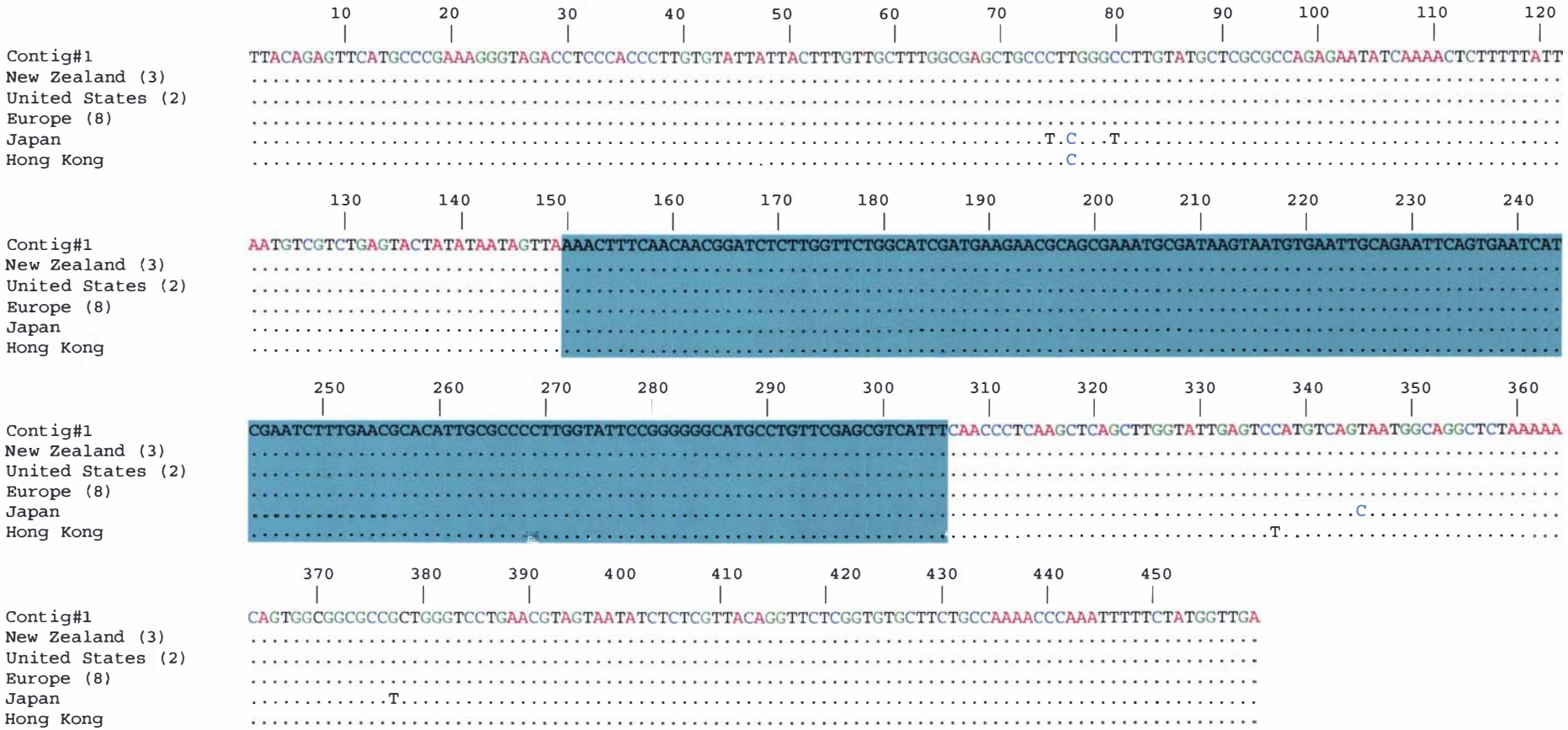


Figure 5.4
Comparison of ITS1, 5.8S and ITS2 sequences from 15 isolates of *C. camelliae* (New Zealand isolates=NEW, PALM, WELL; United States isolates=11160, 12996; European isolates=990038, MHC13, II, 793, Spain1, Spain2, 2001835, 2001980). Each sequence is a contig of the forward and reverse sequence. Sequences from duplicate PCR reactions (Hong Kong and Japan) identical and not shown. The 5.8S region is shown in blue.

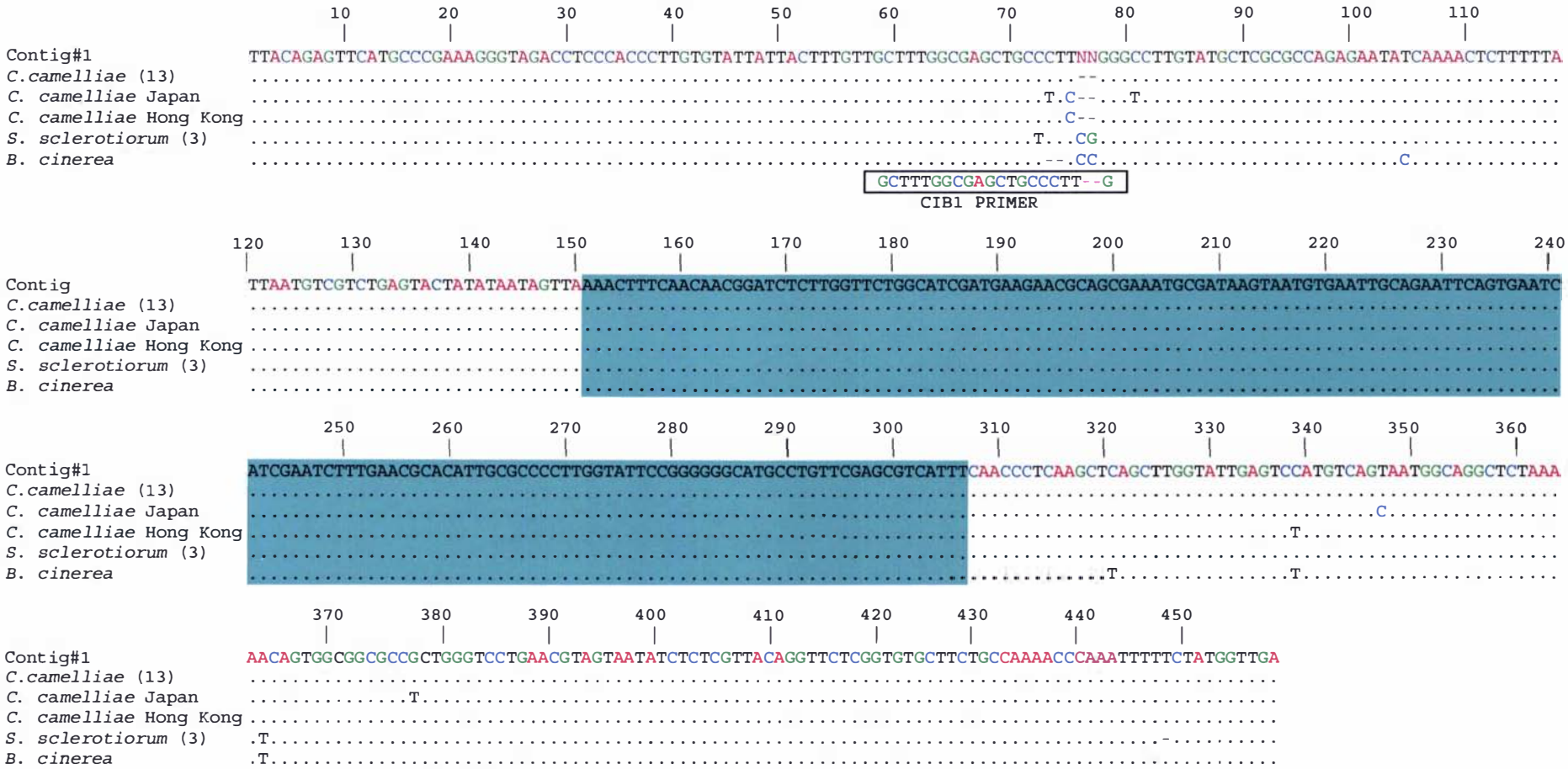


Figure 5.5
Comparison of ITS1, 5.8S and ITS2 sequences from the three *C. camelliae* sequence variations and the New Zealand isolates of *S. sclerotiorum* (GORE, SSCL, WAIR) and *Botrytis cinerea*. The location of the *C. camelliae*-specific primer CIB1 is also indicated (primer ITS4 not shown). Each sequence is a contig of the forward and reverse sequence. The 5.8S region is shown in blue.

Figure 5.6
Petals of *C.* x *williamsii* 'E. G. Waterhouse' 4 d after inoculation with agar plugs of (left) *S. sclerotiorum* and (right) *C. camelliae.* The lesion colour and pattern of spread was distinctly different between the two. The *S. sclerotiorum* lesion failed to spread further than shown, while the lesion caused by *C. camelliae* rapidly engulfed the entire petal.

5.3.4 Test of *C. camelliae* isolate-specific Primers

Specificity of the CIB1/ITS4 primer pair was tested using pure cultures of *C. camelliae, S. sclerotiorum, B. cinerea,* and *Monochaetia* spp. DNA, *C. camelliae*-infected twigs and petals, *C. camelliae*-infected and S. *sclerotiorum*-infected petals and uninfected controls (Sections 5.2.1.2 and 5.2.1.3). The same isolates were also tested using the ITS1/ITS4 primer pair and with the PCR run at a range of annealing temperatures. Comparison of the two primer pairs at two different annealing temperatures is shown in Figures 5.7 and 5.8.

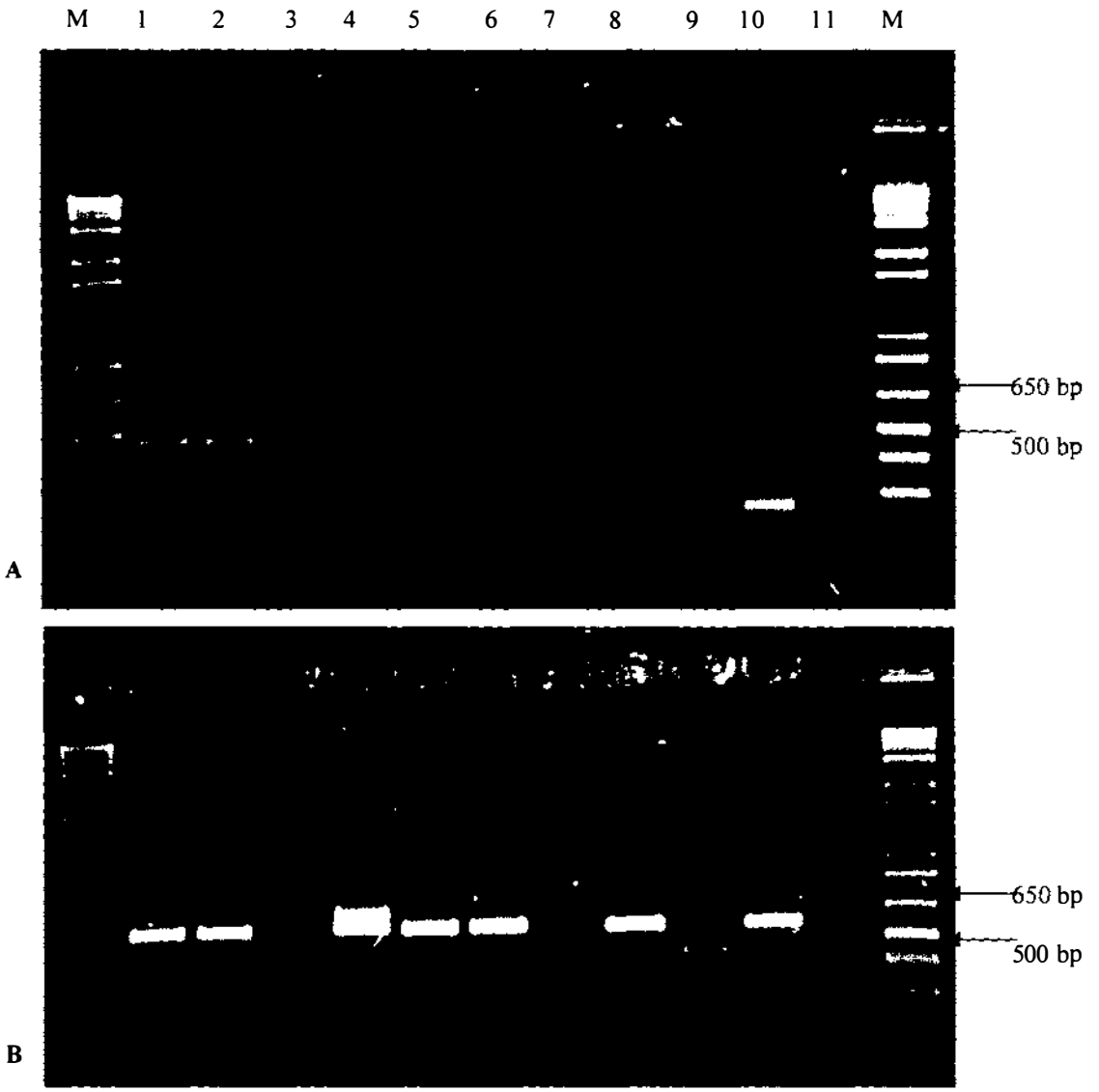


Figure 5.7
Products from PCR at 55°C annealing temperature and 2% agarose gel. (A) *C. camelliae*-specific PCR primers CIB1/ITS4. (B) Universal primers ITS1/ITS4. Lane M, 1Kb plus ladder (Life Technologies Ltd); lanes 1-11: genomic DNA templates. Lane 1 *C. camelliae* TAUP; lane 2 *C. camelliae* Spain1; lane 3 negative control; lane 4 *Monochaetia* sp.; lane 5 *S. sclerotiorum* SSCL; lane 6 *B. cinerea*; lane 7 'Brian' uninfected twig; lane 8 'Brian' *C. camelliae*-infected twig; lane 9 'Brian' uninfected petal; lane 10 'Brian' *C. camelliae*-infected petal; lane 11 'Brian *S. sclerotiorum*-infected petal.

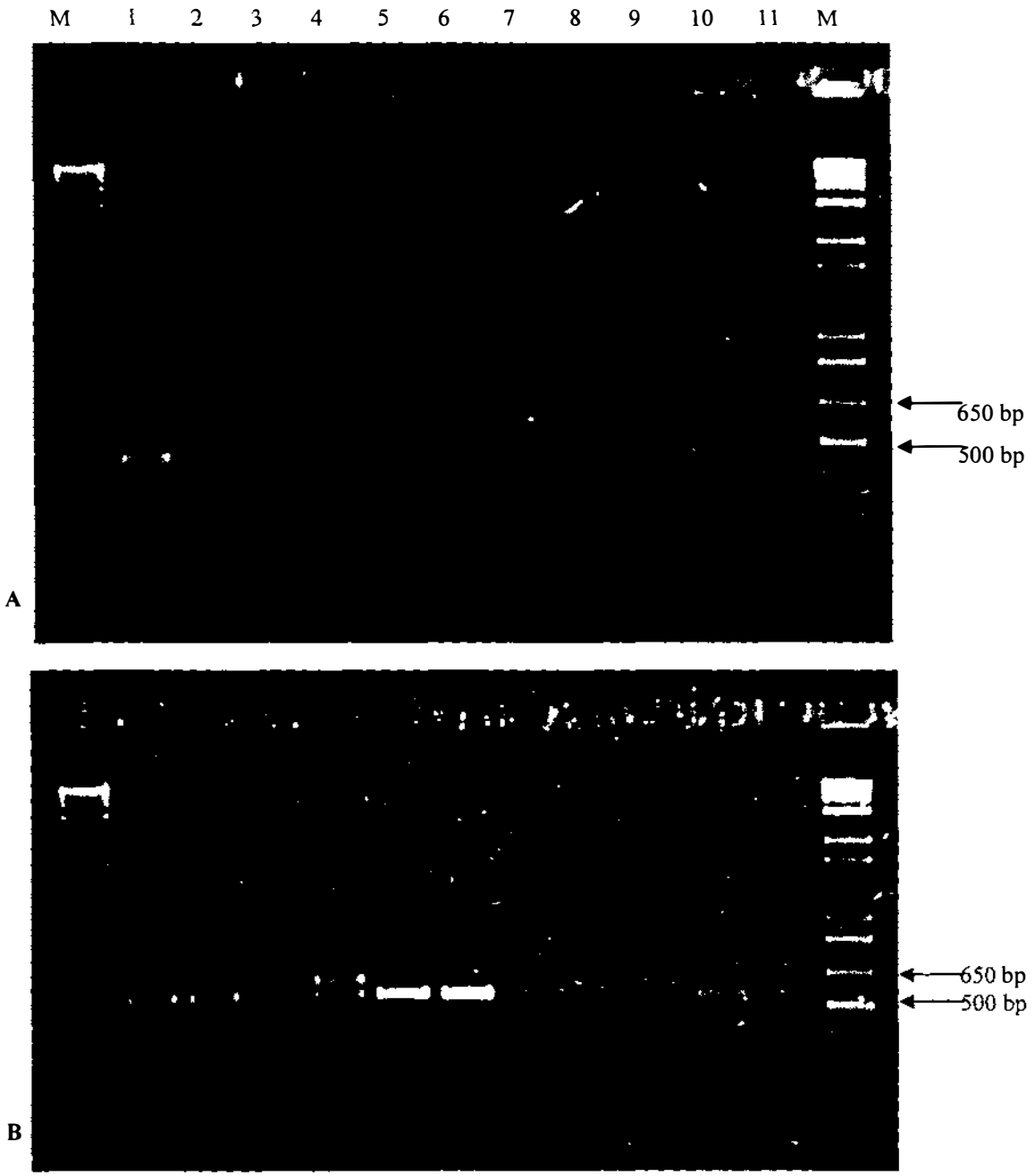


Figure 5.8
Products from PCR at 60°C annealing temperature and on 2% agarose gel. (A) *C. camelliae*-specific PCR primers CIB1/ITS4. (B) Universal primers ITS1/ITS4. Lane M, 1Kb plus ladder (Life Technologies Ltd); Lanes 1-11: genomic DNA templates. Lane 1 *C. camelliae* TAUP; lane 2 *C. camelliae* Spain1; lane 3 negative control; lane 4 *Monochaetia* sp.; lane 5 *S. sclerotiorum* SSCL; lane 6 *B. cinerea*; lane 7 'Brian' uninfected twig; lane 8 'Brian' *C. camelliae*-infected twig; lane 9 'Brian' uninfected petal; lane 10 'Brian' *C. camelliae*-infected petal; lane 11 'Brian *S. sclerotiorum*-infected petal.

The universal primer pair ITS1/ITS4 amplified a band approximately 550 bp from most samples containing fungal DNA. The primers did not detect *S. sclerotiorum* in *S. sclerotiorum*-infected petal tissue, probably because the amount of fungal DNA relative to petal DNA was very low. The *C. camelliae* isolate-specific primer pair CIB1/ITS4 amplified a band approximately 480 bp in length in those samples containing *C. camelliae* DNA, and detected *C. camelliae* in petal and stem tissue. The higher annealing temperature of 60°C eliminated many of the extra bands found in the CIB1/ITS4 PCR. PCR using CIB1/ITS4 on pure cultures of *S. sclerotiorum* sometimes produced faint bands in the 480 bp region, but never from *S. sclerotiorum*-infected plant tissue, presumably because the amount of *S. sclerotiorum* DNA present was too small to be detected.

5.3.5 β-tubulin Sequence Alignment of *C. camelliae* and *S. sclerotiorum* Isolates

Comparison of the partial β-tubulin sequence of five *C. camelliae* isolates, two New Zealand *S. sclerotiorum* isolates and *B. cinerea* (Z69263) is shown in Figure 5.9. A product 425 bp in length was sequenced from *C. camelliae* isolates and 424 bp from *S. sclerotiorum* isolates. There was no sequence variation within either of the species, but there were 32 substitutions and one deletion difference between *C. camelliae* and *S. sclerotiorum*. *B. cinerea* sequence varied from *C. camelliae* by 60 substitutions, two additions and five deletions.

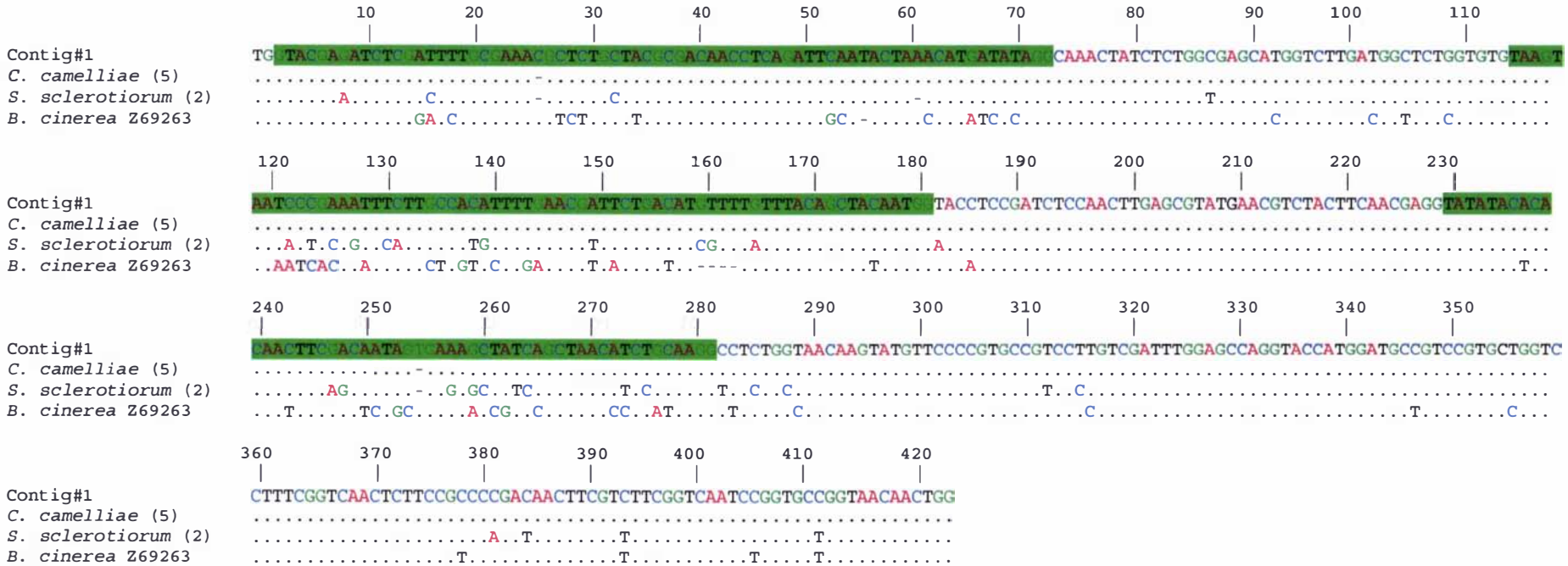


Figure 5.9

Comparison of the partial β-tubulin sequence of *C. camelliae* isolates (12996, Spain2, 2001980, NEW, PALM) with New Zealand *S. sclerotiorum* isolates (GORE, SSCL) and *B. cinerea* (Z69263). Introns C, D and E shown in green.

5.3.6 ITS Phylogenetic Analysis

The phylogenetic tree based on ITS sequence is shown in Figure 5.10. All three *C. camelliae* isolates grouped mostly closely with isolates of *S. trifoliorum, S. minor, S. sclerotiorum, S. glacialis, B. cinerea, B. fuckeliana* and *B. calthae.* The three *C. camelliae* isolates did not group together, with the Japanese isolate forming a group with *S. glacialis*, and the Hong Kong and New Zealand isolates individual branches. In comparison with other *Ciborinia* species, *C. camelliae* appeared to be more closely related to these *Sclerotinia* and *Botrytis* species and the five *Ciborinia* species each appeared to be as closely related to each other as to other species (e. g. *M. oxycocci* and *S. pirolae*). The New Zealand isolates of *S. sclerotiorum* formed a group with the Norwegian isolates of *S. sclerotiorum*, while the *S. sclerotiorum* isolates from Austria grouped together, but were not distinct from, some *B. cinerea* isolates and *B. calthae.* The New Zealand isolate of *B. cinerea* grouped closely with other isolates of *B. cinerea* and *B. calthae* but was distinct from them.

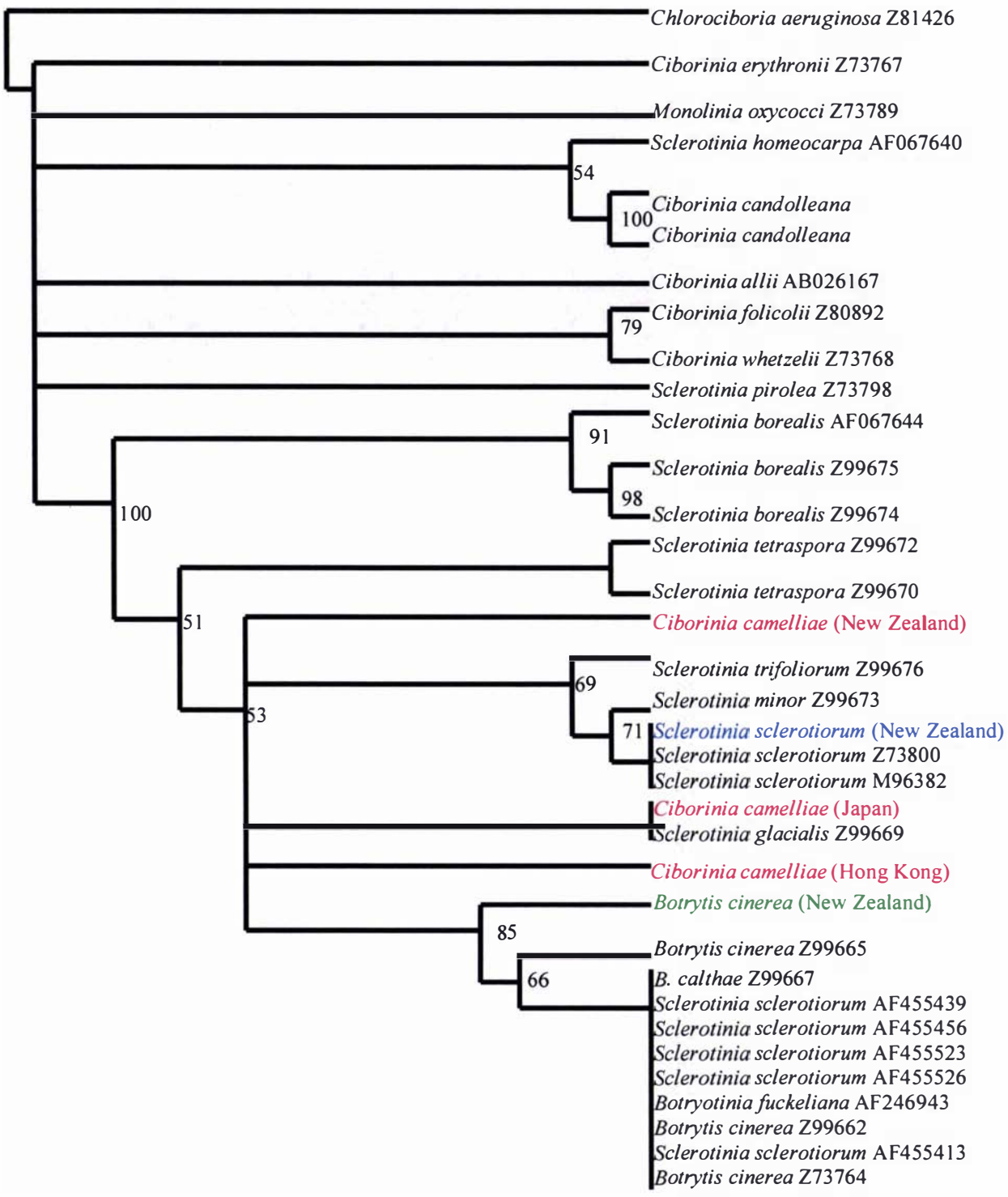


Figure 5.10
Phylogenetic relationship based on ITS regions of rDNA of *C. camelliae* isolates (red) with other *Ciborinia*, *Sclerotinia* and *Botrytis* species (the New Zealand *S. sclerotiorum* isolate is shown in blue and the *B. cinerea* isolate in green). Numbers on branches are % bootstrap values from 1000 replications. Branches with less than 50% support were collapsed

5.4 DISCUSSION

There was little variation in the ITS sequence of isolates within *C. camelliae* species. The two isolates that did vary were from Hong Kong and Japan. Since the fungus is assumed to have originated in east-Asia and to be widespread there, it is likely that more isolates from this region would show greater variation. Two sequences were obtained from independent PCR reaction products of these isolates to ensure that the differences were not due to PCR or sequencing errors. In addition, high fidelity *Pwo* DNA polymerase was used to amplify all *C. camelliae* isolates, reducing the base substitution error rate. The identical sequences obtained from isolates in the United States, New Zealand, the United Kingdom, France, Spain, Italy and Switzerland may also indicate that the fungus spread from one initial introduction to California, in the United States, to New Zealand and to Europe. Further sequencing from regions within Japan, from where the fungus was believed to be imported to California, may identify the source. Myburg *et al* (2002), for example, could group isolates of *Cryptonectria cubensis* according to geographic regions, but Cruse *et al* (2002) found no correlation between genetic isolation and geographic distance for *Stachybotrys chartarum*.

In general, ITS sequence variability has been found between species, but with little or no variation in sequence within a species (Lee & Taylor 1992; Bulman & Marshall 1998) and *C. camelliae* appears to conform to this pattern. Based on the 15 isolates sequenced in this study, variation in the ITS sequence varied from 0-1.1%. The amount of variation in the ITS sequence (number of base differences, usually expressed as a %) has been shown to vary from 0-15.8% within a species (Seifert *et al* 1995). For example, the ITS sequences of ten isolates of *Ampelomyces quisqualis* (a hyperparasite of powdery mildew) showed high sequence variability (4.3-14.2%) and relationships with other closely related taxa indicated that the isolates did not represent a single species (Kiss & Nakasome 1998). Morphological, cultural (Kiss & Vajna 1995) and RFLP (Kiss 1997) support this data. In contrast, three species of *Trichoderma* which are readily distinguished by conidial ornamentation and conidium morphology showed very low ITS sequence variation (0.3-2%) (Kinderman *et al* 1998). The conflict between morphological and molecular data for classifying ascomycete fungi is discussed by Seifert *et al* (1995).

Van Toor (2002) found that New Zealand isolates were distinctly different from the United States isolates using UP-PCR banding patterns. He found the majority of the genetic variation (87%) associated with the difference between the two countries, and only 9% associated with variation within the countries. He suggested that since the genotypes were distinctly different between the two countries, that the most likely alternative source of the pathogen was Japan, contradicting the introduction pattern indicated by ITS sequences in this study. At the time *C. camelliae* was discovered in New Zealand, Japan and the United States were the only countries known to have the pathogen, however, it is possible given its widespread distribution in Europe and the United Kingdom (discovered later) that the pathogen was present but unidentified in one or more of these countries. Van Toor (2002) tested only three isolates from the United States, one from Oregon on the west coast and two from the east coast (Virginia and Georgia) and none from California. Since the state of California provides many tourists to New Zealand, further testing of ITS variation within the United States, or of different genes, may resolve from which country the pathogen entered New Zealand.

The similarity of ITS sequences of New Zealand isolates of *S. sclerotiorum* and *B. cinerea* with *C. camelliae* encouraged further analysis in order to determine the relationship of *C. camelliae* to other members of its genus and *Sclerotinia* and *Botrytis* genera. Phylogenetic analysis of the ITS region showed that the *C. camelliae* isolates were more closely related to members of the genera *Sclerotinia* and *Botrytis* than to other members of the genus *Ciborinia*. *C. camelliae* was originally described as *S. camelliae* (Hara 1919; Hansen & Thomas 1946) until Whetzel (1945) created the genus *Ciborinia* to contain species that incorporated host tissue into their sclerotia. In their study of the Sclerotiniaceae, Carbone & Kohn (1993) concluded that the *Ciborinia* were not a monophyletic group and were in need of revision. The results of this study support this view and it may be that a future taxonomic revision alters these genera. Members of the genus *Ciborinia* are similar in many respects however, and it may be that their commonality of life cycle is more unifying than molecular data. Similarities between the *Ciborinia* species are discussed in Taylor & Long (2000).

Phylogenetic analysis of the partial β-tubulin gene was intended to clarify the relationship of *C. camelliae* and *S. sclerotiorum,* but there were too few β-tubulin sequences of relevant species on the NCBI database to undertake a phylogenetic analysis. Sequence comparison of the partial β-tubulin gene of *C. camelliae* and New Zealand isolates of *S. sclerotiorum* did indicate that the two were distinct species, and that contamination or misidentification of *C. camelliae* was unlikely.

Initial phylogenetic studies utilised one gene for relationship comparisons (e.g. rDNA phylogeny of the Sclerotiniaceae (Holst-Jensen *et al* 1997a)), but there is an increasing trend to use multiple gene loci (Baldauf & Doolittle 1997; O'Donnell *et al* 1998; Schoch *et al* 2000; Groenewald *et al* 2001; Articus *et al* 2002; Cruse *et al* 2002; Yli-Mattila *et al* 2002; Wyand & Brown 2003). This is because the selected gene may have undergone horizontal gene transfer, lineage sorting or gene duplications that produce incongruencies when compared to trees based on different genes (Maddison 1997). In their study of the *Gibberella fujikuroi* species complex, O'Donnell *et al* (1998) found the phylogenies based on β-tubulin, mtSSU and 28S genes were largely concordant, while the ITS phylogeny was not. This was due to the presence of two highly divergent nonorthologous ITS2 types. Taylor *et al* (2000) reviewed phylogenetic species recognition based on multiple loci and concluded that single gene phylogenies were not sufficient for species identification. In addition, Wainwright *et al* (1993) suggested that 1000 bases is the minimum length on which analysis can be based, as the shorter the sequence, the more significant any difference would become. Thus, the more genes and the longer the sequence, the more confidence in the results, although constraints to variation in one gene may be different to another (Down 2002).

The *C. camelliae* isolate-specific primers (CIB1/ITS4) detected *C. camelliae* DNA in petal and twig tissue. Long (unpubl. data) had previously isolated *C. camelliae* from bud scars of infected *Camellia* flowers, and the results from experiments reported here confirm that the fungus is able to infect more than petal tissue, so that the pathogen is not confined to floral parts as previously thought. The distance the fungus penetrates into the twig and its long-term viability within the twig are two areas for future investigation. If fungal mycelium in the twig were found to survive from one camellia flowering season to the next, then it may provide a potential

source of inoculum, as the pathogen may be able to infect a new flower from within the plant. In regions where the pathogen is already present, this would probably not be a significant source. If the fungus were found to survive long-term within the twig, then a review of biosecurity protocols/procedures is required for those countries in which the pathogen is absent.

It was not possible to design species–specific primers based on the ITS region of *C. camelliae* and isolate-specific primers were constructed instead. The use of these isolate-specific primers is intended mainly for use in laboratory experiments for accurate identification and detection of *C. camelliae in planta*, but they could be used by quarantine staff for screening camellia plant imports/exports. Their usefulness, however, is limited since they do not detect the two Asian isolates of *C. camelliae,* and it is unknown how much variation exists in their region of origin. Although ITS DNA sequence of *S. sclerotiorum* is very similar to *C. camelliae*, raising the possibility of a close relationship, infection of camellias by *S. sclerotiorum* was not observed in the field and the likelihood of a false positive result unlikely. Again, while the ITS DNA sequence of *C. camelliae* and *B. cinerea* were very similar, *B. cinerea* infection of camellia petals is more easily identified, as infections occur in older petals (i. e. post-experiment) and it is simple to culture and identify.

In future work, the β-tubulin gene may be a more appropriate gene with which to design *C. camelliae*-specific primers and also to investigate phylogenetic relationships of *Ciborinia* and other closely related species.

CHAPTER SIX – ASCOSPORE INOCULATION TECHNIQUES AND RESISTANCE TESTING

6.1 INTRODUCTION

C. camelliae ascospores infect the petals of camellia flowers, but very little is known about the infection process (pre-penetration, penetration and post-penetration events). Prior to the study of Taylor (1999), no camellia species or varieties were known to be resistant (Baxter & Segars 1989). Hansen & Thomas (1940) reported that of the more than 50 varieties of camellia in a nursery, all appeared to be equally susceptible and Baxter & Berly (1956) reported more than 100 varieties (in private gardens) showed no resistance to *C. camelliae*. Presumably these were the more common species and varieties (i.e. *C. japonica, C. reticulata, C. saluenensis* and hybrids) that are widely distributed as ornamental plants. Baxter (unpublished, cited in Baxter & Epps 1981) reported that *C. japonica* 'Betty Sheffield' and 'Rev. John G. Drayton' did not appear to be quite as susceptible as other varieties, although they could be infected by *C. camelliae*. Opinions differed as to whether the autumn-flowering species were susceptible to the disease. Both Frampton (1994) and Haasis (1953) stated that *C. sasanqua* was susceptible, whereas Matsumoto (1995) stated that it was immune. He also reported that wild japonicas (in Japan) were not "as damaged as other cultivars" by *C. camelliae.*

Preliminary studies in the pre-blight season investigated the infection of autumn-flowering species using mycelial plug inoculum, and were followed during the blight season by ascospore inoculation of spring-flowering species (Taylor 1999). Wounding was required for infection from mycelial plug inoculum but not from ascospore inoculum and younger flower buds appeared to have more resistance than older flowers with both types of inocula (Taylor 1999). Ascospore inoculations of various species and varieties also showed that there were levels of resistance within the genus, but this was not quantified (Taylor 1999).

The majority of *C. japonica, C. reticulata* and hybrids were susceptible to infection by ascospores of *C. camelliae* and the autumn-flowering *C. sasanqua* cultivars were susceptible to infection by mycelial plug inoculation. The resistant and susceptible

species, as indicated by the results of ascospore inoculation testing, are shown in Table 6.1. The species that appeared to have some level of resistance are not commonly grown and are generally available only from specialist nurseries. Species with resistant characteristics were found in the Subgenera Metacamellia, Protocamellia and Camellia (Chang & Bartholomew 1984 classification system).

Table 6.1 Resistance Rating Summary of Camellia Species tested by Airborne Inoculation of Ascospores (from Taylor 1999)

Camellia Species/Cultivar	Susceptible/Resistant
all *C. japonica* varieties tested	S
all *C. reticulata* varieties tested	S
all hybrids tested except *C. japonica* x *C. lutchuensis* 'Spring Mist'	S
C. chekiangoleosa	S
C. cuspidata[a]	R
C. euphlebia[b]	S
C. forrestii[a]	R
C. fraterna	R
C. gigantocarpa	S
C. grijsii[a]	R
C. longicarpa[a]	R
C. lutchuensis	R
C. pitardii var. *pitardii*	S
C. polydonta	S
C. rosiflora	S
C. transarisanensis[a]	R
C. transnokoensis	R
C. yuhsienensis	R
C. yunnanensis[a]	R

[a] atypical symptoms, such as pitting or small brown lesions which failed to develop further
[b] *C. euphlebia* was incorrectly labeled and it is not known what species this is

Further research is required to investigate these apparently resistant species. In order to study resistance in *Camellia* species, it is necessary first to develop, then adapt and assess inoculation techniques that allow resistance to be quantified.

6.1.1 Methods of Inoculation

Apart from the inoculation technique developed by Taylor (1999), there were no published protocols for inoculating camellias with *C. camelliae* ascospores.

The method of Taylor (1999) involved the application of 'dry' ascospores in a settling chamber. The apparatus consisted of an aquarium pump, which blew air through water in a Drechel bottle (to hydrate it), over several apothecia. Ascospores released by the apothecia were blown into the settling chamber where they settled out on flowers or petals laid on the floor of the chamber. The number of ascospores

released into the chamber could not be controlled, making comparisons possible within a run but not between runs. Further details are given in Section 6.2.2.

Dry ascospores may also be applied using cotton wool swabs, a needle, a paintbrush or other implements that transfer ascospores from the surface of the apothecium (or stored medium) to the host surface. These methods all result in qualitative data, as the number of ascospores can not be controlled. Application using dry spores can render quantitative data if the spores are applied as a dust in a settling tower (Eyal *et al* 1968; Coertze & Holze 1999) but Eden *et al* (1996) noted in their work with *B. cinerea*, that this method was not used because of problems of dilution and calibration with talc as the carrier, and the impossibility of applying spores accurately and evenly in a settling tower.

Inoculation techniques that use spores suspended in water (or other solutions) are the most common method for inoculating spores onto host plants, even if those spores are normally dry-dispersed (Anonymous 1983). Spore suspensions can provide quantitative data where the concentration of the inoculum is known and the application method applies the suspension in uniformly sized droplets with an even distribution over the host surface.

The simplest method is to apply droplets of a standard inoculum concentration using a micropipette. For resistance testing, this allows the assessment of both the % disease incidence i.e. number of samples to develop lesions/total number of samples and the % disease severity i.e. area of lesion after a defined incubation period). Vloutoglou *et al* (1999) used this method for their study of *Alternaria linicola* infection of linseed and Denoyes-Rothan *et al* (1999) for their study of fruit resistance of strawberries to *Colletotrichum acutatum*.

Devices that spray spore suspensions range from non-quantitative applicators e.g. atomisers and Devilbis sprayers to controlled droplet applicators e.g. spinning discs and Potter towers. None of these devices, however, produce quantitative data.

6.1.2 Factors Affecting Germination, Penetration and Lesion Development

There are a number of factors that affect the infection process. This study considered only those that affected the development of suitable resistance testing techniques. A brief review of the main factors is given below.

6.1.2.1 Inoculum Concentration

In general, the higher the concentration of spores applied to the host tissue, the greater the incidence or severity of disease (Deverall & Wood 1961; Eisensmith *et al* 1982; Jeger *et al* 1985; Eden *et al* 1996; Coertze & Holz 1999; Hartman *et al* 1999; Vloutoglou *et al* 1999). Sirjusingh *et al* (1996) reported that *Botrytis cinerea* conidia required threshold concentrations in order for infection to occur on geranium flower parts. For petals, a minimum of 1×10^4 conidia/ml was required, while for sepals, stamens, pistils and pedicels it was between 10-100 conidia/ml. On grapes, Coertze & Holz (1999) found that a single conidium of *B. cinerea* could cause infection. Disease incidence or severity did not increase after a critical concentration of spores was reached. For *A. linicola* on linseed, this critical concentration was $>1 \times 10^5$ conidia/ml (Vloutoglou *et al* 1999), while for *Venturia inaequalis* on apples this was $>8.12 \times 10^4$ ascospores/ml (Hartman *et al* 1999). It is thought that at high concentrations there may be some self-inhibition of spore germination (Brown 1922; Vloutoglou *et al* 1999).

6.1.2.2 Relative Humidity

Relative Humidity (RH) is a measure of the saturation of air by water vapour at a given temperature. As RH increases, the number of infections usually increases (Harrison 1984; Arauz & Sutton 1989; Eden *et al* 1996; Jhorar *et al* 1998). Various workers have reported different critical RH values for different plant/pathogen combinations by inoculation with dry spores or drying the spore suspension immediately after inoculation. For example, Williamson *et al* (1995) reported that *B. cinerea* caused lesions on roses at RH 94% or above, while for *B. fabae* (on beans), lesions were rarely seen at RH 86% but were numerous at RH 92-100% (Harrison 1984). For *Botryosphaeria obtusa*, most spore germination occurred at RH's above 95% (Arauz & Sutton 1989), for *Sclerotinia sclerotiorum* on kiwifruit flowers above RH 90% (Hoyte *et al* 2002) and for *Didymella rabiei* on chickpeas above RH 95%

(Jhorar *et al* 1998). The RH at which spores will germinate and cause infection is, however, greatly influenced by temperature and, in the absence of free water, higher temperatures may be required for germination and infection at lower RHs. Precise measurement and control of RH is extremely difficult, however, and literature on the subject should be carefully evaluated (Harrison *et al* 1994).

6.1.2.3 Temperature

The optimum temperature for the infection process varies and the optimum temperature for one stage may not be the optimum for another (Hong & Michailides 1998). The optimum temperature for conidial germination of *Sphaerotheca* macularis f. sp. *fragariae* was 20°C, but the maximal lesion expansion rates occurred at 25°C (Miller *et al* 2003). Germination of *B. obtusa* was less at lower than higher temperatures (Arauz & Sutton 1989). Gadoury & Pearson (1990) found that the percentage of ascospores of *Uncinula necator* that germinated and formed appressoria increased as the temperature increased from 10°C to 23°C, with no appressoria or infections at 5°C and 31°C, similarly Huang *et al* (2003) found the percentage of germinated ascospores of *Leptosphaeria maculans* that penetrated stomata of oilseed rape increased with increasing temperature from 5°C to 20°C.. The optimum temperature for infection of strawberries by *Colletotrichum acutatum* was between 25-30°C (Wilson *et al* 1990) and for *A. linicola* on linseed, disease severity was lower at 15°C than at 25°C (Vloutoglou *et al* 1999).

6.1.2.4 Free Water

For most fungal spores, germination and penetration events require water, and lack thereof changes or halts the infection process. Dry-inoculated conidia of *B. cinerea* produce short germ tubes only, with little extracellular adhesive (Williamson *et al* 1995; Cole *et al* 1996) whereas conidia in suspension produce long germ tubes (Cole *et al* 1996) and an extracellular adhesive matrix (Williamson *et al* 1995; Cole *et al* 1996). Braun & Howard (1994) showed that in *Cochliobolus heterostrophus*, this matrix was produced or secreted within the zone of hydration and was involved in the non-specific attachment of germlings to the surface, and free water (or high humidity) improved the adhesion of *Uromyces viciae-fabae* spores to the substratum (Beckett *et al* 1990). Similarly, Spotts & Holz (1996) found that *B. cinerea* conidia

adhered more strongly when applied in suspension (or to a wet surface) of grapes than when dry conidia were applied to a dry surface.

For powdery mildews, however, free water is not required for germination and infection (Butt 1978) and Yamaoka & Takeuchi (1999) noted that in *Erysiphe graminis*, the frequency of abnormal appressoria actually increased in water compared to dry inoculations.

6.1.2.5 Wetness Duration

Disease incidence is likely to increase with increasing wetness duration (excluding powdery mildews). For *D. rabiei* on chickpea, germination and germ tube penetration increased linearly with increased wetness duration, up to 18 h (Jhorar *et al* 1998). Mills (1944) developed a table showing the number of hours of leaf wetness required (at various temperatures) for infection of apples by *Venturia inaequalis* and many workers since then have shown that disease incidence increases with increased duration of leaf wetness (e.g. Hartman *et al* 1999; Nita *et al* 2003). Temperature also influences wetness duration, so that while 8 h was sufficient to initiate disease at 25°C by *A. linicola* in linseed, 10 h was required when the temperature was 15°C (Vloutoglou *et al* 1999).

6.1.2.6 Nutrients

Germination of *Botrytis* conidia may also be aided by the addition of nutrients. Various sugars, particularly glucose, have been shown to improve germination (Deverall & Wood 1961; Blakeman 1975; van den Heuval & Waterreus 1983) and increase the number of lesions and their rate of spread (Deverall & Wood 1961), but van den Heuval & Waterreus (1983) reported that glucose was not important for penetration. Amino acids (except taurine and lysine) stimulated germination (Blakeman 1975), xylose caused a decrease (Deverall & Wood 1961) and nitrogen compounds had little effect (Deverall & Wood 1961). Conidial germination may also be affected by nutrient leaching from the conidium by water (Brodie & Blakeman 1977). Leachates from the epidermis or pollen grains on the plant surface also provide nutrients for the spore (Blakeman 1980).

6.1.2.7 Light and Dark Periods

A continuous dark period following inoculation increased disease severity for *D. rabiei* and chickpea (Jhorar *et al* 1998) and *A. linicola* on linseed (Vloutoglou *et al* 1999).

6.1.3 Objectives

The objectives of this study were to:

a) develop and adapt inoculation techniques for use with *C. camelliae*/camellia petals.
b) assess these techniques on the basis of:
    1) data collected (quantitative or qualitative)
    2) reproducibility
    3) similarity to field observations
    4) ease of use (quick, simple, low technology).
c) produce a resistance rating scale.
d) test species for resistance using this rating scale.
e) investigate inconsistent and atypical results.

6.2 MATERIALS & METHODS

Camellia flowers were collected from plants at five sites around Palmerston North, three sites in the Wairarapa and three sites in Los Angeles, California. The sites are referred to by letter (Section 2.2.3) and indicated for each flower species in each experiment.

Flowers were fully opened (~2 d old) but still largely undamaged by weather and bird or insect activity; visibly damaged petals were discarded. Unless otherwise stated, flowers were collected in the morning and inoculated before evening on the day of collection. For single petal inoculation, petals were removed from the flower in order and placed in each of the control and treatment rows (or columns). Petaloids and the outer petals with sepal-like qualities were discarded. One flower from a species with a large number of petals (e. g. *C. japonica* 'Alba Plena') might comprise the total number of petals for an experiment, but for most species and varieties, the petals of several flowers were required. The petals of *C.* x *williamsii* x *C. japonica*

'Night Rider' were not used after initial tests, as *C. camelliae* symptoms did not show clearly on the black-red petals.

