Epidemiology of ascochyta blight of chickpea in Australia

by

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Progress of ascochyta blight in a commercial chickpea crop (Desavic) at Balaklava, South Australia

A) A few patches of plants with symptoms of ascochyta blight are visible in August 1998.

B) The same crop in September 1998, with no chickpea plants remaining alive. Green plants visible are weeds.

(Photographs Courtesy of Mark Ramsey)

Dedicated to Dr Janice Fletcher Head of the Metabolic Unit of the Adelaide Women's and Children's Hospital, in great appreciation of her involvement and invaluable support in the successful treatment of my son, Shehik Khan

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DECLARATION

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

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Approx.	approximately
°C	degrees Celsius
cm	centimetre
CMI	Commonwealth Mycological Institute
CMSA	chickpea seed meal agar
CSIRO	Commonwealth Scientific and Industrial Research Organisation
3-D	three dimentional
ddH_2O	double distilled water
et al.	and others
FAO	Food and Agriculture Organisation
Fig.	figure
g	grams
h	hour
ha	hectare
HPLC	high performance liquid chromatography
ICARDA	International Centre for Agricultural Research in the Dry Areas
ICRISAT	International Crop Research Institute for the Semi-Arid Tropics
INRA	Institut National Agronomique
ISPAVE	I stituto Sperimentale per la Patologia Vegetale
Kg	kilogram
L	litre
Max.	maximum
Min.	minimum
mm	millimetre
mL	millilitre
MS	mass spectrometry
m/z	mass to charge ratio
µm	micrometre
nm	nanometre
OMA	oat meal agar
PAS	periodic acid/Schiffs
PCR	polymerase chain reaction
PARC	Pakistan Agriculture Research Council
PDA	potato dextrose agar
%	percentage

RAPD	randomly amplified polymorphic DNA
RH	relative humidity
RIC	reconstructed ion current
RO	reverse osmosis
SARDI	South Australian Research and Development Institute
SDW	sterilised distilled water
t	tons (metric)
TBO	toluidine blue O
TLC	thin layer chromatography
Temp.	temperature
TS	transverse section
UC	University of California
USA	United States of America
UV	ultra violet
v/v	volume per volume
vwk	very weak
wk	weak

Summary

In Australia, chickpea is a relatively new, but rapidly expanding, grain legume crop that has received considerable attention in the cereal belt of southern Australia. The present study was conducted to determine the etiology of a blight disease of chickpea in south-eastern Australia and the factors affecting disease development. The disease had previously been identified as phoma blight.

A range of *Phoma*-like isolates from chickpea in South Australia were tested for pathogenicity to roots and foliage in a series of preliminary experiments. Inoculation techniques were standardised and conidial suspension was found to be the most appropriate inoculum. Conidial suspensions were used in the subsequent experiments. Pathogenicity testing of nine *Phoma*-like isolates revealed two isolates that were highly pathogenic. They were subsequently identified as *Ascochyta rabiei* based on morphological characters, and by collaborators using RAPD-PCR in France and mating type studies in Italy. These isolates were accessioned in the Agricultural Scientific Collections Unit, NSW Agriculture as DAR 71767 and DAR 71768. This is the first time that *A. rabiei* has been conclusively identified in commercial crops in the southern hemisphere. Additional isolates of *A. rabiei* were obtained from commercial crops in South Australia during 1996 and 1997 and South Australia, Victoria, New South Wales and Queensland during the 1998 growing season.

Screening of chickpea varieties in the greenhouse revealed that, of the desi types, Dooen was the most resistant to ascochyta blight, followed by Tyson, Norwin and Heera, whereas Desavic was highly susceptible, and of the kabuli types, Kaniva, Garnet and Bumper ranged from