Apothecia were collected from three sites in New Zealand and two sites in Los Angeles, California. The sites are referred to by letter (Section 2.2.4) and indicated for each experiment. They were stored in containers containing wet paper towels at 4°C and remained in good condition for around three weeks, but were generally discarded after two weeks and fresh apothecia collected. Old apothecia turn from tan to darker brown, wither and/or grow various fungal infections. Alternatively, apothecia were collected as required from the sclerotial storage site at the end of the Agricultural Engineering Building, Massey University (Turitea Campus). Each apothecium could be used for approximately five days, depending upon the inoculation technique. Specific details of each inoculation technique are given with each Section.

After inoculation, the flowers or petals were placed in containers and incubated at 20°C in 12/12 L/D. The containers were 420 x 300 x 130 mm and lined on the base with wet paper towels. Insect screen mesh, cut to fit the base of the container, was laid on bottle tops to prevent the mesh from contacting the wet paper towels and the petals were laid on the mesh. The entire container was placed in a clear plastic bag and sealed to keep the container humid.

Control petals (either uninoculated or inoculated with water only) were a check on the current state of natural infections, which varied day-to-day and it ensured that the ascospores used for inoculation of each experiment were able to cause disease lesions. They were not generally used in the statistical analyses but where they were included details are given with the relevant experiments.

Data were recorded at 24 h intervals up to 120 h or when all the petals were completely diseased, which ever came first. An estimate was made of % disease area or a measure taken of the diameter of the lesion area. The variation in the rate at which lesions developed could be quite large between different experiments on different days. The day on which lesion development had not quite covered the

entire petal of the most susceptible species or variety was generally the day chosen for analysis, usually the 48 h or 72 h assessment data.

Data were analysed as described in Section 2.4. Specific tests and transformations are given with each experiment. Examples of SAS code are in Appendix II and statistical tables in Appendix IV (raw data on CD).

6.2.1 Inoculation by Ascospore Suspension

Ascospores were collected by suspending an apothecium upside down in a 28 ml McCartney bottle. The stipe of the apothecium was wrapped in moist facial tissues and plugged the mouth of the bottle. Ascospores were ejected onto the glass bottom and sides and suspended in water by rinsing the bottle with ~ 1 ml RO water. The ascospores from several apothecia were combined and the suspension diluted to the required ascospore concentration after counting on a haemocytometer. The suspension was agitated by hand and swirled with the micropipette tip between the application droplets to ensure the ascospores remained in suspension.

All petals were inoculated in the centre with a 3 μl droplet of a 1 x 10^6 ascospore/ml suspension unless stated otherwise (Experiments 6.2, 6.6, 6.7, 6.8, 6.20). Petals on flowers were also inoculated in the centre, but petals were selected for their exposure between other petals (Figure 6.1).

Figure 6.1
Diagram of six petal inoculation sites on the intact flowers. Inoculations with RO water shown in blue, inoculation with ascospore suspension in brown.

Inoculations of stamens and carpels (Experiments 6.25 and 6.26) used a 3 μl droplet of a 1 x 10^6 ascospore/ml suspension inoculated as shown in Figure 6.2. Browning at, and spread from, the inoculation site were considered to be likely *C. camelliae* infection.

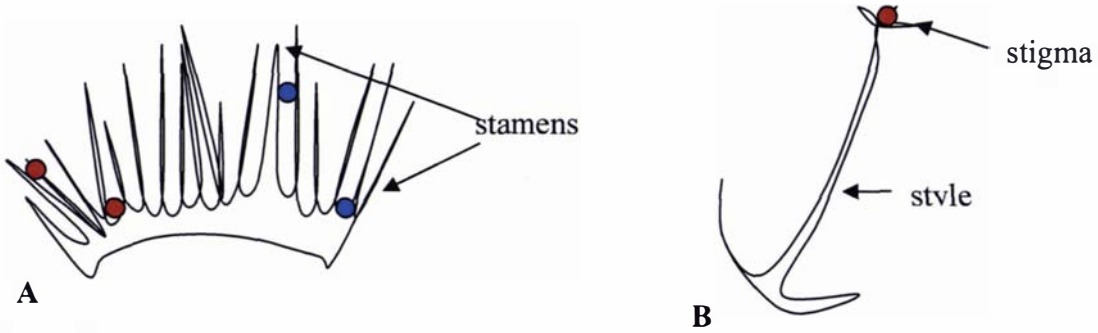


Figure 6.2
Illustration of inoculation positions on stamens and anthers. Blue circles indicate control droplets (water only), while brown circles indicate spore suspension droplets (A) Group of stamens with two water control inoculations and two spore inoculation sites. For each treatment, one inoculation site near the tip of the stamen and the other at the base. (B) Carpel with single inoculation site on stigma.

Inoculation of stamens and petals on intact flowers (Experiment 6.26) used a 3 µl droplet of a 1 x 10^6 ascospore/ml suspension inoculated as shown in Figure 6.3. Browning at, and spread from, the stamen inoculation site were considered likely *C. camelliae* infection, while petal lesions were measured as described below.

Figure 6.3
Side view of flower showing inoculation sites on petals and stamens: blue=RO water control; brown=spore suspension.

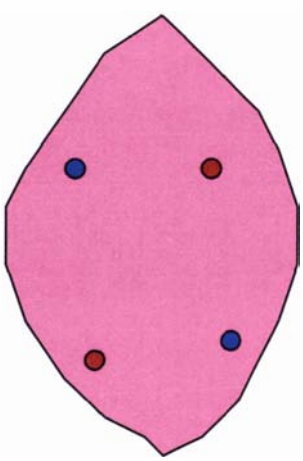


Figure 6.4
Opposing inoculation sites on a single petal: blue=RO water control, brown=ascospore suspension.

Inoculation of petals to study colour change (Experiments 6.27 and 6.28) used either a 3 µl droplet of 1 x 10^6 ascospores/ml or a 3 µl droplet of RO water (control). Each petal was inoculated at 4 points, two with water droplets and two with spore suspension droplets (Figure 6.4)

The length and width of the petal lesion was measured at 24 h intervals and the area of the disease lesion was calculated as the area of an oval:

$$A = \pi r_1 r_2 - \pi r_h^2$$

where A = lesion area

$r_1 r_2$ = radii of oval

r_h = radius of inoculation droplet.

Petals that developed natural infections were not included in the analysis, unless the infection did not interfere with the progress of the inoculated lesion. Experiments in which the ascospore suspension did not cause lesions, or in which natural infections were numerous, were discarded. Generally, natural infections could be distinguished from treatment inoculations by their location on the petal and their size (either more or less advanced than the treatment inoculations or more irregularly shaped).

Lesion area (using natural data or log transformed) at 48, 72, or 96 h was used for the analysis, in single- or two-factor ANOVAs, except for Experiments 6.27 and 6.28 where a logistic regression was fitted. Specifics are detailed with each experiment.

6.2.1.1 Validation of Inoculation Technique

Preparation of Ascospore Suspensions

Experiment 6.1

Aim:

a) to determine whether ascospore infectivity is affected by the centrifugation and vortexing required to concentrate weak ascospore suspensions.

The two treatments were ascospore suspensions (apothecia WBG) that had been 1) hand-shaken or 2) centrifuged at 13 000 rpm for 5 min then vortexed, applied to 20 petals each of *C. saluenensis* x *C. reticulata* 'Brian' (V).

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h data.

Short-term Storage of Ascospore Suspensions

Experiment 6.2

Aim:

a) to determine infectivity of 24 h old ascospore suspensions.

The five treatments were 1) 1 x 10^8, 2) 1 x 10^7, 3) 1 x 10^6, 4) 1 x 10^5, and 5) 1 x 10^4 ascospores/ml suspensions (apothecia WBG) applied to 10 petals per treatment of *C. saluenensis* x *C. reticulata* 'Brian' (V).

Each petal was inoculated with the ascospore suspension prepared 24 h earlier (stored at 4°C) and used in Experiment 6.6. Petals were incubated and lesions measured as described in Section 6.2. Data at 72 h was used for comparison.

Long-term Storage of Dry Ascospores or Ascospores in Suspension

Experiment 6.3

Aim:

a) to determine infectivity of ascospores over a period of two weeks.

The five treatments were 1) 24 h, 2) 6 d, 3) 9 d, 4) 13 d, and 5) 15 d old ascospores (apothecia WBG) applied to 10 petals per treatment of *C. pitardii* x *C. japonica* 'Nicky Crisp' (M).

Ascospores ejected onto the bottom of a McCartney bottle were stored dry at room temperature before preparing a suspension immediately prior to use. Petals were inoculated, incubated and lesions measured as described in Section 6.2. Data at 48 h was used for comparison.

Experiment 6.4

Aim:

a) to assess the infectivity of ascospores after long-term storage.

The four treatments were ascospores (apothecia WBG) stored for one year in 1) RO water at 4°C, 2) on base of McCartney bottle at 4°C, 3) on Pore etch filters + CaCl at

4°C (Steadman & Cook 1974) and 4) freshly collected ascospores applied to 10 petals per treatment of *C. saluenensis* x *C. reticulata* 'Brian' (V).

Petals were inoculated, incubated and lesions measured as described in Section 6.2. Data at 72 h was used for comparison.

Drying of Ascospore Suspensions

Experiment 6.5

Aim:

a) to determine whether drying the ascospore suspension after inoculation affects the infection process.

The two treatments were 1) wet inoculation (the normal inoculation method) and 2) dry (after inoculation, the droplet was dried using a hairdryer waved approximately 150 mm from the petals, with a temperature at the petals of around 50°C) (apothecia GL) applied to 20 petals per treatment of *C. japonica* x *C. saluenensis* 'E. G. Waterhouse'.

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data.

Concentration of Ascospore Suspensions

Experiment 6.6

Aim:

a) to determine the critical ascospore concentrations for resistance testing (i. e. minimum, maximum and optimum number of ascospores/ml).

The six treatments were 1) control (RO water), 2) 1×10^8, 3) 1×10^7, 4) 1×10^6, 5) 1×10^5, and 6) 1×10^4 ascospores/ml suspensions (apothecia WBG) applied to 10 petals per treatment of *C. pitardii* x *C. japonica* 'Nicky Crisp' (M).

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data.

Experiment 6.7

Aims:

a) to determine the critical ascospore concentrations for resistance testing (i. e. minimum, maximum and optimum number of ascospores/ml)
b) to evaluate lesion growth rates on a susceptible cultivar inoculated with ascospore suspensions
c) to compare between plant variation (of the same variety) with ascospore concentration.

The seven treatments were 1) control (RO water), 2) 1×10^8, 3) 1×10^7, 4) 1×10^6, 5) 1×10^5, 6) 1×10^4, and 7) 1×10^3 ascospores/ml suspensions (apothecia DG) applied to 10 petals per treatment of two *C. japonica* 'Alba Plena' plants (D) (Run 1: Plants 1 and 2: Run 2: Plants 3 and 4).

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A two-factorial ANOVA was performed on the 96 h log transformed data. The experiment was done twice (Run 1 and Run 2).

Inoculation by Potter Spray Tower

Potter (1952) developed the Potter Spray Tower for the precise and accurate application of insecticides in aerosol form but this equipment can also be used for applying fungal ascospore suspensions. The Potter Spray Tower consists of a) the atomiser connected to the liquid reservoir containing the fluid, b) a spray tube through which the atomised fluid is sprayed and c) the spray table. Compressed air sprays the fluid down the spray tube onto the spray table laid with sample/s. An even distribution of fluid is achieved by weighing the amount falling on microscope coverslips that are distributed over the spray table and adjusting the levelling of the apparatus.

Experiment 6.8

Aim:

a) to test the suitability of the Potter Spray Tower for inoculation of *Camellia* petals.

For this initial test, no attempt was made to calibrate the machine. The Potter Precision Laboratory Spray Tower (Burkhard Manufacturing Co. Ltd, Richmansworth, Herts. England) was used at 100 mm Hg to apply ascospores in suspension on to petals of *C. japonica* 'Desire'. Four applications were made, with six petals in each application. Each concentration was the ejecta of four apothecia (apothecia WBG). The four ascospore concentrations were: 1.4×10^5, 7.6×10^4, 7.1×10^4, and 8.0×10^3 ascospores/ml. The petals were incubated as described in Section 6.2.

Flower Variability – Overnight Storage

Experiments 6.9 and 6.10

Aim:

a) to determine whether overnight storage of flowers affects infection and lesion development.

The three treatments were petals inoculated 1) the day of collection (0 h), 2) stored at 4°C for 24 h or 3) stored at 20°C for 24 h (apothecia WBG) applied to 40 petals per treatment of *C. saluenensis* x *C. reticulata* 'Brian' (V).

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 48 h log transformed data.

Flower Variability – Variation between Flowers and between Plants

Experiment 6.11

Aim:

a) to determine variation between flowers from the same plant.

Eight petals from each of five flowers of *C. saluenensis* x *C. reticulata* 'Brian' (M) were inoculated (apothecia (WBG).

Petals were incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 48 h log transformed data. The experiment was done twice (Run 1 and Run 2).

Experiment 6.12

Aim:

a) to determine variation between flowers from different plants of the same cultivar.

The five treatment plants of *C. saluenensis* x *C. reticulata* 'Brian' were 1) Plant 1 (M), 2) Plant 2 (M), 3) Plant 3 (V), 4) Plant 4 (AgResearch) and 5) Plant 5 (HortResearch). The ascospore suspension (apothecia WBG) was applied to 20 petals per treatment.

Petals were inoculated, incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data.

Experiment 6.13

Aims:

a) to determine variation between flowers from the same plant

b) to determine variation between flowers from different plants of the same cultivar.

Six-eight petals from each of four flowers from each of four *C. japonica* 'Covina' plants (Plants 1, 2, 3 and 4) (D) were inoculated (apothecia DG).

Petals were inoculated, incubated and lesions measured as described in Section 2. A two-factorial ANOVA was performed on the 48 h data.

Experiment 6.14

Aims, inoculation and analysis (72 h log transformed data) as for Experiment 6.13.

Ten petals from each of six flowers from each of five *C. japonica* 'Alba Plena' plants (Plants 1, 2, 10, 11 and 12) (D) were inoculated (apothecia DG).

Experiment 6.15

Aims, inoculation and analysis as for Experiment 6.13.

Ten petals from each of three or four flowers from each of four *C. japonica* 'Alba Plena' plants (Plants 1, 2, 4 and 12) (D) were inoculated (apothecia DG).

6.2.1.2 Assessment of Species, Hybrid and Cultivar Resistance

Variation in Species Considered Susceptible

Aim of Experiments 6.16-6.19

a) to determine infection and lesion growth in susceptible species, hybrids and cultivars.

Experiment 6.16

Petals of four susceptible species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions measured as described in Section 6.2.

Experiment 6.17

Petals of nine susceptible species and hybrids (listed in results Section 6.3.1.2) were inoculated (apothecia GL), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data ('Bob's Tinsie' and 'Peach Blossom') and 96 h natural data ('Little Slam' and 'Prince Frederick William'). The analysis was split in two, as there was a large difference in lesion size between the two data sets.

Experiment 6.18

Petals of 11 susceptible species and hybrids (listed in results Section 6.3.1.2) were inoculated (apothecia WBG), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data ('Brian' (M) and 'E. G. Waterhouse' (M)) and 96 h data ('E. G. Waterhouse' (M) and (VN), 'Desire' (VC) and 'Prince Frederick William' (DP)). The analysis was split in two, as there was a large difference in lesion size between the two data sets.

Experiment 6.19

Petals of two susceptible species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data.

Differences between Species Considered Susceptible and Resistant

Aim for Experiments 6.20-6.24

a) to test infection and lesion growth in suspected resistant and susceptible species and cultivars.

Additional aim for Experiment 6.23

b) to test infection and lesion growth on intact flowers of suspected resistant and susceptible species and cultivars.

Experiment 6.20

Petals of four susceptible and three suspected resistant species (listed in results Section 6.3.1.2) were inoculated with a 1 x 10^5 ascospores/ml suspension (apothecia DG), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h data.

Experiment 6.21

Petals of seven susceptible species, three suspected resistant species and one non-Camellia species (listed in results Section 6.3.1.2) were inoculated (DG), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 48 h log transformed data (*C. saluenensis* and *C. japonica* 'Alba Plena') and 72 h data (*C. hiemalis* 'Shôwa-no-sakae' and *C. japonica* 'Alba Plena'). The analysis was split in two, as there was a large difference in lesion size between the two data sets

Experiment 6.22

Petals of two susceptible species and four suspected resistant species (listed in results Section 6.3.1.2) were inoculated (DG), incubated and lesions measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data.

Experiment 6.23

Petals of five susceptible species and one suspected resistant species and intact flowers of three susceptible species and one suspected resistant species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions

measured as described in Section 6.2. A single-factorial ANOVA was performed on the 72 h log transformed data (except for *C. saluenensis* at 48 h) (petal data only).

Experiment 6.24

Petals of five susceptible species and hybrids and one suspected resistant hybrid (listed in results Section 6.3.1.2) were inoculated (apothecia GL), incubated and lesions measured as described in Section 6.2. A single factor ANOVA was performed on the 72 h log transformed data.

Stamens and Stigma

Aim:

a) to determine susceptibility of stamens and stigma of a suspected resistant cultivar.

Experiment 6.25

Stigma and stamens of two suspected resistant species (listed in results Section 6.3.1.2) were inoculated (apothecia DG) and incubated as described in Section 6.2.

Experiment 6.26

Aim:

a) to determine susceptibility of stamens in suspected resistant and susceptible species.

Stamens and petals of four susceptible and three suspected resistant species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions measured as described in Section 6.2.

Colour Change at Inoculation Site

Aim for Experiments 6.27 and 6.28:

a) to examine colour change at site of inoculation.

Petals of two susceptible and two suspected resistant species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions measured as described in Section 6.2 (Figure 6.4).

A logistic regression was fitted to the number of 'Ginryû' petals at 48 h.

Experiment 6.28

Petals of three susceptible and one suspected resistant species (listed in results Section 6.3.1.2) were inoculated (apothecia DG), incubated and lesions measured as described in Section 6.2 (Figure 6.4).

A logistic regression was fitted to the number of 'Spring Mist' and 'Ginryû' petals at 48 h.

6.2.2 Inoculation by Airborne Ascospores

Dry ascospore inoculation of petals was achieved using the apparatus developed by (Taylor 1999). The apparatus consisted of an enclosed 330 x 330 x 360 mm container, an enclosed 120 x 80 x 65 mm box modified to fit inflow and outflow tubes, and an aquarium pump (1 L/min) and Dreschel bottle. The assembled apparatus is shown in Figure 6.5.

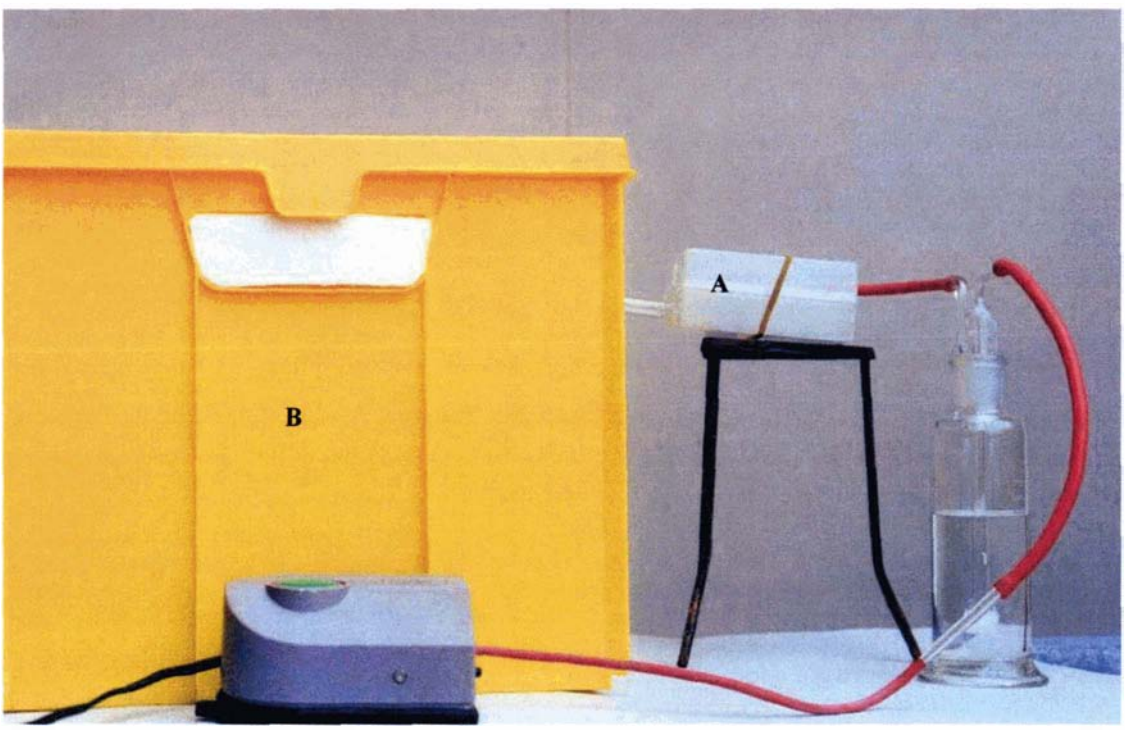


Figure 6.5
Ascospore inoculation apparatus developed by Taylor (1999). The aquarium pump blows air through water to saturate it, then over apothecia in the translucent box (A). Ascospores are blown into the yellow box (B) and settle out on petals laid in the bottom.

6.2.2.1 Calibration of a Settling Chamber

Experiment 6.29

Deposition of ascospores onto the floor of the settling chamber was assessed using glass slides and petals from cultivars known to be susceptible to infection of *C. camelliae*.

Five rows of five glass microscope slides (26 x 76 mm) were placed evenly on the base of the settling chamber and inoculated with ascospores. The number of ascospores/cm^2 on one square cm on each of the glass slides was counted. This was repeated five times.

Five rows of five petals of *C. japonica* 'Desire' were placed evenly on the base of settling chamber and inoculated with ascospores. This was repeated six times.

6.2.2.2 Resistance Assessment

Twenty-four suspected resistant and susceptible species and cultivars were tested in each of three experiments. Petals were inoculated using the airborne inoculation chamber. Petals were randomly assigned to one of 24 squares on the base of the chamber (Figure 6.6). Twelve replicate chambers were run (each replicate was called a 'block'), with one petal from each species or cultivar in each chamber (four flowers from each species or cultivar and three petals taken from each flower). A coverslip in the centre of the squares was used to count ascospores/cm^2.

1	2	3	4	5
6	7	8	9	10
11	12	coverslip	13	14
15	16	17	18	19
20	21	22	23	24

Figure 6.6
Layout of the petal chamber for inoculation by airborne ascospores. Each petal of each species or cultivar was assigned by random numbers to one of the 24 squares. The coverslip was laid in the centre square.

Three experiments of 12 blocks each was run with a total of 63 species and cultivars tested. Some species and cultivars were tested in more than one experiment. The percent area lesion was assessed at 24 h intervals after inoculation for 5 or 6 d.

The initial data for each experiment were inspected and six groups of response identified on the basis of mean infection level of different species/cultivars over time (this was to simplify the analysis). A repeated measures ANOVA was performed to test for differences between groups, species and cultivars within groups, and the effect of ascospore density on the progress of infections. The percentage data were transformed to logits (logit(p%)=log((p+1)/101-p) in order to keep them in the range 0-100%. Group and Cultivar-Within-Group were treated as categorical factors (as was Time); Spore Density was treated as a linear covariate. To allow for non-linear effects, Spore Density^2 was also included in the model. To test for different effects of ascospore density on different Groups, Group*Spore Density and Group*Spore Density^2 interaction were also included; as were Cultivar-Within-Group*Spore Density and Cultivar-Within Group*Spore Density^2.

Experiments 6.30-6.32

Species and cultivars used are shown in Tables 6.2-6.4 (apothecia WBG). The resistance or susceptibility found in prior testing is indicated where known.

Table 6.2 Species, Hybrids and Cultivars Tested in Experiment 6.30

Species/Hybrid/Cultivar	Resistant/Susceptible/Unknown
C. cuspidata (A)	R
C. fraterna (A)	R
C. grijsii (A)	R
C. japonica 'Aquarius' (A)	S
C. japonica 'Dixie Knight' (A)	S
C. joponica 'Mark Allan' (A)	S
C. longicarpa (A)	R
C. lutchuensis (A)	R
C. reticulata 'Simpatica' (A)	S
C. rosiflora 'Cascade' (A)	S
C. trichocarpa (A)	U
C. yunnanensis (A)	R
Hybrids	
C. hybrid 'Gay Baby' (A)	U
C. hybrid 'Fairy Wand' (A)	U
C. cuspidata x *C. saluenensis* 'Cornish Snow' (A)	U
C. cuspidata hybrid 'Spring Festival' (A)	U
C. japonica x C. lutchuensis 'Scentuous' (M)	U
C. lutchuensis x *C. japonica* 'Nymph' (M)	U
C. lutchuensis hybrid 'Fairy Blush' (A)	U
C. saluenensis x *C. reticulata* 'Brian' (M)	S
C. rusticana x *C. lutchuensis* 'Fragrant Joy' (A)	U
C. x *williamsii* 'E. G. Waterhouse' (M)	S
C. x *williamsii* 'Mirage' (A)	U
C. x *williamsii* 'Softly' (A)	S

Table 6.3 Species, Hybrids and Cultivars Tested in Experiment 6.31

Species/Hybrid/Cultivars	Resistant/Susceptible/Unknown
C. japonica 'Blood of China' (DP)	U
C. japonica 'Dahlohnega' (DP)	U
C. japonica 'Easter Morn' (JV)	U
C. japonica 'Elegans Supreme' (DP)	U
C. japonica 'Guilio Nuccio' (DP)	U
C. japonica 'Laurie Bray' (A)	U
C. japonica 'Lemon Drop' (DP)	U
C. japonica 'Little Red Riding Hood' (DP)	U
C. japonica 'Mary Paige' (DP)	U
C. japonica 'Nuccio's Jewel' (DP)	U
C. japonica 'Roger Hall' (DP)	U
C. japonica 'Silver Chalice' (DP)	U
C. japonica 'Sugar Babe' (DP)	U
C. japonica 'The Czar' (DP)	U
C. japonica 'Twilight (DP)	U
'Cox's Fancy' (DP) (not found in ICR)	U
'Diorella' (DP) (not found in ICR)	U
'Melody Time' (DP) (not found in ICR)	U
Hybrids	
C. hybrid 'Punkin' (DP)	U
C. reticulata hybrid 'Lovely Lady' (DP)	U
C. saluenensis x *C. reticulata* 'Brian' (M)	S
C. x *williamsii* 'E. G. Waterhouse' (M)	S
C. x *williamsii* 'Les Jury' (DP)	U
C. x *williamsii* 'Rose Holland' (DP)	U

Table 6.4 Species, Hybrids and Cultivars Tested in Experiment 6.32

Species/Hybrid/Cultivars	Resistant/Susceptible/Unknown
C. fraterna (A)	R
C. japonica 'Dixie Knight Supreme' (A)	U
C. japonica 'Dolly Dyer' (A)	U
C. japonica 'Grand Slam' (DP)	U
C. japanica 'Lady Loch' (M)	U
C. japonica 'Leonora Novick' (A)	U
C. japonica 'Maroon and Gold' (A)	U
C. japonica 'R. L. Wheeler' (A)	U
C. japonica 'Ville de Nantes' (M)	U
C. japonica 'Zambo' (A)	U
C. pitardii var. *pitardii* 'Sprite' (V)	U
C. reticulata 'Zhangjia Cha' (A)	U
C. rosiflora 'Cascade' (A)	S
'Ameniflora' (M) (not found in ICR)	U
'Ole Hybrid' (DP) (not found in ICR)	U
Hybrids	
C. cuspidata x *C. saluenensis* 'Cornish Snow' (A)	U
C. cuspidata hybrid 'Spring Festival' (A)	U
C. pitardii x *C. japonica* 'Nicky Crisp' (M)	U
C. saluenensis x *C. reticulata* 'Warwick Berg' (M)	U
C. x *williamsii* 'E. G. Waterhouse' (M)	S
C. x *williamsii* 'Mirage' (A)	U
C. x *williamsii* 'Rendezvous' (A)	U
C. x *williamsii* 'Rose Bouquet' (A)	U
C. x *williamsii* 'Softly' (A)	U

6.2.2.3 Field Inoculation

This technique was designed to evaluate the use of dry ascospores to infect flowers *in situ* on *Camellia* plants outdoors.

Experiment 6.33

Apothecia (D) were suspended in 28 ml McCartney bottles with the stipe and sclerotium wrapped in wet tissue and blocking the bottle entrance. To release the ascospores the apothecium was removed from the bottle and held close to the flower. After several seconds a visible cloud of ascospores was released. Each treated flower was marked by a twist tie and assessed at 72 h. Inoculations were carried out at Descanso Gardens and The Huntington over January and February. The species and varieties tested were: *C. cuspidata* (D), *C. fraterna* 15476-D (H), *C. hiemalis* 'Shishigashira' (D), *C. japonica* 'Alba Plena' (D), *C. japonica* 'Alba Plena Imp' 11838 (H), *C. japonica* 'Katherine Nuccio' 11466-A (H), *C. japonica* 'Pink Snow' 13634-E (not found in any reference book), *C. japonica* 'Otto Hopfer' 43722 (H), *C. maliflora* 12851-K (H), *C.* x *vernalis* 'Ginryû' 17871 (H).

6.3 RESULTS

6.3.1 Inoculation by Ascospore Suspension

6.3.1.1 Validation of Inoculation Technique

Preparation of Ascospore Suspensions

Experiment 6.1

There was no evidence that infection and growth were affected by centrifuging and vortexing the ascospore suspension ($F_{1,\ 32}$=0.00, p<0.9906). At 72 h the average lesion size on the control petals was 658 mm^2 (SE=38) (n=17) compared to 658 mm^2 (SE 37) (n=18) on the petals inoculated with treated ascospore suspension.

Short-term Storage of Ascospore Suspensions

Experiment 6.2

Few lesions developed at inoculation sites in any of the treatments. No lesions (except natural infections) developed on petals inoculated with 1 x 10^8 or 1 x 10^4 ascospores/ml suspensions. At 1 x 10^7 and 1 x 10^5 ascospores/ml infections developed on 2/10 petals, and at 1 x 10^6 4/10 petals developed infections.

Long-term Storage of Dry Ascospores or Ascospores in Suspension

Experiment 6.3

No lesions (except natural infections) developed on petals inoculated with 15 d, 13 d, 9 d, or 6 d old spore suspensions while half the petals inoculated with the 24 h old spore suspension developed lesions.

Experiment 6.4

None of the ascospore suspensions prepared from dry-stored ascospores initiated disease lesions. Natural infections were visible on some petals in all treatments. The freshly collected ascospores caused disease lesions in 6/10 petals.

Drying of Ascospore Suspensions

Experiment 6.5

There was evidence for a difference between wet and dry inoculation treatments ($F_{1, 22}$=84.76, p<0.0001). Symptom development was quicker and spread more quickly on the wet inoculated petals (912 mm^2, SE 73, n=17) than on the petals on which the spore suspension was dried immediately after inoculation (122 mm^2, SE 58, n=7).

Concentration of Ascospore Suspensions

Experiment 6.6

There was evidence that ascospore concentration affected lesion development and growth ($F_{4, 23}$=5.61, p<0.0027) (analysis on 72 h data). LS Means showed that the biggest lesions were from the 1 x 10^6 ascospores/ml concentration, followed by 1 x 10^7, 1 x 10^5, and then 1 x 10^4 and 1 x 10^8 which were not significantly different from each other (Figure 6.7).

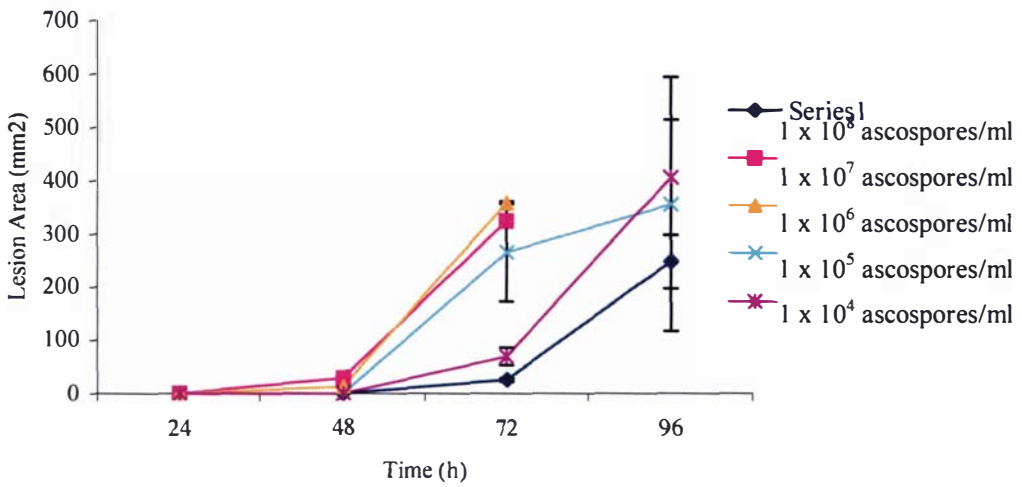


Figure 6.7
Effect of ascospore concentration on lesion growth on 'Nicky Crisp' (natural data) in Experiment 6.6.

Experiment 6.7

Run 1: there was evidence for differences in lesion growth between plants of the same cultivar ($F_{1, 66}$=6.35, p<0.0142) with the average lesion area of Alba Plena' Plant 1 273 mm^2 (SE 31) compared with 149 mm^2 (SE 31) for Plant 2. There was no evidence that different ascospore concentrations affected lesion development and growth ($F_{5, 66}$=1.16, p<0.3380) or of a plant*concentration interaction ($F_{4, 66}$=1.95,

$p<0.1125$). The average lesion area for each plant at each ascospore concentration is shown in Table 6.5

Table 6.5 Average Lesion Area for Each Plant of 'Alba Plena' at a Range of Ascospore Concentrations (natural data) in Experiment 6.7 (Run 1)

Ascopore Concentration (ml)	'Alba Plena' Plant 1			'Alba Plena' Plant 2		
	Average Lesion Area (mm^2)	SE	n	Average Lesion Area (mm^2)	SE	n
1×10^8	-	-	-	69	28	8
1×10^7	201	53	8	48	22	4
1×10^6	235	79	9	163	44	5
1×10^5	260	75	9	416	121	5
1×10^4	340	78	9	160	65	6
1×10^3	322	64	9	59	38	5

Run 2: no lesions developed on petals of Plants 3 and 4 inoculated with 1×10^3, 1×10^4, 1×10^5 or 1×10^6 ascospores/ml suspensions. Data from 1×10^7 and 1×10^8 ascospores/ml were used in the analysis.

There was no evidence for differences in lesion growth between plants of the same cultivar ($F_{1, 28}=0.10$, $p<0.7535$), or that different ascospore concentrations affected lesion development and growth ($F_{1, 28}=2.28$, $p<0.1425$) but there was evidence for a plant*concentration interaction ($F_{1, 28}=14.43$, $p<0.0007$) (Figure 6.8).

For 'Alba Plena' Plant 3, lesion area was greater at the lower ascospore concentration of 1×10^7 ascospores/ml (647 mm^2, SE 51, n=6) with the average lesion area 325 mm^2 (SE 48, n=9) at 1×10^8 ascospores/ml. In contrast, 'Alba Plena' Plant 4. average lesion area (376 mm^2, SE 56, n=8) was smaller from 1×10^7 ascospores/ml and larger (489 mm^2, SE 64, n=9) from 1×10^8 ascospores/ml.

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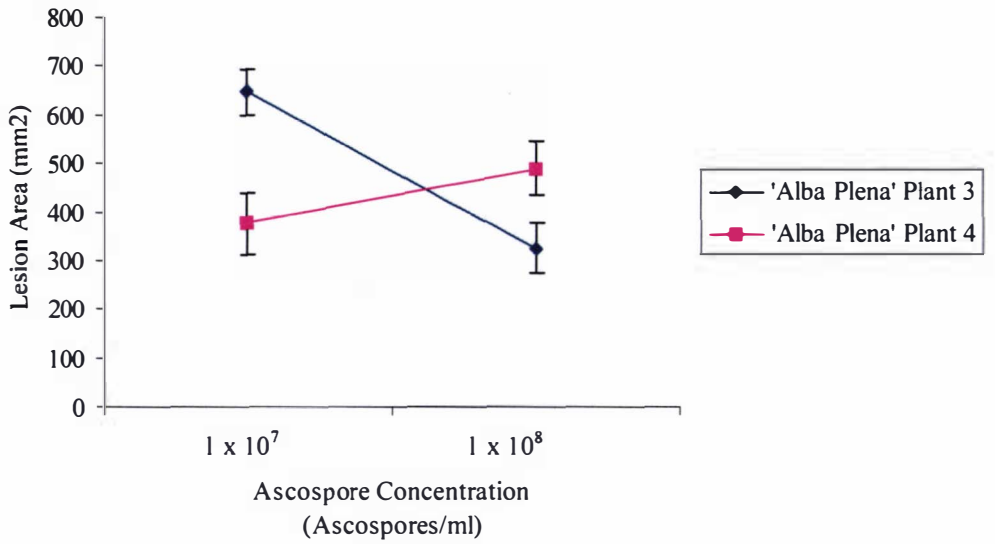


Figure 6.8
Significant plant*concentration interaction for Experiment 6.7 (Run 2).

Inoculation by Potter Spray Tower

Experiment 6.8

All petals in all four inoculations developed multiple disease lesions, with the first small brown spots visible 24 h after inoculation. At 1.4 x 10^5 and 7.1 x 10^4 ascospores/ml concentrations the lesions were especially large on the wings of the petals (Figure 6.9). It was not known whether this was because of the aerodynamics of the curved petal affecting ascospore settlement or whether these outer thinner petal sections were more susceptible or showed lesions more quickly.

Figure 6.9
Petals of *C. japonica* 'Desire' 48 h after inoculation with a 7.1 x 10^4 ascospores/ml suspension by Potter Tower, Experiment 6.8.

Flower Variability – Overnight Storage

Experiments 6.9 and 6.10

In both experiments, flowers stored for 24 h at 20°C did not develop lesions but were paler at the inoculation site (Figure 6.10). There was a significant difference in lesion size of flowers stored for 24 h at 4°C and flowers that were inoculated the day of collection (Experiment 6.9 $F_{1, 76}=3.98$, $p<0.0496$; Experiment 6.10 $F_{1, 62}=7.17$, $p<0.0095$). Lesions on freshly collected flowers were larger (Experiment 6.9 96 mm^2, SE 10, n=4; Experiment 6.10 32 mm^2, SE 3, n=36) than lesions on flowers that had been stored at 4°C (Experiment 6.9 69 mm^2, SE 6, n=38; Experiment 6.10 18 mm^2, SE 2, n=28) (natural data). Natural infections were present in all three treatments.

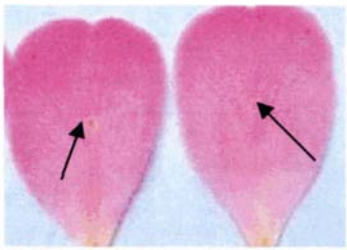


Figure 6.10
Petals stored for 24 h at 20°C, Experiment 6.9. At the inoculation site (arrows), the petals were bleached of colour.

Flower Variability – Variation between Flowers and between Plants

Experiment 6.11

Run 1: there were significant differences in lesion growth and development between petals of flowers from the same plant ($F_{4,35}$=18.35, p<0.0001).

Run 2: there were no differences in lesion growth and development between flowers from the same plant ($F_{4,31}$=1.39, p<0.2616).

Experiment 6.12

There was evidence for differences between flowers on different plants from the five plants of hybrid 'Brian' in Palmerston North ($F_{4,77}$=10.72, p<0.0001) (Table 6.6)

Table 6.6 Average Lesion Area 72 h after Inoculation in Experiment 6.12 (natural data)

Location of *C. saluenensis* x *C. japonica* 'Brian' Plants	No. Infected/No. of Petals (%Disease Incidence)	Average Lesion Area (mm^2) (SE)
VN	19/20 (95)	95 (10)
M – near Humanities Building	14/20 (70)	30 (6)
AgResearch	7/20 (35)	35 (10)
HortResearch	19/20 (95)	82 (9)
M – near Student Union	19/20 (95)	45 (5)

Experiment 6.13-6.15

In all three experiments, the Mean Square for the difference between plants was not significantly higher than the Mean Square for the difference between flowers on the same plant, indicating that there was as much variation between several flowers from the same plant as there was between flowers from different plants (Experiment 6.13 1718.93/2432.15=0.71, compared to a $F_{3,\ 7}$ df distribution p=0.5761; Experiment 6.14 93856.86/16882.76=5.56, compared to a $F_{25,257}$ df distribution p=1.1803;

Experiment 6.15 423.86/57.30=7.40, compared to a $F_{3,11}$ df distribution p=0.0055) (ANOVA tables Appendix IV).

Similarly, in all three experiments, the Mean Square for the difference between flowers was considerably larger than the Mean Square for the difference between petals from the same flower indicating that the variation between flowers (due to age, position on plant etc.) was considerably greater than the variation between petals from the same flower. (Experiment 6.13 2432.15/218.83=11.11, compared to a $F_{7,13}$ df distribution p=0.0001; Experiment 6.14 93856.86/16882.76=5.56, compared to a $F_{25,257}$ df distribution p=1.1803; Experiment 6.15 57.30/18.30=3.13, compared to a $F_{11,114}$ df distribution p=0.0010) (ANOVA tables Appendix IV).

6.3.1.2 Assessment of Species, Hybrid and Cultivar Resistance

Variation in Species Considered Susceptible

Experiment 6.16

Of the four species inoculated, disease lesions developed on only one (Table 6.7).

Table 6.7 Disease Lesion Area at 72 h after Inoculation in Experiment 6.16 (natural data)

Species/Cultivar	No. Infected/No. of Petals (%Disease Incidence)	Average Lesion Area (mm^2) (SE)
C. japonica 'Alba Plena' #4 (D)	10/20 (50)	245 (74)
C. sasanqua 20727 (H)	0/20	-
C. sasanqua 'Moon Moth' 14894-G (H)	0/10	-
C. x *vernalis* 'Ginryû' 12624F (H)	0/20	-

Experiment 6.17

There was a significant difference between the average lesion size of *C. japonica* 'Bob's Tinsie' and *C. japonica* 'Fleur Dipater' at 72 h ($F_{1,35}$=8.79, p<0.0054), but at 96 h, there was no significant difference between the average lesion size of *C. japonica* 'Prince Frederick William' and *C. japonica* 'Little Slam' ($F_{1,10}$=0.01, p<0.9204) (ANOVA tables Appendix IV) (Table 6.8). After 96 h no infections from inoculation were observed on 'Bizarre', 'Botanyuki' 'Lily Pons', 'Hagoromo' or 'Tiny Princess'. One natural infection was observed on each of 'Lily Pons' and 'Tiny Princess'.

Table 6.8 Disease Lesion Area at 72 or 96 h after Inoculation in Experiment 6.17 (natural data)

Species/Hybrids/Cultivar	No. Infected/No. of Petals (%Disease Incidence)	Average Lesion Area (mm^2) (SE)
C. japonica 'Bizarre'[a]	0/20	-
C. japonica 'Bob's Tinsie' (DP)	17/20 (85)	140 (19)[b]
C. japonica 'Fleur Dipater' (VC)	20/20 (100)	284 (29)[b]
C. japonica 'Lily Pons' (VC)	0/20	-
C. japonica 'Little Slam' (DP)	5/20 (25)	215 (68)[c]
C. japonica 'Hagoromo' (VC)	0/20	-
C. japonica 'Prince Frederick William' (DP)	7/20 (35)	205 (64)[c]
C. rusticana 'Botanyuki' (VC)	0/20	-
Hybrid		
C. japonica x *C. fraterna* 'Tiny Princess' (VC)	0/20	-

[a] name not found in any reference book, most likely a *C. japonica*
[b] analysis at 72 h
[c] analysis at 96 h

Experiment 6.18

There was no significant difference between the average lesion size of *C. saluenensis* x *C. reticulata* 'Brian' and *C.* x *williamsii* 'E. G. Waterhouse' (M) at 72 h ($F_{1, 22}$=3.92, p<0.0603) while at 96 h there were significant differences between the average lesion size of 'Brian', 'Desire' (VC), 'Prince Frederick William' and *C.* x *williamsii* 'E. G. Waterhouse' (VN) ($F_{3, 23}$=3.57, p<0.0300) (ANOVA tables Appendix IV) (Table 6.9). The Tukey's groupings are shown in Table 6.10. After 96 h no infections from inoculation were observed on 'Sweet Emily Kate', 'Cinnamon Cindy', 'Desire' (A), 'Softly', and 'Cornish Snow'. Natural infections were observed on 'Desire' (A).

Table 6.9 Disease Lesion Area at 72 or 96 h after Inoculation in Experiment 6.18 (natural data)

Species/Hybrids/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
C. japonica 'Desire' (VC)	10/20 (50)	179 (61)[b]
C. japonica 'Desire' (A)	0/20	-
C. japonica 'Prince Frederick William' (DP)	2/20 (10)	447 (120)[b]
C. japonica 'Softly' (A)	0/20	-
Hybrids		
C. cuspidata x *C. saluenensis* 'Cornish Snow' (A)	0/20	-
C. japonica x *C. lutchuensis* 'Cinnamon Cindy' (DP)	0/20	-
C. japonica x *C. lutchuensis* 'Sweet Emily Kate' (DP)	0/20	-
C. saluenensis x *C. reticulata* 'Brian' (M)	12/20 (60)	150 (70)[a]
		706 (134)[b]
C. x *williamsii* 'E. G. Waterhouse' (VN)	12/20 (60)	657 (279)[b]
C. x *williamsii* 'E. G. Waterhouse' (M)	12/20 (60)	203 (41)[a]

[a] analysis at 72 h
[b] analysis at 96 h

Table 6.10 Tukey's Test for Significant Differences In Lesion Area Between *Camellia* species at 96 h after Inoculation with a *C. camelliae* Ascospore Suspension, Experiment 6.18

Tukey's Grouping[a]	Mean	N	Species/Cultivar
a	2.70	3	*C. saluenensis* x *C. reticulata* 'Brian
a	2.66	12	*C.* x *williamsii* 'E. G. Waterhouse' (VN)
a	2.63	2	*C. japonica* 'Prince Frederick William'
b	1.98	10	*C. japonica* 'Desire'

[a] numbers followed by the same letter are not significantly different by Tukey's test ($p<0.05$)

Experiment 6.19

There was no evidence for a difference in lesion size between *C. japonica* 'Alba Plena' Plant 15 and *C. japonica* 'Bella Rosa' at 72 h ($F_{1,21}=0.37$, $p<0.5473$) (ANOVA table Appendix IV) (Table 6.11).

Table 6.11 Lesion Area at 72 h after Inoculation in Experiment 6.19 (natural data)

Species/Hybrids/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
C. japonica 'Alba Plena' Plant 15 (D)	14/20 (70)	878 (214)
C. japonica 'Bella Rosa'[a] (D)	9/20 (45)	1,150 (630)

[a] name not found any reference book, most likely *C. japonica*

Differences between Species Considered Susceptible and Resistant

Experiment 6.20

There were significant differences in lesion size between the three susceptible species that developed lesions ($F_{2,16}=120.28$, $p<0.0001$) (ANOVA table Appendix IV) (Table 6.12). The Tukey's groupings are shown in Table 6.13.

Table 6.12 Lesion Area at 72 h after Inoculation in Experiment 6.20 (natural data)

Species/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
Susceptible		
C. hiemalis 'Shishigashira' (D)	5/40 (5)	82 (8)
C. hiemalis 'Shôwa-no-sakae' (D)	3/30 (10)	60 (3)
C. japonica 'Alba Plena' Plant 10 (D)	11/20 (55)	452 (35)
C. saluenensis (D)	1/10 (10)	90[a]
Resistant		
C. cuspidata (D)	0/20	-
C. fraterna (D)	0/30	-
C. lutchuensis (D)	0/30	-

[a] at 48 h

Table 6.13 Tukey's Test for Significant Differences In Lesion Area Between *Camellia* species at 72 h after Inoculation with a *C. camelliae* Ascospore Suspension, Experiment 6.20

Tukey's Grouping[a]	Mean	N	Species/Cultivar
a	2.64	11	*C. japonica* 'Alba Plena' Plant 10
b	1.91	5	*C. hiemalis* 'Shishigashira'
b	1.77	3	*C. hiemalis* 'Shôwa-no-sakae'

[a] numbers followed by the same letter are not significantly different by Tukey's test ($p<0.05$)

Experiment 6.21

Overall, lesions developed on three of the seven susceptible species, while no lesions developed on the three resistant species (*C. cuspidata*, *C. fraterna* and *C. lutchuensis*) or non-host *Magnolia salicifolia.* The rate of lesion growth on petals of *C. saluenensis* was much greater than that on *C. japonica* 'Alba Plena' and *C. hiemalis* 'Shôwa-no-sakae'. Since lesions were evident 24 h after inoculation, the maximum lesion area was measured at 48 h, with 100% of the petal area diseased 72 h after inoculation. At 48 h, there was evidence for significant differences in lesion area between *C. saluenensis* 12851-H and on *C. japonica* 'Alba Plena' ($F_{1,31}=69.57$, $p<0.0001$) (ANOVA table Appendix IV) (Table 6.14). There was also evidence for significant differences in lesion growth between *C. japonica* 'Alba Plena' and *C. hiemalis* 'Shôwa-no-sakae' at 72 h ($F_{1,23}=19.10$, $p<0.0002$) (ANOVA table Appendix IV).

Table 6.14 Lesion Area at 48 or 72 h after Inoculation in Experiment 6.21 (natural data)

Species/Hybrid/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
Susceptible		
C. hiemalis 'Shôwa-no-sakae' (D)	5/20 (25)	51 (18)[b]
C. japonica 'Alba Plena' Plant 1 and 10 (D)	13/20 (65)	13 (3)[a]
	20/20 (100)	220 (28)[b]
C. rosiflora (D)	0/20	-
C. saluenensis 12851-H (H)	20/20 (100)	97 (9)[a]
C. sasanqua 'Moon Moth' 14894-G (H)	0/20	-
C. x *vernalis* 'Ginryû' 14897-M (H)	0/20	-
C. saluenensis x *C. cuspidata* 'Cornish Snow' 15426 (H)	0/20	-
Resistant		
C. cuspidata (D)	0/20	-
C. fraterna (D)	0/20	-
C. lutchuensis (D)	0/20	-
Non-Camellia Species		
Magnolia salicifolia 14398 (H)	0/10	-

[a] analysis at 48 h
[b] analysis at 72 h

Experiment 6.22

There was evidence for significant differences in lesion size between *C. japonica* 'Alba Plena' and *C. japonica* 'Covina' at 72 h ($F_{1,22}$=42.46, p<0.0001) (ANOVA table Appendix IV) (Table 6.15). Disease lesions did not form on any of the suspected resistant species.

Table 6.15 Lesion Area 72 h after Inoculation in Experiment 6.22 (natural data)

Species/Hybrid/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
Susceptible		
C. japonica 'Alba Plena' Plant 2 (D)	4/20 (20)	479 (33)
C. japonica 'Covina' Plant 4 (D)	20/20 (100)	96 (38)
Resistant		
C. grijsii 'Juhuacha' (D)[a]	0/16	-
C. grijsii 'Zhenzhu Cha' (D)[a]	0/4	-
C. lutchuensis x *C. japonica* 'Koto-no-kaori' (D)	0/19	-
C. lutchuensis x *C. japonica* 'Minato-no-akebono' (D)'	0/10	-

[a] name not found in any reference book, named by Tim Thibault, Descanso Gardens

Experiment 6.23

No disease lesions developed on *C. cuspidata* x *C. saluenensis* 'Cornish Snow' or *C. grijsii* 'Juhuacha' from either single petal inoculations or intact flower inoculations. At 48 h the lesions on *C. saluenensis* petals were more advanced than those on other species, and by 72 h the petals were 100% infected.

There was evidence for significant differences in lesion size between *C. hiemalis* 'Showa Supreme', *C. japonica* 'Alba Plena' and *C.* x *vernalis* 'Ginryû' at 72 h ($F_{2,36}$=17.33, p<0.0001) (ANOVA table Appendix IV) (Table 6.16). There was no difference in lesion areas on *C. japonica* 'Alba Plena' and *C.* x *vernalis* 'Ginryû', but those on *C. hiemalis* 'Showa Supreme' were significantly smaller than on the other two (Table 6.17).

Table 6.16 Lesion Area at 48 or 72 h after Inoculation in Experiment 6.23 (natural data): Petal Inoculations

Species/Hybrid/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
Susceptible		
C. hiemalis 'Shôwa-Supreme' 13634C (H)	13/20 (65)	43 (9)
C. japonica 'Alba Plena' #4 (D)	22/30 (73)	197 (27)
C. *saluenensis* 12851 (H)	20/20 (100)	46 (5)[a]
C. x *vernalis* 'Ginryû' 14897-M (H)	4/20 (20)	165 (26)
C. cuspidata x *C. saluenensis* 'Cornish Snow' 15426 (H)	0/20	-
Resistant		
C. grijsii 'Juhuacha' (D)	0/18	-

[a] 48 h data

Table 6.17 Tukey's Test for Significant Differences In Lesion Area on Petals of *Camellia* species at 72 h after Inoculation with a *C. camelliae* Ascospore Suspension, Experiment 6.23

Tukey's Grouping[a]	Mean	N	Species/Cultivar
a	5.05	22	*C. japonica* 'Alba Plena' (D)
a	5.08	4	*C.* x *vernalis* 'Ginryû' (H)
b	3.55	13	*C. hiemalis* 'Showa Supreme' (H)

[a] numbers followed by the same letter are not significantly different by Tukey's test ($p<0.05$)

While four of the twenty inoculated *C.* x *vernalis* 'Ginryû' petals developed lesions, sixteen developed pinkish blotches at and around the inoculation site. This was the second time colour change had been observed at or around the inoculation site, and as it was apparently not an aberration, this phenomenon was explored further in Section 6.2.1.2 Colour Change.

Experiment 6.24

Of the four species that developed disease lesions, there was no significant difference in lesion size between species ($F_{3,34}=2.24$, $p<0.1012$) (ANOVA table Appendix IV) and no infections from inoculation were observed on 'Dahlohnega' and 'Fairy Blush', but natural infections were present on 'Dahlohnega' (Table 6.18).

Table 6.18 Disease Lesion Area 72 h after Inoculation in Experiment 6.24 (natural data)

Species/Cultivar	No. Infected/No. of Petals (% Disease Incidence)	Average Lesion Area (mm^2) (SE)
Susceptible Species		
C. japonica 'Blood of China' (DP)	8/20 (40)	190 (41)
C. japonica 'Dahlohnega' (DP)	0/20	-
C. japonica 'Great Eastern' (JV)	11/20 (55)	354 (109)
C. reticulata x *C. japonica* 'Hulyn Smith' (DP)	9/20 (45)	655 (144)
C. saluenensis x *C. reticulata* 'Brian' (JV)	10/20 (50)	392 (108)
Resistant		
C. lutchuensis hybrid 'Fairy Blush' (A)	0/20	-

Stamens and Stigma

Several experiments (not reported) using intact flowers of *C. yunnanensis* infected by airborne ascospores showed that the infection first began in the stamens (Figure 6.11 A) and then spread down through the stamens and into the petals (Figure 6.11 B).

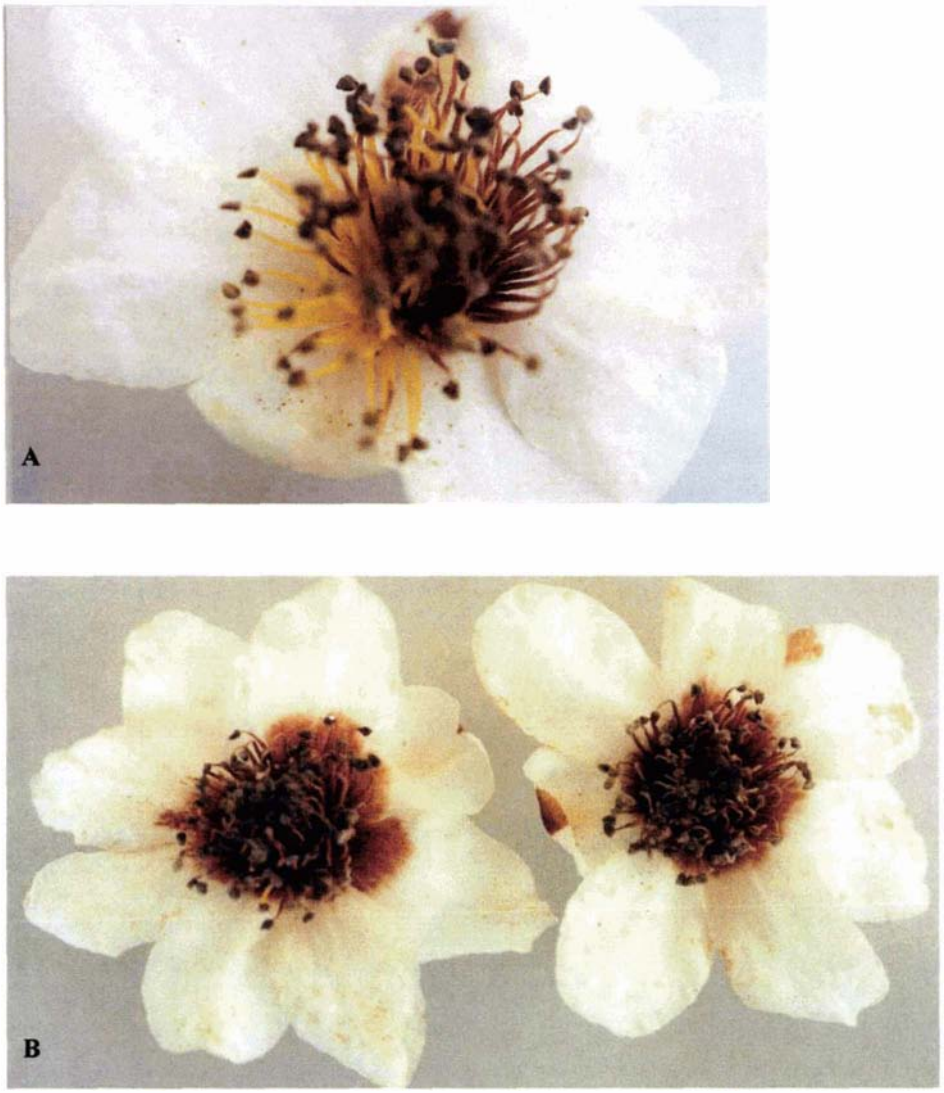


Figure 6.11
C. yunnanensis inoculated by airborne ascospores. (A) No infections were visible on the petals, but many of the stamens had turned brown (48 h). (B) 96 h after inoculation by airborne ascospores all the stamens are brown and disease symptoms are spreading from the base of the petals where they meet the stamens, up into the rest of the petal. Note that symptoms are not seen on any other area of the petals

Experiments 6.25

The results were not statistically analysed, as the browning symptoms could not definitively be identified as *C. camelliae*. Attempts to reisolate the pathogen from brown sections were unsuccessful due to contamination by other fungi. Browning

symptoms developed on 1/28 water-only control inoculations compared to 12/28 ascospore-inoculated stamens (Table 6.19).

Table 6.19 Browning Symptoms on Stamens and Stigma 72 h after Inoculation in Experiment 6.25

Hybrids	No. Brown/ No. of Stigma	No. Brown/No. of Stamens (Control)	No. Brown/No. of Stamens
C. japonica x *C. lutchuensis* 'Minato-no-haru'	0/7	0/14	6/14[a]
C. japonica x *C. lutchuensis* 'Koto-no-kaori'	4/4	1/14[a]	6/14[a]

[a] all browning symptoms developed on inoculations at the base of the stamen on the 'skirt' rather than those higher up the stamen

Experiment 6.26

At 24 h after inoculation, many natural infections were visible on the petals and by 48 h these had overtaken many of the petal inoculation sites. On the stamens it was difficult to determine whether browning symptoms originated from the inoculation site (Table 6.20).

Table 6.20 Browning Symptoms on Stamens 48 h after Inoculation in Experiment 6.26

Species/Cultivar	No. Brown/ No. of Stamens (Control)	No. Brown/ No of Stamens	No. Brown/No of Petals (Control)	No. Brown/No. of Petals
C. cuspidata	9/9	9/9	5/9	6/9
C. fraterna	6/7	4/7	0/7	0/7
C. japonica 'Flame'	6/6	6/6	6/6	6/6
C. japonica 'Sarasa'	7/7	6/7	6/7	7/7
C. japonica 'Stella Sewell'	0/3	0/3	3/3	3/3
C. lutchuensis	0/12	2/12	0/12	0/12
C. saluenensis x C. *cuspidata* 'Cornish Snow'	4/6	5/6	8/8	8/8

Colour Change at Inoculation Site

Experiments 6.27 and 6.28

In Experiment 6.27 only petals of 'Ginryû' developed a pink colour change at the spore inoculation site (Figure 6.12). Petals of 'Alba Plena' developed typical disease lesions but petals of 'Minato-no-haru' and 'Koto-no-kaori' did not become infected (Table 6.20). Similarly, petals of 'Ginryû' and 'Spring Mist' in Experiment 6.28 (Figure 6.13) developed a pink colour change at the ascospore inoculation site while petals of 'Alba Plena' developed typical disease lesions (Table 6.21).

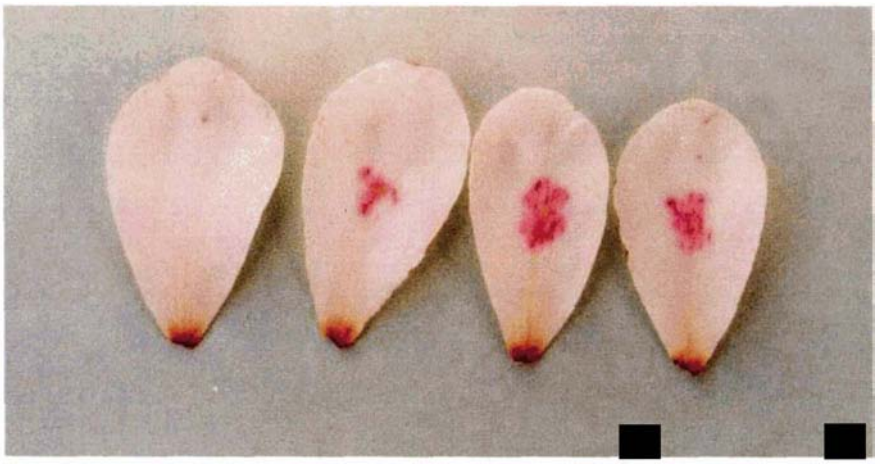


Figure 6.12
Example of colour change on *C.* x *vernalis* 'Ginryû' in Experiment 6.21. Water-inoculated control (left), three petals inoculated with ascospore suspension (right). Around the inoculation site, uneven sections of the petal developed pink colouring. Small brown 'threads' were visible in these sections, but over six days, neither the pink nor the brown 'threads' visibly changed their intensity or shape.

Table 6.21 Colour Change at Inoculation Site for Experiment 6.27

Species/Hybrid/Cultivar	No. Infected/No. Inoculated (water-only[a])	No. Infected/No. Inoculated[a]
C. japonica 'Alba Plena' #4 (D)	0/10	9/12 (disease lesions)
C. japonica x *C. lutchuensis* 'Minato-no-haru' (D)	0/16	0/16
C. japonica x *C. lutchuensis* 'Koto-no-kaori' (D)	0/16	0/16
C. x *vernalis* 'Ginryû' (H) 13624F	0/14	12/13 (colour change)

[a] two inoculation sites per petal

In both experiments, there was evidence that ascospore inoculation caused the colour change at the inoculation site ($p<0.0001$) (Logistic regression tables Appendix IV). In Experiment 6.28, colour change was observed at two control inoculation sites on 'Ginryû' (Table 22). The experiments were observed over six days, during which the size, shape and colour of the 'spots' on 'Ginryû' and 'Spring Mist' did not change after first developing at 48 h.

Figure 6.13
Colour change on 'Spring Mist' in Experiment 6.28. The petals exhibit the deep pink colour change around the spore inoculation site (lower left and upper right of each petal). The water control inoculations have no effect (upper left and lower right of each petal).

Table 6.22 Treatment and Control Petals of Species and Cultivars for Experiment 6.28

Species/Hybrid/Cultivar	No. Infected/No. Inoculated (water-only[a])	No. Infected/N. Inoculated[a]
C. japonica 'Alba Plena' Plant 15 (D)	0/16	16/16 (disease lesions)
C. japonica x *C. lutchuensis* 'Spring Mist' (N)	0/12	12/12 (colour change)
C. x *vernalis* 'Ginryû' (H) 13624F	2/16	11/12 (colour change)

[a] two inoculation sites per petal

Both experiments gave evidence that the colour change was in response to ascospore inoculation. While colour change was consistent, however, the pattern of colour was not similar between species. While 'Spring Mist' formed single dark rings around the inoculation site, on 'Ginryû' the colour change was more amorphous with several darker centres surrounded by lighter regions (Figure 6.14).

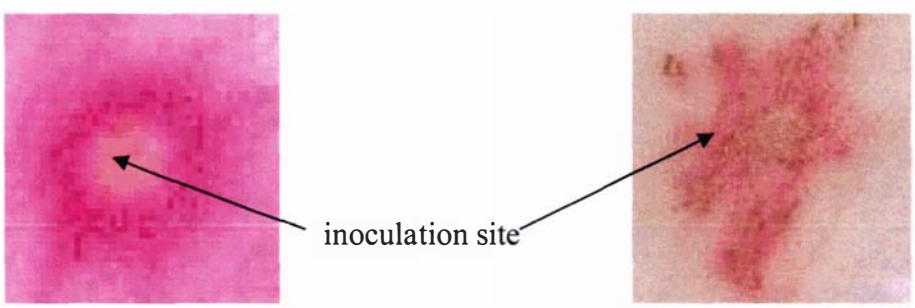


Figure 6.14
Comparison of colour change site on 'Spring Mist' (left) and 'Ginryû' (right) after inoculation with an ascospore suspension.

At two control inoculation sites, colour change was also observed in 'Ginryû'. It is unknown why this occurred, but given the high natural infection rate at that time, it is possible ascospores were already on the petal surface and that the water inoculation in some way activated either these ascospores or the plant's resistance mechanism.

Lesion Growth on Susceptible Species

The combined results of experiments reported in this Chapter for ascospore inoculation of susceptible species are shown in Table 6.23. Although lesion area data from different experiments is not directly comparable, it is presented here in order to a) collate often disparate lesion growth data over time and b) create a reference for others doing similar testing. For example, the table shows that *C. saluenensis* lesion development was rapid in comparison to other susceptible species tested, and that lesion measurements were not possible after 48 h. This information is useful when designing experiments, and for comparison of results of future researchers.

Table 6.23 Lesion Area from Combined Results for Resistance Testing of Susceptible Species

Highly Susceptible			
Species/Cultivar	48 h mm^2 (SE, n)	72 h mm^2 (SE, n)	96 h mm^2 SE, n)
C. saluenensis	103 (28, 49)	-	-
Susceptible			
Species/Cultivar	48 h mm^2 (SE, n)	72 h mm^2 (SE, n)	96 h mm^2 (SE, n)
C. japonica 'Alba Plena'	13 (3, 13)	395 (78, 506)	-
C. japonica 'Bella Rosa'	-	1150 (630, 9)	-
C. japonica 'Blood of China'	-	190 (41, 8)	-
C. japonica 'Bob's Tinsie'	-	140 (19, 17)	-
C. japonica 'Covina'	29 (10, 24)	96 (38, 20)	-
C. japonica 'Desire'	-	-	179 (61, 10)
C. japonica 'Fleur Dipater'	-	-	284 (26, 20)
C. japonica 'Great Eastern'	-	354 (109, 11)	-
C. japonica 'Little Slam'	-	-	215 (68, 5)
C. japonica 'Prince Frederick William'	-	-	326 (121, 9)
C. reticulata x *C. japonica* 'Hulyn Smith'	-	655 (144, 9)	-
C. saluenensis x *C. reticulata* 'Brian'	64 (32, 76)	203 (41, 12)	706 (134, 12)
C. x *williamsii* 'E. G. Waterhouse'	-	557 (355, 29)	681 (25, 15)
Moderately Susceptible			
Species/Cultivar	48 h mm^2 (SE, n)	72 h mm^2 (SE, n)	96 h mm^2 (SE, n)
C. hiemalis 'Shishigashira'	-	82 (8, 5)	-
C. hiemalis 'Shôwa-no-sakae'	-	55 (4, 8)	-
C. hiemalis 'Showa Supreme'	-	43 (9, 12)	-
C. x *vernalis* 'Ginryû'	-	217 (52, 7)	-

6.3.2 Inoculation by Airborne Ascospores

6.3.2.1 Calibration of a Settling Chamber

Experiment 6.29

The distribution of ascospores was fairly even, with ascospores counted in each cm^2, but the number of ascospores/cm^2 was always higher in one corner of the box. The number of ascospores/cm^2 varied greatly between tests because the release of ascospores could not be controlled.

All petals developed 'pock' marks within 24-48 h and these rapidly grew into lesions that covered the entire petal. As described in Section 6.3.2 (Inoculation by Potter Tower), lesion spread, or ascospore number, was greater on the wings of the petals. There was little difference in lesion area between petals at 48 h and no apparent difference after 72 h.

6.3.2.2 Resistance Assessment

Experiment 6.30

An initial inspection of the effect of species/cultivar and ascospore density on disease progress showed that the effects differed between species/cultivars (Figure 6.15) although some common patterns emerged. In 'Brian' (Figure 6.15 A), disease lesions were visible within 24-48 h and lesions spread rapidly until 100% of the petal area was infected. In *C. cuspidata* (Figure 6.15 B), only 3/12 petals developed lesions, with symptoms first visible after 48-72 h but then spreading rapidly until they covered the petal. In *C. trichocarpa* (Figure 6.15 C), 1/12 petals became infected and the infection was not visible until 144 h after inoculation.

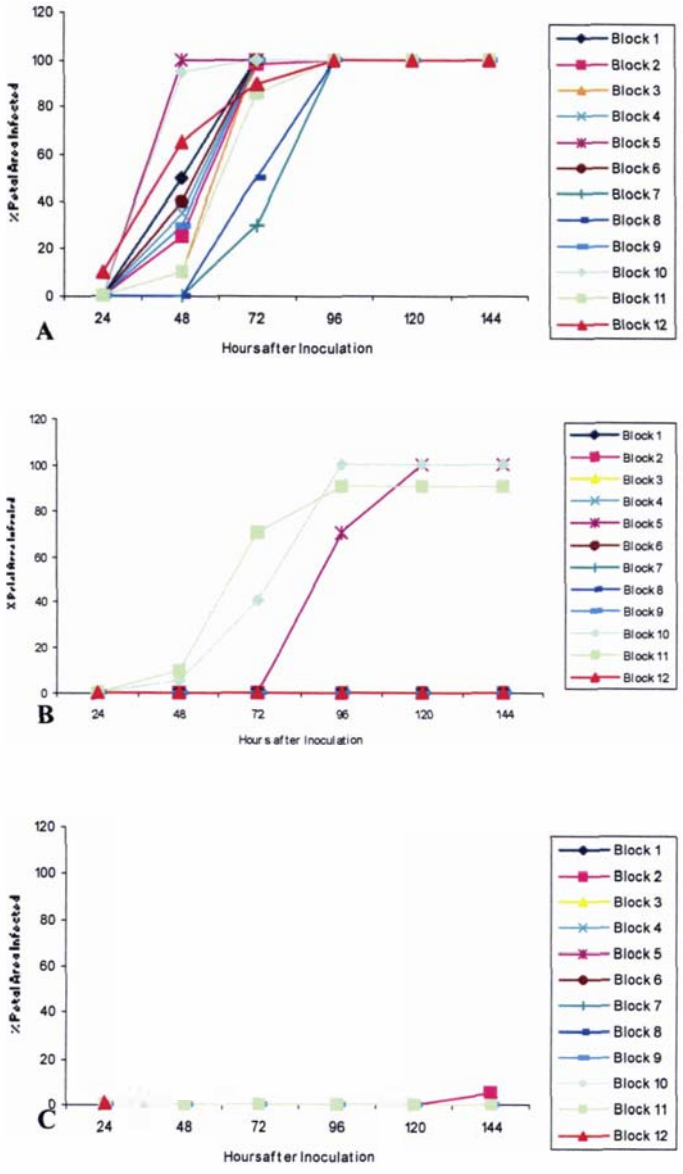


Figure 6.15 A-C
Responses to infection shown by three species/cultivars in Experiment 6.29 (natural data). (A) *C. saluenensis* x *C. japonica* 'Brian', early and rapid spread of infection. (B) *C. cuspidata*, slower symptom development. (C) *C. trichocarpa*, late symptom development and low incidence of infection.

Six groups of species/cultivar were identified on the basis of mean lesion spread over time (Figure 6.16). Group 1 had the most rapid response, with lesions covering the petals within 96 h of inoculation. Group 2 lesion spread was less rapid than that of Group 1 and Group 3 slower still, not reaching 100% petal coverage even after 144 h incubation. Members of Group 4 had medium spread of lesion development by 144 h, while low rates of spread were found in Group 5 and very little lesion development or no disease development in members of Group 6 (Table 6.24).

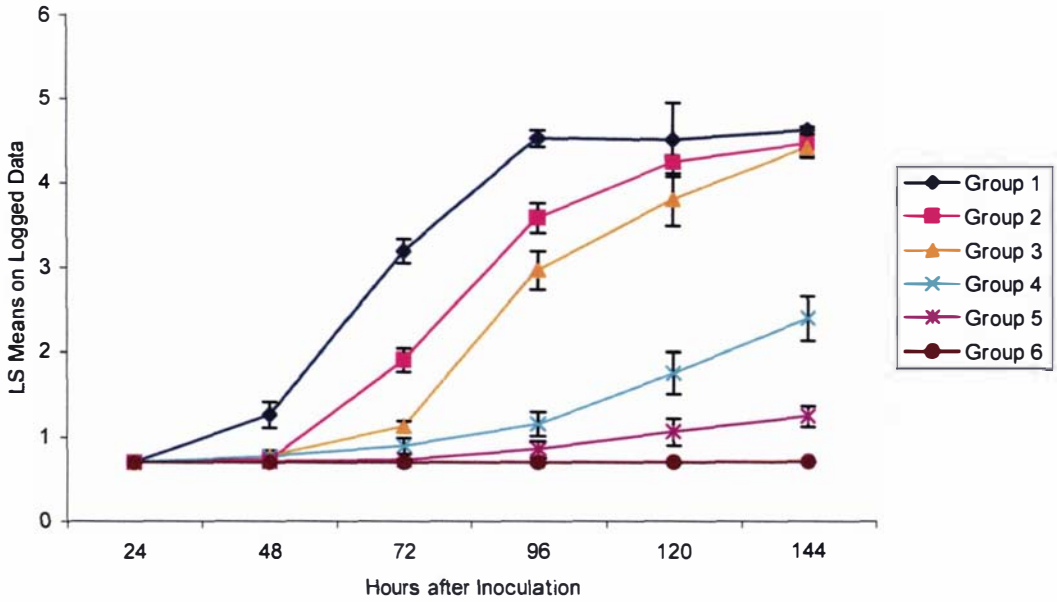


Figure 6.16
(Group average) LS Means of species and cultivars grouped by response to lesion development over time, Experiment 6.30. Six main patterns of response were identified.

Table 6.24 Response of each Species/Cultivar by Group over Time, Experiment 6.30

Species/Cultivar	% Lesion Area (LS Means on Logged Data)					
	24 h	48 h	72 h	96 h	120 h	144 h
Group 1						
'Brian'	0.69314	0.69764	1.3871	3.32314	4.62497	4.62497
'E. G. Waterhouse'	0.69314	0.70296	0.96097	2.90615	4.33110	4.49326
'Simpatica'	0.69314	0.69314	1.43456	3.34374	4.62497	4.62497
Group 2						
'Aquarius'	0.69314	0.69314	0.70846	1.76820	3.40660	4.29732
'Fairywand'	0.69314	0.69314	0.73376	2.04084	3.75642	4.17597
Group 3						
'Mark Allen'	0.69314	0.69314	0.81993	1.24960	3.06285	3.74976
'Nymph'	0.69314	0.69314	0.81057	0.93825	3.50447	4.62497
'Dixie Knight'	0.69314	0.69314	0.72548	1.10320	2.40402	3.11469
'Gay Baby'	0.69314	0.69314	0.72009	1.18962	2.87955	3.70073
Group 4						
'Spring Festival'	0.69314	0.7171	1.04478	1.18707	1.5110	2.43849
'Fragrant Joy'	0.69314	0.69314	0.69314	0.71150	0.82329	1.28180
'Mirage'	0.69314	0.69314	0.70451	1.027663	1.065733	1.5393
'Scentuous'	0.69314	0.69314	0.713	0.83904	1.45732	2.23736
C. rosaeflora 'Cascade'	0.69314	0.69314	0.69314	0.69531	0.89891	1.245321
Group 5						
C. japonica 'Softly'	0.69314	0.69314	0.69981	0.78146	1.22723	1.490514
C. cuspidata	0.69314	0.69314	0.71150	0.72629	0.88850	1.39035
C. grijsii	0.69314	0.69314	0.69314	0.69314	0.70934	0.75237
C. longicarpa	0.69314	0.69314	0.69314	0.69314	0.72160	0.85391
'Cornish Snow'	0.69314	0.69314	0.69314	0.695311	0.70197	0.78900
Group 6						
C. fraterna	0.69314	0.69314	0.69314	0.69314	0.69314	0.69314
C. lutchuensis	0.69314	0.69314	0.69314	0.69314	0.69314	0.69314
C. trichocarpa	0.63914	0.69314	0.69314	0.69314	0.69314	0.69314
C. yunnanensis	0.69314	0.69314	0.69314	0.69314	0.69314	0.69314
'Fairy Blush'	0.69314	0.69314	0.69314	0.69314	0.69314	0.69314

The tests of sphericity were significant ($p<0.0001$) indicating strong correlations between observations at different times. Because of this the multivariate tests of repeated effects (ANOVA and MANOVA tables Appendix IV) were used.

Averaged across the six observation periods, the Groups had significantly different mean levels of disease ($F_{5,216}=149.24$, $p<0.0001$), and the mean level was also (marginally) influenced by the spore density (in a non-linear fashion, as both the linear ($F_{1,216}=3.08$, $p<0.0800$) and quadratic ($F_{1,216}=3.09$, $p<0.0800$) Spore Density terms were marginally significant).

However, looking at the Within-Subject effects, it was clear that the overall mean disease development varied across Time (F_5, 199.93, $p<0.0001$), that this pattern varied between Groups (F_{25}, 24.33, $p<0.0001$) (Time*Group interaction although the variation of Cultivars-Within-Groups was not significant), and that the effect of Spore Density varied with Time and Group (linear F_{25}, 2.40, $p<0.0002$ and quadratic F_{25}, 1.56, $p<0.0394$).

To investigate these differences further, the ANOVAs for each observation period's data (which covered the same factors and interactions as the Between-Subjects effects, but applied to a single period's data) were inspected.

An examination of disease progress showed no significant differences between the six groups of camellias after 24 h but Groups 1 and 4 had significantly more disease than the other groups after 48 and 72 h ($F_{5,\ 216}=10.72$, $p<0.0001$ and $F_{5,\ 216}=54.12$, $p<0.0001$ respectively). By 96 h, Groups 1, 2, 3 and 4 had higher levels of infection than the others ($F_{5,\ 216}=96.29$, $p<0.0001$) and at 120 h, Groups 1, 2 and 3 had the greatest amount of disease, Group 4 less, and Groups 5 and 6 the least ($F_{5,\ 216}=114.23$, $p<0.0001$). At this time the Cultivar-within-Group effect was significant ($F_{18,\ 216}=1.71$, $p<0.0400$) and Table 6.22 shows that while members of Group 3 were closely grouped during earlier days, by 120 h, the group is less concentrated, particularly 'Nymph'. At 144 h there were significant differences in the overall levels of disease between Group ($F_{5,\ 216}=128.13$, $p<0.0001$) with Groups 1, 2 and 3

with the highest levels of disease, followed by Group 4. Group 5 had little disease development and Group 6 very little disease development.

The relationship between ascospore density and the level of infection for each day are shown in Appendix VI (Experiments 6.30-32). While some groups showed an increase in disease development with increasing ascospore density, others decreased or did not change. Overall the results were not consistent.

Experiment 6.31

The initial inspection of disease progress in both ascospore density and the species or cultivar being tested showed significant differences. Examples of the response are similar to those in Experiment 6.30 (Figure 6.15) with some common patterns of response. Six groups of camellias were again identified by plotting the mean lesion area of different species and cultivars over time (Figure 6.17). Group 1 had the most rapid response, reaching 100% petal diseased by 120 h. Group 2 was somewhat less rapid. The initial rapid response of Group 3 slowed and did not reach 100% lesion area by 144 h. Members of Group 4 attained medium disease development by 144 h while members of Group 5 attained mid-low disease development by 144 h. No or low levels of disease were seen in members of Group 6 (Table 6.25).

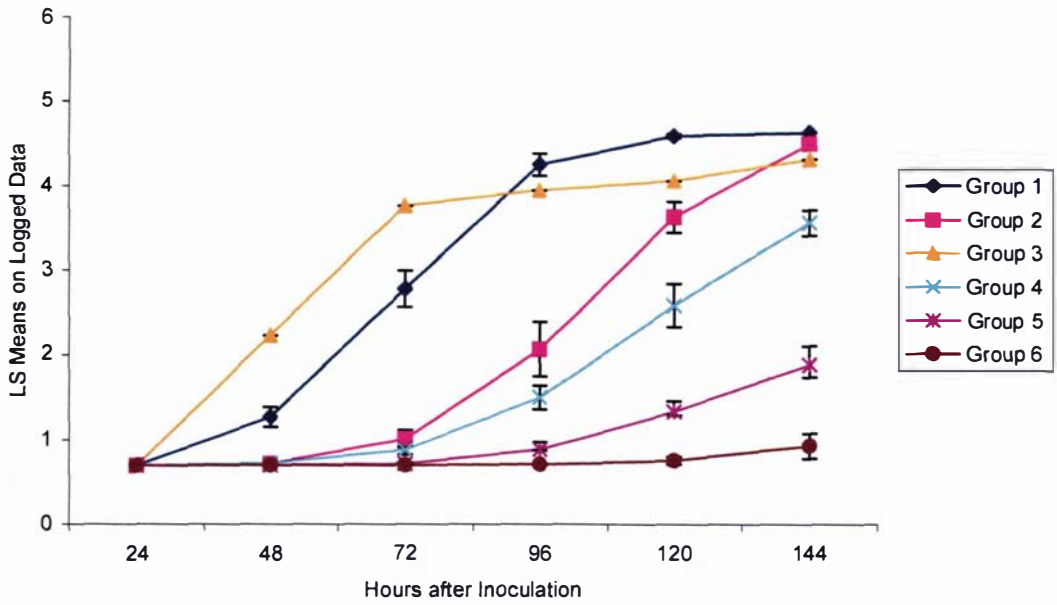


Figure 6.17
(Group average) LS Means of species and cultivars grouped by response to lesion development over time, Experiment 6.31. Six main patterns of response were identified.

Table 6.25 Response of each Species/Cultivar by Group over Time, Experiment 6.31

Species/Cultivar	% Lesion Area (LS Means on Logged Data)					
	24 h	48 h	72 h	96 h	120 h	144 h
Group 1						
'Diorella'	0.69314	1.53144	3.23367	4.62497	4.62497	4.62497
'E. G. Waterhouse'	0.69314	1.69818	2.49358	4.31015	4.62497	4.62497
'Elegans Supreme'	0.69314	0.72346	1.69836	3.39118	4.43938	4.62497
'Lemon Drop'	0.69314	0.94364	2.310895	4.18956	4.49326	4.62497
'Mary Paige'	0.69314	1.68623	3.18112	4.62497	4.62497	4.62497
'Punkin'	0.69314	0.887681	2.163273	4.067701	4.62497	4.62497
'Roger Hall'	0.69314	1.37944	3.49	4.62497	4.62497	4.62497
'Silver Chalice'	0.69314	1.36557	3.00885	4.08864	4.62497	4.62497
'Sugar Babe'	0.69314	1.19923	3.42671	4.30767	4.62497	4.62497
Group 2						
'Little Red Riding Hood'	0.69314	0.71333	0.81428	1.43333	3.31372	4.56721
'Lovely Lady'	0.69314	0.70450	1.16085	2.49513	3.948111	4.43938
'Nuccio's Jewel'	0.69314	0.72234	1.05199	2.28258	3.61870	4.49326
Group 3						
'Brian'	0.69314	2.23053	3.75831	3.94093	4.05604	4.30767
Group 4						
'Cox's Fancy'	0.69314	0.73213	0.99320	1.78037	3.08589	3.97886
'Easter Morn'	0.69314	0.72053	1.01609	1.69868	2.97587	3.446176
'Laurie Bray'	0.69314	0.71080	0.80409	1.67259	3.19814	3.99657
'Melody Time'	0.69314	0.71059	0.74921	1.13781	2.48553	3.57468
'Rose Holland'	0.69314	0.69531	0.71800	0.98451	1.63486	3.23595
'Twilight'	0.69314	0.75235	0.98394	1.71362	2.12678	3.100204
Group 5						
'Dahlohnega'	0.69314	0.69531	0.72142	1.03232	1.15911	1.45010
'Les Jury'	0.69314	0.69314	0.69747	0.71999	1.27851	2.12218
'The Czar'	0.69314	0.71261	0.723958	0.89810	1.56900	2.08896
Group 6						
'Blood of China'	0.69314	0.69314	0.69314	0.69747	0.70720	0.77478
'Guillio Nuccio'	0.69314	0.69531	0.69963	0.71395	0.79821	1.076087

The tests of sphericity were significant ($p<0.0001$) and the multivariate tests of repeated effects (ANOVA and MANOVA tables Appendix IV) were used for analysis.

Averaged across the six observation periods, the Groups had significantly different disease development ($F_{5,216}=128.46$, $p<0.0001$), and the disease was also influenced by the ascospore density in a linear fashion ($F_{1,216}=5.13$, $p<0.0246$).

The overall mean disease development for Within-Subject effects varied across Time (F_5, 551.24, $p<0.0001$), between Groups (F_{25}, 25.03, $p<0.0001$), between Cultivars-Within-Groups (F_{90}, 1.41, $p<0.0094$). The effect of Spore Density^2 varied with Time (F_5, 3.14, $p<0.0094$) and the effect of Spore Density varied with Time and Group (F_{25}, 2.22, $p<0.0006$).

To investigate these differences further, the ANOVAs were inspected for each observation period's data (which covered the same factors and interactions as the Between-Subjects effects, but applied to a single period's data).

An examination of disease progress showed no significant differences between the six groups of camellias after 24 h but at 48 and 72 h, Groups 1 and 3 had greater amounts of disease development than the others ($F_{5,216}$=19.91, $p<0.0001$ and $F_{5,216}$=56.68, $p<0.0001$ respectively). The significant Cultivar-Within-Group effect ($F_{18,216}$=2.10, $p<0.0069$) related to 'Lovely Lady' in Group 2. On this day, 'Lovely Lady' had significantly lower disease development than the other members of the group, but by Day 5 had rejoined the main group. By 96 h, Groups 1 and 3 again had greater disease development than the others ($F_{5,216}$=124.51, $p<0.0001$), and as at 72 h, 'Lovely Lady' in Group 2 had less disease development than the other members of Group 2, resulting in the significant Cultivar-Within-Group effect ($F_{18,216}$=1.72, $p<0.0380$). By 120 and 144 h, Groups 1, 2, 3, and 4 had the greatest disease development ($F_{5,216}$=88.96, $p<0.0001$ and $F_{5,216}$=86.35, $p<0.0001$ respectively).

Experiment 6.32

The initial inspection of disease development between both ascospore density and the species or cultivar being tested showed significant differences. Examples of the response are similar to those in Experiment 6.30 (Figure 6.15) with some common patterns of response. Six groups of camellias were again identified by plotting the mean disease development of different species and cultivars over time (Figure 6.18). Group 1 had a rapid response, reaching 100% petal area diseased by 120 h. Group 2 initially had the most rapid response but after 72 h disease development plateaued. Members of Group 3 had an intermediate response of disease development, while Group 4 had a slow response. Group 5 initially tracked Group 4, but disease development increased rapidly after 96 h. Group 6 had consistently little disease development (Table 6.26).

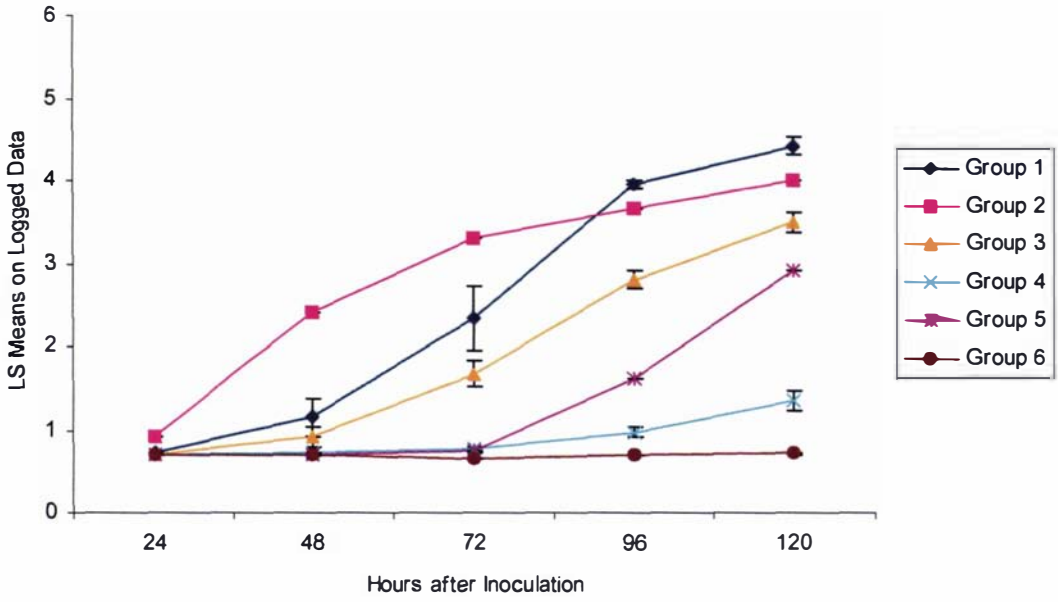


Figure 6.18
(Group average) LS Means of species and cultivars grouped by disease development over time, Experiment 6.32. Six main patterns of response were identified.

Table 6.26 Response of each Species/Cultivar by Group over Time, Experiment 6.32

Species/Cultivar	% Lesion Area (LS Means on Logged Data)				
	24 h	48 h	72 h	96 h	120 h
Group 1					
'Lady Loch'	0.698058	0.785365	1.600948	4.009969	4.272445
'Rose Bouquet'	0.736498	1.098875	2.534771	3.852278	4.624973
'Warwick Berg'	0.700815	1.564053	2.877765	3.97497	4.350833
Group 2					
'Zhangjia Cha'	0.906909	2.420748	3.291095	3.667127	3.993395
Group 3					
'Ameniflora'	0.703855	0.772699	1.10097	2.604092	3.361816
'Dolly Dyer'	0.693147	0.790977	1.702226	3.116416	3.707634
'E. G. Waterhouse'	0.693147	0.800161	1.466599	2.984994	3.723534
'Nicky Crisp'	0.707148	1.671204	2.412073	3.094017	3.589671
'Ole Hybrid'	0.693147	0.92027	1.744306	2.521539	2.8883
'R. L. Wheeler'	0.639147	0.751157	1.835989	2.919491	3.712544
'Zambo'	0.693147	0.710238	1.378867	2.381529	3.439518
Group 4					
'Cornish Snow'	0.693147	0.693147	0.703855	0.906909	1.050586
'Grand Slam'	0.714804	0.850484	1.066947	1.22795	1.576769
'Leonora Novick'	0.693147	0.703855	0.765429	1.111567	1.802324
'Maroon and Gold'	0.693147	0.693147	0.695507	0.760858	1.193345
'Mirage'	0.693147	0.695507	0.717894	0.926281	1.651735
'Rendezvous'	0.693147	0.693147	0.709391	0.872141	1.160015
'Spring Festival'	0.693147	0.693147	0.695507	0.906909	1.050586
'Sprite'	0.693147	0.709508	0.805599	1.305437	1.767823
'Ville de Nantes'	0.693147	0.693147	0.698058	0.763367	0.906909
Group 5					
'Dixie Knight Supreme'	0.693147	0.705725	0.758499	1.613295	2.910223
Group 6					
C. fraterna	0.693147	0.693147	0.693147	0.698058	0.744021
C. rosaeflora 'Cascade'	0.693147	0.693147	0.693147	0.693147	0.693147
'Softly'	0.693147	0.693147	0.693147	0.693147	0.695507

The tests of sphericity were significant ($p<0.0001$) the multivariate tests of repeated effects were used (ANOVA and MANOVA tables Appendix IV) for analysis.

Averaged across the six observation periods, the Groups had significantly different mean disease development ($F_{5,192}=52.69$, $p<0.0001$), and the mean disease development was also influenced by ascospore density, in both a linear ($F_{1, 192}=22.57$, $p<0.0022$) and non-linear fashion ($F_{1, 192}=7.79$, $p<0.0058$).

The overall average disease development for Within-Subject effects varied over Time (F_4, 122.26, $p<0.0001$)), and this pattern varied between Groups (F_{20}, 14.65, $p<0.0001$). The effect of ascospore density depended on time (F_4, 4.26, $p<0.0025$) and the pattern of disease development between Groups was also affected by ascospore density and time, in both a linear (F_{20}, 3.10, $p<0.0001$) and quadratic (F_{20}, 2.13, $p<0.0029$) fashion.

To investigate these differences further, the ANOVAs for each observation period's data (which covered the same factors and interactions as the Between-Subjects effects, but applied to a single period's data) were inspected.

At 24 h, Group 2 had greater disease development than the other groups ($F_{5, 192}=259.21$, $p<0.0001$) while within Group 1, 'Warwick Berg' did not initially form a good group ($F_{18, 192}=2.63$, $p<0.0006$) but from 72 h onwards, the levels of infection on this variety matched those of the rest of the Group. At 48 and 72 h, Groups 1 and 2 had greater disease development than other groups ($F_{5, 192}=22.53$, $p<0.0001$ and $F_{5, 192}=25.01$, $p<0.0001$ respectively) while disease development at 48 h in 'Nicky Crisp' in Group 3 varied from the other members that Group ($F_{18, 192}=2.14$, $p<0.0061$). By 96 h, Groups 1, 2 and 3 had the greatest disease development ($F_{5, 192}=45.83$, $p<0.0001$) and by 120 h, Groups 1, 2, 3 and 5 had the greatest disease development, Group 4 had moderate disease development and Group 6 had little disease development ($F_{5, 192}=56.05$, $p<0.0001$).

6.3.2.3 Field Inoculation

Experiment 6.33

Known susceptible species such as *C. japonica* 'Alba Plena' and 'Katherine Nuccio' were highly susceptible as shown by the percent infection in Table 6.27. Autumn-flowering species such as *C. hiemalis*, *C. malifora* and *C.* x *vernalis* had a medium disease incidence (33-66%) while of the two suspected resistant species, *C. cuspidata* had no lesions and *C. fraterna* 10.5%.

Table 6.27 Infection of Flowers by Inoculation with Airborne Ascospores from a Hand-Held Apothecium, Experiment 6.33

Species/Cultivar	No. Flowers Infected/No. Flowers Treated (% Infection)
C. cuspidata (D)	0/6 (0)
C. fraterna 15476-D (H)	2/19 (10.5)
C. hiemalis 'Pink Snow' 13634-E	7/18 (39)
C. hiemalis 'Shishigashira' (D)	10/15 (66.5)
C. japonica 'Alba Plena' (D)	30/32 (93.7)
C. japonica 'Alba Plena Imp' 11838 (H)	4/5 (80)
C. japonica 'Katherine Nuccio' 11466-A (H)	12/12 (100)
C.japonica 'Otto Hopfer' 43722 (H)	4/4 (100)
C. maliflora 12851-K (H)	5/14 (35.7)
C. x *vernalis* 'Ginryû' 17871 (H)	4/12 (33.3)

6.4 DISCUSSION

6.4.1 Inoculation by Ascospore Suspension

The preferred method of studying resistance in *Camellia* species was by ascospore suspension inoculations since they resulted in quantitative, rather than qualitative, data. Experiments using ascospore suspensions on known susceptible species were only partially successful. Disease incidence was often low, or non-existent. This led to a series of experiments investigating ascospore longevity and infectivity, the effect of nutrients, wet and dry inoculations and other factors in order to understand the infection process. Preparation of an ascospore suspension from freshly ejected ascospores, followed by inoculation on to freshly collected test petals greatly improved disease incidence compared with earlier experiments. There were, however, a number of experiments that were discarded because of low numbers of infections and it appears that other factors, such as temperature conditions prior to flower collection, may influence the infection process.

Many more experiments were also discarded because of the high number of natural infections (Figures 6.19 and 6.20). These natural infections would have occurred before the flowers were collected, but were too recent for symptoms to be visible at collection time. Within 24-48 h of inoculating petals for an experiment, a large number of infections on the petals - but not at the inoculation site - would be evident. By 48-72 h after inoculation these natural infections would have overgrown the lesion developing at the inoculation site and the experiment would be discontinued. Thus, although natural infections showed a cultivar was susceptible, efforts to determine the effect of ascospore concentration on infection or variation between flowers and plants were stymied time after time by these natural infections.

Figure 6.19
Forty-eight hours after inoculation, this experiment to determine the effect o f ascospore concentration on infection was discarded because of the high number of natural infections. Small brown lesions from ascospore suspension inoculations are just visible in the centre of some petals (arrows). The natural infections are larger and spread irregularly over the petal

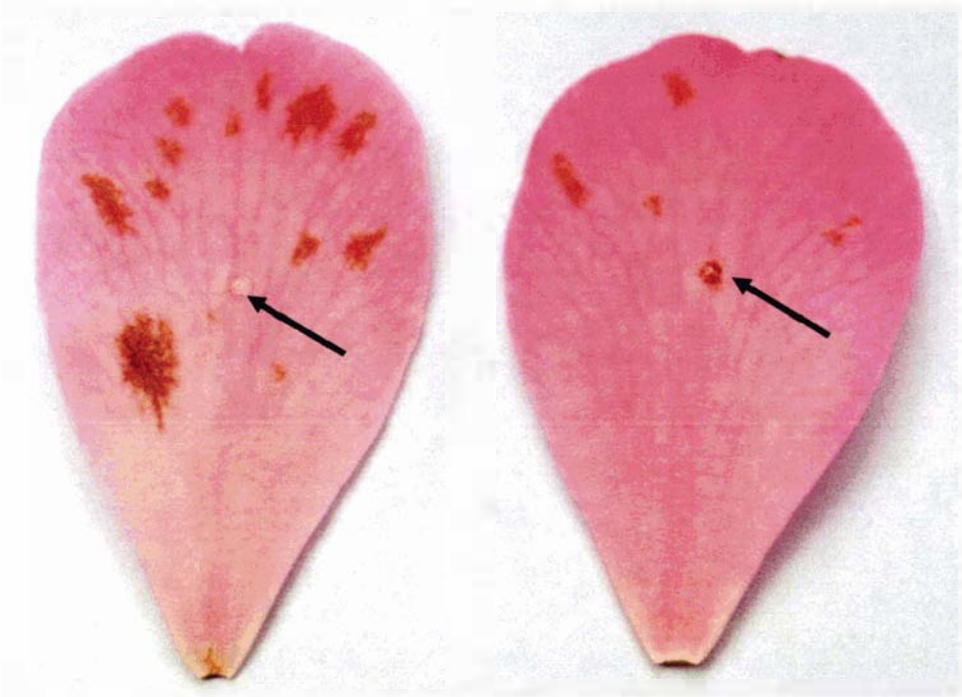


Figure 6.20
Close up of two petals from the same experiment. Lesions from the inoculation (arrows) are just visible or beginning to form lesions while natural infections are larger and spreading rapidly.

The highest natural infection levels coincided with the period in which most *Camellia* species and varieties were flowering and there was the greatest availability of apothecia. In Los Angeles, a desert climate, it was particularly noticeable that after long dry periods followed by substantial rain, the number of natural infections would increase from a low level to experiment-ruining levels. In fact, I stopped preparing experiments in the week following heavy rains because they would all be ruined by natural infections. The maturation of apothecia may be triggered by rain and this was discussed in Section 4.3.7.

6.4.1.1 Validation of Inoculation Technique

Ascospore Suspensions

None of the preserved ascospores (either dry or in suspension) remained infective. Ascospores that had been stored for up to 24 h were infective, but resulted in fewer infections than when the suspension was inoculated within hours of preparation. This may have been due to ascospores losing their infectivity, bursting in the water, or perhaps clumping and sticking to the glass during storage. Since storage of ascospores was not of importance to this project it was not investigated further. During the disease season, many ascospores were available and outside the disease

season ascospores were not required. For other studies – e. g. microscope studies of germination behaviour - successful storage of ascospores would be advantageous, as this work could then be done outside the natural disease season as long as camellia flowers were available.

Subjecting the ascospore suspension to centrifugation and vortexing to increase the ascospore concentration did not affect their infectivity. Preparation of standardised ascospore suspensions sometimes required that the ascospores be subject to these treatments.

Wet inoculation of spores produced more petal lesions and faster symptom development than petals whose inoculation droplets were dried immediately after inoculation in the single experiment carried out. Many fungal spores require free water (or high RH) for the germination and penetration process to begin or continue. Similarly, this experiment indicated that disease incidence and severity was greater when free water was present, although it may be that the drying process dried out or killed spores. Subsequent to this experiment, Vingnanasingam (2002) showed that the presence or absence of free water was not critical for germination but did appear to affect germ tube growth and penetration, thus explaining why symptom development may have been slower on dried petals.

The results from ascospore concentration experiments were conflicting. One experiment (6.6) indicated that there was a concentration effect, with higher and lower ascospore concentrations causing smaller lesions compared to the intermediate concentrations trialled. The second experiment (6.7 Run 1) indicated that there was no difference in lesion area resulting from different ascospore concentrations and in the third experiment (6.7 Run 2) only the 1×10^8 and 1×10^7 ascospores/ml concentrations caused lesions. More concentration experiments were initiated, but due to many natural infections or poor infection incidence (for whatever reason), none of the results were usable. The majority of experiments carried out during the course of this research used a concentration of 1×10^6 ascospores/ml, occasionally dropping to 1×10^5 ascospores/ml when apothecia were scarce or the number of petals in an experiment was unusually large.

Flower Variability – Overnight Storage

The overnight storage of flowers, for inoculation the following day, was not a viable option as shown by the results of Experiments 6.9 and 6.10. In some cases, flower collection could take all day, especially where flowers were collected from different towns around the Manawatu or in Wellington or Wanganui. In many initial experiments (not reported), no infections occurred at the inoculation site and some of these poor results can now be attributed to flowers stored at room temperature overnight. Storage at 4°C overnight is a viable option, as lesions did develop as a result of inoculation, but in both experiments where this option was used, the lesion area was significantly smaller than that of inoculations of freshly collected flowers. Presumably chilling the petals slowed the subsequent infection process. Thus, while not optimal, flowers could be stored at 4°C and an allowance made for the reduced lesion size.

Natural infections developed on all treatments, including those stored at 4°C overnight, and illustrate the problem faced by people wishing to store flowers for display at camellia shows. Flowers picked when perfect and stored cold will still develop lesions if infection has already occurred, although whether the cold slows ascospore germination or penetration of petal tissue is unknown.

Of greatest interest was the discovery that flowers (of a susceptible variety) stored for 24 h at 20°C did not develop lesions. This indicated that picking the flowers and holding them at 20°C before inoculation activated some resistance process. Picking the flower may trigger some physiological response, as flowers on the plant continue to become infected, but cold storage must either slow or prevent this response. The bleached area at the site of inoculation is further evidence that some physiological event occurred. That this occurred in a susceptible variety may indicate that at least some may have a resistant response (to *C. camelliae*), but that the response is too delayed to effective in the field. Colour change is discussed further in Section 6.4.1.7.

Flower Variability – Variation between Flowers and between Plants

Three experiments (6.13, 6.14 and 6.15) showed that there was little variation in lesion growth and development between petals of the same flower. The variation

that did occur, was presumably due to small differences between large and small, older and younger, and inner and outer petals and laboratory technique.

There were large differences between flowers from the same plant (Experiments 6.11, 6.13, 6.14 and 6.15), presumably because of differences in the age of the flowers and of their position on the plant (high or low, inner or outer branches, exposure to the sun etc.). This large variation was expected, and for this reason, when flowers were amalgamated for an experiment, equal numbers of petals from each flower were placed in each treatment to minimise this effect.

Experiments 6.13, 6.14 and 6.15 showed that within plant variation was greater than between plant variation of the same cultivar. When flowers were picked for these experiments, care was taken to collect flowers that were similar in age (fully opened perhaps 2 d), avoiding those that were older (damaged petals, blight infected) and younger (petals smaller, more turgid and unfaded). Presumably this care resulted in less variation between plants, as the flowers were all of a similar age and had experienced similar environmental conditions.

Experiment 6.12 demonstrated that between plant variation of the same variety can not be deduced from single-flower experiments. In New Zealand, 'Brian' was the only cultivar planted in sufficient numbers to enable a comparison of variation between plants of a single cultivar. The extensive plantings at Descanso Gardens, particularly of the many-petaled cultivars, allowed for a proper experimental design, with sufficient plants to compare petals per flower, flowers per plant and plants per cultivar.

Many of the apparently resistant species have few petals, and it was always necessary to amalgamate petals from several flowers in order to have sufficient petals for an experiment. These experiments show that as long as petals from each flower were assigned to each of the treatments in an experiment (i.e. a blocked design), compensation was made for the large variation between flowers from the same plant (or different plants).

Inoculation by Potter Spray Tower

Only one test was made using the Potter Spray Tower to inoculate ascospores in suspension. This showed that the technique would not be suitable for large scale testing of camellia petals for several reasons.

First, to make sufficient volume of each ascospore suspension was costly in terms of ascospores per number of petals inoculated. Second, it was found that infection was greater if ascospore suspensions were prepared and used quickly, something that would be difficult to achieve with this technique. Third, few petals fitted on the spray table, making screening of large numbers of different species and cultivars, or replicates of the same, impractical. And fourth, petals moved during the spray application due to the force of the air. The petals of *C. japonica* 'Desire' are large compared to those of many species and the smaller petals would be blown over or off the spray table. Finally, this technique would not be suitable for camellia growers and breeders due to the cost of the Potter Tower.

6.3.1.2 Assessment of Species, Hybrid and Cultivar Resistance

Variation in Species Considered Susceptible

The first ascospore suspension inoculation experiments used known susceptible species in order to test and improve this method of inoculation. Initially, the results were poor, with many species failing to become infected from the inoculation suspension, or with low disease incidence. Further experiments were instigated to investigate factors that might have contributed to this low or no disease incidence.

Disease incidence was improved by using ascospore suspensions made from freshly ejected ascospores, inoculating the petals as soon as possible after flower collection and preparation of the ascospore suspension, and maintaining the ascospores in suspension during the inoculation process by continued vigorous shaking. It remained, however, an imperfect indicator of resistance and susceptibility because, even within an experiment (e. g. Experiment 6.12) disease incidence on susceptible cultivars could greatly vary, meaning low or no disease incidence on suspected resistant species could not solely be attributed to its supposed resistance.

When no infections resulted from the ascospore inoculation, then the presence of natural infections on the experimental petals, field observations of infected flowers, and other methods of testing (concurrent with this work) confirmed that that species or cultivar was susceptible, and that the inoculation had failed to cause disease lesions because of experimental conditions. Despite some problems with infection rates using ascospore suspension inoculation, there were never any false positives i.e. no resistant species developed disease lesions, while there were many false negatives ie. susceptible species which did not develop disease lesions. These were easily distinguished, first on the basis of field observations when collecting the flowers and secondly with repeat testing, both with ascospore suspensions and other inoculation techniques.

Categories of Disease Expression

Four broad categories of host response to susceptibility were observed using ascospore suspensions. These were: highly susceptible; susceptible; moderately susceptible and resistant.

Highly Susceptible

In all experiments using *C. saluenensis*, the development of lesions and their spread was faster than that of other susceptible species such as *C. japonica.* At 48 h after inoculation the disease lesion size on C. *saluenensis* petals was more advanced than that of any other species and at 72 h 100% of the petal area was diseased. In contrast, on *C. japonica* species at 48 h, lesions were (generally) just beginning to spread from the inoculation site and lesion size was more accurately measured at 72 or 96 h after inoculation. *C. pitardii* var. *pitardii* also appears to be highly susceptible (data not reported).

Susceptible

On all *C. japonica* cultivars tested and the hybrids *C. reticulata* x *C. japonica* 'Hulyn Smith', *C. saluenensis* x *C. reticulata* 'Brian' and *C.* x *williamsii* 'E. G. Waterhouse' lesion development was best measured at 72 or 96 h after inoculation. The combined results of lesion growth on susceptible species are shown in Table 6.23. This table includes only those experiments reported in this Section, which included approximately one quarter of the total ascospore suspension inoculation experiments

conducted. The remainder were discarded because of poor disease incidence or the large number of natural infections, but observations of partial results from these unreported experiments, and observations of infections in the field, indicate that probably all varieties of *C. japonica* and *C. reticulata* species could be included in this category.

Moderately Susceptible/Moderately Resistant

A few lesions caused by *C. camelliae* were seen on *C. cuspidata, C. fraterna*, and *C. cuspidata* x *C. saluenensis* 'Cornish Snow' petals and were similar to field observations. Flowers on plants of *C. cuspidata* and hybrid *C. cuspidata* x *C. saluenensis* 'Cornish Snow' and fallen flowers of *C. fraterna* were observed to have lesions similar in colour to those caused by *C. camelliae* infections on white *C. japonica* flowers. Attempts to isolate the pathogen in culture from diseased petals were unsuccessful. Flowers that had fallen to the ground were old and more likely to have been damaged by birds, insects, rain etc., perhaps providing entry wounds for the pathogen (whether *C. camelliae*, *Botrytis* or other).

Low disease incidence was not used as an indicator of resistance unless all known susceptible species and cultivars in an experiment had a high disease incidence, natural infections were not present or were few compared to control species and field observations indicated that there were none/fewer infected flowers than on known susceptible species nearby. This rather complicated assessment was used for only the autumn-flowering camellias, *C. hiemalis, C. sasanqua, C.* x *vernalis,* studied at Descanso Gardens and The Huntington, Los Angeles. These species did not flower during the *C. camelliae* ascospore season in New Zealand. Casual field observations indicated that these species were not as heavily infected as nearby *C. japonica* and *C. reticulata* plants. Few disease lesions resulted when tested by ascospore suspension, e.g. in Experiment 6.21 all 20 *C. japonica* 'Alba Plena' petals were infected by the ascospore suspension, compared to only 5/20 *C. hiemalis* 'Shôwa-no-sakae' petals. Lesion area also tended to be somewhat smaller than those of *C. japonica* lesions (Table 6.23). Ascospore suspension testing indicated that *C. hiemalis* 'Shishigashira', 'Shôwa-no-sakae', 'Showa Supreme' and *C.* x *vernalis* 'Ginryû' were moderately susceptible and field observations indicate that other varieties of

these species and other autumn-flowering species (e.g. *C. sasanqua*) were also moderately susceptible.

Resistant

At no time, in reported or unreported experiments, did the flowers of species *C. forrestii*, *C. grijsii*, C. grijsii 'Juhuacha' and 'Zhenzhu Cha', *C. longicarpa*, *C. lutchuensis, C. rosiflora, C. transnokoensis, C. yuhsienensis*, *C. yunnanensis*, or hybrids *C. lutchuensis* hybrid 'Fairy Blush', *C. lutchuensis* x *C. japonica* 'Koto-no-kaori' and 'Minato-no-akebono' develop disease lesions at the inoculation site.

Infection of Stamens and Stigma

Susceptibility of stamens and stigma was investigated because of some inconsistent results from intact flower inoculations (experiments not reported) using the airborne ascospore inoculation chamber (Section 6.2.2) and observations made in the field. Intact flowers of the resistant species *C. yunnanensis* were occasionally observed to have symptoms of infection by *C. camelliae*.

Experiments with stamens and stigma were difficult to conduct and the design of the experiments and the results show some of these handicaps (other experiments not reported because of poor infection in control inoculations). Carpels with stigma are difficult to collect in large enough numbers for an experiment, and many of the cultivars available have no reproductive parts (e.g. *C. japonica* 'Alba Plena'). Both stamens and stigma are fine and easily bruised or crushed when handled. Inoculation of stamens and stigma on intact flowers does avoid this problem but ensuring the droplets adhere to the stamen or stigma was rather difficult whether on intact or dissected reproductive parts, as there was not enough surface tension to hold the droplet in position at the inoculation site. Another problem for dissected reproductive parts was that they dried out very quickly in the humid chambers, thus making browning symptoms from dehydration or senescence difficult to distinguish from browning symptoms caused by the pathogen. Further experiments using the isolate-specific primers (Chapter Five – Molecular Studies) should elucidate whether the reproductive parts are infected by *C. camelliae.*

Colour Change at Inoculation Site

These are the first reports of a pigmentation increase around the inoculation site in some species of camellia. The colour change was visible from 48 h onwards but these reactions failed to develop further over 6 days of observation. I had observed bleaching of colour around inoculation sites on 'Brian' in New Zealand inoculation trials, but they were not investigated at that time.

'Spring Mist' is a hybrid (*C. japonica* x *C. lutchuensis*) with suspected resistance and its response to inoculation was not unexpected. However, 'Ginryû' is considered a 'moderately susceptible' cultivar of *C.* x *vernalis,* and both in the laboratory and in the field, natural infections occur regularly, though at a lesser rate than in other species (e.g. *C. japonica*). If colour change were in fact a resistance response, then the factors that determine whether a resistant or susceptible response occurs would be important for understanding the host/pathogen interaction. The infrequent infections that are observed on resistant species may be the result of microscopic damage to the petals (from wind, rain, insects, birds) that allows the fungus entry through wounds, thus avoiding one line of plant defences.

Of similar importance is determining whether resistance mechanism(s) cause the colour change. In tomato fruit, colour change – ghost spotting – is seen when spores of the fungus *Botrytis cinerea* germinate and produce necrotic lesions (Verhoeff 1970). These lesions fail to develop. Analysis of infected cells has shown that they contain a higher concentration of phenolic compounds, materials which have anti-fungal effects and which are components of cell wall strengthening constituents. In tomatoes, lignification of the cell walls could form a barrier to infection by *B. cinerea,* and this defence response is seen as colour change on the fruit. Cytokinin, a plant-growth hormone, is also involved with colour change in a number of plant/pathogen interactions (Angra-Sharma & Sharma 1999). 'Green islands' of healthy tissue, high in cytokinins, were closely correlated with resistance in some plant species (Angra-Sharma & Sharma 1999). Thus, colour change of infected sites is seen in other plant/pathogen interactions, and appears to be the by-product of defence reactions.

6.4.2 Inoculation by Airborne Ascospores

6.4.2.1 Resistance Assessment

The initial inspection of the data for all three experiments with the settling chamber indicated that there were very different responses to inoculation by the species and cultivars tested, from the rapid and total infection shown by those in Group 1, to the low or no infections seen in those of Group 6. Analysis showed that species could be grouped by these differences in infection rate response and this allowed the analyses to be simplified.

In Experiment 6.30, species in Groups 1, 2 and 3 were all known to be susceptible, or had parents which were known to be susceptible, except for 'Nymph', a *C. lutchuensis* x *C. japonica* hybrid. In Group 4 with low levels of infection, was the susceptible *C.* x *williamsii* 'Mirage' and *C. rosiflora* 'Cascade' and three hybrids with one resistant parent: a *C. cuspidata* hybrid 'Spring Festival', *C. rusticana* x *C. lutchuensis* 'Fragrant Joy' and *C. japonica* x *C. lutchuensis* 'Scentuous'. Group 5 had species with low levels of disease development that were observed at 120 and 144 h after inoculation. As well as low disease development, the resistant species *C. cuspidata, C. grijsii* and *C. longicarpa* had very low disease incidence. Despite its resistant parent, *C. cuspidata* x *C. saluenensis* 'Cornish Snow' was known from field observations to be moderately susceptible and along with the susceptible *C.* x *williamsii* 'Softly', the apparent resistance of these two species is difficult to explain. Three known resistant species, *C. fraterna, C. lutchuensis and C. yunnanensis,* occurred in Group 6, along with an untested species, *C. trichocarpa* and the hybrid *C. lutchuensis* hybrid 'Fairy Blush'. In the field neither *C. trichocarpa* nor 'Fairy Blush' were observed with disease symptoms.

The majority of species and varieties in this experiment responded in ways that agreed with observations of disease in the field. The results from several species, e.g. 'Cornish Snow' and 'Softly' were anomalous with the results of previous observations and indicate that the inoculation method does not entirely reproduce patterns of infection seen in the field.

In Experiment 3.31, no known resistant species were included and all the species and varieties chosen were expected to show medium to high levels of disease severity in

concordance with field observations. The range of response, however, was just as great as that shown by the mixture of known resistant and susceptible species in Experiment 6.30. Many of the species and varieties were collected at DP (Mangatainoka) the day before they were inoculated and stored at 4°C overnight. Overnight storage of flowers was shown to affect infection of *C. saluenensis* x *C. reticulata* 'Brian' in an ascospore suspension experiment conducted after this series of experiments and this may explain the anomalous results.

In Experiment 3.32, species and cultivars in Groups 1-4 were all known susceptible species or suspected susceptible species. The sole member of Group 5 was *C. japonica* 'Dixie Knight Supreme' and along with *C. rosiflora* 'Cascade' and *C.* x *williamsii* 'Softly' in Group 6, developed low levels of infection despite their susceptible rating. The only known resistant species in this experiment, *C. fraterna,* was also in Group 6.

Conclusion

This series of experiments established another technique for screening *Camellia* species and cultivars for resistance/susceptibility and quantifying the disease response. In most cases, the results were as expected, i.e. resistant species failed to develop disease lesions or developed much smaller lesions much later than susceptible species and susceptible species developed disease lesions. Excluding the results of Experiment 6.31 in which flowers were stored overnight, there were, however, some anomalous results. For example, *C.* x *williamsii* 'Softly' did not generally develop disease lesions and these did not spread rapidly over the petal as would be expected of a *C. saluenensis* x *C. japonica* hybrid. Field observations of this hybrid indicated that it would be as susceptible as other hybrids of its type (e. g. 'E. G. Waterhouse' and 'Rendezvous'). In this case, inoculation with airborne ascospores in a chamber, perhaps the method most similar to that which occurs naturally, does not replicate what is seen in the field and indicated that there was some other important factor not considered in this technique.

These experiments showed that browning did occur on resistant species if they were incubated for more than five days. These browning symptoms, however, could not definitively be identified as *C. camelliae* lesions. Neither the colour of the

lesion/spot, nor the manner in which it developed on the petal, was typical of *C. camelliae* lesions on susceptible species. These brown spots generally appeared around 120 h, but failed to increase greatly in size. By 120 and 144 h, the smaller and thinner petals (which included many of the resistant species) in these experiments were beginning to dry out (despite the humid chamber) and curl up anyway.

Ascospore density had variable effects, depending on the Group and over the course of each Experiment, indicating that no single response applied to all species and cultivars. The data, however, remains semi-quantitative because the number of ascospores per square cm could not be controlled.

The experimental design used in this report was too time consuming for general purposes. The collection of four flowers from each of 24 species or varieties, preparation of petals and inoculation of 12 replicate blocks took around 14 h of one day, plus ~3 h for daily data observations. Statistical analysis was initially unwieldy until species with similar responses were grouped together. To save time where a formal analysis was required the experimental design may be modified, perhaps running fewer species, with fewer replicate blocks, but using several petals of each species in each block.

The airborne ascospore chamber is an inexpensive and simple piece of equipment to buy, build and set up. For small scale testing without formal analysis, this technique is suitable for growers and breeders who wish to check the resistance or susceptibility of new camellia species and varieties. Based on the results of these experiments, plus other less formal tests using this apparatus, a simple resistance rating system was developed (Figure 6.21).

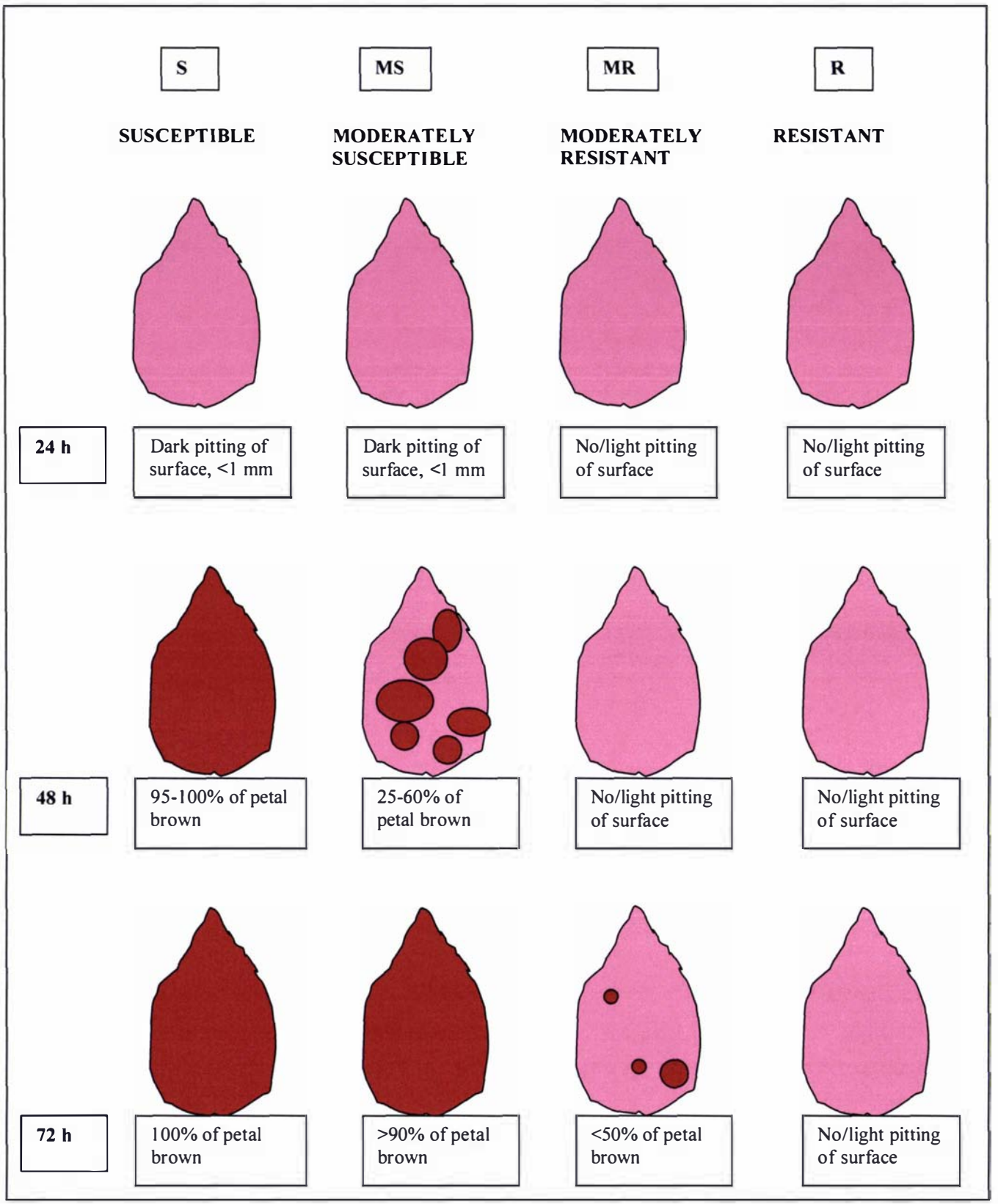


Figure 6.21
Resistance rating scale developed through multiple tests of species and cultivars using the airborne ascospore inoculation chamber. The times indicated are provisional, as symptom development was variable.

6.4.2.2 Field Inoculation

Fewer flowers were inoculated than planned for this experiment due to lack of consistent release of ascospores. Apothecia and bottles were prepared the night before, and provided the difference in temperature and humidity was high between the bottle and the field, the apothecia discharged readily. In practice this meant that apothecia would discharge on fine days but not when the weather was cooler and/or wet.

Susceptible species always developed disease lesions on some of their flowers, whereas known resistant species developed no symptoms, or with fewer and no-progressing lesions than comparably treated susceptible flowers.

This technique could be used by camellia growers and breeders to test new species and varieties for resistance and susceptibility, particularly in public gardens where flowers cannot be picked. It is probably easier, however, to monitor the species and cultivars of interest on a daily basis for natural infections and assess disease severity.

6.4.3 General Discussion

Overall, comparison and evaluation of experiments was difficult due to the confounding effect of variability in pathogenicity of the fungus and variability in environmental conditions experienced by the flowers prior to collection. In this study neither factor could be controlled for, and there was no prior information.

If the introduction of *C. camelliae* to New Zealand was an isolated event, then it is likely that variation in pathogenicity between isolates is relatively low, with only one or several genotypes introduced. No variation was found in the ITS and partial ß-tubulin sequences of New Zealand isolates in this study, and van Toor (2002) also found less genetic variability within isolates in New Zealand (9%) compared to those from the United States (17%) using UP-PCR.

Camellia plants and flowers are exposed to a wide range of climatic conditions during their flowering season, whether it be autumn- or spring-flowering, or in Palmerston North, New Zealand, or Los Angeles, the United States. During this

study rainfall, hail, wind, frost, diurnal and seasonal temperature fluctuations, and RH varied greatly (some seasons recorded by Tiny Tag Datarecorder, unpubl.) and I suspect that the greatest amount of variability in disease response in this study was due to these environmental factors.

To reduce the variability possible in subsequent experiments, species of interest could be grown in pots in greenhouses with some ability for climate control. This would increase the uniformity of conditions experienced by the plant between experiments by removing the extreme conditions (wind, frost, very high or low temperatures) while also reducing the effect of different soils and/or mineral deficiencies between plants. In addition, once cultured sclerotia can be induced to germinate and produce ascospores, then one genotype can be selected for inoculation work.

The inoculation techniques trialled in this section gave quantitative (ascospore suspensions) or qualitative data (airborne ascospores). Quantitative data is best for defining the degree of the resistance reaction, and the ascospore suspension gave data on a) disease incidence and b) disease severity and were useful for working with species which were susceptible. i.e. yes, no and maybe results. It did not help clarify the atypical symptoms shown by some of the resistant species. Qualitative data, while not as precise, allowed the development of the Rating Scale, which in combination with the quick and simple testing procedure (required no complicated machinery or analysis) permitted species to be assessed for resistance by amateurs such as plant breeders and species/hybrids/cultivars with resistant qualities to be identified.

The Rating Scale developed from airborne ascospore inoculation experiments differed slightly from that developed from ascospore suspension inoculation experiments. A comparison of the two is shown in Figure 6.22. Single point inoculation by ascospore suspension discriminated between highly susceptible species, where disease lesions spread much faster than those of 'normal' susceptibility. Discrimination between the moderately susceptible and moderately resistant was less clear using ascospores in suspension. Results for resistant species were in accordance in both techniques. The small differences in rating were

attributable to the type of ascospore inoculation, as infection by ascospores in suspension permitted the disease lesion to be measured from a single point (thus distinguishing highly susceptible and susceptible), whereas the widespread distribution of airborne ascospores on petals obscured this. Airborne ascospore inoculation did distinguish between moderately susceptible and moderately resistant species. Concentrated ascospores in water cause slightly different infections to widely dispersed 'dry' inoculated ascsospores.

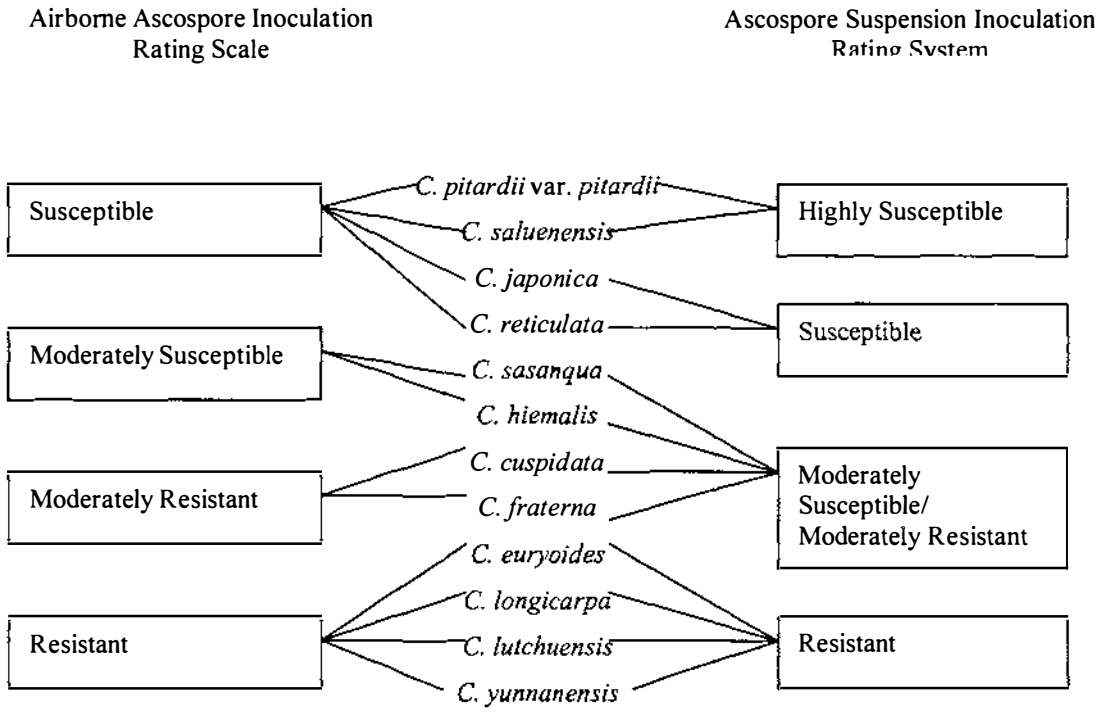


Figure 6.22
Comparison of resistance/susceptibility ratings of a selection of *Camellia* species by two inoculation methods.

For future investigations, or use by *Camellia* growers and breeders, I would recommend that either (or both) pipetted ascospores in suspension or the airborne ascospore chamber inoculation techniques be used. Both techniques are relatively cheap and simple to use and do not necessarily require extensive analysis of results.

There were some anomalous results whichever testing technique was used, but resistant species repeatedly failed to become infected, or occasionally become infected, while susceptible species almost always showed disease symptoms. Of particular interest to *Camellia* breeders is that some hybrids with a resistant parent

were resistant themselves, e.g. *C. japonica* x *C. lutchuensis* 'Minato-no-haru' and 'Koto-no-kaori', although not all such hybrids were resistant, e.g. *C. saluenensis* x *C. cuspidata* 'Cornish Snow'.

Apart from validating that resistant species and hybrids exist and which ones they are, these experiments have shown three interesting new paths for investigation of resistance in *Camellia* species. The first was the discovery that the susceptible hybrid 'Brian' failed to become infected after the petals were stored overnight at 20°C prior to inoculation. This indicates that there may be some resistance mechanism in these petals. Second, colour change at the inoculation site indicates another form of resistance mechanism. Third, the apparent susceptibility of *C. yunnanensis* stamens. Sirjusingh *et al* (1996) showed that stamens of geranium required fewer *B. cinerea* conidia to infect them than leaves which may explain this result. Stamens may also possess different chitinase enzymes to those in petals, but this is discussed further in Chapter Nine - Resistance Mechanisms: Chitinase Enzymes.

Free water is not critical for germination but does appear to affect subsequent germ tube growth and penetration (Vingnanasingam 2002). In the New Zealand disease season (late July-mid November) free water in the form of rain and dew is commonly present on the petals, increasing the ability of ascospores to cause infection. Vingnanasingam (2002) also observed that ascospores released from an apothecium have a small amount of liquid associated with them, thus providing a reservoir of moisture for the early stages of infection. During the two seasons working in Los Angeles (a desert environment), it was very noticeable that the vast majority of infections occurred in the week following rain, when water was present on the plants and in the atmosphere. Out side this period, infections were few. This, however, is also a function of the water requirements for the germinating sclerotia, as apothecia were only abundant during and after rain periods.

An incubation temperature of 20°C was the only temperature used in these experiments. Lesions would develop on flowers stored at 4°C, but very slowly and it is unknown whether the low temperature prevented germination and penetration (i.e. if only established infections went on to cause lesions), but it certainly slowed lesion

development. Interestingly, Denoyes-Rothan *et al* (1999) found that an incubation temperature of 18°C allowed better discrimination between resistant and susceptible varieties of strawberries to *Colletotricum acutatum* than 25°C.

Denoyes-Rothan *et al* (1999) also found that infection of strawberries by *Colletotrichum acutatum* was influenced mainly by wetness duration and disease development by RH, but I have made no distinction between factors that affect germination of *C. camelliae*, infection or lesion development on camellias.

CHAPTER SEVEN – DEFENCE MECHANISMS: ALUMINIUM HYPERACCUMULATION

7.1 INTRODUCTION

Plants that accumulate inordinate amounts of heavy metals are termed hyperaccumulators (Brooks *et al* 1977). In general, high concentrations of metals are toxic to organisms; however, some metals (e.g. Mg, Mn, Mo and Cu) are essential plant nutrients (Marschner 1995) and are usually taken up in small quantities. Non-essential elements (e.g. Al, Cd, Se and Tl) are not necessary for plant growth but in both cases, in at least a few plant species, both essential and non-essential metals are taken up and stored in excess of nutritional requirements (Brooks 1998). The metal-organic complex is usually stored within vacuoles, and often in epidermal cells of leaves (Zhao *et al* 2000).

7.1.1 Hyperaccumulation and Plant Resistance

Why do some plant species hyperaccumulate metals that are phytotoxic? Boyd & Martens (1992) presented five hypotheses to account for this phenomenon:

1. Tolerance to, or disposal of, the element from the plant;
2. A drought-resistance strategy;
3. A means of avoiding competition from less metal-tolerant plants;
4. Inadvertent uptake of heavy metals;
5. Defence against herbivores or pathogens.

There has been little or no work to investigate the first four hypotheses, but there is evidence that, for some plant-pathogen/herbivore interactions, high metal concentrations may deter grazing by herbivores or defend against fungal or bacterial pathogens.

Boyd *et al* (1994) showed that the nickel-accumulating brassica, *Streptanthus polygaloides*, inhibited the growth of selected bacterial and fungal pathogens. *S. polygaloides* plants were grown in high- and low-nickel amended soil, the high-nickel plants yielded an average 5,630 $\mu g\ g^{-1}$ dry weight, the low-nickel plants, 124 $\mu g\ g^{-1}$ dry weight. Growth of the two fungi *Erysiphe polygoni* and *Alternaria*

brassicicola was reduced (but not prevented) on high-nickel plants relative to low-nickel plants. The bacterial pathogen *Xanthomonas campestris* pv. *campestris* failed to grow and produce symptoms on high-nickel plants.

For insect herbivores, nickel was shown to be toxic to larvae of *Pieris rapae* on *Thlaspi montanum* var. *montanum* (Boyd & Martens 1994) and to repel the fruit fly *Drosophila melanogaster* on *Sebertia acuminata* trees (Sagner *et al* 1998). Hyperaccumulation of zinc deterred locusts (*Schistocerca gregaria*), slugs (*Deroceras caruanae*) and caterpillars (*Pieris brassicae*) from feeding on *Thlaspi caerulescens* (Pollard & Baker 1997; Jhee *et al* 1999).

7.1.2 Aluminium

Aluminium is a light metal, occurring in nature only as compounds, which comprise 8.1% of the earth's crust. It is the most abundant element after oxygen and silicon. It is usually unavailable for chemical and biological reactions, being bound in crystalline aluminosilicates, oxyhydroxides and non-silicate containing compounds (Driscoll & Postek 1996: McLaren & Cameron 1996). In acid soils, however, aluminium can be present as the toxic trivalent cation Al^{3+}. It forms strong bonds with oxygen-donor compounds (Martin 1986) and this binding is probably an important factor in its toxicity as it can interact with multiple sites in the apoplasm and symplasm of root cells (Ma *et al* 2001). Aluminium toxicity is a major limiting factor for plant productivity on acidic soils and these soils account for ~40% of the world's arable land (Ma *et al* 2001).

7.1.3 Aluminium Hyperaccumulation

The 'normal' aluminium content of a plant is <300 $\mu g\ g^{-1}$ but certain aluminium-accumulating plant species can contain >1 000 $\mu g\ g^{-1}$ (1%) on a dry weight basis (Brooks *et al* 1977).

Aluminium hyperaccumulators are widely scattered among the major subdivisions of dicotyledenous plants (Chenery & Sporne 1976). The phenomenon is statistically correlated with seven primitive characters (Chenery & Sporne 1976) which occur more frequently in plant families that were present in early fossil history and among tropical rainforest families (Sporne 1973). Primitive plant characteristics that are

associated with these aluminium hyperaccumulators include having a woody habit (i.e. arborescent), vessels with scalariform endplates and/or sidewalls, apotracheal parenchyma and unstoreyed wood, as well as tropical origins (Chenery & Sporne 1976). Elevated concentrations of soluble aluminium are present in highly leached tropical soil. Plants that occur in these regions have therefore evolved mechanisms for tolerating or excluding this non-essential element.

7.1.4 Aluminium Hyperaccumulation in *Camellia*

Studies of aluminium hyperaccumulation in the genus *Camellia* have been limited to *C. sinensis*, the species from whose leaves tea is made (Matsumoto *et al* 1976; Konishi *et al* 1985; Fairweather-Tait *et al* 1987; Ishikawa *et al* 2000; Ruan & Wong 2001; Xie *et al* 2001; Carr *et al* 2003). Tea is the most important economic product of *Camellia* and aluminium is a known neurotoxin (Ganrot 1986) that has been implicated in the development of Alzheimer's, a brain disease (Ganrot 1986; McLachlan & Walker 1987) (reviewed by Wong *et al* 2003).

Aluminium is preferentially accumulated in the epidermis of camellia leaves and because it is a continuous process the concentration is greater in older leaves (Matsumoto *et al* 1976; Memon *et al* 1981; Ruan & Wong 2001; Carr *et al* 2003; Fung *et al* 2003). Carr *et al* (2003) found that aluminium preferentially accumulated in the upper epidermis of young and old leaves in particular the cell walls. Dong *et al* (1999) and Fung & Wong (2002) found that soil pH was the major factor controlling aluminium uptake from soils, with higher levels of aluminium as the pH decreased.

7.1.5 Objectives

There have been no reports of aluminium accumulation in ornamental *Camellia* species. The objectives of this study were to:

a) determine aluminium accumulation by various ornamental *Camellia* species and compare this with *C. sinensis.*
b) relate aluminium accumulation by *Camellia* spp. to soil pH, total and extractable aluminium concentrations.
c) determine if a relationship exists between plant aluminium uptake and resistance to *C. camelliae.*

7.2 MATERIALS & METHODS

7.2.1 Sample Collection

Soil, stems, leaf and flower samples were collected from six locations, two in Los Angeles, USA and four in New Zealand (Table 7.1). Where possible, the identical cultivar of the same species was collected, however, some cultivars grown in New Zealand are not commonly grown in the USA (and visa versa) and substitutions were made. Similarly, the range of species grown at each location differed and a collection of many species was made, a few of which were not duplicated at other sites. Only one plant of each species was available at each site.

Table 7.1 Collection of Species and Location of Sites (l=leaf, s=stem, f=flower, e=soil, x=sclerotia)

Species	Descanso Gardens, Los Angeles	The Huntington Los Angeles	Auckland Botanic Gardens	Massey University, Palmerston North	Seafield, Wanganui	Wellington Botanic Gardens
Susceptible Species						
C. granthamiana	l, s, e	l, s, f, e	l, s, e	l, s, e	l, s, e	l, s, e
C. japonica[a]	l, s, f, e, x	l, s, f, e, x	l, s, f, e, x	l, s, f, e, x	l, s, f, e	-
C. pitardii var. *pitardii*	-	-	l, s, f, e	l, s, f, e	l, s, e	-
C. sasanqua[b]	l, s, f, e	l, s, f, e	l, s, e	l, s, e	l, s, e	-
Resistant Species						
C. cuspidata	l, s, f, e	l, s, f	l, s, f, e	l, s, f, e	-	-
C. fraterna	l, s, f, e	l, s, f, e	l, s, f, e	l, s, f, e	-	-
C. grijsii	-	-	l, s, e	l, s, f, e	l, s, f, e	l, s, f, e
C. lutchuensis	-	-	l, s, f, e	l, s, f, e	l, s, f, e	-
C. transnokoensis	-	-	l, s, f, e	l, s, f, e	l, s, f, e	l, s, f, e
C. yunnanensis	-	-	l, s, f, e	l, s, f, e	l, s, f, e	-
Unknown Resistance Rating						
C. sinensis[c]	-	-	l, s, e	l, s, e	l, s, e,	-

[a] 'Alba Plena' from USA: 'Elegans Champagne' and sclerotia from New Zealand sites
[b] 'Setsugekka' from Auckland Botanic Gardens: 'Shishi Gashira' from other sites
[c] *C. sinensis* was included in order to compare this data with other studies

7.2.2 Sample Preparation and Aluminium Determination

Leaves, stems and flowers were separated and placed in a drying cabinet at 80° C until a constant weight was reached. Samples were weighed, and ground. Approximately 0.2 g of material from each sample was accurately weighed into 50 ml Erlenmeyer flasks. Concentrated nitric acid (10 ml) was added to each flask and the mixtures heated on a heating block until a final volume of ~3 ml was reached. The samples were then diluted to 10 ml using deionized water and stored in polythene containers.

Soil samples were dried at 80°C and sieved to <1 mm size using a nylon sieve. Approximately 0.2 g quantities of sieved soil were accurately weighed into boiling tubes. Ten ml of concentrated nitric acid was then added and the mixtures boiled until a final volume of ~3 ml was reached. The solutions were diluted to 10 ml with deionized water and stored in polythene containers.

An estimation of the soluble Al in the soil was made by weighing 2 g of sieved soil into 50 ml centrifuge tubes and adding 20 ml of 1 *M* ammonium acetate (pH 7.0) to each. The tubes were agitated for 24 h and the mixtures filtered, and the filtrate stored for analysis.

The total concentration of Al ($\mu g\ g^{-1}$) of each sample was calculated using the formula:

$$[Al] = \frac{MV}{w}$$

where *Al* is the total concentration of aluminium ($\mu g\ g^{-1}$ dry weight), *M* is the concentration measured by the atomic absorption spectrometer ($\mu g\ ml^{-1}$), *V* is the sample volume (ml) and *w* is the weight of the sample (g).

Soil pH was measured by weighing 4 g of sieved soil into 35 ml polythene containers and adding 10 ml of distilled water. After shaking, the mixtures were left overnight and re-shaken before the pH was determined using a pH meter.

Chemical analyses on the plant and soil solutions were performed using a GBC 904 atomic absorption spectrometer. Single factor ANOVAs (Minitab) were performed on the data and Fischer's tests used to separate the means.

As quality assurance, fifteen samples were sent to a commercial laboratory (AgResearch, Palmerston North) for analyses of Al and other elements by inductively coupled plasma emission spectroscopy (ICPES). A regression analysis comparing our results to those of the commercial laboratory gave an r^2 value of 0.95 for $y=x$.

7.3 RESULTS

All the *Camellia* species tested were found to hyperaccumulate aluminium. The highest concentrations were found in the leaves, with lower but significant concentrations in the flowers and stems (Table 7.2).

Table 7.2. Resistance to *C. camelliae* and Aluminium Concentrations (μg g^{-1}) in Samples of each Species

Species	Resistance	Leaves (SE)	Stems (SE)	Flowers (SE)
C. cuspidata	resistant	1368 (458)	239 (55)	223 (39)
C. fraterna	resistant	3159 (1730)	547 (343)	357 (160)
C. grijsii	resistant	2984 (855)	461 (86)	333 (64)
C. lutchuensis	resistant	2080 (921)	220 (51)	142 (38)
C. transnokoensis	resistant	3623 (1371)	277 (81)	114 (28)
C. yunnanensis	resistant	1510 (760)	144 (22)	147 (17)
C. granthamiana	susceptible	5285 (2467)	185 (163)	210
C. japonica	susceptible	2441 (1045)	478 (110)	386 (59)
C. pitardii var. *pitardii*	susceptible	3959 (1777)	306 (170)	307 (71)
C. sasanqua	susceptible	1368 (888)	328 (129)	187 (4)
C. sinensis	unknown	3565 (1040)	245 (24)	N.D.

There were no significant differences in leaf, stem, and petal Al concentration between species, and no significant difference between susceptible and resistant species (ANOVA tables Appendix IV). Al concentration in the flowers of each species at each site is shown in Table 7.3.

Table 7.3 Aluminium Concentration (μg g^{-1}) in Flowers of each Species Tested

Species	Descanso Gardens, Los Angeles	The Huntington, Los Angeles	Auckland Botanic Gardens	Massey University, Palmerston North	Vonnie Cave, Wanganui	Wellington Botanic Gardens
C. granthamiana	-	210.49	-	-	-	-
C. japonica[a]	414.94	242.94	449.82	396.66	292.71	-
C. pitardii var. *pitardii*	-	-	377.55	235.75	-	-
C. sasanqua[b]	182.39	191.22	-	-	-	-
Resistant Species						
C. cuspidata	282.39	321.12	237.06	149.28	-	-
C. fraterna	121.85	198.09	826.95	281.62	-	-
C. grijsii	-	-	-	460.61	265.64	273.29
C. lutchuensis	-	-	215.67	119.52	91.47	-
C. transnokoensis	-	-	174.76	143.30	49.84	86.21
C. yunnanensis	-	-	165.46	163.37	113.59	-

[a] 'Alba Plena' from Descanso and Huntington: 'Elegans Champagne' from New Zealand sites
[b] 'Setsugekka' from Auckland Botanic Gardens: 'Shishi Gashira' from other sites

The average Al concentration in sclerotia from *C. japonica* (4 samples) was 157 μg g^{-1} (SE 35).

There were significant differences ($p<0.05$) in leaf and stem Al concentrations between sites (Table 7.4). For comparison, the metal concentrations in leaves and stems of poplar (*Populus deltoides* x *P. yunnanensis* 'Kawa') at Massey University are compared with the metal concentrations in leaves and stems from camellia samples taken at Massy University (Table 7.5).

Table 7.4 Soil pH and Plant Aluminium Concentrations (μg g^{-1}) (SE) in Samples from the Six Sites Investigated

Location	pH	Total	Extractable	Leaves	Stems	Flowers
Descanso	7.1	13199 (588)	4.9 (1.3)	527 (118)	149 (39)	250 (64)
Huntington	6.4	10440 (1464)	4.8 (1.7)	792 (289)	167 (58)	211 (11)
Auckland Botanical Garden	4.5	20757 (2750)	7.9 (1.1)	5093 (805)	355 (56)	379 (83)
Massey University	4.6	11949 (806)	4.9 (0.5)	2294 (466)	301 (69)	244 (45)
Vonnie Cave	5.2	7215 (295)	5.7 (1.2)	1030 (217)	240 (42)	163 (49)
Wellington Botanical Gardens	4.3	12019 (418)	7.9 (1.3)	8383 (2696)	378 (194)	180 (94)

Table 7.5 Metal concentrations (μg g^{-1}) in leaves and stems of *Camellia* spp. and poplar (*Populus deltoids* x *P. yunnanensis* 'Kawa') grown on the same soil. (Al highlighted for comparison)

	Al	B	Ca	Cd	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	Zn
Camellia													
Leaves	2294	41	9998	<0.1	1.5	6.6	127	5710	1532	1588	504	6.3	13
Stems	301	13	3777	<0.1	<0.5	19.1	164	3358	1538	265	361	7.4	37
Populus													
Leaves	82	33	8908	4.1	1.4	46.6	185	13670	719	52	198	2.9	223
Stems	7	3	570	0.4	0.4	19.3	21	1442	165	5	71	0.3	34
Soil	15544	<3	4538	1.8	20.6	16.5	17306	2329	3399	520.4	216	14	106

There was no relationship between soil pH and flower Al concentration (Figure 7.1 A) but there was a highly significant negative correlation (r=-0.48 $p<0.01$) between pH and leaf Al concentration (Figure 7.1 B). There was a linear relationship between leaf aluminium concentration and soil H^+ concentration:

$$M = 10^8 H + 335 \qquad r^2=0.98$$

where M is leaf Al concentration (μg g^{-1} dry weight) and H is the concentration of H^+ ions in soil solution (mol L^{-1}).

Specimens of the sclerotia taken from *C. japonica* ('Alba Plena' and 'Elegans Champagne') had an average aluminium concentration of 157 μg g^{-1} (SE 35).

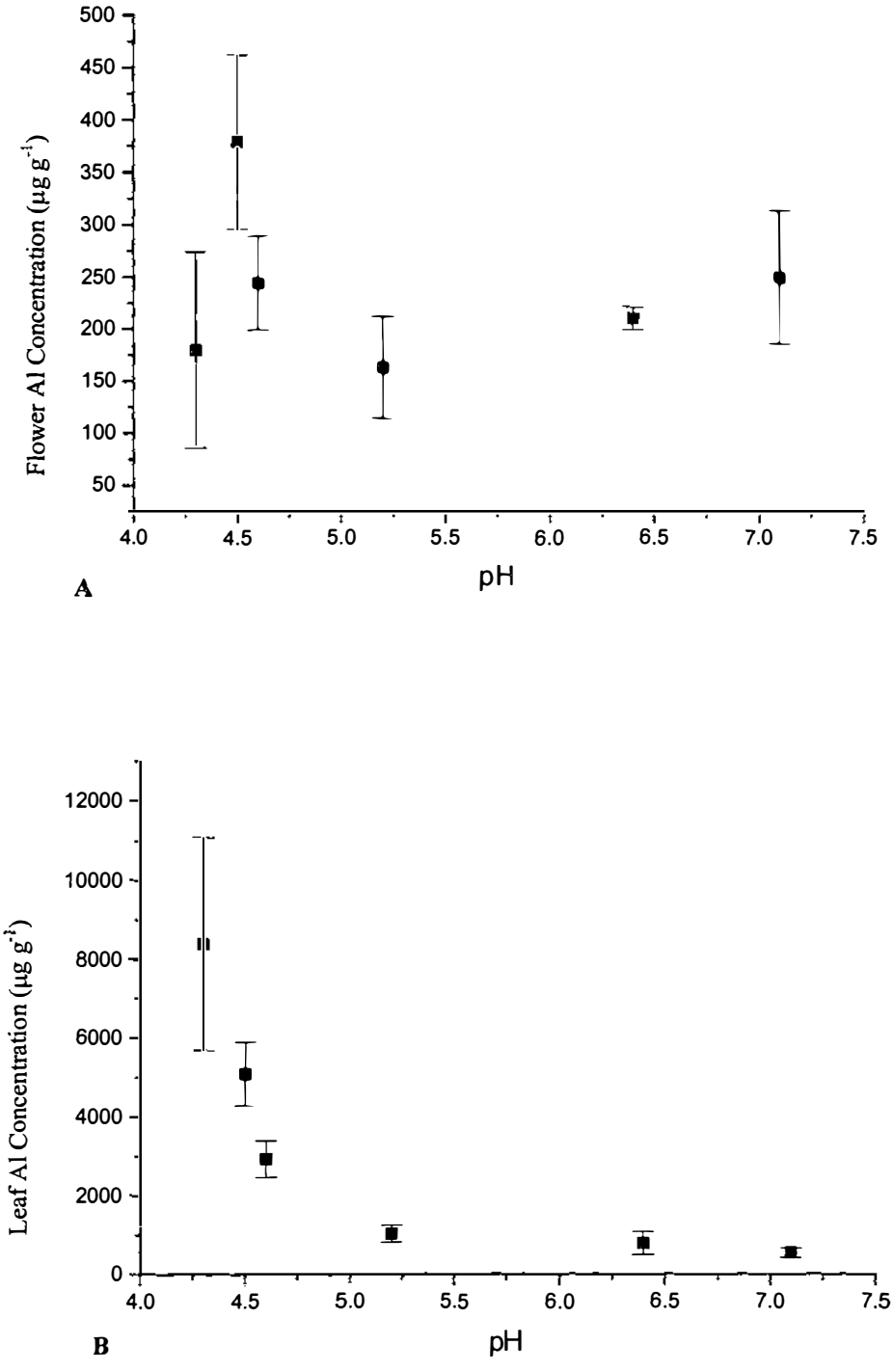


Figure 7.1
Aluminium concentration in *Camellia* spp. flowers (A) and leaves (B) (µg g^{-1}) vs soil pH. There was no relationship between soil acidity and flower aluminium concentrations (A), but leaves taken from acid soils accumulated higher aluminium concentrations (B).

7.4 DISCUSSION

All the ornamental *Camellia* spp. tested were found to hyperaccumulate aluminium to levels similar to that reported for *C. sinensis* (e. g. Dong *et al* 1999; Carr *et al* 2003). As reported in previous studies (Dong *et al* 1999; Fung & Wong 2002), greater amounts of aluminium in leaves were found in camellias growing in low pH soils. This is consistent with the hypothesis that the solubility of aluminium in soil, which is dependent on pH, was the major factor controlling aluminium uptake. There was, however, no relationship between soil acidity and flower aluminium concentrations. The greater variability in aluminium concentration of camellia flowers may be explained by large interspecific variability of flower traits. Unlike leaves, which were relatively uniform in the species tested here, camellia flowers are variable in size, petal number, thickness and the time of the year when they develop. These differences will all affect their aluminium concentration. This explanation could be tested by examining the Al concentration in the flowers of a single species grown in a wide range of soils.

There were no significant differences in flower aluminium concentration between *C. camelliae*-resistant and *C. camelliae*-susceptible species of *Camellia* in aluminium concentration in the flowers indicating that aluminium hyperaccumulation in *Camellia* spp. is not involved in species resistance. *C. camelliae* appears to tolerate the elevated aluminium concentrations in the flowers. Nevertheless, aluminium hyperaccumulation may provide protection against insect herbivores and other species of fungal or bacterial pathogens that infect the leaves. If this were the case, then some degree of plant resistance may be obtained by acidifying the soil with fertilisers such as flowers of sulphur or ammonium sulphate. This could be the focus of a future study.

7.4.1 Phytoremediation Potential

There was a serendipitous outcome of this study that may define a new role for *Camellia* species both in New Zealand and overseas: the *in situ* decontamination of aluminium contaminated wastewater, leachates or runoff. This type of clean-up technology, termed *phytoremediation*, is already well established for other types of contamination; however, there is a lack of suitable species to tackle aluminium contamination.

Aluminium-contaminated sites (from mine tailings, factory waste etc) are a global problem and remediation of these sites is a multi-million dollar industry. Most existing remediation technologies (removal, capping, leaching) do not provide a satisfactory solution, because of excessive costs and high secondary environmental impacts. Phytoremediation uses plants to improve degraded environments. As well as reducing toxic metal concentrations, plants create organic matter and enhance soil microbiological activity. While this technique may take many years to return a site to pristine condition, this 'green' option is a permanent solution that can be an order of magnitude cheaper than alternative technologies.

HortRearch (Environmental Group, Palmerston North) is using data from this study for inclusion in their phytoremediation programme. Depending on the problem and the solution required it is likely that *Camellia* species will be employed to clean up contaminated soils and beautify the site simultaneously. Faster growing species would be preferred and it is likely that *C. japonica* and *C. reticulata* species would be chosen. In addition to removing aluminium from the soil, *C. sasanqua* leaves have been shown to absorb ozone and two organic pollutants, methyl ethyl ketone and acrolein (Omasa *et al* 2000) which may increase their usefulness in certain situations.

7.4.2 Phytoremediation

There are currently three categories for application of phytoremediation technology. *Phytostabilisation* and hydraulic control use transpiration and root growth – particularly of phreatophytic species (e. g. willows and poplars) – to immobilise contaminants by reducing the amount of leaching, controlling erosion, and adding organic matter to the substrate that binds the contaminant, from a site. An example of this technique is through the reduction of saline toxicity in soil (an increasing problem in Australia) through deep-rooting, high water use, evergreen trees that lower the saline water table (Bell 1999).

Plants can also degrade contaminants (*phytodegradation* or *phytodetoxification*) through uptake and metabolism, direct action or plant root exudates or through enhanced microbial activity in the root-zone. Hannink *et al* (2001) created

transgenic tobacco plants that express the bacterial nitroreductase enzyme, which reduces 2,4,6-trinitrotoluene (TNT) to hydroxyaminodinitrotoluene (HADNT), to detoxify soils contaminated by this military explosive. Plant roots have also been shown to degrade other toxic compounds such as perchlorates (Nzengung *et al* 1999) but as yet, nothing is effective on dichlorodiphenylthrichloroethane (DDT) (Chaudhry *et al* 2002).

Metals and contaminants in soils can be removed using hyperaccumulating plants that translocate inordinate amounts into their above-ground biomass in technology called *phytoextraction.* Repeated cropping of the plants allows the metal or contaminant to be removed from the site, where it can then be burned to reduce its volume and/or stored in a contained landfill. A further refinement of this technology is called *phytomining*, where the metal extracted – such as gold – is sufficiently valuable to process from the plant (Anderson *et al* 1998). There are, however, few practical demonstrations of successful phytoextraction, as this field of research is still relatively new.

A New Zealand case study using hydraulic control was undertaken on the Kopu sawdust pile at the base of the Coromandel Peninsula. Natural vegetation had failed to establish on the pile and it was leaching boron (B) in excess of the New Zealand Drinking Water Standard (NZDWS) into a local stream. Trials found that two *Populus deltoides* hybrids were the best candidates for phytoremediation based on survival, biomass production and B uptake (Robinson *et al* 2003). The poplars controlled water leaching from the site, and extracted B from the sawdust (Robinson *et al* 2002). B was returned to the sawdust through leaf-fall, but if the plants were harvested, they could be used as an organic B supplement to trees in orchards that are B-deficient, thus also acting as phytoextractors.

CHAPTER EIGHT – RESISTANCE MECHANISMS: BASED ON THE PHENYLPROPANOID PATHWAY

8.1 INTRODUCTION

The phenylpropanoid pathway in plants produces several major classes of plant products that are used in normal growth and development, as well as products used in disease resistance. It is named for the three-carbon side chain on an aromatic ring derived from L-phenylalanine (Whetten & Sederoff 1995. Phenylalanine is the first step in the pathway and is deaminated by Phenylalanine Ammonia Lyase (PAL), further steps producing alcohols such as *para*-coumaryl, caffeoyl-CoA, lignin, various phenolic compounds, flavonoids, coumarins, stilbenes and benzoic acid derivatives (Tena & Valbuena 1982; Goodman *et al* 1986; Whetten & Sederoff 1995).

The presence and production of lignin and phenolic compounds are discussed in relation to plant resistance.

8.1.1 Phenolic Compounds/Phytoalexins

Some phenolic compounds are found in both healthy and diseased plants, but their synthesis and accumulation seems to be accelerated after infection in resistant plants (Agrios 1988). Other phenolic compounds, called phytoalexins, are not present in healthy plants, but are 'low molecular weight antimicrobial compounds that are synthesised by, and accumulate in, plants after their exposure to microorganisms' (Paxton *et al* 1980). Phytoalexins play an important role in plant defence mechanisms (Bailey 1982; Mansfield 1982; Hahlbrock & Scheel 1989).

Phytoalexins are a chemically diverse group of compounds. Members of the same plant family often produce chemically related phytoalexins, for example, terpenoid compounds are commonly produced by members of the Solanaceae (Deverall 1982). The same plant, however, can produce chemically diverse phytoalexins, e.g. broad bean (*Vicia faba)*, in which wyerone acid (an acetylenic aliphatic compound) (Letcher *et al* 1970) and the isoflavonoid medicarpin (Hargreaves *et al* 1976) have been identified. The same phytoalexin may be produced by more than one plant

species, for example, scopoletin is found in the London plane tree (*Platanus acerifolia*) (El Modafar *et al* 1993) and the rubber tree (*Hevea brasiliensis*) (Jayasuriya *et al* 2003).

As with any plant defence mechanism, the speed of reaction to the invading pathogen is important. Two antifungal phenolic compounds, umbelliferone and scopoletin, were produced when the London plane tree was attacked by *Ceratocystis fimbriata* f. sp. *platani*, but accumulation occurred too late to totally inhibit conidial germination (El Modafar *et al* 1993).

8.1.1.1 Free vs. Glycosylated Phenolic Compounds

Phenolic compounds may exist in a free state within the plant or as inactive glycosylated conjugates, for example, the cucurbitacins produced by the Cucurbitaceae occur in both free and glycosidic combinations (Harborne & Baxter 1993).

Morandi (1996) speculated that phenolic compounds are stored and/or transported as glycosylated conjugates *in vivo*, to be enyzmatically degraded when bioactive aglycones are required. In the laboratory, these glycosylated conjugates can be degraded with acid hydrolysis (Daayf *et al* 1997; Fawe *et al* 1998).

8.1.1.2 Host/Pathogen Examples of Phenolic Compounds in Plant Defence

Attack by *Ceratocystis polonica* on Norway spruce induced both polyphenolic accumulation and lignification around the damaged area (Nagy *et al* 2000). In resistant clones, Franceschi *et al* (1998) observed 40% more polyphenolic parenchyma cells, and these phenolic bodies were reduced in size and density, or disappeared completely, 12 h after wounding indicating that the phenolic compounds were hydrolysed into active antimicrobial products.

Various phenolic compounds and flavonoids have been identified in cucumber infected with *Sphaerotheca fuliginea* or treated with Milsana (bioprotectant extract of *Reynoutria sachalinensis*) (Daayf *et al* 1995; 1997; Fawe *et al* 1998; Daayf *et al* 2000). Similarly, accumulation of phenolic compounds and/or their antimicrobial activity have been studied in barley/*Drechslera graminea* (Paul & Sharma 2002),

chickpea/*Ascochyta rabiei* (Höhl *et al* 1990), coffee/*Hemileia vastatrix* (Silva *et al* 2002) and wheat/*Fusarium* head blight (Siranidou *et al* 2002).

8.1.2 Lignification of Existing Cell Walls

Plant cell walls provide a structural barrier to pathogen penetration. In addition to being a constitutive barrier to infection, induced modifications to the cell wall at the site of pathogen penetration have been documented (Agrios 1988).

8.1.2.1 Definition

Lignin is a phenylpropenol polymer found in the cell walls of mono- and di-cotyledonous plants. Within the wall, lignin forms a mesh with hemicelluloses (Wardrop 1971). Lignin biosynthesis begins with the conversion of phenylalanine to cinnamic acid catalysed by the enzyme PAL and finishes with the oxidation of cinnamyl alcohols by peroxidase to form lignin (Vance *et al* 1980). Different plants and different plant parts produce different amounts and types of lignin through the esterification of various aromatic acids.

Lignification of cell walls in response to pathogen attack has been demonstrated for several host/pathogen systems, e. g. coffee/*Hemileia vastatrix* (Silva *et al* 2002); pearl millet/*Sclerospora graminicola* (Kumudini & Shetty 2002); wheat/*Pyrenophora tritici-repentis* (Dushnicky *et al* 1998); carnations/*Fusarium oxysporum* f. sp. *dianthi* (Baayen *et al* 1996); rice/*Pyricularia oryzae* (Schaffrath *et al* 1995); muskmelon/*Sphaerotheca fuliginea* (Cohen *et al* 1990); and cowpea/*Uromyces* nonpathogenic species (Fink *et al* 1991).

8.1.2.2 Lignification as a Mechanism of Resistance

There are five ways in which lignification may hinder fungal growth (Ride 1978).

1. Lignin may make cell walls more resistant to mechanical penetration.
2. Lignification of the cell wall at the site of attack may make it resistant to the action of fungal enzymes such as polysaccharidases.
3. Lignification of the cell walls may prevent the movement of fungal enzymes and toxins into the host, and of water and nutrients from the host to the fungus.
4. The phenolic precursors of lignin and production of free radicals during polymerisation may inactivate fungal membranes, enzymes, toxins and elicitors.

5. The growing hyphal tip may become lignified and lose the elasticity necessary for further growth.

8.1.3 Cell Wall Appositions

8.1.3.1 Definitions

'Cell wall apposition' is an all-inclusive general term used to describe extra deposits of material laid down at, or near, the site of pathogen penetration. Various workers have described the deposits as "electron dense" (Huang *et al* 2001a) or "electron opaque" (Picard *et al* 2000; Benhamou & Garand 2001) and they may contain callose (β-1,3-glucans) (Rodriguez & Mendgen 1995; Benhamou *et al* 1996; Cordier *et al* 1998; Huang *et al* 2001a) and phenolic compounds including lignin (Benhamou *et al* 1996; Huang *et al* 2001a), plus other substances.

The terminology of 'papillae' and 'lignitubers' as cell wall appositions, and the application of these terms, is less clear. Both Agrios (1988) and Isaac (1992) distinguish between these two structures, but it is unclear whether others make the same distinction or use the terms interchangeably.

Fellows (1928) proposed the name lignituber for the structures he observed in wheat attacked by *Gaumannomyces* (*Ophiobolus*) *graminis* when his tests showed they were largely composed of lignin. Isaac (1992) and Agrios (1988) defined them as a sheath that forms around the hyphal tip as it penetrates the cell wall. From the diagram in Agrios (1988), it appears that the lignituber is an in-pouching of the cell wall as the hyphal tips "growing into the cell lumen are enveloped by cellulosic materials that later become infused with phenolic substances" (Agrios 1988).

A papilla is a body of heterogenous materials deposited between the plasma membrane and the cell wall at the site of fungal attack (Aist 1976; 1983). Papillae occur on the opposite side of the wall to the invading hypha (Rey *et al* 1996) and are hemispherical in shape (Aist 1976; 1983).

The terms 'halo' and 'collar' have also been used in conjunction with papillae. Halos are "roughly circular areas (when viewed from above) of the cell wall which form around the point of incipient fungal penetration" (McKeen *et al* 1968; Sargant

& Gay 1977). Collars surround the base of the fungal haustorium only (Hohl & Stössel 1976; Hohl & Suter 1976) and are thought to be incomplete papillae that have failed to prevent penetration (Hohl & Suter 1976).

8.1.3.2 Host/Pathogen Examples of Cell Wall Appositions in Plant Defence

Huang *et al* (2001a) identified all three cell wall modifications in wheat. They found lignitubers present in wheat infected by *G. graminis* var. *tritici*, whereas papillae and appositions were present in wheat infected by *Phialophora graminicola*. Bordallo *et al* (2002) identified appositions, papillae and lignitubers in tomatoes attacked by *Verticillium chlamydosporium.*

Lignitubers were found in the 'Russet-Burbank' potato infected by *Verticillium dahliae* (Perry & Evert 1984), and in wheat infected by *G. graminis* var. *tritici* (Faull & Campbell 1979; Kang *et al* 2000; Huang *et al* 2001a). Huang *et al* (2001b) also refer to papillae in wheat infected by *G. graminis* var. *tritici* in a separate study.

Some host/pathogen interactions in which papillae have been identified are: lentil/*Ascochyta fabae* f. sp. *lentis* (Roundhill *et al* 1995); cucumber/*Colletotrichum lagenarium* (Siegrist *et al* 1994); wheat/*Puccinia striiformis* (Kang *et al* 2002); barley/*Magnaporthe grisea* (Jarosch *et al* 2003); and barley/*Blumeria graminis* f. sp. *hordei* (Hueckelhoven *et al* 2001).

8.1.3.3 Composition of Cell Wall Appositions, Lignitubers and Papillae

Bordallo *et al* (2002) detected callose, lignin and proteins in cell wall deposits, phenolics, callose, lignin and proteins in papillae, but only lignin in lignitubers. Kang *et al* (2000) found callose, cellulose, xylan and lignin in lignitubers of wheat challenged by *G. graminis* var. *tritici.* Various studies have detected callose (Hächler & Hohl 1982; Benhamou *et al* 1996; Cordier *et al* 1998), cellulose (Hächler & Hohl 1982), pectic substances (Smith & McCully 1978; Bonello *et al* 1991), lignin (or phenolics) (Bonello *et al* 1991; Huang *et al* 2001a; Huang *et al* 2001b; Bordallo *et al* 2002) and silicon (Carver *et al* 1987; Blaich & Grundhoefer 1998) in papillae. When Russo & Bushnell (1989) compared the composition of papillae with wound plugs of barley, they found that both contained carbohydrates, callose and protein,

wound plugs contained cellulose and pectin while only papillae contained phenolic compounds. Lignin was not detected in either, contrary to many other studies. They concluded that fungal attack generates inducing factors not produced by mechanical penetration.

The difference in substances detected in papillae reported here may occur because protective substances are host dependent, or because of differences in techniques or stains used to detect various compounds.

8.1.4 Objectives

The objectives of this study were to:

a) detect whether free or glycosydically-bound phenolic compounds are present or induced in flowers of *Camellia* species challenged with *C. camelliae.*

b) determine whether structural defences such as wall appositions and/or lignification are induced in flowers of *Camellia* species challenged with *C. camelliae.*

c) determine whether such defences are correlated with resistance of flowers of *Camellia* species to *C. camelliae.*

8.2 MATERIALS & METHODS

8.2.1 Phenolic Compounds

Petals of susceptible and resistant camellia flowers were collected for inoculation and extraction (Table 8.1). For each specimen, four samples of 20 g of petals were weighed out. Three of the samples were inoculated using the airborne inoculation chamber (Section 6.2.2). After inoculation (assessed 2 hourly using cover slips), the petal samples were transferred to a humid chamber and incubated at 20°C. The first sample was removed after 24 h for extraction, the second at 48 h and the third at 72 h. The uninoculated sample was extracted immediately. Four petals of susceptible *C. saluenensis* x *C. reticulata* 'Brian' were included in every inoculation to ensure that the *C. camelliae* ascospores were infective. These petals were transferred to the humid chamber along with the sample petals and incubated until large *C. camelliae* lesions were evident. Sample petals may have been infected naturally prior to collection.

8.2.1.1 Extraction, Fractionation and Storage of Samples

The method of Daayf *et al* (1997) was used for extraction, fractionation and acid hydrolysis of samples. Extraction: 20 mg of petal tissue and 200 ml of acidified 80% methanol (pH 2.0 with HCl) were placed in prechilled 250 ml conical flasks and placed on ice. Samples were homogenised using a high speed blender (Polytron, Kinematica, GmbH), flushed immediately with nitrogen (~ 90 sec) and covered with aluminium foil and placed on an orbital shaker at 175 rpm at 4°C in darkness. After 48 h, extracts were clarified under suction through moistened filter paper (Whatmann #1, 11 cm diameter) in a Buchner funnel. A volume of 20 ml 80% acidified methanol was used to wash the residue. Fractionation: extracts were thrice partitioned against 125 ml of light petroleum ether (Ajax Chemicals, UNIVAR) in separating funnels, with the extract and ether shaken vigorously together and allowed to settle (60-90 sec). The methanolic extracts were kept, while the lighter petroleum ether Fraction I extracts were discarded. The methanolic extracts were rotary-evapourated under pressure at 38°C to leave 50 ml of extract then partitioned thrice against 30 ml of diethyl ether in separating funnels, with the extract and diethyl ether shaken vigorously together and allowed to settle. The portion in diethyl ether containing free phenolics was kept as Fraction II extracts, while the other portion became the Fraction III extracts containing glycosidically-bound phenolics post-acid hydrolysis. Acid hydrolysis: extracts were diluted with an equal volume of 4N HCl, and placed in conical flasks in a 100°C water bath for 90 min. The extracts were filtered under suction through moistened filter paper (Whatmann #1, 11 cm diameter) in a Buchner funnel. They were then partitioned thrice against 30 ml of diethyl ether (as described above) and the Fraction III extracts retrieved from the diethyl ether portion. Concentration of extracts *in vacuo*: Fractions II and III were rotary evapourated under pressure at 38°C to a volume of 5-10 ml, then placed in a fumehood for final evapouration to 1 ml. Samples were stored at 4°C in stoppered soda glass specimen tubes.

8.2.1.2 Detection of Phenolic Compounds

Samples were spotted onto silica gel (10-40 μm) (Sigma) plates using capillary tubing. Two spots of each sample (one of 20 μl and one of 30 μl) were run on the same plate and each plate had a control spot of 30 μl diethyl ether. The spots were placed 2.5 cm from the base of the plate. Dried plates were placed in the saturated

solvent chamber with dichloromethane:*n*-hexane:methanol (6:4:1 v/v) and developed for ~20 min. The solvent front was marked on removal from the chamber, and the plate dried for 1 ½ -2 h.

The presence or absence of phenolic compounds of each species and sample was assessed using up to six methods:

a) bioassay (conidial suspension of *Cladosporium* spp.) for detection of antifungal compounds (Daayf *et al* 1997) (*Cladosporium* spp. was maintained on Difco PDA. A concentrated conidial suspension was prepared by scrapping the conidia from the surface of the plate and mixed (1:1, v/v) with a 20 g/l solution of PDA, then sprayed onto silica gel plates.)

b) visible light (430-790 nm) for anthocyanins, quinones and deeply coloured aurone and chalcone pigments (Seikel 1962)

c) short-wave ultraviolet light (254 nm) for flavonoid compounds (Seikel 1962)

d) long-wave ultraviolet light (365 nm) for flavonoid compounds (Seikel 1962)

e) Prussian blue reaction (1% aqueous $K_2Fe(CN)_6$ and 1% $FeCl_3.6H_2O$) for phenolic compounds of all types (Randerath 1970; Harborne 1989)

f) acid vanillin (10% vanillin w/v in conc. HCl) for phenols, especially resorcinol and phloroglucinol derivatives, flavans and leucoanthocyanins (Harborne 1989)

The R_f (Relative front) value was calculated for each band via the methods b-f, or zone of inhibition (bioassay) and averaged over the two spots per sample. This was a measure of the distance travelled by the compound from the point of origin in a given solvent system and was defined as:

$$R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent front}}$$

The R_f value allowed bands from different chromatograph plates, and different seasons, to be compared.

8.2.1.3 Collection and Treatment of *Camellia* Petals

The experiment was conducted over three camellia flowering seasons (Table 8.1). The first season tested whether the method was suitable for camellia/*C. camelliae*

samples, and whether the (lengthy) preparation process could be duplicated. In the second and third seasons, testing was expanded to cover more susceptible and resistant species. The large number of flowers required for each species (80 g – a very large number of flowers for the small-flowered species), and the time required to process the samples, limited the scale of this experiment.

In Season 1 two samples from each species, consisting of an uninoculated sample and a sample inoculated with ascospores of *C. camelliae* were incubated at 20°C for 5 d before extraction and tested for Fractions II and II. In Seasons 2 and 3, four samples were taken from each species: an uninoculated sample extracted immediately (0 h) and three samples inoculated with ascospores of *C. camelliae* then incubated at 20°C for 24 h, 48 h or 72 h before extraction. They were assessed for Fractions II (except *Cladosporium* spp. bioassay) and III in Season 2, but Fraction III only in Season 3. Assessments were made using a *Cladosporium* spp. bioassay, visible light, short- and long-wave ultraviolet light and vanillin acid test.

Table 8.1 Summary of *Camellia* Species and Assessment of Fraction II and II Phenolic Compounds

Species (Season)	Fraction II Free Phenolics[a]						Fraction III Aglycones[a]					
	a	b	c	d	e	f	a	b	c	d	e	f
Susceptible												
C. japonica 'Desire' (1)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
C.japonica 'Elegans Champagne' (3)	-	-	-	-	-	-	✔	✔	✔	✔	-	✔
C. pitardii var. *pitardii* (3)	-	-	-	-	-	-	✔	✔	✔	✔	-	-
C. saluenensis x *C. reticulata* 'Brian' (2)	-	✔	✔	✔	-	✔	✔	✔	✔	✔	-	✔
Resistant												
C. forrestii (1)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
C. forrestii (3)	-	-	-	-	-	-	✔	✔	✔	✔	-	-
C. fraterna (2)	-	✔	✔	✔	-	✔	✔	✔	✔	✔	-	✔
C. lutchuensis (3)	-	-	-	-	-	-	✔	✔	✔	✔	-	-
C. yunnanensis (3)	-	-	-	-	-	-	✔	✔	✔	✔	-	-

[a] letters a-f refer to assessment method as described on the previous page

8.2.1.4 Analysis

Zones of inhibition found in the bioassay were considered the most important indication that antifungal phenolic compounds were present in a sample and all other assessment methods were compared against this. Because of likely variability between samples prepared in different seasons and differences occurring during the development of each chromatograph plate, bands present within $R_f \pm 0.10$ of

inhibition zones were considered to be of further interest as possibly containing antifungal phenolic compounds.

A visual comparison of developed chromatograph plates with each assessment method was made initially. The various bands and zones of inhibition produced appeared to be similar between resistant and susceptible species. No bands were produced by Fraction II free phenolic samples and testing of this Fraction was discontinued in Season 3.

R_f values of each species over the four time samples were compared for each assessment method to determine whether bands were constitutively present or increased/decreased over time. R_f values of each species at each time sample for each assessment method were compared to determine whether similar bands were present or absent in resistant and susceptible species. Small imperfections in the production of the silica gel plates led to some variation in R_f values between samples on the same plate (Figure 8.1). Where bands were obviously similar but the R_f value distorted by these imperfections is indicated in the result tables.

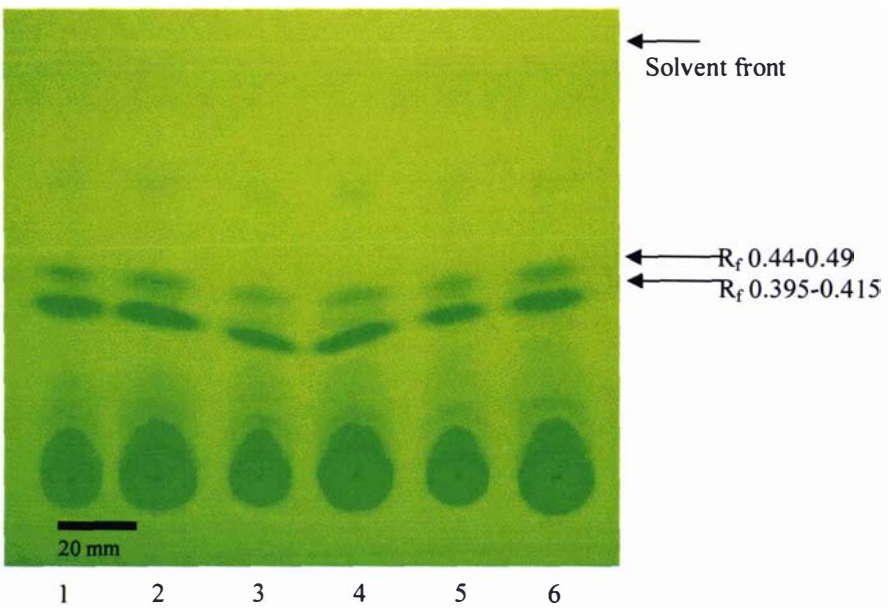


Figure 8.1
Developed chromatograph plate under short-wave ultraviolet light showing the effect of imperfections in the silica gel on bands of the same R_f value. Samples are from *C. fraterna*. Lane 1 and 2 0 h; Lanes 3 and 4 24 hpi and Lanes 5 and 6 48 hpi samples. The two 24 hpi samples show considerable distortion in both orientation and R_f value when compared to 0 hpi and 48 hpi samples.

8.2.2 Cell Wall Appositions and Lignification of Existing Cell Walls

8.2.2.1 Preparation and Storage of Samples

Petals of susceptible and resistant camellia flowers, rhododendron and magnolia flowers (Table 8.2) were collected and processed during the week of 10.09.02.

Table 8.2 Resistance Rating of Species and Cultivars of Camellias Used in a Study of Cell Wall Modifications

Species/Cultivar	Resistance Rating[a]
C. euryoides (M)	U/R
C. forrestii (A)	R
C. fraterna (A)	R
C. grijsii (A)	R
C. japonica 'Desire' (A)	S
C. japanica 'Elegans Champagne' (A)	S
C. lutchuensis (A)	R
C. lutchuensis hybrid 'Fairy Blush' (A)	R
C. pitardii var. *pitardii* (A)	S
C. polydonta (A)	S
C. reticulata 'Zhangjia Cha' (A)	S
C. saluenensis x *C. reticulata* 'Brian' (M)	S
C. transnokoensis (A)	R
C. trichocarpa (M)	U/R
C. yuhsienensis (A)	R
C. yunnanensis (A)	R
Magnolia stellata (K)	non-host resistance
Rhododendron 'Chrysomanicum' (M)	non-host resistance

[a] S=susceptible, R=resistant, U/R=untested, observations indicate resistant

For each species, there were four treatments: 0 h (uninoculated control), 24 h, 48 h and 72 h after inoculation with ascospores of *C. camelliae*. Petals were inoculated using the airborne inoculation chamber (Section 6.2.2) then transferred to a humid chamber and incubated at 20°C. At 24 h intervals, five to six petal discs (12 mm diameter) were cut using a cork borer (or whole petals were used for *C. forrestii*). Discs were cut from fresh uninoculated petals (0 h control) immediately after collection. The discs were cleared in 150 ml beakers containing 50 ml of 1 M KOH which were autoclaved for 15 min at 121°C and stored at 4°C until after the camellia season had finished. Four petals of susceptible *C. saluenensis* x *C. reticulata* 'Brian' were included in every inoculation to ensure that the *C. camelliae* ascospores were infective. These petals were transferred to the humid chamber along with the sample petals and incubated until large *C. camelliae* lesions were evident. Sample petals may have been infected naturally prior to collection.

8.2.2.2 Toludine Blue O to Stain Lignin

Prior to staining, petal discs were rinsed thrice in RO water for 5 min each rinse. Discs were not blotted and were handled as little as possible since they were extremely fragile.

After rinsing, two discs from each sample were stained in Toludine Blue O (Section 2.5.2) for between 40 min and two hours. Discs were removed from the stain and rinsed once in RO water. Excess stain and water were removed using a tissue laid next to the disc to drain liquid by capillary action. Discs were floated on to microscope slides, a drop of Shears Mounting Fluid added and then a coverslip. Slides were viewed with brightfield microscopy using an Olympus BH-2 microscope.

8.2.2.3 Analysis

Each disc was scanned at 400x magnification for the presence of ascospores, papillae, lignitubers and signs of cell wall lignification.

8.3 RESULTS

8.3.1 Phenolic Compounds

Control diethyl ether spots run on each silica gel plate did not produce bands on any of the plates.

8.3.1.1 Fraction II Samples

Testing of Fraction II free phenolic samples was discontinued after the results of the first two seasons' tests. None of the samples from the two susceptible or two resistant species produced zones of inhibition in the *Cladosporium* spp. bioassay or bands using the other assessment methods. Occasionally a short smear was present at the spot site, or a very faint band, but as seen in Figure 8.2, the band was barely distinguishable.

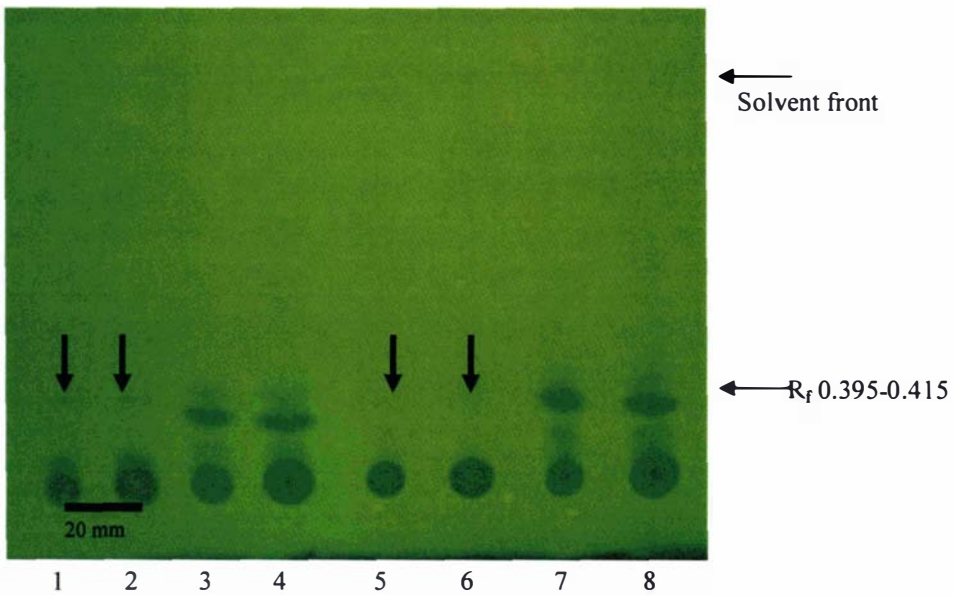


Figure 8.2
Developed chromatograph plate under short-wave ultraviolet light comparing Fraction II and III samples from *C. fraterna*. Lanes I and 2 Fraction II 0 hpi, Lanes 3 and 4 Fraction III 0 hpi, Lanes 5 and 6 Fraction II 24 hpi; and Lanes 7 and 8 Fraction III 24 hpi samples. A smear above the inoculation spot is visible in both Fractions, and a faint band, corresponding to one band in Fraction III, is visible in Fraction II samples (arrows).

8.3.1.2 Fraction III Samples

Cladosporium spp. Bioassay

Where antifungal compounds were present in samples, zones where *Cladosporium* spp. failed to grow were clearly visible (Figure 8.3). In Season 1, zones of inhibition of similar R_f values were seen in both the resistant and susceptible species (Appendix IV). Neither of the species tested in Season 2 produced zones of inhibition, and only two species in Season 3, the susceptible *C. japonica* 'Elegans Champagne' and resistant *C. yunnanensis*. Of these, the R_f values of the inhibition zones of *C. japonica* 'Elegans Champagne' were comparable to those of *C. japonica* 'Desire' and *C. forrestii*, while *C. yunnanensis* produced zones of inhibition only in the 0 hpi samples and this zone extended round the inoculation site.

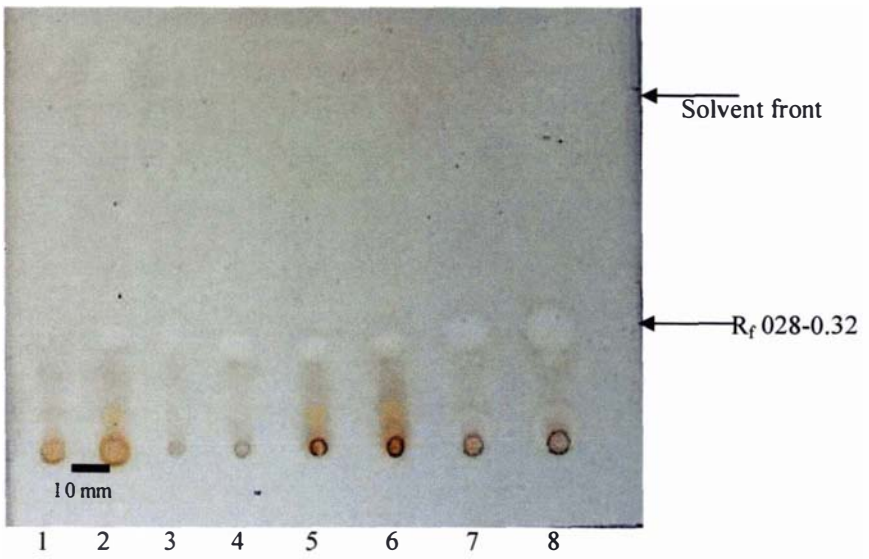


Figure 8.3
Bioassay chromatograph plate showing zones of inhibition where *Cladosporium* spp. has not colonised the silica gel/PDA. Lanes 1 and 2 *C. forrestii* 96 hpi; Lanes 3 and 4 *C. japonica* 'Desire' 96 hpi; Lanes 5 and 6 *C. forrestii* 0 hpi and Lanes 7 and 8 *C. japonica* 'Desire' 0 hpi.

For comparison with other assessment methods, the R_f value of the zone of inhibition was considered to be 0.28-0.32 (excluding the silica gel-distorted zones of *C. japonica* 'Elegans Champagne' and the unusual result for *C. yunnanensis*). Bands with R_f values of between 0.18 and 0.42 were considered to be of further interest when comparing results with other assessment methods.

Visible Light

Of the nine species tested only three produced bands that were visible under normal light conditions (Appendix IV). All other species produced orange-brown smears of varying lengths in which no distinct bands were visible. Figure 8.4 shows one species in which smears and distinct bands were visible.

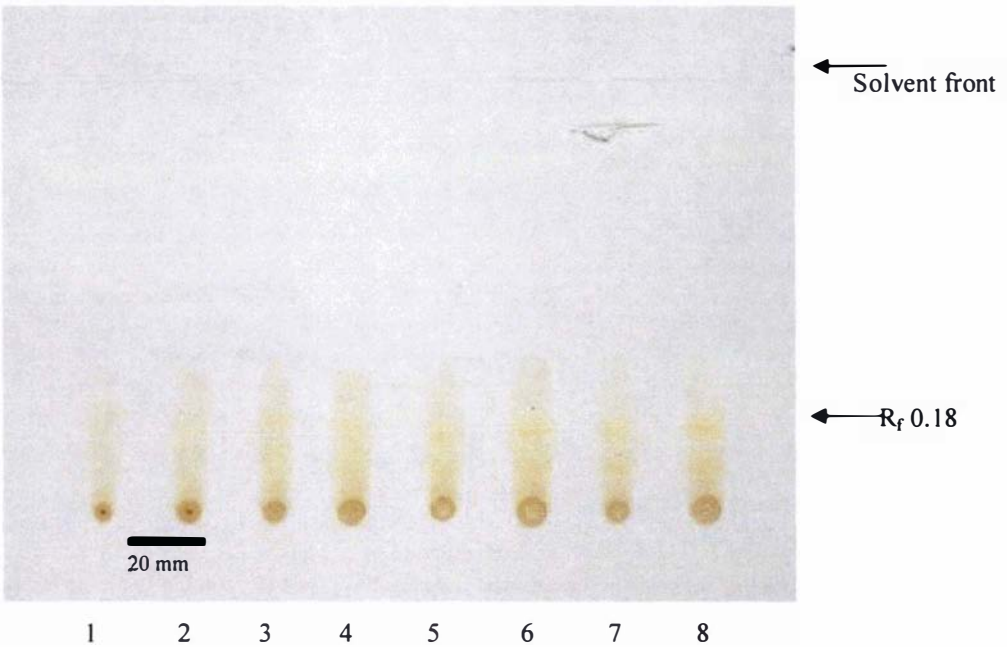


Figure 8.4
Developed chromatograph plate in visible light of *C. yunnanensis*. Lanes 1 and 2 0 hpi, Lanes 3 and 4 24 hpi, Lanes 5 and 6 48 hpi and Lanes 7 and 8 72 hpi samples. Distinct bands are visible in the 48 and 72 hpi samples.

While none of the bands corresponded exactly with the zone of inhibition seen in the bioassay, the R_f values were at the lower end of those considered to be of further interest.

Shortwave Ultraviolet Light

There was some variation in the number of bands, and their R_f value, between samples on different plates and different seasons. The results from Season 2 (*C. saluenensis* x *C. reticulata* 'Brian' and *C. fraterna*) were quite different to those of Seasons 1 and 3. These samples were subsequently re-run, and these results were more similar to those of Seasons 1 and 3, indicating that the silica gel plates and/or solvent system had probably been at fault in Season 2.

For most samples, there were three zones of banding, with the number of distinguishable bands varying slightly. Within the first zone there were 1-2 bands visible, in the second zone two close bands, and in the third zone, a single, more diffuse, band, or three diffuse bands in the case of *C. yunnanensis* (Figure 8.5) and *C. pitardii* var. *pitardii* (Appendix IV).

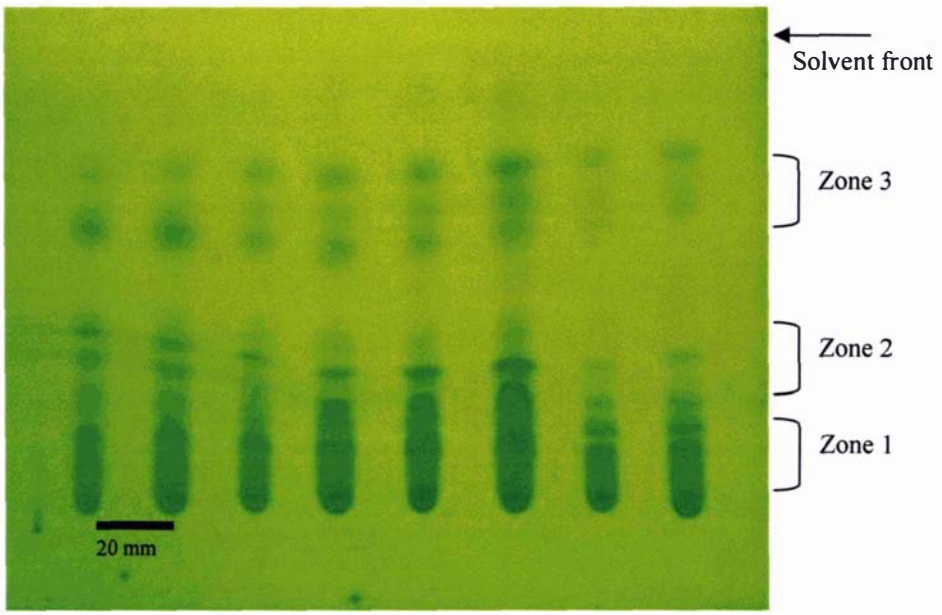


Figure 8.5
Developed chromatograph plate of *C. yunnanensis* under shortwave ultraviolet light showing multiple banding pattern. From left to right; two spots each of 0 hpi, 24 hpi, 48 hpi and 72 hpi time samples. (R_f values Zone 1 0.15-0.1.25; Zone 2 0.225-0.32 and 0.295-0.36; Zone 3 0.575-0.635; 0.65-0.707 and 0.74-0.78)

There was little variation between time samples of the same species, indicating that these phenolic compounds were present constitutively, and comparison of banding patterns of resistant and susceptible species did not show any correlation with resistance.

For most species, the R_f values of the third band (R_f 0.18-0.475) corresponded closely with the zones of inhibition seen in the bioassays and bands 2 and 4 often occurred within the lower and higher limits of bands considered to be of interest.

Longwave Ultraviolet Light

Under longwave UV light there was little variation in the number and size of bands seen with each sample. Each species' samples had two prominent fluorescent bands and there was similarity between the R_f values (Appendix IV). Typically the first band was between R_f 0.09-0.15 and the second between R_f 0.16-0.27. While there was some variation in R_f values between samples on different plates and between seasons, the overall pattern of the two prominent bands was similar.

There was little variation between time samples of the same species, indicating that these phenolic compounds were present constitutively, and comparison of banding

patterns of resistant and susceptible species did not show any correlation with resistance (Figure 8.6).

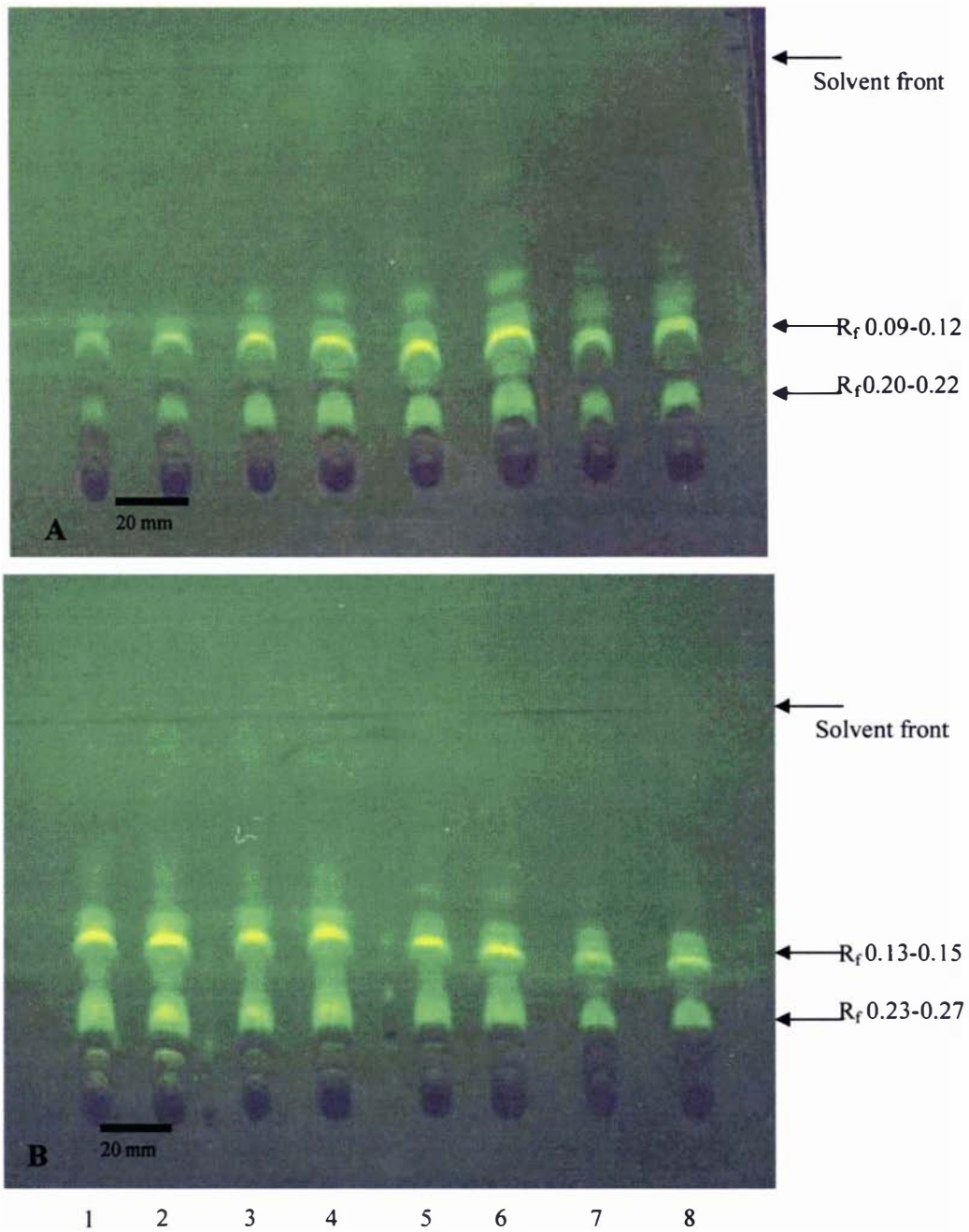


Figure 8.6
Developed chromatograph plates under longwave ultraviolet light showing the two prominent fluorescent bands. (A) *C. pitardii* var. *pitardii* and (B) *C. lutchuensis*. Lanes 1 and 2 0 hpi, Lanes 3 and 4 24 hpi, Lanes 5 and 6 48 hpi and Lanes 7 and 8 72 hpi samples.

For most species, the R_f value of the second band was at the lower end of values considered to be of interest in the bioassay.

Prussian Blue Reaction

Samples from the first season only were tested with the Prussian Blue reagent. In *C. forrestii*, two bands were present, while in *C. japonica* 'Desire', only the second band was present, and that was very faint in comparison (Appendix IV and Figure 8.7).

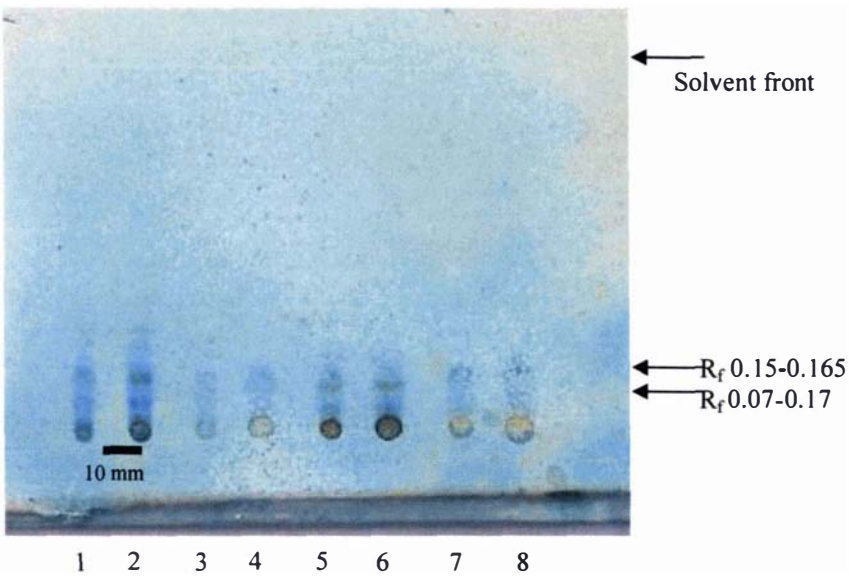


Figure 8.7
Developed chromatograph plate with Prussian Blue reagent comparing the banding patterns. Lanes 1 and 2 *C. forrestii* 96 hpi, Lanes 3 and 4 *C. japonica* 'Desire' 96 hpi, Lanes 5 and 6 *C. forrestii* 0 hpi and Lanes 7 and 8 *C. japonica* 'Desire' 0 hpi samples.

There was little variation between the time samples of *C. forrestii*, indicating that the phenolic compounds were present constitutively. There was, however, a difference in banding pattern and intensity of the reaction between the resistant and susceptible species, with the susceptible species producing only one, very faint band in comparison with the two distinct bands of the resistant species.

Neither of the bands visible with Prussian Blue reagent corresponded with zones of inhibition seen in the bioassay.

Acid Vanillin Test

Tests with *C. saluenensis* x *C. reticulata* 'Brian', *C. japonica* 'Elegans Champagne' and *C. fraterna* produced no distinct bands.

Results from the tests with acid vanillin reagent were very similar to those described for Prussian Blue. In *C. forrestii*, two bands were present, while in *C. japonica* 'Desire', only the second band was present, and that was very faint in comparison (Appendix IV and Figure 8.8).

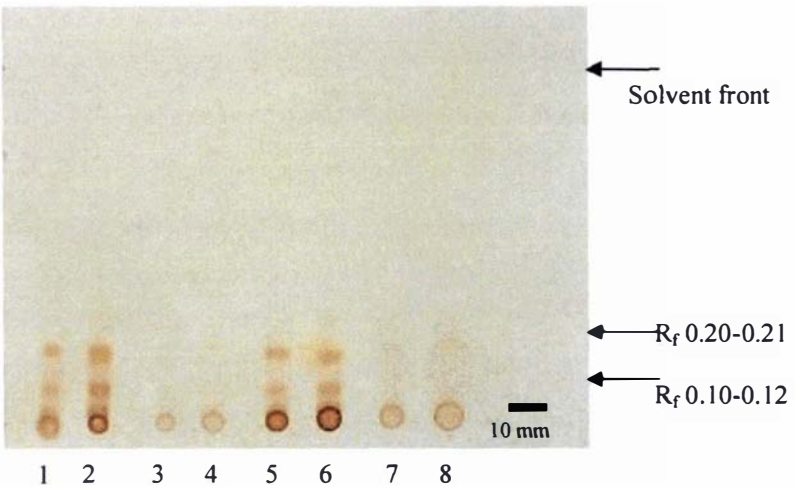


Figure 8.8
Developed chromatograph plate with the acid vanillin test comparing the banding patterns. Lanes 1 and 2 *C. forrestii* 96 hpi, Lanes 3 and 4 *C. japonica* 'Desire' 96 hpi, Lanes 5 and 6 *C. forrestii* 0 hpi and Lanes 7 and 8 *C. japonica* 'Desire' 0 hpi samples.

There was little variation between the time samples of *C. forrestii*, indicating that the phenolic compounds were present constitutively. There was, however, a difference in banding pattern and intensity of the reaction between the resistant and susceptible species, with the susceptible species producing only one, very faint band in comparison with the two distinct bands of the resistant species.

Neither of the bands visible with acid vanillin corresponded with zones of inhibition seen in the bioassay, although the second band was in the lower range of those considered to be of interest.

8.3.2 Cell Wall Appositions and Lignification of Existing Cell Walls

Petal discs were very fragile and often disintegrated while moving between stains and water rinses. For this reason, no data is available from *C. forrestii* at 48 h after inoculation as none of the petals survived the staining process. A disc would sometimes split leaving two epidermal layers, or partially separate, or tear. Occasionally a disc would take up too much stain, however destaining was not undertaken because of the difficulty in handling discs without further destruction.

No ascospores were observed on any of the petal discs in either of the treatment stains.

8.3.2.1 Toludine Blue O to Stain Lignin

Structures resembling papillae/lignitubers and cell wall lignification were observed in some specimens (Table 8.3).

Table 8.3 Species and Hybrids of Camellias with Cell Wall Modifications 72 h after Inoculation with Ascospores of *C. camelliae*

Species/Cultivar		Hours after Inoculation	Modification[a]	Resistance Rating[b]
C. euryoides (M)		48 h	L, P	U/R
C. fraterna	(A)	48 h	P	R
		72 h	L, P	
C. grijsii	(A)	48 h	L, P	R
		72 h	L	
C. lutchuensis	(A)	0 h	L	R
		24 h	L	
		48 h	L	
		72 h	L, P	
C. lutchuensis hybrid 'Fairy Blush'	(A)	48 h	L	R
		72 h	L	
C. polydonta	(A)	0 h	L	S
		24 h	L	
C. trichocarpa	(M)	24 h	L, P	U/R
		48 h	L, P	
		72 h	L, P	
C. yuhsienensis	(A)	48 h	P	R
C. yunnanensis	(A)	48 h	L, P	R
		72 h	L, P	

[a] P=papillae/lignituber; L=lignificaiton
[b] S=susceptible, R=resistant, U/R=untested, observations indicate resistant

Of the nine species where papillae/lignitubers (Figures 8.10-12) or lignification (Figure 8.9) were detected, six were previously known to be resistant to *C. camelliae.* Field observations and concurrent experiments indicated that *C. euryoides* and *C. trichocarpa* were resistant species and both lignification and papillae/lignitubers were observed in these two species. Lignification was detected in one susceptible species, *C. polydonta.*

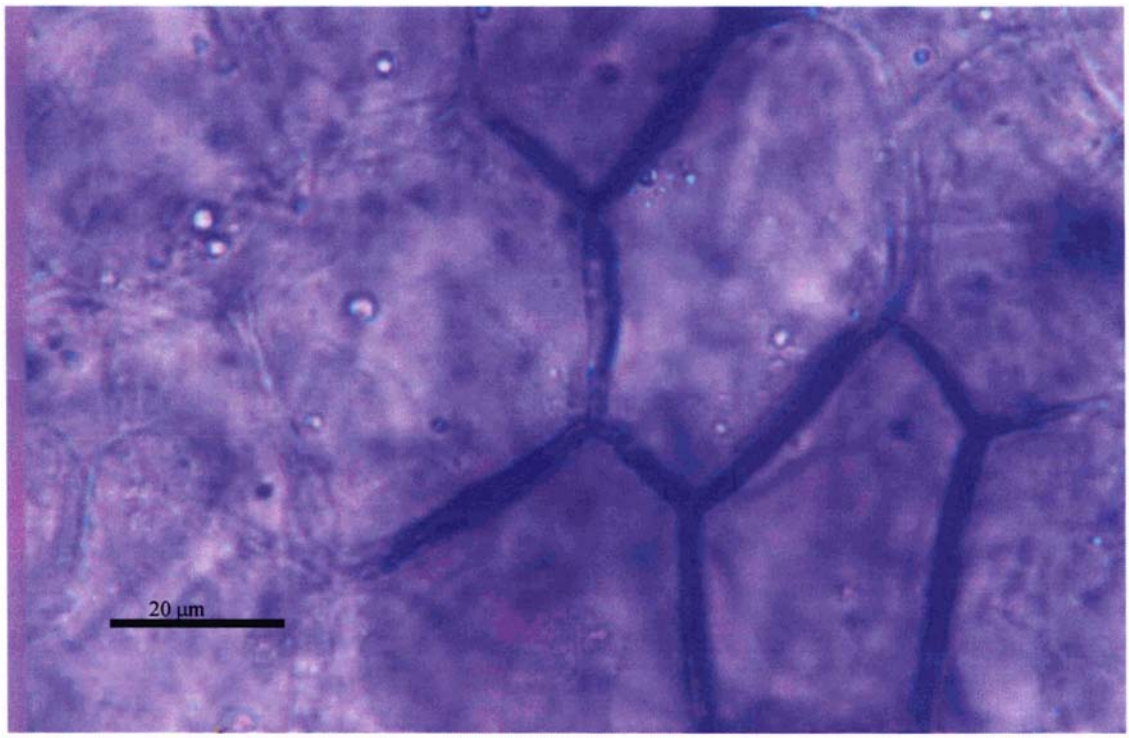


Figure 8.9
C. yunnanensis petal showing lignified cell walls 48 h after inoculation with ascospores of *C. camelliae.* Specimen cleared in KOH and stained with Toludine Blue O.

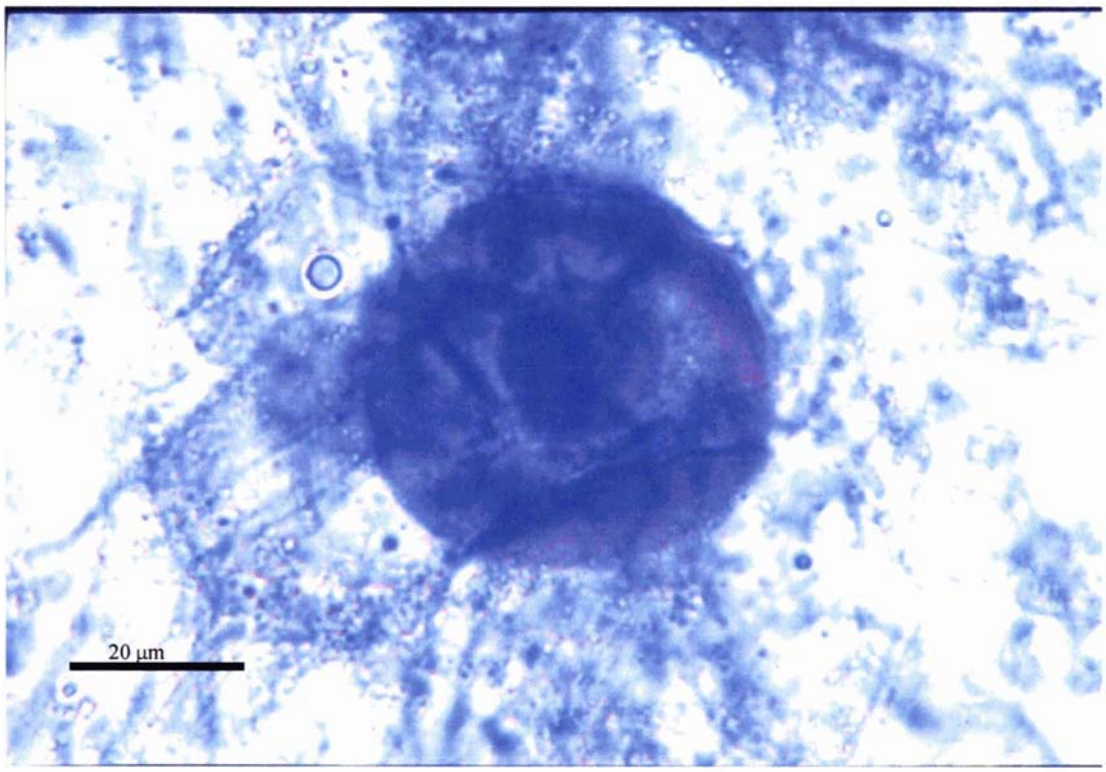


Figure 8.10
C. lutchuensis petal showing papilla/lignituber 72 h after inoculation with ascospores of *C. camelliae.* Specimen cleared in KOH and stained with Toludine Blue O.

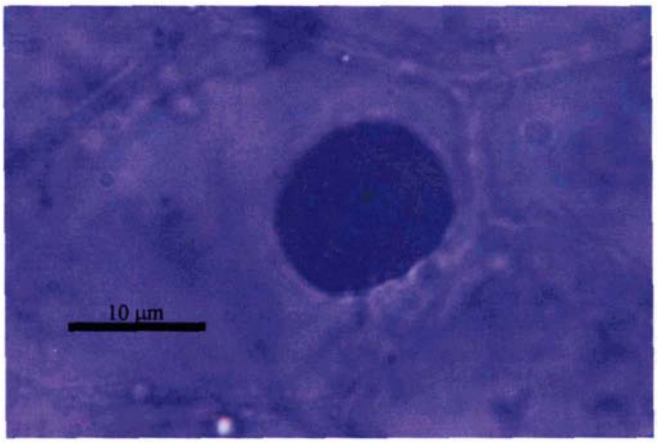


Figure 8.11
C. yunnanensis petal showing papilla/lignituber 72 h after inoculation with ascospores of *C. camelliae*. Specimen cleared in KOH and stained with Toludine Blue O.

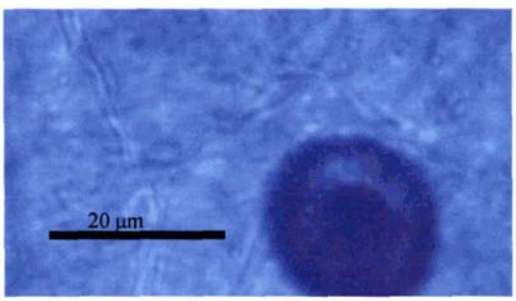


Figure 8.12
C. trichocarpa petal showing papilla/lignituber 24 h after inoculation with ascospores of *C. camelliae*. Specimen cleared in KOH and stained with Toludine Blue O.

Papillae, lignitubers or cell wall lignification were not observed in the two non-host species or in seven *Camellia* species (including two resistant species, *C. forrestii* and *C. transnokoensis*) (Table 8.4).

Table 8.4 Species and Cultivars of Camellia in which Papillae/Lignitubers or Lignification were not Observed

Species/Cultivar	Resistance Rating[a]
C. forrestii (A)	R
C. japonica 'Desire' (A)	S
C. japonica 'Elegans Champagne' (A)	S
C. pitardii var. *pitardii* (A)	S
C. reticulata 'Zhangjia Cha' (A)	S
C. saluenensis x *C. reticulata* 'Brian' (M)	S
C. transnokoensis (A)	R
Magnolia stellata (K)	non-host resistance
Rhododendron 'Chrysomanicum' (M)	non-host resistance

[a] S=susceptible, R=resistant

8.4 DISCUSSION

8.4.1 Phenolic Compounds

Interpretation of the results for phenolic compounds in *Camellia* spp. was complicated by the fact that the experiment was conducted over a period of three years. Some of the variation seen in the results was probably due to differences that accumulated over this time, from differing weather conditions experienced by the

flowers prior to collection, through to small, but cumulative, differences in preparation, extraction of samples, development on chromatograph plates and assessment methods. The first recommendation, therefore, is that future experiments be conducted in one season (see Section 8.4.1.1).

Initial tests of Fraction II (free phenolic) samples did not produce zones of inhibition in the bioassay or distinct bands in any of the other assessment methods and there appeared to be no reason to continue testing for Fraction II free phenolics. There are several points at which the method of preparation and extraction of samples could be improved including running Fraction II samples in future experiments. Improvements to the method are discussed in Section 8.4.1.1

The first season's tests of Fraction III (glycosidically-bound phenolics) samples showed that while there appeared to be no difference between resistant and susceptible species in the bioassay, visible, shortwave or longwave light assessment methods, there was a difference when tested with Prussian Blue and acid vanillan reagents. In the susceptible species *C. japonica* 'Desire', only very faint bands were visible in both 0 hpi and 96 hpi samples, compared to the two distinct bands seen in the resistant *C. forrestii*. These results were not replicated in later season's samples and these two methods of assessment were discontinued. Improvements to the preparation and extraction method may clear up this point in future experiments, but for the purposes of this experiment, zones of inhibition in the bioassay were considered the most important indication of whether antifungal phenolic compounds were present.

Antifungal phenolic compounds were present in four of the species tested by the *Cladosporium* spp. bioassay, but they were spread evenly between resistant and susceptible species, indicating that while they are antifungal to *Cladosporium* spp., they are possibly not antifungal to *C. camelliae*. The unusual result of *C. yunnanensis*, where inhibition was observed in the 0 h time sample only, and just around the site of spot inoculation is more difficult to interpret and future experiments may clarify this anomaly.

None of the bands observed by the other assessment methods corresponded exactly with R_f values of the inhibition zone but a close match was made with the third band seen under shortwave ultraviolet light, indicating that the compound may be a flavonoid (Seikel 1962). A more accurate identification of the antifungal compound could be made using High Performance Liquid Chromotography (HPLC).

The other assessment methods (visible, short- and longwave light) demonstrated that various phenolic compounds were present in *Camellia* species, but because the compounds appeared in both resistant and susceptible species, they were not likely to be a prime factor in resistance to *C. camelliae*. In addition, all the phenolic compounds detected in this experiment were constitutively expressed, indicating that they were not phytoalexins.

8.4.1.1 Improvements to the Method

Secondary plant metabolites, such as phytoalexins, are difficult to study because they are extremely bioactive, unstable and produced in very small quantities. In 2002, McNally *et al* published a methodology for the study of induced glycosylated plant phenolics in cucumber in which a simple, reproducible and standardised methodology was developed. This method involved making the plant material, and its extraction, uniform, optimised the hydrolysis reaction for maximum concentration of antifungal aglycones and suggested improved purification procedures. Relevant changes to improve the results in future experiments with phenolic compounds in *C. camelliae* are described below.

Plant material was first freeze-dried to stabilise phenolic compounds, to standardise the mass of the extract, as water content of plant tissues varies, to disable phenolic-degrading enzymes such as phenolases, peroxidases and polyphenoloxidases reduction of water content (Ferreres *et al* 1997; Dupont *et al* 2000) and inhibit microbial growth that can cause degradation or alteration of compounds.

Major variability between experiments and results can occur since some phenolic compounds, such as flavonoid aglycones (Merken *et al* 2001), are easily degraded by acid hydrolysis, while others, such as the C-flavonoid glycosides are unaffected (Markham 1982). Optimisation of the hydrolysis conditions for camellia/*C.*

camelliae, (through determining the best acid, acid concentration and duration of reaction) would ensure that the highest concentrations of antifungal aglycones are retained.

Silica gel plates, as used in these experiments, are time consuming to prepare and use and contribute to variable results (McNally *et al* 2002). Instead, McNally *et* al (2002) recommend Sep-Pak® cartridges or reverse phase silica to fractionate the samples, then analysis with HPLC using a gradient to identify induced compounds.

The results from this experiment indicate that phenolic compounds, in particular phytoalexins, were not induced in response to infection of camellia flowers by *C. camelliae.* The results were, however, a little inconsistent, partly because the experiment was conducted over three seasons, and partly because of the methodology chosen to investigate it. In view of the improvements that could be made to the method, it is recommended that this experiment be repeated in order to more reliably determine whether phenolic compounds are involved in resistance.

First, the experiment should be conducted in one season. Without the extra load of concurrent experiments (chitinase etc.) it would be possible to prepare four resistant and four susceptible species for analysis. Second, the adoption of the extraction and acid hydrolysis steps as described by McNally *et al* (2002) and possibly the purification and HPLC steps. In addition, a more definitive bioassay could be developed using blended mycelium of *C. camelliae* (instead of *Cladosporium* spp. spores), perhaps using food colouring in the silica gel to distinguish zones of inhibition.

8.4.2 Cell Wall Modifications and Lignification of Existing Cell Walls

In eight of the ten resistant species in this study, lignification and/or papillae/lignitubers were observed in at least one of the time series, whereas cell wall modifications were observed in only one of the six susceptible species samples. This indicates that cell wall modifications may be one form of resistance operating in *Camellia* species resistant to *C. camelliae.* In a concurrent study, Vingnanasingam (2002) identified two resistant *Camellia* species, *C. cuspidata* (not included in my

study) and *C. lutchuensis*, which formed papilla-like thickenings of the cell wall at the penetration point.

In this study of camellia/*C. camelliae* interaction, the majority of cell wall modifications were observed in the 48 h and 72 h samples. Modifications were present in some 0 h and 24 h samples, possibly indicating that infection occurred prior to flower collection.

As anticipated, ascospores were not seen on the surface of the petals. Ascospore suspension inoculation experiments (not cited) showed that ascospores were rarely seen in RO water suspensions more than 7 d old, presumably because the ascospores burst in water. Since the petal discs had been autoclaved in 1 M KOH and stored for two months prior to staining, it was expected that any ascospores would have long since disintegrated.

The long length of storage may also have contributed to the poor preservation of some the petal discs, particularly those with thin petals (i.e. most resistant species). Over-stained specimens could not be destained without further destruction, and disintegrating specimens could not be rearranged on the microscope slide without total destruction. Thus, those specimens in which no cell wall modifications were observed may owe more to poor preservation or overstaining than absence of these structures. Storage was necessary because of the need to continue resistance testing studies, and processing samples for other resistance mechanism studies. Future microscopic studies could be carried out at the time of preparation, perhaps avoiding some of the problems described here, although petal tissue is inherently more delicate than the leaf tissue for which most of the staining techniques have been developed.

The timing of these plant-induced responses may be the key to whether a species is resistant or susceptible. Aist & Israel (1977) suggested that the effectiveness of papillae as defence structures might depend on the ability of the host to complete papillae deposition and consolidation before penetration of the host wall becomes too advanced. Hächler & Hohl (1984) reported that the *Phytophthora infestans*-susceptible potato 'Bintje' began collar development later than resistant variety 'Eba'

while von Ropenack *et al* (1998) found that the accumulation of a phenolic conjugate (p-CHA) and speed of papillae compaction played an important role in the non-race specific resistance of barley to powdery mildew. In a study of coffee/*H. vastatrix* Silva *et al* (2002) found that PAL activity was stimulated later in the susceptible species, while regression analysis showed that accumulation of polymers in cell walls of susceptible pearl millet cultivars was slower than that for cultivars resistant to *S. graminicola* (Kumudini & Shetty 2002). In the susceptible *Picea abies*/*Pythium dimorphum* interaction, lignification occurred too late to effectively prevent the pathogen from spreading (Børja *et al* 1995).

Studies have linked increased activity at two enzymatic steps in the metabolic pathway of lignin synthesis with increasing resistance. Silva *et al* (2002) studied species of coffee tree resistant and susceptible to the coffee leaf rust fungus (*Hemileia vastatrix*). They found that the percentage of germinated urediospores, number of appressoria and amount of fungal growth inside leaf tissues were similar in resistant and susceptible species until the third day after inoculation. Two peaks of PAL activity were detected at days 2 and 5 after inoculation in resistant species. The first peak coincided with the early accumulation of phenolic compounds and the beginning of HR cell death, while the second peak coincided with the later accumulation of phenols and lignification of the host cell walls. Similarly, Fink *et al* (1991) noted that an increase in PAL activity indicated the involvement of lignification in the early defence of cowpea against the non-host pathogen *Uromyces* spp. When Moerschbacher *et al* (1990) inhibited PAL in wheat against *Puccinia graminis* f. sp. *tritici*, the amount of lignified necrotic tissue decreased and fungal growth increased.

Various superoxidases have also been shown to form an effective barrier against fungal penetration by the hydrogen-peroxide mediated lignification of cell walls (Finke *et al* 1991; Thordal-Christensen *et al* 1997; Koike *et al* 2001; Lane 2002).

Localisation and quantification of β-1,3-glucan (callose) levels at the site of appositions and papillae has also been shown for the resistant wheat/*Phialophora graminicola* interaction (Huang *et al* 2001a) and in the normally susceptible

wheat/*G. graminis* var. *tritici* interaction where it was found a fungicide indirectly enhanced structural and biochemical reactions (Huang *et al* 2001b).

In this preliminary study, Toludine Blue O was used to detect if lignin was present in a selection of resistant and susceptible *Camellia* species. More extensive studies of papillae/lignitubers and lignification and their presence or absence in resistant or susceptible species of *Camellia* is recommended, together with more detailed biochemical studies to detect other activation stages, such as PAL activity, in the phenylpropanoid pathway.

CHAPTER NINE – RESISTANCE MECHANISMS: CHITINASE ASSAY

9.1 INTRODUCTION

Chitinase enzymes are found in a wide variety of plants and there is strong correlative evidence that they are defence proteins with antifungal activity (Graham & Sticklen 1994). Chitinase expression patterns (Meins & Ahl 1989; Anguelova-Merhar *et al* 2001; McFadden *et al* 2001), *in vitro* studies of fungal growth inhibition (Schlumbaum *et al* 1986) and enhanced resistance by transformed plants to fungal pathogens (Broglie & Broglie 1994; Tabaeizadeh *et al* 1999; Takatsu *et al* 1999; Yamamoto 2000; Datta *et al* 2001; O'Brien *et al* 2001) indicate that these proteins are a part of the plant's defence systems.

9.1.1 Role of Chitinase *in planta*

Chitinases (poly (1,4-*N*-acetyl-β-D-glucosaminide)) glycano-hydrolase, EC3.2.1.14) catalyse the hydrolysis of chitin polymers (β-1,4-*N*-acetyl-glucosamine), liberating oligomers such as chitobiose, chitotriose, chitotertraose and higher homologues (Wurms 1996). Chitin is one of the most ubiquitous polymers in nature (Flach *et al* 1992) found in insects, marine invertebrates and is a major component of cell walls of many fungi. Chitinase has been shown to associate with hyphal walls *in planta* (Wubben *et al* 1992), and in combination with β-1,3-glucanase, has been shown to degrade cell walls and/or inhibit growth (Boller 1985; Schlumbaum *et al* 1986; Arlorio *et al* 1992; Mathivanan 2000; Radhajeyalakshmi *et al* 2000; El-Katatny *et al* 2001).

While many studies show evidence (some circumstantial) that chitinases are associated with increased levels of resistance, not all studies show a positive correlation between resistance and chitinase activity (Nielsen *et al* 1993; Kim & Hwang 1994; Lange *et al* 1996; Simmons *et al* 2001). Care is therefore required to interpret cause and effect.

In addition to their suspected role in plant defence, chitinases have a role in normal plant growth and development (Collinge *et al* 1993; Graham & Sticklen 1994; Ponath *et al* 2000; Zhong *et al* 2002). They are expressed constitutively in plant organs not challenged by pathogens or abiotic factors and specific chitinases can be found in certain organs only during certain stages of development (Graham & Stricklen 1994). Chitinase activity is higher in roots and flowers compared to other plant tissues. In roots, it is hypothesised that the enzyme kills or slows the growth of soil pathogens that are in constant contact with the rhizosphere (Graham & Sticklen 1994). In flowers, however, differential accumulation during development (Neale *et al* 1990; Lawton *et al* 1994) and its specificity in certain flower parts (Trudel *et al* 1989; Leung 1992; Takei *et al* 2000) indicate that specific chitinases play a role in sexual reproduction.

9.1.2 Characteristics and Classification

Plant chitinases are typically monomeric 25-35 kDa proteins and nearly all are endolytic, cleaving within the chitin polymer rather than at the terminus (Graham & Sticklen 1994). Enzyme Commission (EC) numbers for enzymes involved in hydrolysis of chitin are EC.3.2.1.14 (endochitinases), EC.3.2.1.17 (lysozymes) and EC.3.2.1.52 (exochitinases) (www.expasy.ch/enzyme/).

Chitinases may be acidic or basic, contain a catalytic domain, and a putative chitin-binding domain. They are classified on the presence or absence of an N-terminal and sequence similarity of the catalytic domain (Shinshi *et al* 1990; Collinge *et al* 1993). Other chitinase nomenclature systems are shown in Table 9.1.

Table 9.1 Comparison of Chitinase Nomenclature Terminology (from Neuhaus *et al* 1996)

Family of Glycosy 1 Hydrolase (Henrissat 1991)	PR Proteins	Class (Shinshi *et al* 1990; Collinge *et al* 1993; Melchers *et al* 1994)	Old Gene Name 1 (Meins *et al* 1994)	New Gene Name 2 (Neuhaus *et al* 1996)
19	PR-3	I	Chi 1	Chia 1
		II	Chi 2	Chia 2
		IV	Chi 4	Chia 4
		V	Chi 5	Chia 5
		VI		Chia 6
18	PR-8	(III)	Chi 3	Chib 1
18	PR-11	(VI)	Chi 6	Chic 1
-	PR-4	I		Chid 1
		II		Chid 2

9.1.2.1 Class I

Class I chitinases are generally basic, with a conserved cysteine-rich terminal domain with putative chitin-binding properties and a highly conserved chitinolytic catalytic domain separated by a variable proline-rich hinge (Shinshi *et al* 1990; Raikhel & Lee 1993). They are usually located in the vacuole (Mauch & Staehelin 1989; Kunze *et al* 1998; Suarez *et al* 2001). Class I chitinases tend to have higher specific activities than Class II chitinases because the chitin-binding domain increases catalytic efficiency (Graham & Sticklen 1994). Class I chitinases are strongly antifungal, particularly in combination with β-1,3-glucanases (EC.3.2.1.39) (Sela-Buurlage *et al* 1993). It is hypothesised that vacuolar localisation and accumulation allows large quantities of the enzyme to be released on lysis of the cell when challenged by an invading pathogen or HR response in a non-specific manner.

9.1.2.2 Class II

Class II chitinases are generally acidic and found in the extracellular space (Broglie & Broglie 1994; Graham & Sticklen 1994). Class II chitinases lack a carboxyl-terminal extension that is necessary for the targeting of chitinases to the vacuole (Neuhaus *et* al 1991; Melchers *et al* 1993; Neuhaus *et al* 1994). Within the catalytic domain they have high sequence similarity (60-64%) to Class I chitinases (Punja & Zhang 1993), but lack the cysteine-rich terminal chitin-binding domain (Graham & Sticklen 1994). They demonstrate no antifungal activity alone (Sela-Buurlage *et al* 1993; Joosten *et al* 1995) but moderate activity in combination with class I β-1,3-glucanases (Sela-Buurlage *et al* 1993). Mauch & Staehelin (1989) suggest that Class II chitinases may act as signalling molecules, releasing elicitors, which induce plant defences. Their lower catalytic activity (Sela-Buurlage *et al* 1993), low/no antifungal activity (Sela-Buurlage *et al* 1993; Joosten *et al* 1995) and extracellular location (Broglie & Broglie 1994; Graham & Sticklen 1994) may thus reflect their role in early detection and induction of plant defences, rather than the outright antifungal activity of Class I chitinases.

9.1.2.3 Class III

Class III chitinases may be acidic or basic and are found in the extracellular space (Graham & Sticklen 1994). The conserved catalytic domain differs from that of

Class I and Class II catalytic domains and they lack a cysteine-rich chitin-binding domain (Broglie & Broglie 1994; Graham & Sticklen 1994). Class III chitinases are quite different from other chitinase Classes in that a) the proteins appear to be derived from a different ancestral sequence (Hamel *et al* 1997); b) the secondary structure is completely different (Henrissat 1991) and c) some function as both chitinases and lysozymes (Métraux *et* al 1989; Jekel *et al* 1991; Shih *et al* 2001). Lysozymes cleave the peptidoglycan polymers of prokaryote cell walls, thus they may be involved in defence against bacterial pathogens (Brunner *et al* 1998). Multiple functions for Class III chitinases have been reported. Studies by Lawton *et al* (1994); Kästner *et al* (1998); Yeboah *et al* (1998); Jacobs *et al* (1999); Regalado *et al* (2000) indicate that they are involved in plant defences, while other studies show that they may be involved in storage (Clendennen *et al* 1998), establishment of mycorrhizal relationships (Salzer *et al* 2000) and development and/or differentiation (Hamel *et al* 1997).

9.1.2.4 Class IV

The Class IV chitinases (proposed by Collinge *et al* 1993) are similar to Class I chitinases in that they possess a chitin-binding domain, a proline-rich hinge and catalytic domain. They lack a carboxyl-terminal extension that targets chitinase to the vacuole (Neuhasu *et al* 1991) and are assumed to accumulate extracellularly. The presence and position of sequence substitutions and deletions in the chitin-binding and catalytic domain are characteristic of this Class. Class IV chitinases are associated with defence responses to pathogens (Nielsen *et al* 1996; Gerhardt *et al* 1997; Li *et al* 2001), fruit ripening (Robinson *et al* 1997), senescence (Hanfrey *et al* 1996) and embryo development (Dong & Dunstan 1997; Gerhardt *et al* 1997; Kragh *et al* 1996).

9.1.2.5 Class Exceptions

While the majority of chitinases studied thus far fall into one of the above classes, they do not represent absolute divisions. Classes V-VII have now been assigned for newly studied chitinases (Melchers *et al* 1994) and there are yet other chitinases which do not fit any current Class (Ary *et al* 1989; Lerner & Raikhel 1992).

9.1.3 Objectives

The objectives of this study were to:

a) determine whether chitinase activity could be detected in *Camellia* species

b) determine whether chitinase activity was correlated with resistance or susceptibility.

9.2 MATERIALS & METHOD

Endochitinase activity was detected by staining undegraded glycol chitin in agar plates with Calcofluor (Sharrock & Gill 1991 unpubl. data; modified from Trudel & Asselin 1989).

Petals of susceptible and resistant camellia flowers, rhododendron, azalea, magnolia and tulip flowers were collected for inoculation and extraction (Table 9.2). For each specimen, four samples of 1.5 g of petal were weighed out. Three of the samples were inoculated using the airborne inoculation chamber (Section 6.2.2). After inoculation (assessed 2 hourly using cover slips), the petal samples were transferred to a humid chamber and incubated at 20°C. The first sample was removed after 24 h for extraction, the second at 48 h and the third at 72 h. The uninoculated sample was extracted immediately. Four petals of susceptible *C. saluenensis* x *C. reticulata* 'Brian' were included in every inoculation to ensure that the *C. camelliae* ascospores were infective. These petals were transferred to the humid chamber along with the sample petals and incubated until large *C. camelliae* lesions were evident. Sample petals may have been infected naturally prior to collection.

Table 9.2 Flower Species Tested for Endochitinase Activity on Glycol Chitin

Flower			Source[a]	Rating[b]
Genus	Species	Cultivar		
Tulip	(peony form)	'Monsella'	M	U
Rhododendron	-	'red' (unknown)	K	U
	tephropelum	-	M	U
	arboreum spp. *zeylanicum*	'College Pink'	M	U
	-	'Medusa'	M	U
	-	'Chrysomanicum'	M	U
Magnolia	*stellata*	-	K	U
	soulangeaua	-	M	U
Camellia	*cuspidata*	-	A	R
	euryoides	-	M	R
	forrestii	-	A	R
	fraterna	-	A	R
	grijsii	-	A	R
	longicarpa	-	A	R
	lutchuensis	-	A	R
	pitardii var. *pitardii*	-	A	S
	polydonta	-	A	S
	transnokoensis	-	A	R
	trichocarpo	-	A	R
	yuhsienensis	-	A	R
	C. saluenensis x *C. reticulata*	'Brian'	M	S
	C. japonica	'Desire'	A	S
	C. japonica	' Elegans Champagne'	A	S
	C. japonica	'Softly'	A	S
	C reticulata	'Zhangjia Cha'	A	S
	C. japonica x *C. lutchuensis*	'Spring Mist'	E	R
	C. japonica x *C. saluenensis*	'E. G. Waterhouse'	M	S
	C. lutchuensis hybrid (1999 CN) not in Register -	'Fairy Blush'	A	R
	(*C. pitardii* x *C. fraterna*) x unknown	'Alpen Glo'	E	R
	C. cuspidata x *C. saluenensis*	'Cornish Snow'	A	S
	C. cuspidata hybrid	'Spring Festival'	A	S

[a] A=Arboretum; E=Esplanade; M=Massey; K=307 Kahuterawa Rd, Palmerston North

[b] Resistance Rating based on ascospore inoculation tests: S=Susceptible; R=Resistant; U=Unknown/Untested

Each sample was ground (Polytron, Kinematica GmbH, Littau) with 5 ml of extraction buffer (0.1 M sodium acetate pH 5.6) and centrifuged at 12 000 rpm for 20 min. The sample was then filtered through moistened filter paper and glass wool and stored in glass bottles at 4°C.

Thirty microlitres of the sample extract, or 30 µl extraction buffer (control), was placed in each well of the chitin agar plate (Section 2.3.1.2). The plate was sealed with parafilm and incubated at 37°C for 5 h. After incubation, the agar in each plate was covered with 10 ml of 0.01% (w/v) Calcofluor white M2R (Fluorescent brightener 28, Sigma) in 500 mM tris (hydroxymethyl) aminomethane hydrochloric acid (Tric-HCl) buffer pH 8.9 containing 0.02% (w/v) sodium azide, for 10 min. The plate was rinsed with RO water several times and left covered with water for 15 min.

The water was removed and the plate viewed under long wavelength UV light (350 nm). Dark zones around wells indicated that the chitin had been degraded, while the remainder of the plate fluoresced blue/white (Figure 9.1). The diameters of the zones of degradation were marked at two perpendicular points. The reaction zone radial extension (mm) was calculated by averaging the two diameter measurements and dividing by two to get the radius, then subtracting the radius of the well (2.5 mm) from the total radius.

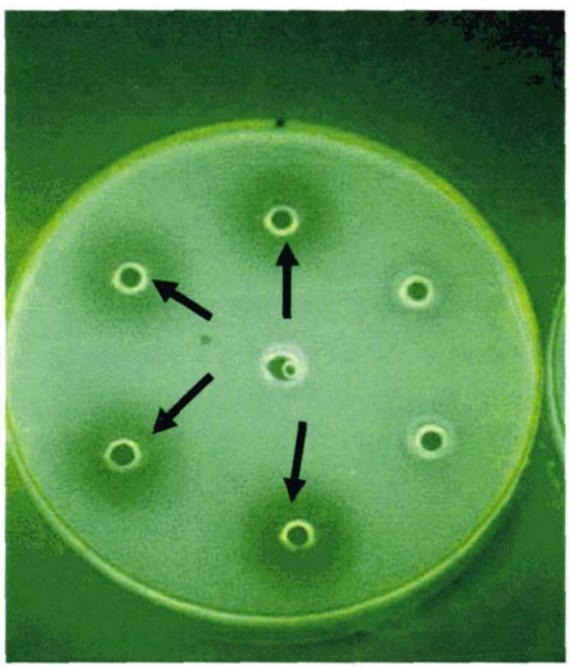


Figure 9.1
Glycol chitin agar plate showing dark zones of degraded chitin at four inoculation sites (arrows) after 5 h incubation at 37°C. The remainder of the plate fluoresced white.

Three replicate wells of each sample were tested; each well on a different plate and each plate contained one control well. Seven species (28 samples) were tested twice from the same extraction (i.e. six replicate wells of each sample; first three wells on 18.9.01, second three on 3.10.01) (*C. euryoides*, *C. grijsii*, *C. japonica* 'Champagne'

and 'Desire', *C. lutchuensis*, *C. pitardii* var. *pitardii*, *C. yuhsienensis* (referred to as Test 1 and Test 2), and six species (24 samples) were extracted twice (*C. cuspidata*, *C. japonica* 'Champagne', *C. longicarpa*, *C. reticulata* 'Zhangjia Cha', hybrids 'E. G. Waterhouse' and 'Brian') (referred to as Extract 1 and Extract 2).

Data were analysed with SAS for Windows Version 8e after log transformation to meet assumptions of normality of distribution and constant variance of residuals. A logistic regression was first fitted to determine reaction and goodness of fit on all data. Second, an ANOVA was performed on data with values >0 to determine differences between classes (Species, Extract, Time and Replicate). SAS code is shown in Appendix II.

9.3 RESULTS

Glycol chitin degradation was not observed in any control well and control data are not reported here.

The only significant effect was in Species where the small P value ($p<0.0001$) indicated a large difference between species. There was no evidence for effects of Extract (different extractions of the same species), Time (of extraction after inoculation), Time effects for different Species or different Time effects for different Extracts of the same Species (Logistic regression table Appendix IV). The Replicate(Extract*Species) term was not required.

There was evidence for differences between species ($F_{21, 176}=33.78$, $p<0.0001$) and Time effects for different Species ($F_{49, 176}=2.28$, $p<0.0001$). There was no evidence for a consistent Time effect ($F_{3, 176}=1.79$, $p<0.1509$), that different Extracts of the same Species ($F_{1, 176}=0.30$, $p<0.5862$) or different Time effects for different Extracts of the same Species ($F_{3, 176}=2.01$, $p<0.1142$) were significant (ANOVA table Appendix IV). The Replicate(Extract*Species) term was not required.

Within the Time(Species) analysis, four species showed significant Time effects. These were *C. fraterna* ($F_{3=5.28}$, $p<0.0016$), *C. transnokoensis* ($F_{3=5.37}$, $p<0.0015$), *C. yuhsienensis* ($F_{3=12.55}$, $p<0.0001$) and *Magnolia soulangeaua* ($F_{2=0.56}$, $p<0.0010$). The Time(Species) effect for these four species is shown in Figure 9.2. For *C. yuhsienensis* and *C. fraterna*, the chitin degradation zone was least 48 h after inoculation with *C. camelliae* and greatest after 72 h. In *C. transnokoensis*, the degradation zone decreased after 24 h to a low at 72 h after inoculation. In *M. soulangeaua*, the degradation zone was least at 0 h and greatest at 48 and 72 h (no degradation zone was visible at 24 h). For all other species, the reaction zone radial extension was consistent between the four sample times.

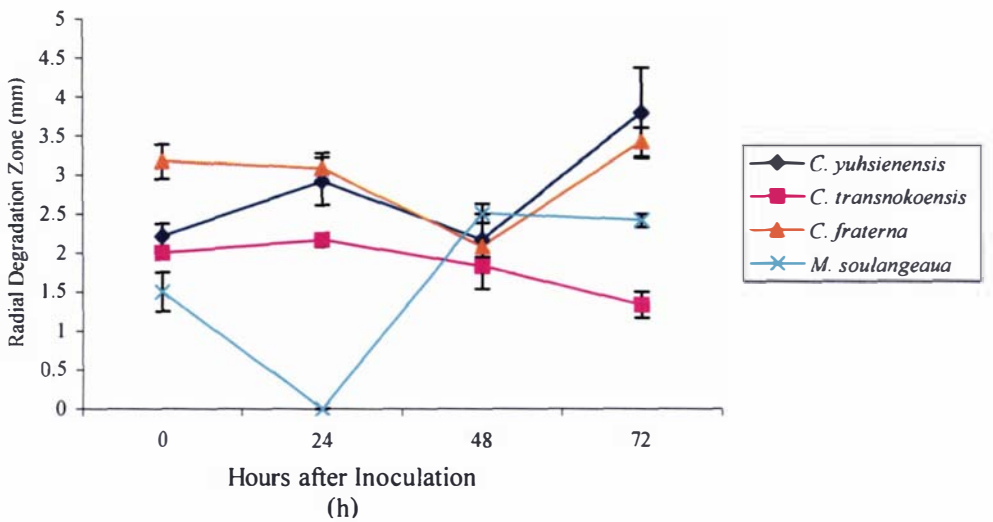
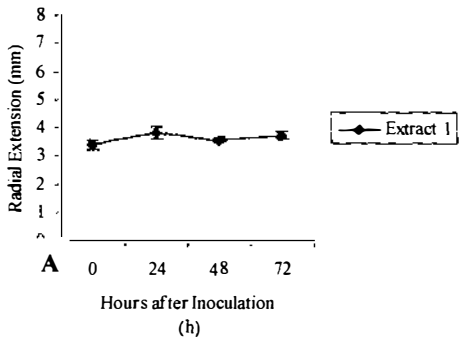
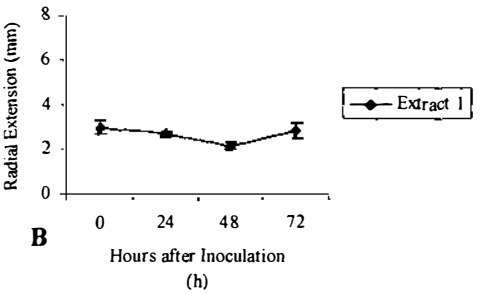
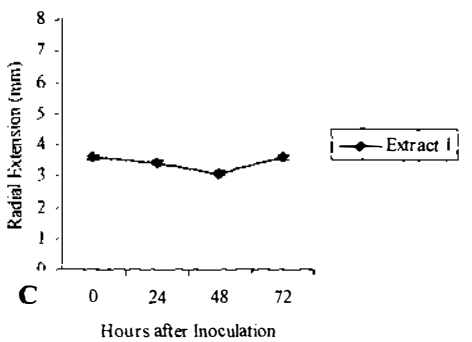


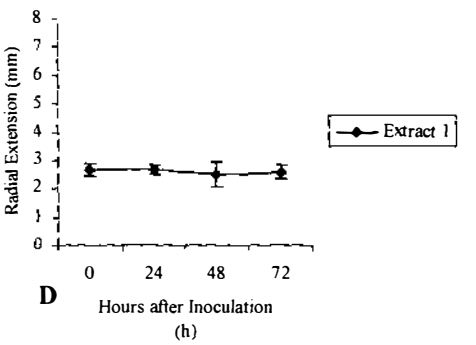
Figure 9.2
Significant time effects on chitinase production of three species of camellia and one of magnolia after inoculation with airborne ascospores of *C. camelliae*.

Of the eight non-host species tested for chitinase activity, none was detected in the four Time samples from *M. stellata* and chitinase was detected in the other seven (Figures 9.3 A-E and 9.4 A-B).









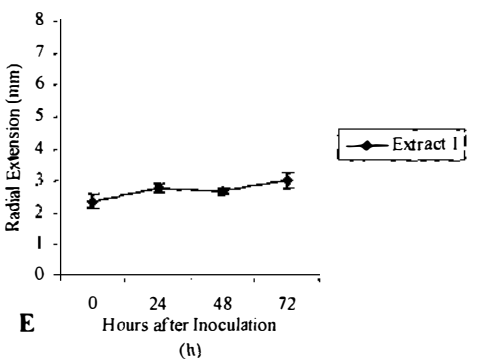


Figure 9.3 A-E
Rhododendron spp. in which chitinase activity was detected at all four Time samples after inoculation with airborne ascospores of *C.camelliae*. (A) *Rhododendron* 'red'; (B) *R. tephropelum*; (C) *R.* 'Chrysomanicum'; (D) *R.* 'College Pink'; (E) *R.* 'Medusa'.

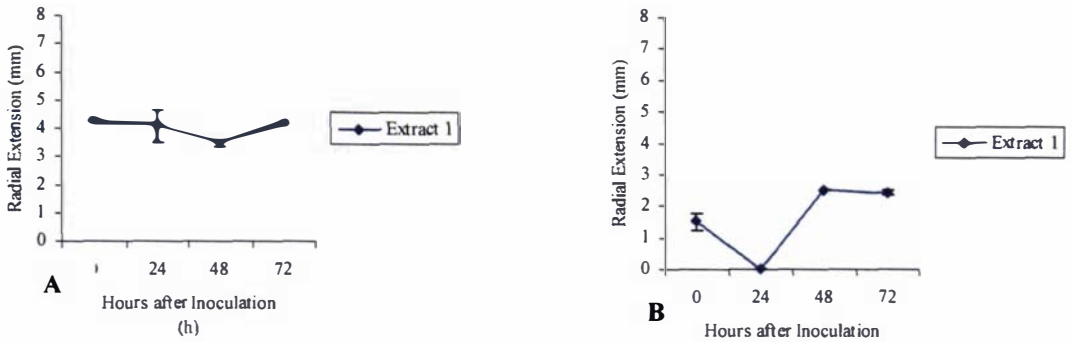


Figure 9.4 A-B
Chitinase activity of (A) *Tulip* 'Monsella' and (B) *M. soulangeaua* after inoculation with airborne ascospores of *C. camelliae*.

Of the thirteen species or hybrids of *Camellia* rated as 'resistant', no chitinase activity was detected in the hybrids *C. lutchuensis* hybrid 'Fairy Blush' and *C. japonica* x *C. lutchuensis* 'Spring Mist'. Of the two extracts made of *C. cuspidata* flowers, none was detected in Extract 1, but chitinase activity was detected in 24 and 72 h Time samples of Extract 2 (Figure 9.5).

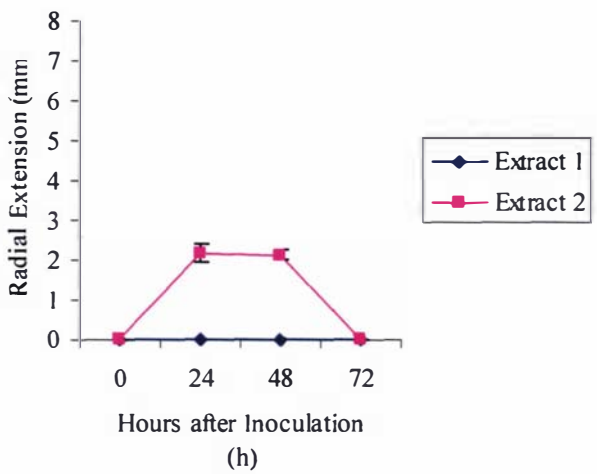


Figure 9.5
Chitinase activity in two Extracts of *C. cuspidata* in petals inoculated with airborne ascospores of *C. camelliae*.

Chitinase activity was detected in the remaining ten 'resistant' *Camellia* species (Figure 9.6 A-J).

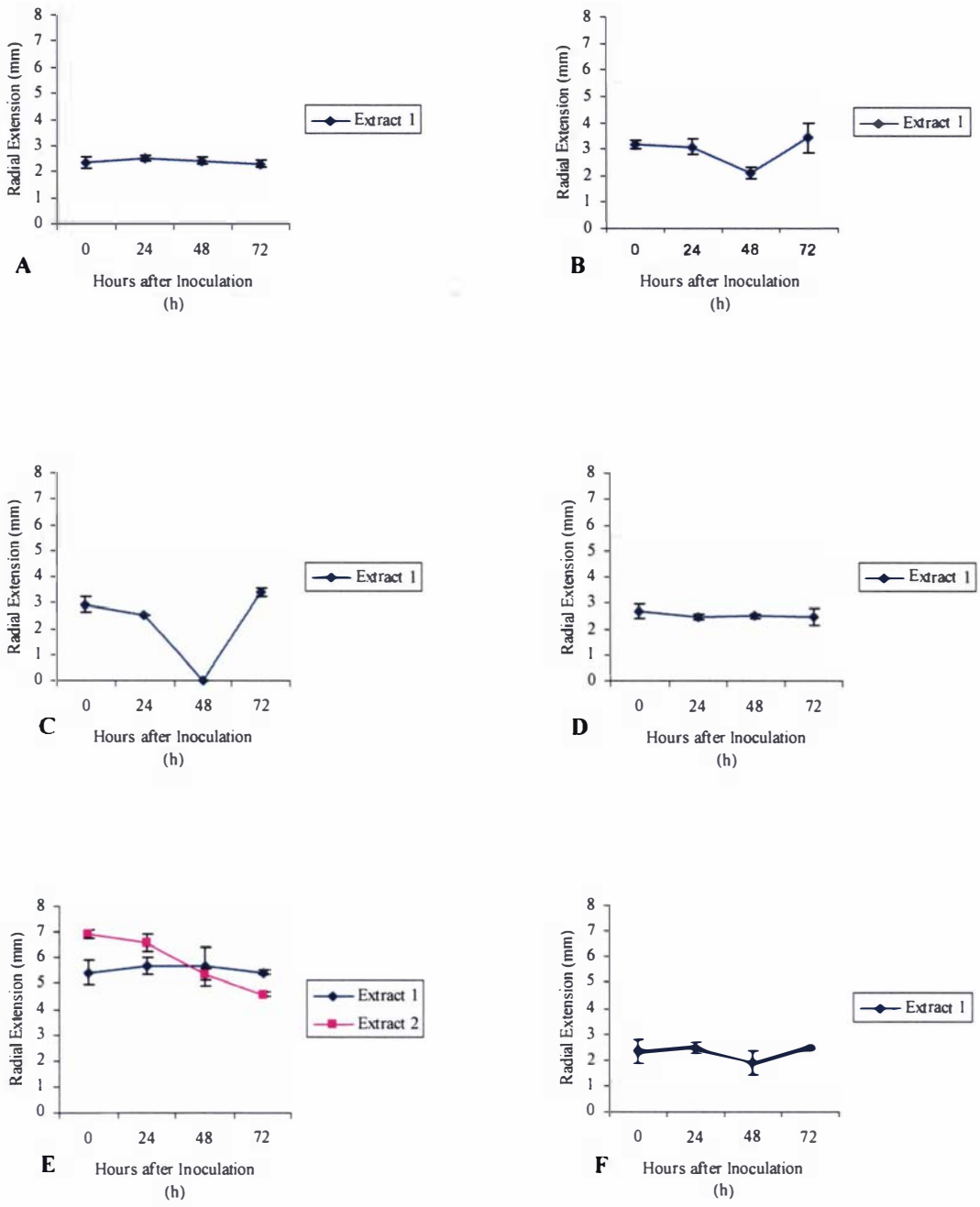


Figure 9.6 A-F
'Resistant' *Camellia* species in which chitinase activity was detected in petals inoculated with airborne ascospores of *C. camelliae*. (A) *C. euryoides*; (B) *C. fraterna*; (C) *C. forrestii*; (D) *C. grijsii*; (E) *C. longicarpa;* (F) *C. lutchuensis*.

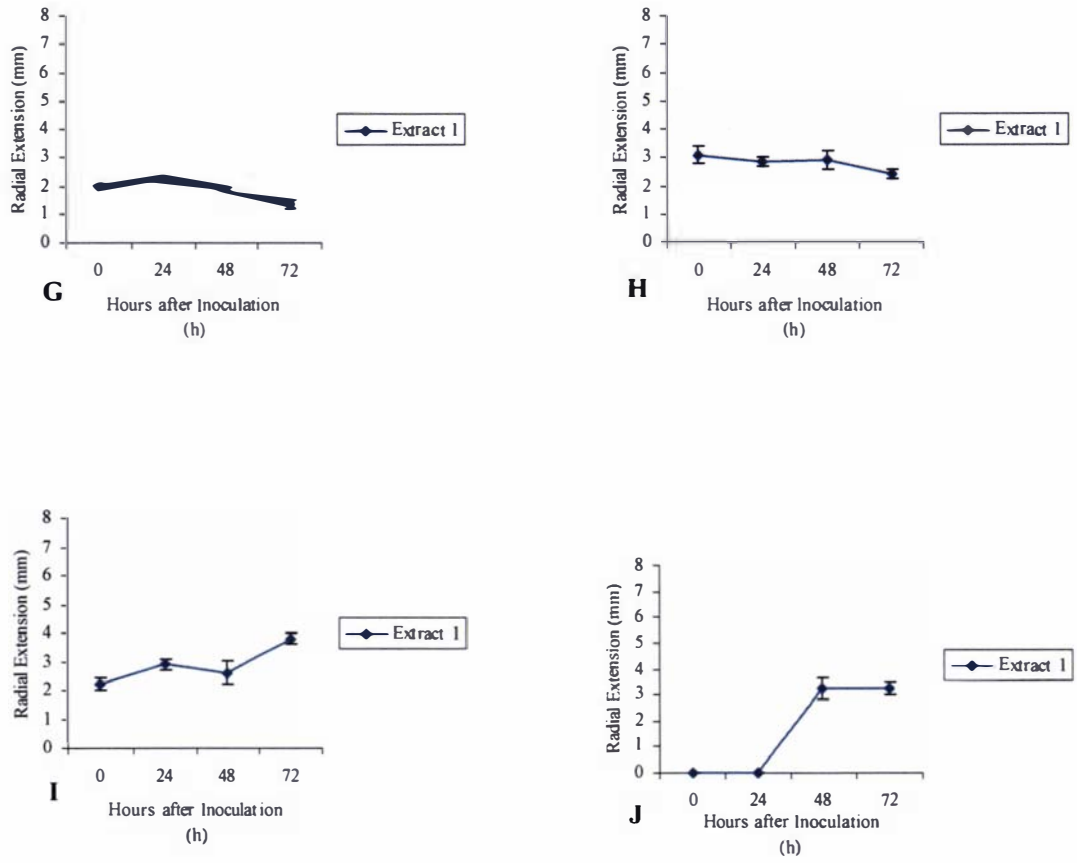


Figure 9.6 G-J (continued)
'Resistant' *Camellia* species in which chitinase activity was detected in petals inoculated with airborne ascospores of *C. camelliae*. (G) *C. transnokoensis*; (H) *C. trichocarpa*; (I) *C. yuhsienensis*; (J) *C. yunnanensis*.

Two of the ten *Camellia* species rated as susceptible produced detectable levels of chitinase activity. In both species, the chitinase was detected in the second test (tested 2 wk after first test) of the same Extract (Figure 9.7 A-B).

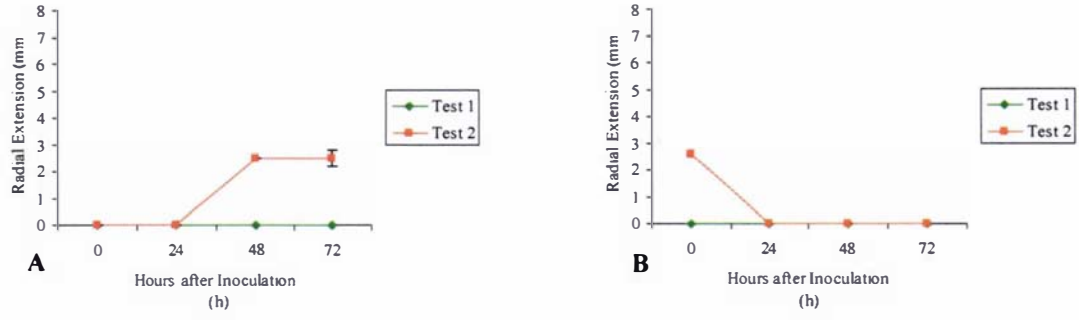


Figure 9.7 A-B
Susceptible *Camellia* species in which chitinase activity was detected in petals inoculated with airborne ascospores of *C. camelliae*. (A) *C. japonica* 'Desire'; (B) *C. pitardii* var. *pitardii*.

No chitinase activity was detected in any Test or Extract in any of the Time samples of *C. cupidata* hybrid 'Spring Festival', *C. cuspidata* x *C. saluenensis* 'Cornish Snow', *C. japonica* 'Elegans Champagne', *C. japonica* 'Softly', *C. japonica* x *C. saluenensis* 'E. G. Waterhouse', *C. polydonta, C. reticulata* 'Zhangjia Cha', *C. williamsii* 'Brian'.

9.4 DISCUSSION

In general, *Camellia* spp. classified as resistant had detectable chitinase activity while in those species classified as susceptible detectable chitinase activity was absent.

Those species in which chitinase activity was detected generally had activity in all four Time samples and the ANOVA did not detect any difference in the amount of chitinase between Time samples. Thus, it would appear that chitinase was produced constitutively, rather than induced by pathogen challenge, although picking the flowers may perhaps be enough to induce chitinase synthesis in those species able to do so. In their study of pepper flowers and roots, Hong & Hwang (2002) found high constitutive levels of a CAChi2 gene. They concluded that while constitutive expression in root tissues may be a pre-emptive defence against root pathogens, the subcellular localisation of CAChi2 transcripts in flowers indicated involvement of chitinase in the differentiation of floral organs.

Of the 13 *Camellia* species rated as 'resistant' chitinase activity was not detected in one extract of *C. cuspidata* or in the hybrids 'Spring Mist' and 'Fairy Blush'. Chitinase activity was detected in the second extract of *C. cuspidata*; this may indicate that there was some variation in environmental and/or laboratory preparation conditions between the Extracts that influenced the result. The lack of detectable chitinase activity in 'Spring Mist' and 'Fairy Blush' is less easy to explain. Both hybrid identifications were uncertain – 'Spring Mist' because it looks very different to that of the same label in Los Angeles and 'Fairy Blush' because it lacks a label (it was identified by Vonnie Cave, editor NZ Camellia Bulletin). Despite identification problems, both hybrids were resistant in petal ascospore tests. Either the assay method did not detect chitinase activity for these two varieties or another form of

resistance was operating. Since chitinase activity was not detected in the first extract of *C. cuspidata*, but was in the second, further testing may detect chitinase activity.

Two of the ten *Camellia* spp. rated as 'susceptible' had detectable levels of chitinase activity. The activity in *C. japonica* 'Desire' and *C. pitardii* var. *pitardii* was only detected when samples of the same extract were tested 13 days after the extraction was first tested. As it was unlikely that the samples had become contaminated, it may be that chitinase is present in these 'susceptible' species but is synthesised more slowly than in 'resistant' species or that the assay method is not sufficiently sensitive. Wurms (1996) tested the method with kiwifruit and found that the glycol chitin assay was "highly sensitive and detected extremely low levels of activity". The difference between a susceptible and resistant reaction however, typically depends on the speed and extent of the defence response (Bowles 1990; Sequeira 1990). In this experiment, the extract and glycol chitin agar plates were incubated for 5 h. In order to detect minute levels of chitinase activity however, the samples could be incubated longer (between 5-48 h). Resolution would be lost, because lower chitinase concentrations would have sufficient time to digest a comparable zone to that of higher concentrations, but sensitivity would be increased. An experiment to test for sensitivity was carried out, but not all the control inoculations were positive, and the results were discarded.

Of the five *Rhododendron*, two *Magnolia* and one *Tulip* spp. tested only one – *M. stellata* – did not produce detectable chitinase activity, again, further testing may detect chitinase activity. These species were included in the assay as they flower at the same time as the main *Camellia* spring flowering season but are not infected by *C. camelliae*. While they are naturally incompatible, it is not necessarily the same defence mechanism(s) in action as in 'resistant' *Camellia* and the detected chitinase activity may have more to do with flower development than resistance.

Most plant chitinases are endochitinases and have been recorded in numerous plant species. Chitinases have also been detected in fungi but because chitinase was regularly detected in samples extracted at 0 h (where *C. camelliae* was absent) it indicated that the chitinase activity detected was of plant rather than fungal origin. An experiment to determine whether fungal chitinases were present was abandoned

as ascospores failed to infect autoclaved petal samples, but a preparation made from pure culture could be tested in future.

The Calcofluor petri dish assay using glycol chitin was a sensitive method to detect endochitinase activity in flower petals. Using this method it was also simple to screen large numbers of samples for the presence of chitinase. Glycol chitin is soluble and a specific endochitinase substrate (Pan *et al* 1991) but non-specific reactions may occur (Hackman & Goldberg 1964; Cabib 1987). Colloidal chitin is used in conventional plate assays and is relatively insensitive (O'Brien & Colwell 1987; Wirth & Wolf 1990; Frändberg & Schnürer 1994).

9.4.1 Future Directions

The correlation between the presence of chitinase activity with resistant *Camellia* species indicated that this resistance mechanism is of interest for further study. Foremost is a need to replicate these results and to expand the number of species tested. Six specific experiments are described below to clarify results already described.

Experiment 1

Quantify the relationship between degraded glycol chitin radial extension zone and enzyme concentration (e. g. dilute samples by 2-, 4-, 8-, 16-, 32-, and 64-fold and plot results against $\log_{10}$ percentage).

Experiment 2

Determine the sensitivity versus resolution (e. g. dilute samples by 2- and 4-fold and incubate at 37°C for 2, 5, 12, 24 and 48 h).

Experiment 3

Determine whether susceptible species of *Camellia* produce low levels of chitinase (by incubating at the time indicated by Experiment 2).

Experiment 4

Determine whether flower parts differentially express chitinase activity as infection work appeared to show that *C. yunnanensis* stamens were susceptible while petals were not (but difficult to collect a sufficient number of stamens for a testing).

Experiment 5

Determine whether spray applications of chitosan reduce infection. Chitosan is a chitinase-specific inducer and an elicitor of host defences, but may also have fungistatic properties on the petal surface.

Experiment 6

Test other chitinase assay methods to determine if they also detect chitinase activity (e. g. radiometric assay of Molano *et al* 1977; colormetric assay of Boller *et al* 1983).

Provided that the results of the experiments suggested still indicate that chitinase is present in resistant species and is rarely detected in susceptible species, then the next step is to identify which class(es) of chitinase(s) are present.

A differential display of mRNA on pathogen- and non-challenged *Camellia* species would identify potential PR protein genes (e.g. Davis *et al* 2002), while *in situ* hybridisation with probes made from cDNA clones would locate the chitinase gene(s) (Hong & Hwang 2002). It is important to consider the results of (Robert *et al* (2002) who found that in *Vitis vinifera* the induction of chitinase genes depended on the infecting pathogen. Thus, what is activated in response to *C. camelliae*, may not be what is activated in response to lesser (e.g. *B. cinerea*) or non-host pathogens.

These molecular studies would clarify which chitinase genes are present and which are activated in resistant *Camellia* species challenged by *C. camelliae*. The correlation of chitinase activity with *Camellia* resistance described in this work could then be studied.

Further elicitor-induced resistance work may also be indicated. The SAR elicitor acibenzolar-S-methyl failed to induce resistance in susceptible *Camellia* (van Toor *et*

al 2001). In *Pinus*, Davis *et al* (2002) found that chitinase homologues in Classes I, II and IV were responsive to SA, whereas JA induced only Class I and IV homologues. Data indicate that biotrophic pathogens tend to elicit a SA-dependent response, while necrotrophs elicit a JA-dependent response (McDowell & Dangl 2000; Thomma *et al* 2001). Microscopic study of the infection process showed that *C. camelliae* was mainly biotrophic until ~60 h post-infection (Vingnanasingam 2002) so that chitosan (a chitinase-specific inducer) or a JA-inducer may be an effective elicitor of chitinase defence responses.

CHAPTER TEN – GENERAL DISCUSSION

10.1 SUMMARY OF RESEARCH OUTCOMES AND FUTURE DIRECTIONS

Camellia flower blight, caused by the pathogen *C. camelliae*, is the most serious disease of ornamental camellias (Raabe *et al* 1978) and chemical and cultural control measures, as yet, fail to give adequate control of the disease. Since control measures have failed to contain the disease, use of resistant species, hybrids and cultivars is considered the most appropriate means of circumventing the pathogen.

This study has provided the basis for further research into the pathogen, and host/pathogen interaction through investigation of some of its basic biology, molecular biology and possible resistance mechanisms, and has given the camellia breeder a starting point for resistance breeding programmes.

The distribution and spread of the pathogen in New Zealand was determined through surveys. These showed that the pathogen had widely dispersed since its initial introduction into Wellington, sometime before its eventual identification in 1993 (Stewart & Neilson 1993). Human activity appeared to be a factor in its spread to some areas (e. g. Auckland and Christchurch), but the main dispersal method was by the seasonal production of airborne ascospores, as each year, the pathogen advanced another ~35 km on its previous season's distribution. In this manner, the pathogen successfully colonised most of the North Island, and the northern and eastern parts of the South Island. The pattern of spread, and its lack of recognition in infected areas, mirrors that of its spread within the United States, and presumably Europe too.

Naturally formed sclerotia were conditioned to germinate out of season. Temperature and light were important factors in germination of *C. camelliae* sclerotia, but the most successful method used the plant growth hormone gibberellic acid. Excessive handling of the sclerotia appeared to inhibit germination for a season. Maturation of the apothecium was achieved using natural light. Ascospores produced out of season can be used to extend resistance testing of autumn-flowering species (e. g. *C. sasanqua*) and those that flower early or late in the main camellia-

flowering season. Such species that are found to be resistant can be included in future experiments for resistance mechanisms. In addition, the availability of ascospores out of season reduces research pressure during the main three month blight season.

New Zealand, and possibly Europe and North America, isolate-specific primers were developed to detect the pathogen *in planta*. Initially, they were intended for better quarantine control of exported/imported camellias, but with the discovery that camellia flower blight was widespread in Europe (after this project began) only South Africa and Australia would benefit from this technology. In addition, variation was found in the ITS region of the two Asian isolates sequenced, limiting their usefulness. The primers do, however, allow the pathogen to be detected in both camellia petal and stem tissue, increasing the accuracy with which the diagnosis can be made in the case of atypical symptoms and further investigation is required to determine whether the pathogen can survive in stem tissue between blight seasons, possibly providing a second source of infection. The isolate-specific primers would also be used to clarify infection of stamens in resistant species. Preliminary work comparing part of the β-tubulin gene indicated that this may be a more suitable target for developing a species-specific primer.

Phylogenetic analysis of the ITS region of *C. camelliae* indicated that it was more closely related to *S. sclerotiorum,* and other *Sclerotinia* spp. than it was to other *Ciborinia* spp. Host-range infection experiments indicated that *C camelliae* and *S. sclerotiorum* were different species. Comparison of partial sequence from the β-tubulin gene also indicated that the two species were dissimilar. More β-tubulin sequences are required, from *Ciborinia* and *Sclerotinia* species, in order to analyse the phylogenetic relationship using this gene and to improve the reliability of any conclusions. Whetzel (1945) set up the genus *Ciborinia*, to hold those that had host tissue embedded in the sclerotium and transferred *S. camelliae* Hara to this genus. It may be time for taxonomists to examine the *Sclerotinia/Ciborinia* division more closely, especially given the advent of molecular data.

Two inoculation techniques, one quantitative and suitable for breeders to use, and one qualitative, were developed and trialled. Results from hundreds of tests of the

airborne ascospore settling chamber produced a simple resistance rating scale with which breeders could easily test the resistance status of new cultivars and hybrids. Both methods confirmed that resistant species exist and quantified that resistance. Several hybrids (e. g. *C. lutchuensis* hybrid 'Fairy Blush') with a resistant parent were shown to be resistant, which is valuable information for camellia breeders.

One difficult aspect of this research, which also applied to the study of resistance mechanisms, was the paucity of research conducted on petals of other flower species. Although many methods for inoculation and assessment of resistance were available for other host/pathogen combinations, the vast majority were developed for leaf and fruit pathogens (e.g. Kim & Huang 1994; Anguelova-Merhar *et al* 2001), and were not readily transferable to the more delicate tissues of petals.

The distribution of resistant and susceptible species within the genus *Camellia*, as determined through this study and by Taylor (1999) showed that resistant and susceptible species did not occur within the same Section (Figure 10.1). The majority of susceptible species belonged to the Section Camellia, while many of the resistant species were in Section Theopsis. Since only 25 of 267 known species were tested, there is much more testing to be done, and many more sources of potentially resistant species for breeding.

Of the four resistance mechanisms trialled in this study, two, possibly three, had results which indicated that they were involved in species resistance to *C. camelliae*. An experiment using reciprocal grafting of susceptible scions (*C. pitardii* var. *pitardii* x *C. japonica* 'Nicky Crisp') onto resistant root stocks (*C. yuhsienensis* and *C. forrestii*) and *vice versa* did not induce resistance in the susceptible scion (nor did the resistant scion become susceptible) (Taylor, unpublished) as it does for soybean and brown stem rot (Bachman & Nickell 1999). Further research into cell wall appositions, lignification and chitinase enzymes is indicated. Chitinase activity and cell wall modifications were detected in most of the resistant species (Table 10.1). The data indicate that at least two resistance mechanisms are operating in resistant species, and that the resistance mechanisms studied in this thesis are active in multiple Sections of the genus *Camellia*. Future research may indicate whether the

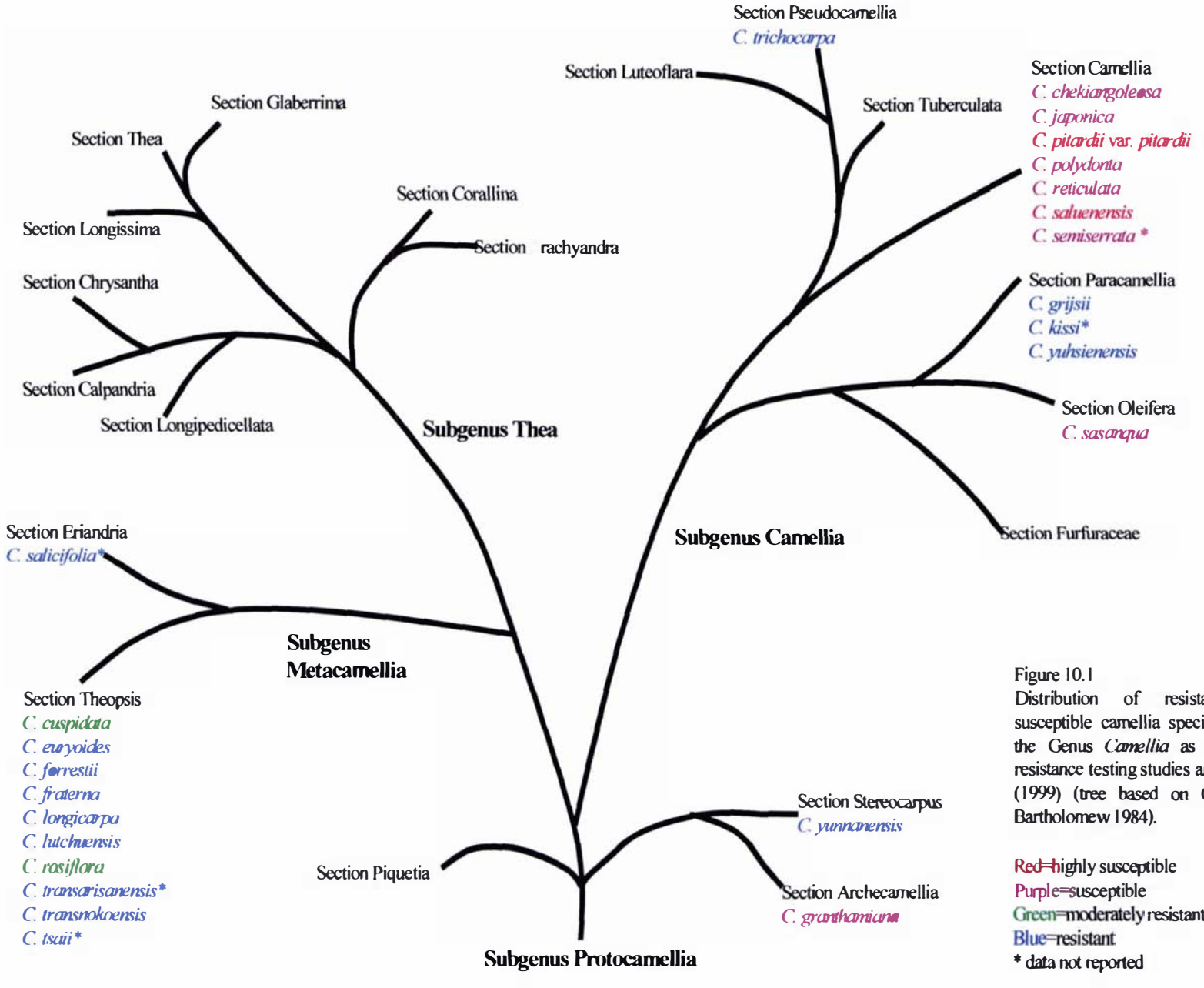


Figure 10.1
Distribution of resistant and susceptible camellia species within the Genus *Camellia* as found in resistance testing studies and Taylor (1999) (tree based on Chang & Bartholomew 1984).

Red=highly susceptible
Purple=susceptible
Green=moderately resistant
Blue=resistant
* data not reported

resistance mechanisms studied in this thesis are absent, turned off, or too slowly induced to be of use in the susceptible species.

Table 10.1 Resistance Mechanisms in Resistant *Camellia* Species by Section

Species	Section	Cell Wall Modificatons[a]	Chitinase Activity
C. euryoides	Theopsis	✔	✔
C. forrestii	Theopsis	-	✔
C. fraterna	Theopsis	✔	✔
C. grijsii	Paracamellia	✔	✔
C. longicarpa	Theopsis	-	✔
C. lutchuensis	Theopsis	✔	✔
C. transnokoensis	Theopsis	x	✔
C. trichocarpa	Pseudocamellia	✔	✔
C. yuhsienensis	Paracamellia	✔	✔
C. yunnanensis	Theopsis	✔	✔

[a] either papillae/lignotubers or lignification or both

Since lignin and phenolic compounds are both produced via the phenylpropenoid pathway, further testing for phenolic compounds using the improvements suggested may also increase support for the production of these substances in *Camellia* resistance. This study provides the basis for future research into species resistance in *Camellia* and the possibility of producing resistant varieties through genetic engineering.

Some preliminary studies of techniques required for genetic engineering of camellia using *Agrobacterium tumefaciens* have been completed (Haldeman 1995; Shults & Haldeman 1995; Haldeman & Locker 1996; Shults & Haldeman 1996; Haldeman & Sprenne 1998). Identification of resistance genes, their stable transformation into susceptible *Camellia* species, and the expression of resistance to *C. camelliae* remain to be explored. Whether the camellia-growing public (outside of the USA) would accept genetically engineered camellias is unknown, but there is still plenty of work to be done before genetically engineered camellias could become a reality.

10.1.1 Breeding for Resistance

Plant breeding by humans for desirable characteristics is an ancient and established practice and has developed efficient procedures. Once developed, a resistant plant is a cheap and easy method of controlling a pathogen. There are two disadvantages, however, which need to be considered during research and production of resistant plants. These are: that resistance genes are usually pathogen-specific, and pathogen

adaptation to the resistance gene. Durable resistance, through multilines, variety mixtures, gene development and multiple-resistance gene barriers, is the new strategy (Parlevliet 1995).

Efficient breeding for resistance requires a) the proper assessment of resistance, b) identification of suitable sources of resistance and c) incorporation of the resistance into commercial varieties. This thesis has focused on the first two points, through the development of two assessment methods (airborne ascospores and ascospore suspensions), and identification of *Camellia* species resistant to *C. camelliae.*

In the case of *Camellia/C. camelliae*, preliminary studies showed that there were at least two resistance mechanisms operating in resistant *Camellia* species, with further research likely to identify more resistant species and more operational resistance mechanisms. As resistance is desired primarily for the *C. camelliae* pathogen, the potential exists for the production of resistant *Camellia* hybrids and cultivars, either through conventional breeding or genetic modification (through the transfer of genes within the Genus *Camellia*, which may or may not make the transgenic plant more acceptable to the public).

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APPENDIX I

APPENDIX II – SAS CODES

Single-factor ANOVA

```
data expt;
input species area;
larea=log(area);
cards;
(data)
;
run;
proc glm data=expt;
model larea=species;
lsmeans (factor)/stderr pdiff;
output out=rdis p=predict r=residual;
run;
proc plot data=rdis;
plot residual*predict;
run;
proc chart data=rdis;
vbar residual/type=freq;
run;
proc univariate data=rdis plot;
var residual;
run;
```

Two-Factor ANOVA

```
data expt;
input conc soak light stipes;
lstipes=log(stipes + 1);
cards;
;
run;
proc print;
var conc soak light lstipes;
run;
proc glm data=xxxx;
class conc soak light;
model lstipes=conc soak light con*soak conc*light soak*light/ssl;
lsmeans conc soak light conc*soak conc*light soak*light/stderr pdiff;
output out=rdis p=predict r=residual;
run;
proc plot data=rdis;
plot residual*precict;
run;
proc chart data=rdis;
vbar residual/type=freq;
run;
```

```
proc univariate data=rdis plot;
var residual;
run;
```

Non-parametric ANOVA

```
data expt;
input temp size rank;
cards;
;
run;
proc print;
var temp size rank;
run;
proc glm data=xxxx;
class temp size;
model rank=temp size;
lsmeans temp size/stderr pdiff;
output out=rdis p=predict r=residual;
run;
proc plot data=rdis;
plot residual*predict;
run;
proc chart data=rdis;
vbar residual/type=freq;
run;
proc univariate data=rdis plot;
var residual;
run;
```

Repeated Measures ANOVA

```
data expt;
input vari spored spored2 day1 day2 day3 day4 day5 day6 grp;
(data)
;
run;
proc glm data=expt;
class vari grp;
model day1 day2 day3 day4 day5 day6=vari(grp) spored spored2 grp*spored
grp*spored2 spored*vari(grp) spored2*vari(grp)/ss1 solution;
run;
lsmean vari(grp)/at (spored spored2)=();
run;
output out=rdis p=p1 p2 p3 p4 p5 p6 r=r1 r2 r3 r4 r5 r6;
run;
proc plot data=rdis;
plot (r1-r6):(p1-p6);
run;
```

Chi Square

```
data expt;
input incub wash n nscl;
cards;
;
run;
proc print;
var incub wash n nscl;
run;
proc genmod data=xxxx;
class incub wash;
model n/nscl=incub wash incub*wash/dist=binomial type1;
run;
```

Poisson

```
data expt;
input wound wash gacon stipes;
cards;
;
run;
proc print;
var wound wash gacon stipes;
run;
proc genmod data=expt;
class wound wash gacon;
model stipes=wound wash gacon wound*wash/dist=poisson type1;
run;
proc plot data=rdis;
plot residual*predict;
run;
proc chart data=rdis;
vbar residual/type=freq;
run;
proc univariate data=rdis plot;
var residual;
run;
```

APPENDIX III – SURVEY SITES

North Island

Town	No. Sites	No. Plants (total)	No. Diseased
Auckland	5	178	8
Cambridge	2	8	1
Clive	2	12	2
Dannevirke	1	5	2
Hamilton	3	17	13
Hastings	1	14	0
Havelock North	3	14	0
Huntly	3	7	5
Katikati	1	2	0
Napier	1	15	1
Ngaruwhahia	1	2	0
Norsewood	1	3	3
Paeroa	1	5	1
Putaruru	2	5	4
Taumaranui	3	7	4
Tauranga	2	4	2
Te Awamutu	2	11	1
Te Kuiti	2	11	11
Te Puke	1	7	0
Thames	1	1	0
Tokaroa	4	4	4
Waihi	1	1	1
Warkworth	1	3	0
Whakatane	2	26	6
Whangarei	2	18	3

South Island

Town	No. Sites	No. Plants (total)	No. Diseased
Ashburton	4	100	3
Balclutha	1	4	0
Dunedin	5	268	0
Geraldine	5	10	0
Gore	5	47	0
Greymouth	1	6	0
Invercargill	1	37	0
Kaikoura	7	9	0
Karamea	1	1	0
Mosgiel	7	32	0
Murchison	2	9	0
Oamaru	2	70	1
Queenstown	2	45	0
Timaru	6	69	8
Ross	1	1	0
Westport	1	5	0

APPENDIX IV – STATISTICAL TABLES

CHAPTER FOUR – SCLEROTIAL GERMINATION

Table IV.1 ANOVA Statistics for Experiment 4.2

Source	DF	SS	MS	F Value	Pr >
Concentration	5	1.22237131	0.24447426	3.89	0.1064
Soak	1	0.02579206	0.02579206	0.41	0.5567
Light	1	2.63598206	2.63598206	41.92	0.0029
Concentration*Soak	4	0.63195882	0.15798971	2.51	0.1970
Concentration*Light	5	1.66060367	0.33212073	5.28	0.06860
Soak*Light	1	0.98160688	0.98160688	15.61	0.0168

Table IV.2 ANOVA Statistics for Experiment 4.4

Source	DF	SS	MS	F Value	Pr >
Temperature	4	157.1458333	39.28645833	6.06	0.0152
Size	2	28.99120370	14.49560185	2.24	0.1693

CHAPTER SIX – ASCOSPORE INOCULATION TECHNIQUES AND RESISTANCE TESTING

Table IV.3 Experiment 6.1

Source	DF	SS	MS	F Value	Pr>
Treatment	1	3.39326729	3.39326729	0.00	0.9906

Table IV.4 Experiment 6.5

Source	DF	SS	MS	F Value	Pr>
Treatment	1	28.08313363	28.08313363	84.76	0.0001

Table IV.5 Experiment 6.6

Source	DF	SS	MS	F Value	Pr>
Treatment	4	435558.5657	108889.6414	5.61	0.0027

Table IV.6 Experiment 6.7, Run 1

Source	DF	SS	MS	F Value	Pr>
Plant (Cultivar)	1	11.72387739	11.72387739	6.35	0.0142
Plant*Concentration	4	14.40511671	3.60127918	1.95	0.1125
Concentration	5	10.71498177	2.14299635	1.16	0.3380

Table IV.6 Experiment 6.7, Run 2

Source	DF	SS	MS	F Value	Pr>
Plant (Cultivar)	1	2570.251	2570.251	0.10	0.7535
Plant*Concentration	1	58199.509	58199.509	2.28	0.1425
Concentration	1	368889.278	368889.278	14.43	0.0007

Table IV.8 Experiment 6.9

Source	DF	SS	MS	F Value	Pr>
Treatment	1	1.90485824	1.90485824	3.98	0.0496

Table IV.9 Experiment 6.10

Source	DF	SS	MS	F Value	Pr >
Treatment	1	4.32637045	4.32637045	7.17	0.0095

Table IV.10 Experiment 6.11, Run 1

Source	DF	SS	MS	F Value	Pr >
Plant	4	12.51234368	3.12808592	18.35	0.0001

Table IV.11 Experiment 6.11, Run 2

Source	DF	SS	MS	F Value	Pr >
Plant	4	3.57829999	0.89457500	1.39	0.2616

Table IV.12 Experiment 6.12

Source	DF	SS	MS	F Value	Pr >
Plant	4	24.64849611	6.16212403	10.72	0.0001

Table IV.13 Experiment 6.13

Source	DF	SS	MS	F Ratio
Plant	3	5156.78	1718.93	0.71[a]
Plant*Fflower	7	17025.08	2432.15	11.11[b]
Petal(Plant*Flower)	13	2844.76	218.83	-

[a] MS(Plant)/MS(Plant*Flower)
[b] MS(Plant*Flower)/MS(Petal(Plant*Flower))

Table IV.14 Experiment 6.14

Source	DF	SS	MS	F Ratio
Plant	4	1754186.28	438546.57	4.67[a]
Plant*Fflower	25	2346421.45	93856.86	5.56[b]
Petal(Plant*Flower)	257	4338869.86	16882.76	-

[a] MS(Plant)/MS(Plant*Flower)
[b] MS(Plant*Flower)/MS(Petal(Plant*Flower))

Table IV.15 Experiment 6.15

Source	DF	SS	MS	F Ratio
Plant	3	1271.57	423.86	7.40[a]
Plant*Fflower	11	630.27	57.30	3.13[b]
Petal(Plant*Flower)	114	2086.59	18.30	-

[a] MS(Plant)/MS(Plant*Flower)
[b] MS(Plant*Flower)/MS(Petal(Plant*Flower))

Table IV.16 Experiment 6.17, 72 h data

Source	DF	SS	MS	F Value	Pr >
Plant	1	6.01359024	6.01359024	8.79	0.0054

Table IV.17 Experiment 6.17, 96 h data

Source	DF	SS	MS	F Value	Pr >
Plant	1	279.4069435	279.4069435	0.01	0.9204

Table IV.18 Experiment 6.18, 72 h data

Source	DF	SS	MS	F Value	Pr >
Plant	1	4.45952509	4.45952509	3.92	0.0603

Table IV.19 Experiment 6.18, 96 h data

Source	DF	SS	MS	F Value	Pr >
Plant	3	3.009	1.003	3.57	0.0300

Table IV.20 Experiment 6.19

Source	DF	SS	MS	F Value	Pr >
Plant	1	0.73180594	0.73180594	0.37	0.5473

Table IV.21 Experiment 6.20

Source	DF	SS	MS	F Value	Pr >
Plant	2	2.8633	1.4316	102.28	0.0001

Table IV.22 Experiment 6.21, 48 h data

Source	DF	SS	MS	F Value	Pr >
Plant	1	7.321	7.321	69.57	0.0001

Table IV.23 Experiment 6.21, 72 h data

Source	DF	SS	MS	F Value	Pr >
Plant	1	10.50653958	10.50653958	19.10	0.0002

Table IV.24 Experiment 6.22

Source	DF	SS	MS	F Value	Pr >
Plant	1	11.34086833	11.34086833	42.46	0.0001

Table IV.25 Experiment 6.23

Source	DF	SS	MS	F Value	Pr >
Plant	2	18.92796836	9.46398418	17.33	0.0001

Table IV.26 Experiment 6.24

Source	DF	SS	MS	F Value	Pr >
Plant	3	7.76265152	2.58755051	2.24	0.1012

Table IV.27 Logistic Regression Statistics for Experiment 6.27

Effect	df	Deviance	p
Plant & Petal	6	27.73	0.7895
Condition	1	0.00	0.0001
Residual	5	0.00	-

Table IV.28 Logistic Regression Statistics for Experiment 6.28

Effect	df	Deviance	p
Plant & Petal	10	53.36	0.9979
Condition	1	10.36	0.0001
Residual	11	10.36	-

Table IV.29 Tests of Hypotheses for Between Subjects' Effects, Experiment 6.30

Source	DF	SS	MS	F Value	Pr>F
Group	5	1320.98	264.20	149.24	0.0001
Cultivar(Group)	18	42.14	2.34	1.32	0.1755
Spore Density	1	5.45	5.45	3.08	0.0800
Spore Density^2	1	5.48	5.48	3.09	0.0800
Spore Density*Group	5	3.36	0.67	0.38	0.8623
Spore Density^2*Group	5	8.58	1.72	0.97	0.4373
Spore Density*Cultivar(Group)	18	36.70	2.04	1.15	0.3045
Spore Density^2*Cultivar(Group)	18	16.24	0.90	0.51	0.9520
Error	216	382.39	1.77	-	-

Table IV.30 Manova Test Criteria and Exact F Statistics of Within-Subject Effects (Wilkes Lambda: Pillai's and H-L Trace give similar results), Experiment 6.30

Effect	F Value	DF	Den DF	Pr>F
Time	199.93	5	212	0.0001
Time*Group	24.33	25	789.05	0.0001
Time*Cultivar(Group)	0.79	90	1033	0.9194
Time*Spore Density	1.73	5	212	0.1283
Time*Spore Density^2	1.34	5	212	0.2492
Time*Spore Density*Group	2.40	25	789.05	0.0002
Time*Spore Density^2*Group	1.56	25	789.05	0.0394
Time*Spore Density*Cultivar(Group)	1.24	90	1033	0.9993
Time*Spore Density^2*Cultivar(Group)	0.58	90	1033	0.9993

Table IV.31 Tests of Hypotheses for Between Subjects' Effects, Experiment 6.31

Source	DF	SS	MS	F Value	Pr>F
Group	5	1239.10	247.82	128.46	0.0001
Cultivar(Group)	18	48.90	2.72	1.41	0.1294
Spore Density	1	9.89	9.89	5.13	0.0246
Spore Density^2	1	4.01	4.01	2.08	0.1507
Spore Density*Group	5	1.79	0.36	0.19	0.9679
Spore Density^2*Group	5	10.55	2.11	1.09	0.3649
Spore Density*Cultivar(Group)	18	16.20	0.90	0.47	0.9695
Spore Density^2*Cultivar(Group	18	7.47	0.41	0.21	0.9998
Error	216	416.69	1.93	-	-

Table IV.32 Manova Test Criteria and Exact F Statistics of Within-Subject Effects (Wilkes Lambda: Pillai's and H-L Trace give similar results), Experiment 6.31

Effect	F Value	DF	Den DF	Pr>F
Time	551.24	5	212	0.0001
Time*Group	25.03	25	789.05	0.0001
Time*Cultivar(Group)	1.41	90	1032.98	0.0094
Time*Spore Density	1.07	5	212	0.3800
Time*Spore Density^2	3.14	5	212	0.0094
Time*Spore Density*Group	0.64	25	789.05	0.9132
Time*Spore Density^2*Group	2.22	25	789.05	0.0006
Time*Spore Density*Cultivar(Group)	0.74	90	1032.98	0.9624
Time*Spore Density^2*Cultivar(Group)	0.96	90	1032.98	0.5855

Table IV.33 Tests of Hypotheses for Between Subjects' Effects, Experiment 6.32

Source	DF	SS	MS	F Value	Pr>F
Group	5	614.97	122.99	52.69	0.0001
Cultivar(Group)	18	26.42	1.47	0.63	0.8743
Spore Density	1	22.57	22.57	9.67	0.0022
Spore Density^2	1	18.19	18.19	7.79	0.0058
Spore Density*Group	5	20.97	4.19	1.80	0.1154
Spore Density^2*Group	5	16.27	3.25	1.39	0.2282
Spore Density*Cultivar(Group)	18	27.02	1.50	0.64	0.8624
Spore Density^2*Cultivar(Group	18	2.82	0.16	0.07	1.0000
Error	192	448.22	2.33	-	-

Table IV.34 Manova Test Criteria and Exact F Statistics of Within-Subject Effects (Wilkes Lambda: Pillai's and H-L Trace give similar results), Experiment 6.32

Effect	F Value	DF	Den DF	Pr>F
Time	122.26	4	189	0.0001
Time*Group	14.65	20	627.79	0.0001
Time*Cultivar(Group)	1.12	72	745.55	0.2402
Time*Spore Density	4.26	4	189	0.0025
Time*Spore Density^2	2.14	4	189	0.0776
Time*Spore Density*Group	3.10	20	627.79	0.0001
Time*Spore Density^2*Group	2.13	20	627.79	0.0029
Time*Spore Density*Cultivar(Group)	0.73	72	745.55	0.9517
Time*Spore Density^2*Cultivar(Group)	0.25	72	745.55	1.0000

CHAPTER SEVEN – RESISTANCE MECHANISMS: ALUMINIUM HYPERACCUMULATION

Table IV.35 ANOVA Statistics by Site

Source	DF	SS	MS	F Value	Pr >
pH	5	26.593	5.319	24.77	0.000
Soil	5	1.018E +09	203686371	6.80	0.000
Extract	5	81.5	16.3	1.61	0.181
Flowers	5	185372	37074	1.52	0.220
Leaves	5	172818032	34563606	7.40	0.000
Stems	5	965734	193147	3.08	0.021

Table IV.36 ANOVA Statistics by Species

Source	DF	SS	MS	F Value	Pr >
pH	11	9.062	0.824	1.05	0.430
Soil	11	232035697	21094154	0.36	0.964
Extract	11	136.1	12.4	1.20	0.327
Flowers	10	356872	35687	1.62	0.171
Leaves	11	85791926	7799266	0.98	0.479
Stems	10	669145	66914	0.83	0.608

CHAPTER EIGHT – RESISTANCE MECHANISMS: BASED ON THE PHENYLPROPANOID PATHWAY

Table IV.37 Average R_f values of Zone of Inhibition in *Cladosporium* spp. Bioassay for Resistant and Susceptible *Camellia* Species

Species	R_f Value of Time Samples (SE)			
	0 h	24 h	48 h	72 h/96 h
C. japonica 'Desire' (1)	0.28 (0)	-	-	0.32 (0)
C. japonica 'Elegans Champagne' (3)[a]	0.24 (0)	0.23 (0)	0.36 (0)	0.375 (0.015)
C. forrestii (1)	0.28	-	-	0.30 (0)
C. yunnanensis (3)	0.0 (0)[b]	0	0	0

[a] distortions from silica gel
[b] zone of inhibition around inoculation spot only

Table IV.38 Average R_f values of Chromatograph Bands seen with Visible Light for Resistant and Susceptible *Camellia* Species

Species	R_f Value of Time Samples (SE)			
	0 h	24 h	48 h	72 h
C.japonica 'Elegans Champagne' (3)	0	0.21 (0)	0	0
C. forrestii (3)	0.22 (0)	0.22 (0)	0.205 (0.005)	0.21 (0)
C. yunnanensis (3)	0	0	0.18 (0)	0.18 (0)

Table IV.39 Average R_f values of Chromatograph Bands seen under Shortwave UV Light for Resistant and Susceptible *Camellia* Species

Species	Zone No.	Band No.	R_f Value of Time Samples (SE)			
			0 h	24 h	48 h	72 h/96 h
Susceptible						
C. japonica 'Desire' (1)	1	1	0.11 (0)	-	-	0.11 (0)
		2	0.16 (0.01)	-	-	0.15 (0)
	2	3	0.23 (0.01)	-	-	0.23 (0.01)
		4	0.28 (0)	-	-	0.305 (0.015)
	3	5	0.55 (0)	-	-	0.55 (0)
C.japonica 'Elegans Champagne' (3)	1	1	0	0.09 (0)	0.09 (0)	0.09 (0)
	2	3	0.20 (0)	0.20 (0)	0.20 (0)	0.20 (0)
		4	0.29 (0)	0.29 (0)	0.28 (0)	0.26 (0)
	3	5	0.56 (0)	0.56 (0)	0.53 (0)	0.56 (0)
C. pitardii var. *pitardii* (3)[a]	1	1	0.10 (0)	0.08 (0)	0.09 (0.01)	0.09 (0.01)
		2	0.16 (0.01)	0.15 (0)	0.14 (0.01)	0.14 (0.01)
	2	3	0.33 (0.01)	0.315 (0.005)	0.305 (0.005)	0.315 (0.005)
		4	0.42 (0)	0.41 (0)	0.415 (0.005)	0.415 (0.005)
	3	5	indistinct	0.59 (0)	0.63 (0.02)	0.685 (0.005)
		6	indistinct	0.68 (0)	0.695 (0.025)	0.73 (0.02)

		7	indistinct	0.76 (0)	0.77 (0.01)	0.805 (0.005)
C. saluenensis x *C. reticulata* 'Brian' (2)	1					
		2	0.18 (0)	0.17 (0.01)	0.15 (0.01)	0.17 (0.01)
	2	3	0.36 (0.025)	0.41 (0)	0.365 (0.005)	0.43 (0.02)
		4	0.52 (0)	0.53 (0.01)	0.50 (0.02)	0.545 (0.005)
	3	5	0.76 (0.01)	0.73 (0)`	0.71 (0.01)	0.735 (0.015)
Resistant						
C. forrestii (1)	1	1	0.08 (0)	-	-	0.08 (0)
		2	0.12 (0)	-	-	0.12 (0)
	2	3	0.18 (0)	-	-	0.18 (0)
		4	0.30 (0)	-	-	0.30 (0)
	3	5	0.56 (0)	-	-	0.57 (0)
C. forrestii (3)	1	1	0.08 (0)	0.08 (0)	0.08 (0)	0.08
		2	0.13 (0)	0.125 (0.015)	0.125 (0.015)	0.13 (0)
	2	3	0.27 (0)	0.295 (0.015)	0.265 (0.015)	0.30 (0)
		4	0.32 (0)	0.33 (0.01)	0.33 (0.01)	0.34 (0)
	3	5	0.52 (0.01)	0.465 (0.005)	0.47 (0)	0
C. fraterna (2)[a]	1	2	0.18 (0)	0.14 (0)	0.17 (0.01)	-[b]
	2	3	0.395 (0.02)	0.34 (0)	0.415 (0.01)	-[b]
		4	0.475 (0.005)	0.44 (0.01)	0.49 (0.01)	-[b]
	3	5	0.72 (0)	0.66 (0)	0.70 (0.02)	-[b]
C. lutchuensis (3)	1	1	0.14 (0)	0.12 (0)	0.13 (0.01)	0.115 (0.005)
		2	0.21 (0)	0.205 (0.005)	0.20 (0)	0.18 (0)
	2	3	0.405 (0.005)	0.40 (0)	0.36 (0.02)	0.32 (0)
		4	0.48 (0)	0.475 (0.005)	0.43 (0)	0.41 (0)
	3	5	0.72 (0)	0.70 (0)	0.70 (0)	0.69 (0.01)
C. yunnanensis (3)[a]	1	2	0.15 (0)	0.125 (0.005)	0.13 (0)	0.125 (0.005)
	2	3	0.32 (0.02)	0.245 (0.025)	0.245 (0.005)	0.225 (0.005)
		4	0.36 (0.01)	0.30 (0.02)	0.295 (0.005)	0.30 (0.02)
	3	5	0.61 (0)	0.575 (0.025)	0.285 (0.005)	0.635 (0.025)
		6	0.65	0.655 (0.005)	0.665 (0.015)	0.705 (0.025)
		7	0.755 (0.005)	0.74 (0.01)	0.755 (0.005)	0.78 (0.02)

[a] distortions from silica gel
[b] no sample left for second run

Table IV.40 Average R_f values of Chromatograph Bands seen under Longwave UV Light for Resistant and Susceptible *Camellia* Species

Species	Band No.	R_f Value of Time Samples (SE)			
		0 h	24 h	48 h	72 h/96 h
Susceptible					
C. japonica 'Desire' (1)	1	0.10 (0)	-	-	0.09 (0)
	2	0.17 (0)	-	-	0.16 (0)
C.japonica 'Elegans Champagne' (3)	1	0.12 (0)	0.12 (0)	0.12 (0)	0.12 (0)
	2	0.23 (0)	0.23 (0)	0.22 (0)	0.22 (0)
C. pitardii var. *pitardii* (3)	1	0.12 (0)	0.11 (0)	0.11 (0)	0.09 (0)
	2	0.22 (0)	0.22 (0)	0.20 (0)	0.21 (0)
C. saluenensis x *C. reticulata* 'Brian' (2)	1	0.12 (0)	0.12 (0)	0.11 (0)	0.12 (0)
	2	0.25 (0)	0.22 (0)	0.24 (0.02)	0.26 (0)
Resistant					
C. forrestii (1)	1	0.09 (0)	-	-	0.11 (0)
	2	0.15 (0)	-	-	0.15 (0)
C. forrestii (3)	1	0.11 (0)	0.11 (0)	0.11 (0)	0.11 (0)
	2	0.18 (0)	0.18 (0)	0.175 (0.015)	0.19 (0)
C. fraterna (2)	1	0.07 (0)	0.07 (0)	0.05 (0)	0.06 (0)
	2	0.09 (0)	0.11 (0)	0.09 (0)	0
C. lutchuensis (3)	1	0.15 (0)	0.145 (0.05)	0.14 (0)	0.13 (0)
	2	0.27 (0)	0.27 (0)	0.26 (0)	0.23 (0)
C. yunnanensis (3)	1	0.12 (0)	0.12 (0)	0.11 (0)	0
	2	0.22 (0)	0.16 (0.05)	0.16 (0)	0

Table IV.41 Average R_f values of Chromatograph Bands seen with Prussian Blue Reaction for Resistant and Susceptible *Camellia* Species

Species	Band No.	R_f Value of Time Samples (SE)	
		0 h	96 h
Susceptible			
C. japonica 'Desire' (1)	1	0.15 (0)[a]	0.17 (0)[a]
Resistant			
C. forrestii (1)	1	0.07 (0)	0.10 (0)
	2	0.15 (0)	0.165 (0.005)

[a] very faint

Table IV.42 Average R_f values of Chromatograph Bands seen with Vanillan Acid Test for Resistant and Susceptible *Camellia* Species

Species	Band No.	R_f Value of Time Samples (SE)	
		0 h	96 h
Susceptible			
C. japonica 'Desire' (1)	1	0.20 (0)	0
Resistant			
C. forrestii (1)	1	0.10 (0)	0.10 (0)
	2	0.20 (0)	0.21 (0)

[a] very faint

CHAPTER NINE – RESISTANCE MECHANISMS: CHITINASE ASSAY

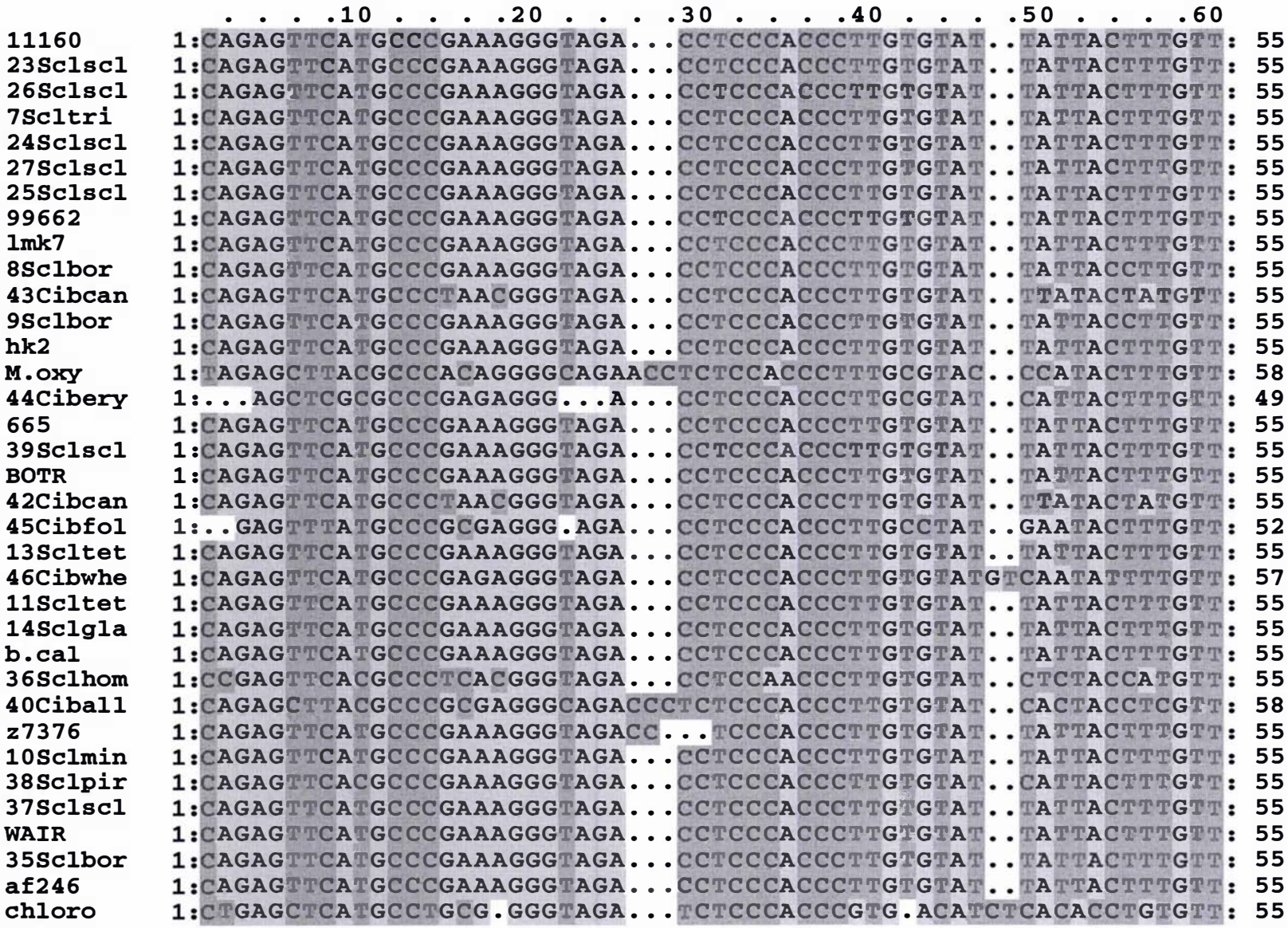
Table IV.43 Logistic Regression Statistics for Type 1 Analysis

Source	Deviance	DF	Chi-Square	Pr > ChiSq
Intercept	746.70			
Species	191.99	32	554.71	<.0001
Extract(Species)	183.72	6	8.26	0.2195
Time	181.39	3	2.33	0.5069
Time(Species)	87.95	95	93.45	0.5258
Time(Species*Extract)	87.95	18	0.0	1.0000

Table IV.44 ANOVA Statistics for Chitinase Assay

Effect	DF	Type III SS	Mean Square	F Value	Pr>F
Species	21	19.29	0.92	33.78	<.0001
Extract(Species)	1	0.01	0.01	0.30	0.5862
Time	3	0.15	0.05	1.79	0.1509
Time(Species)	49	3.04	0.06	2.28	<.0001
Time(Species*Extract)	3	0.16	0.05	2.01	0.1142

APPENDIX V – MOLECULAR STUDIES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36Sclhom 1:CCGAGTTCACGCCCTCACGGGTAGA...CCTCCAACCCTTGTGTAT..CTCTACCATGTT: 55
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WAIR 1:CAGAGTTCATGCCCGAAAGGGTAGA...CCTCCCACCCTTGTGTAT..TATTACTTTGTT: 55
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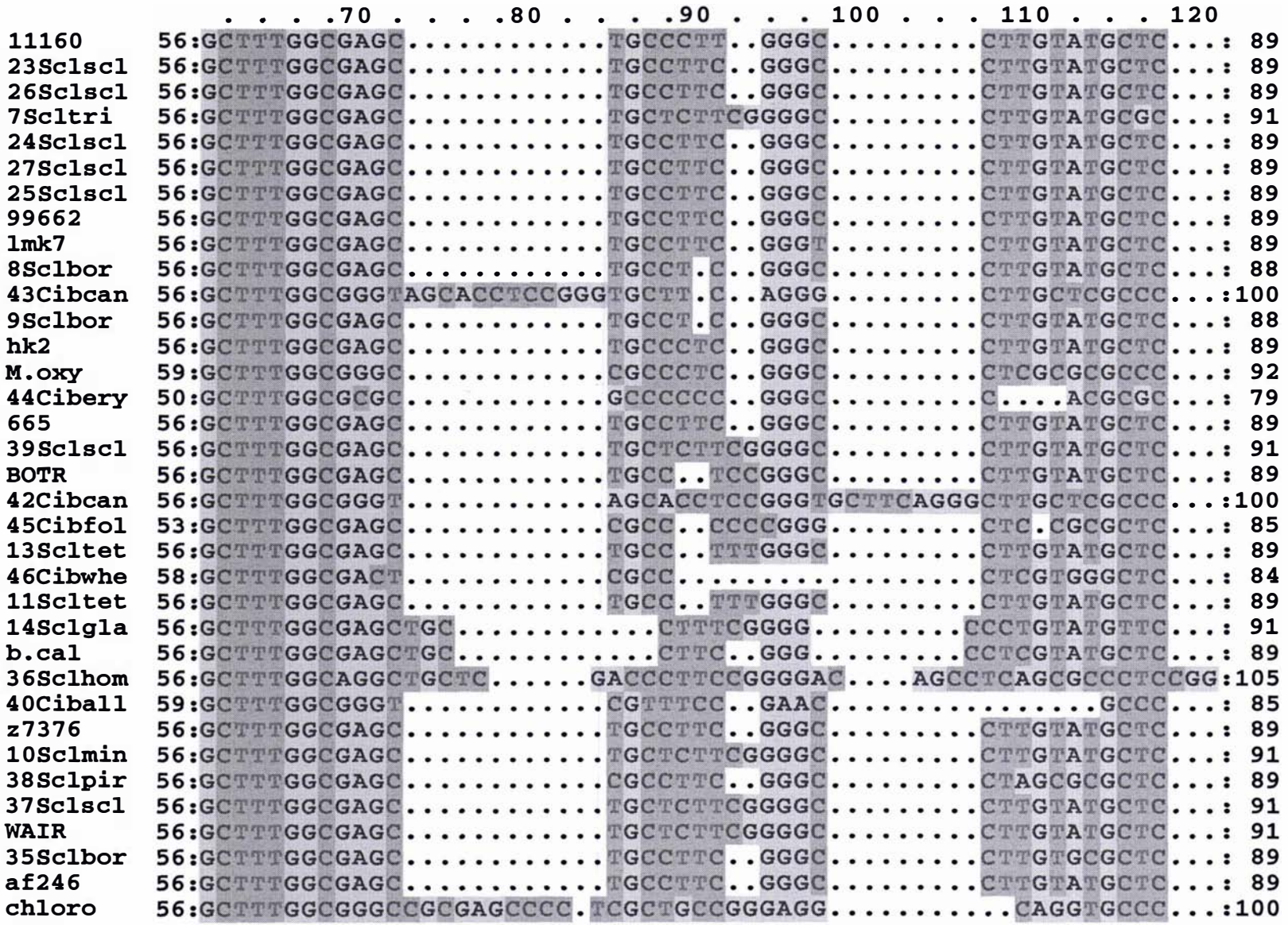
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b.cal 56:GCTTTGGCGAGCTGC.....CTTC..GGG.....CCTCGTATGCTC...: 89
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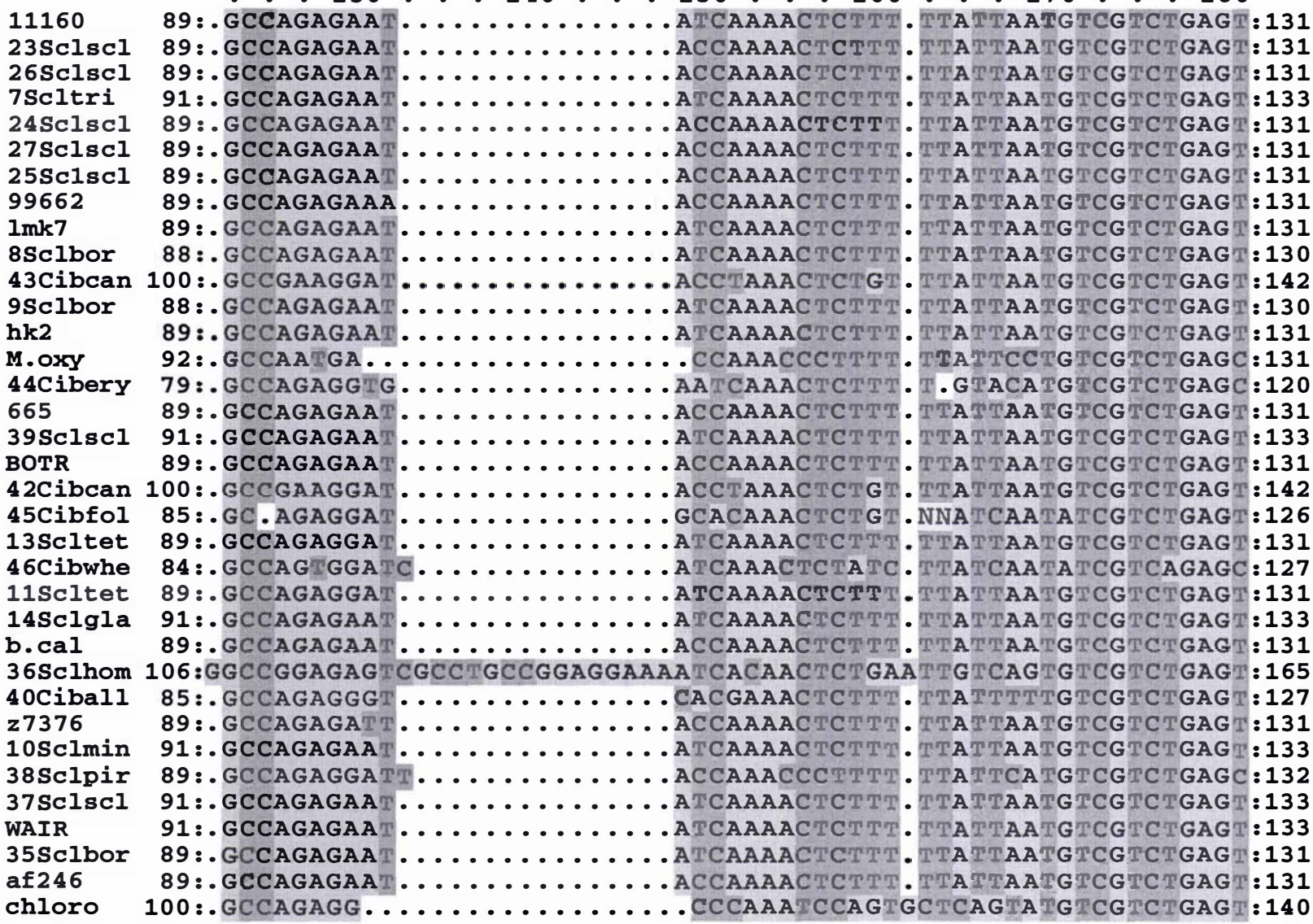
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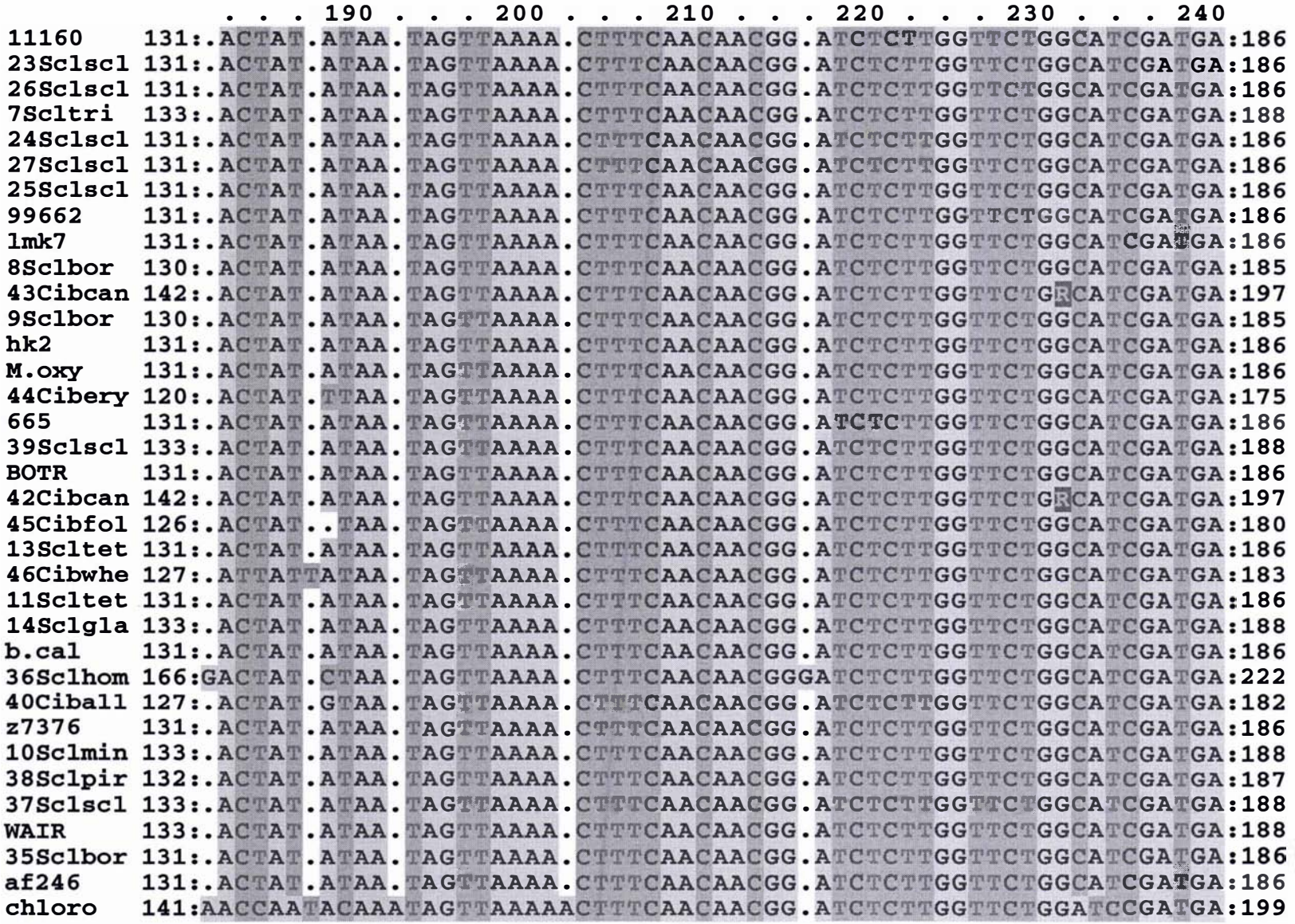
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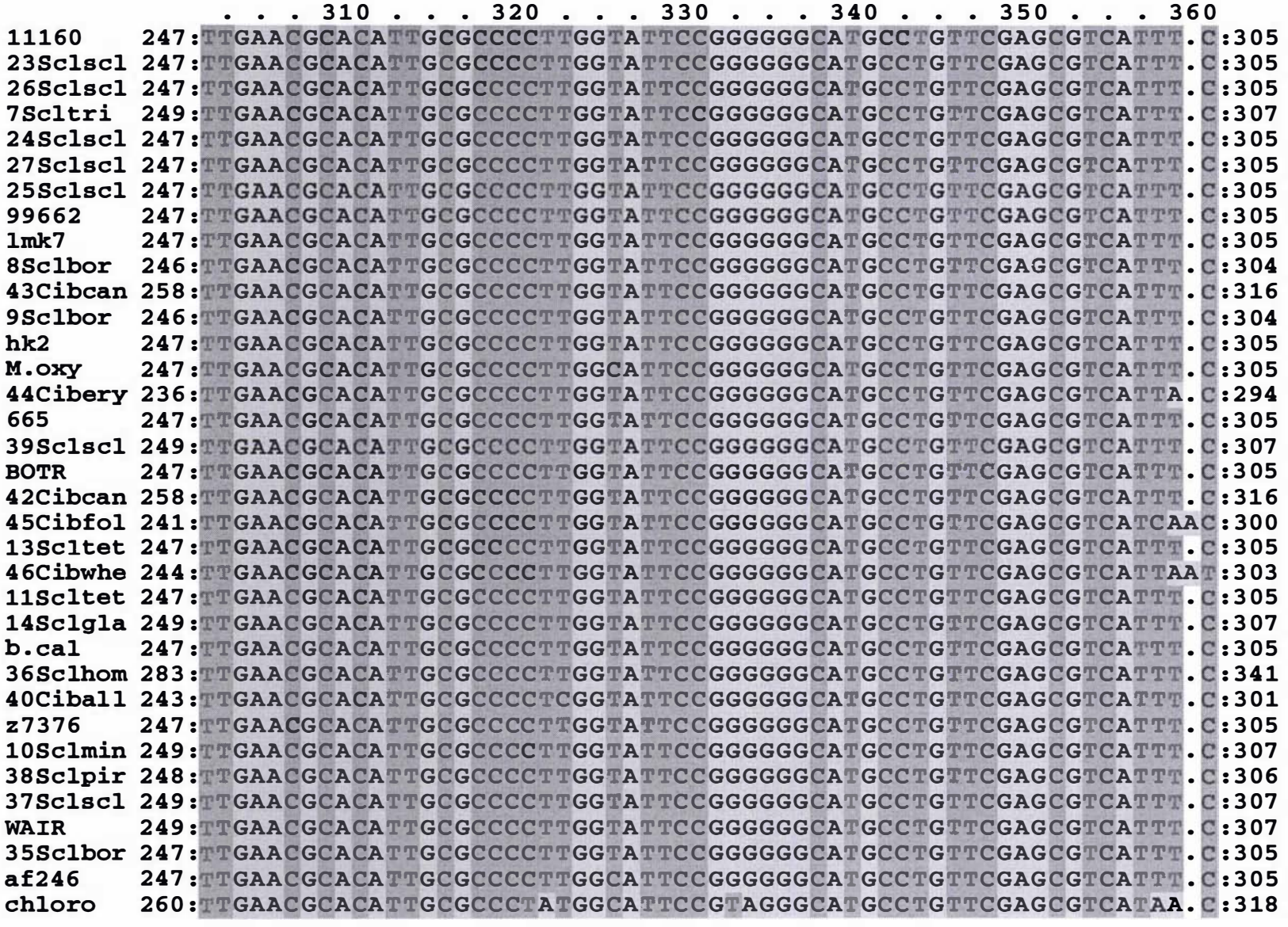
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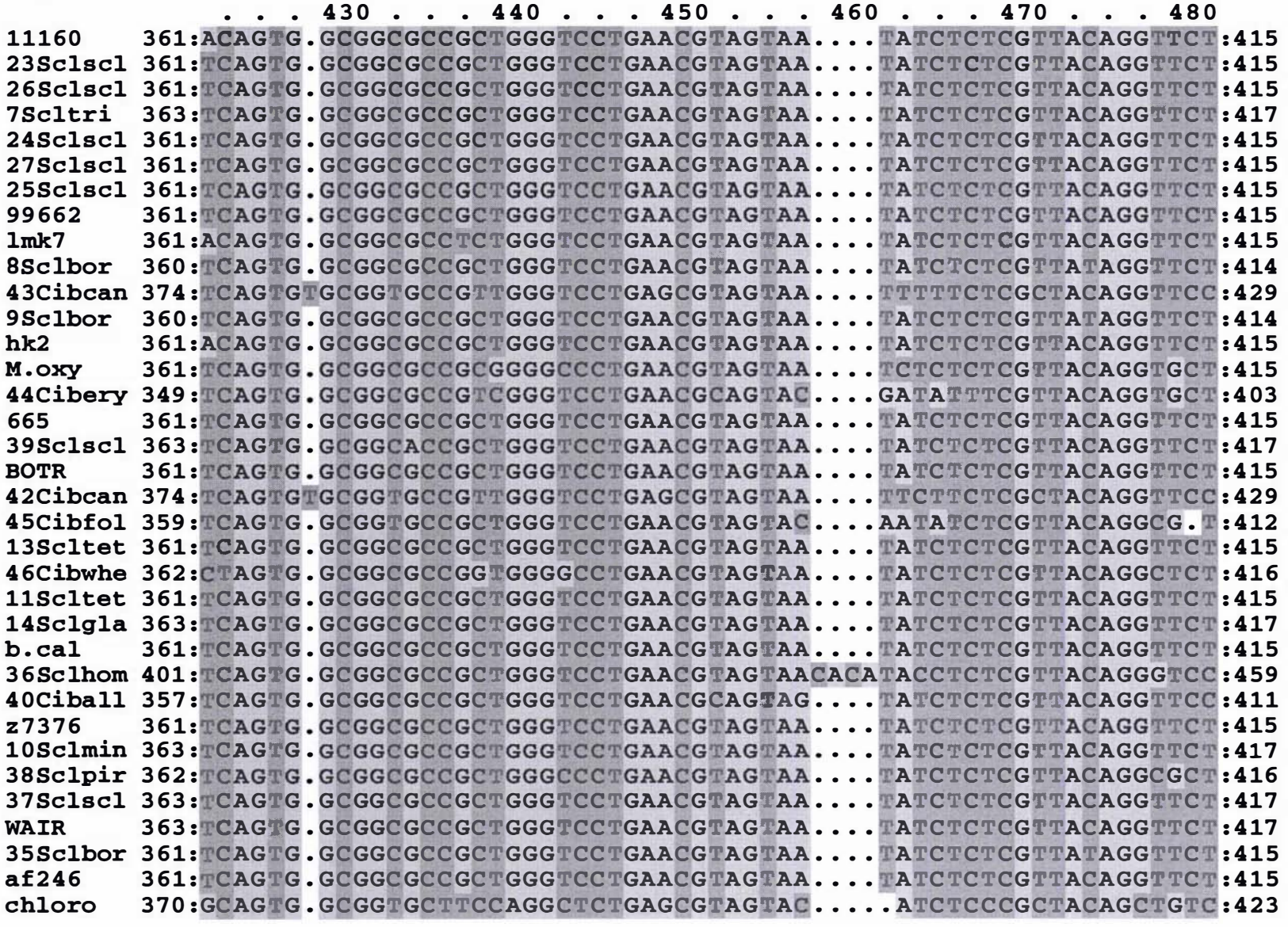
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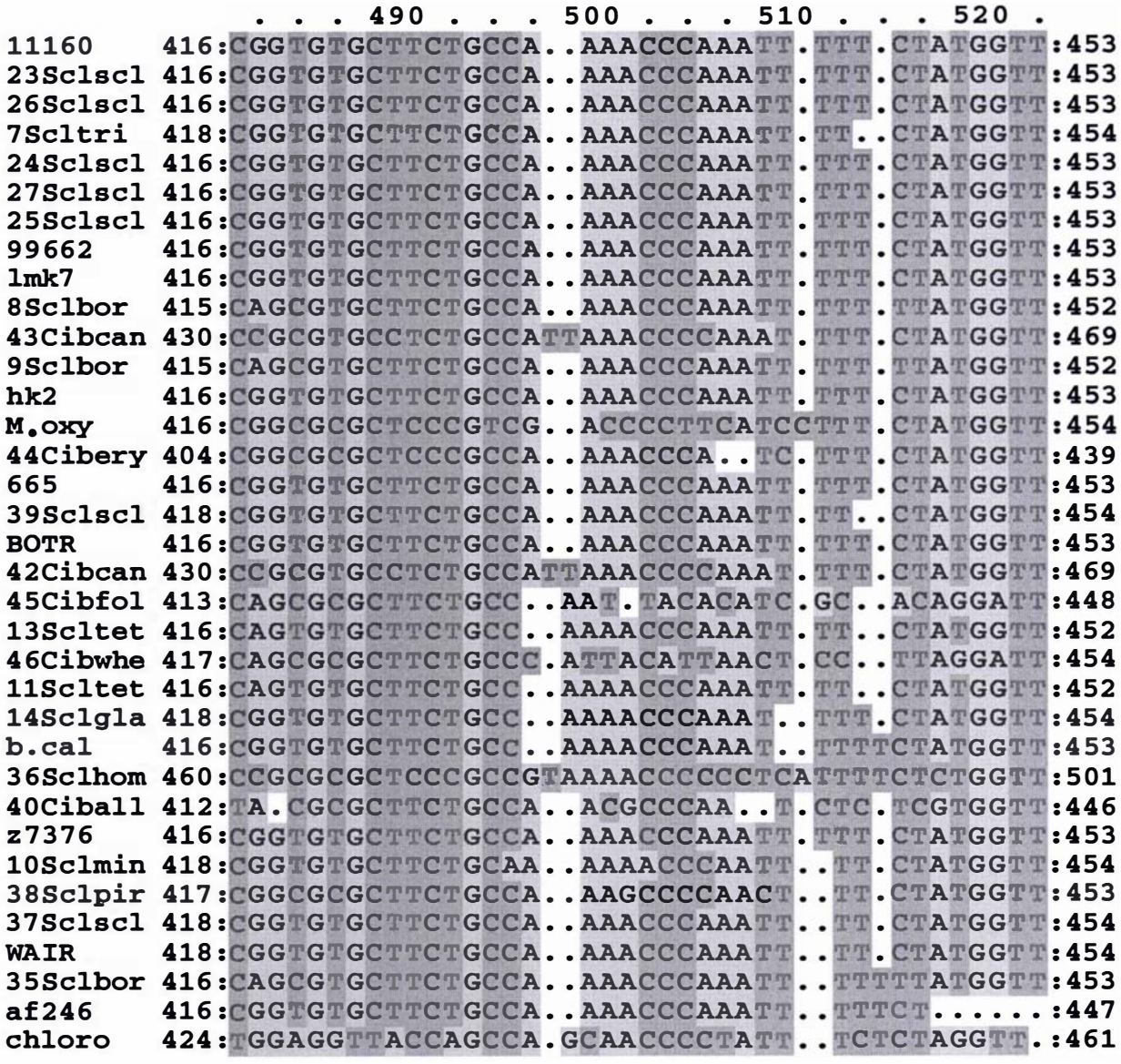
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APPENDIX VI

Effect of Ascospore Density on Disease Development (Experiments 6.30-32)

Experiment 6.30: During the establishment of disease lesions over the first 24 h period (Figure VI.1 A), the data was patchy – overall trends became apparent after 48 h (R-Squared 0.22). Forty-eight hours after inoculation (Figure VI.1 B), members of Group 2 showed increased disease development with increasing ascospore density. Except for Group 4 which had fewer infections at lower spore density, the other groups showed no real change in the level of infection with changing ascospore density (R-Squared 0.39). At 72 h (Figure VI.1 C) Groups 1 and 2 showed a significant relationship between ascospore density and infection level, which decreased at the highest densities (quadratic). The other groups showed no or low levels of disease development with increasing density of ascospores (R-Squared 0.60). At 96 h (Figure VI.1 D) Groups 1, 2 and 3 showed increasing disease development with higher ascospore density but with the quadratic effect at the highest densities. Groups 4, 5 and 6 showed low or no change with higher ascospore densities (R-Squared 0.71). After 120 h (Figure VI.1 E), Groups 1, 2 and 3 have the highest levels of infection regardless of ascospore density, with small linear (Group 3) or quadratic (Group 2) effects. Groups 4 and 5 had low levels of infection at all densities, and little or no infection regardless of spore density in Group 6 (R-Squared 0.75). After 144 h (Figure VI.1 F), Groups 1, 2 and 3 had the highest levels of infection with a small quadratic effect for Group 2. Groups 4 and 5 had minor linear effects with increasing ascospore density and Group 6 was unchanged (R-Square 0.77).

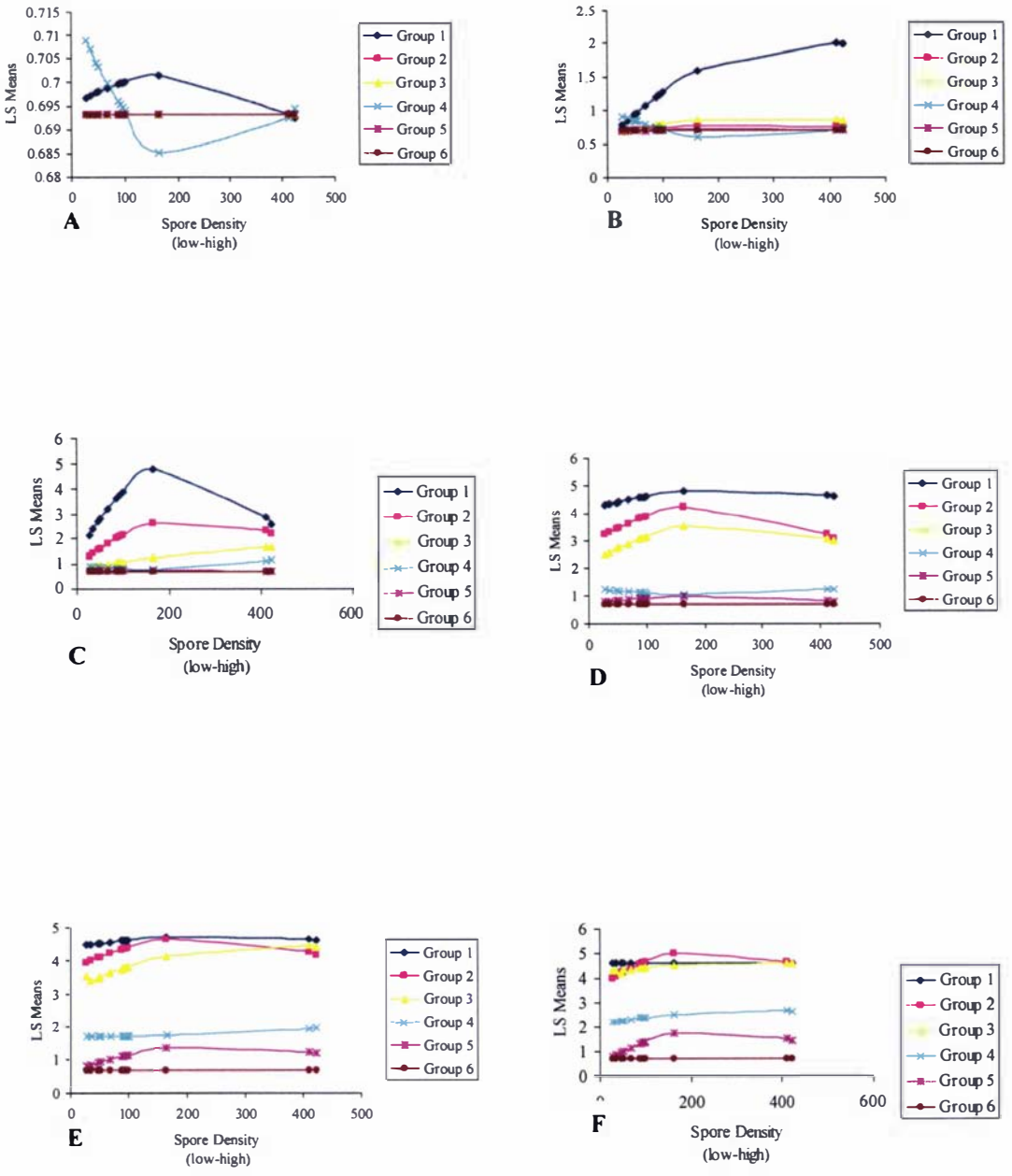


Figure VI.1 A-F
Relationship between ascospore density and disease development over time, Experiment 6.30. LS Means at 24 h (A), 48 h (B), 72 h (C), 96 h (D), 120 h (E) and 144 h (F).

Experiment 6.31: The relationship between spore density and disease development for each day are shown in Figure VI.2 A-F. During the first 24 h period, Group 1 showed a negative relationship between spore density and infection levels. Overall trends became apparent after 48 h (R-Squared 0.09). Forty-eight hours after inoculation, members of Groups 1 and 3 showed decreasing disease development with increasing spore density (quadratic effect). The other groups showed no real change (R-Squared 0.40). At 72 h, Groups 1, 2 and 4 showed a quadratic effect in response to spore density, disease development increasing below 1000 ascospores/ml, decreasing above. Group 3 was more erratic, but generally a linear effect with decreased disease development at higher spore densities. Groups 5 and 6 showed no change (R-Squared 0.63). Group 1 showed a negative linear effect, while Groups 2, 3 and 4 showed quadratic effects, but while Groups 2 and 4 was a positive relationship, Group 3 was a negative relationship. Groups 5 and 6 showed little change in response to changing spore density (R-Squared 0.76). At 120 h, Group 1 showed a slight positive linear effect, whilst Groups 4, 5 and 6 showed negative linear effects. Groups 2 and 3 had a significant relationship between spore density and disease development, which increased (Group 3) or decreased (Group 2) at higher densities (quadratic effect) (R-Squared 0.70). On Day 6, effects of spore density were less pronounced. Groups 1 and 2 showed little change, Group 3 had a negative quadratic effect and in Groups 4, 5 and disease development decreased at higher spore densities (R-Squared 0.69).

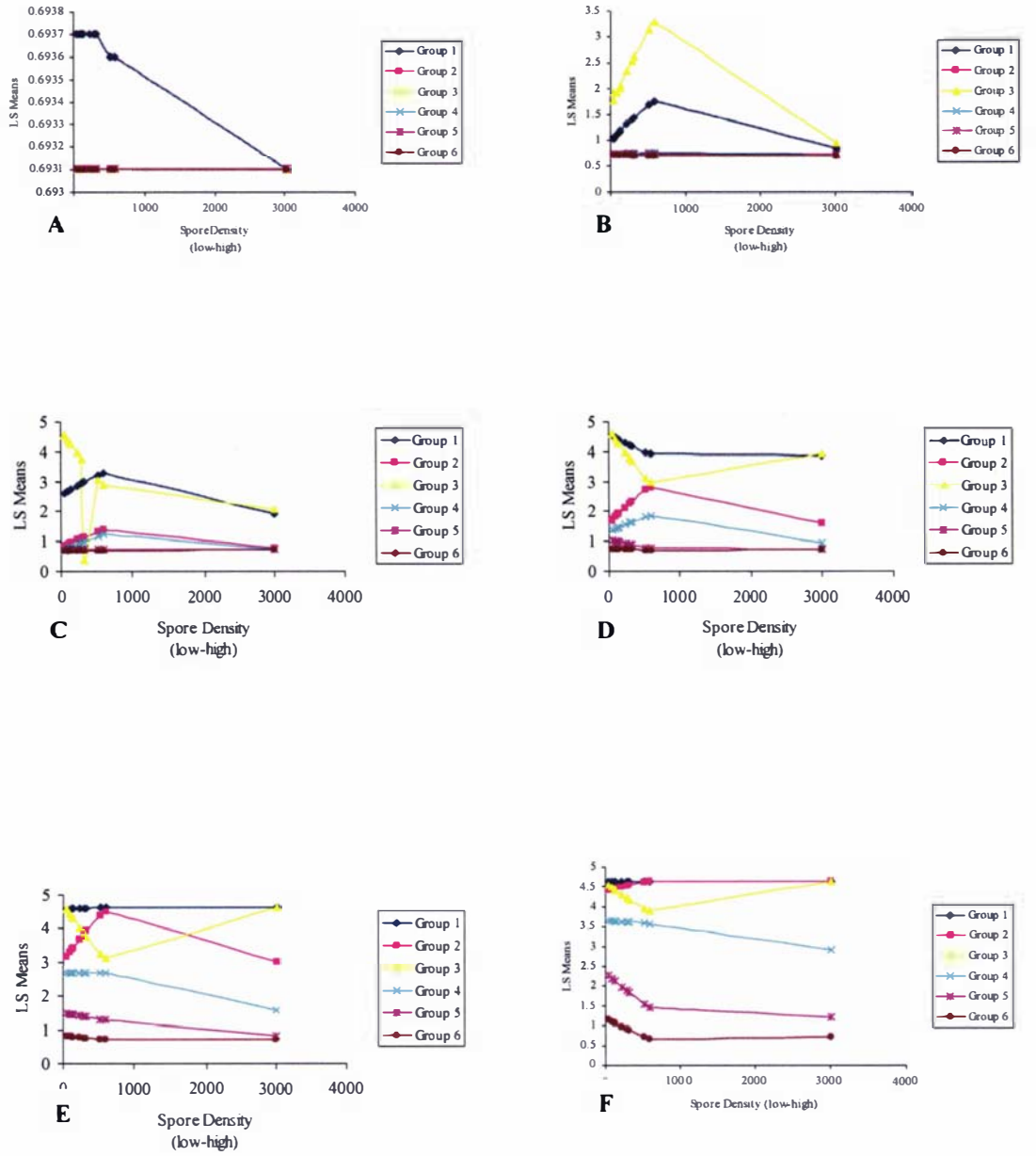
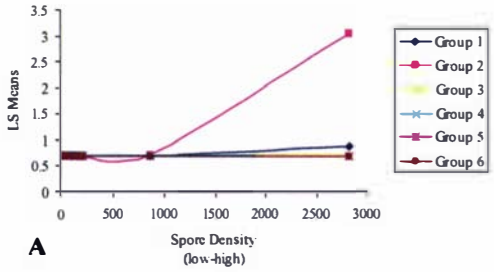
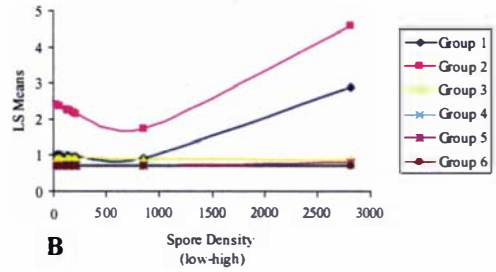
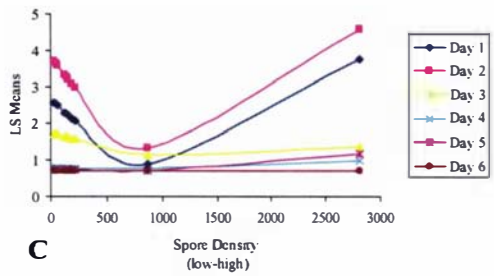


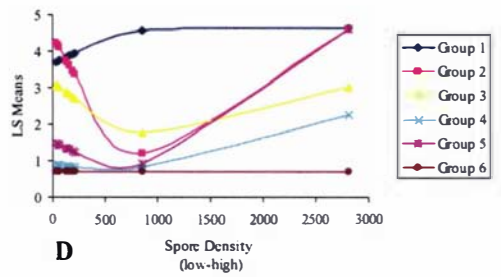
Figure VI.2 A-F
Relationship between ascospore density and disease development over time, Experiment 6.31. LS Means at 24 h (A), 48 h (B), 72 h (C), 96 h (D), 120 h (E) and 144 h (F).

Experiment 6.32: The relationship between spore density and the level of infection for each day are shown in Figure VI.3 A-E. During the first 24 h period, symptoms of disease were usually not visible. Members of Group 2, however, showed that higher ascospore densities produced disease lesions more quickly (R-Squared 0.98). Forty-eight hours after inoculation, Group 1 showed a linear relationship between increasing ascospore density and increasing disease development. Group 2 showed a quadratic relationship and the other groups showed no real change (R-Squared 0.53). At 72 h, both Group 1 and 2 showed a strong quadratic effect between the amount of disease development and ascospore density. Groups 3, 4 and 5 showed small, linear effects and Group 6 showed no change (R-Squared 0.48). Groups 2, 3 and 5 showed quadratic effects, while Groups 1 and 4 showed a linear effect. Group 6 showed no change in the level of infection with changing ascospore density (R-Squared 0.59). At 96 h, Groups 2 and 5 showed a strong quadratic effect, Groups 3 and 4 small quadratic effects and Group 6 no real change (R-Squared 0.65).









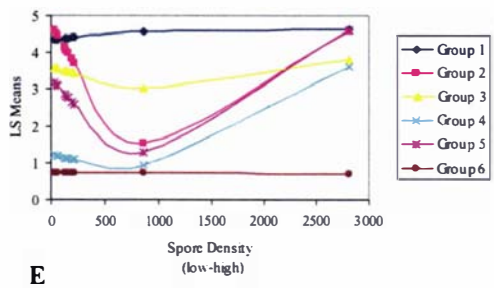


Figure VI.3 A-E
Relationship between ascospore density and disease development over time, Experiment 6.32. LS Means at 24 h (A), 48 h (B), 72 h (C), 96 h (D), and 120 h (E).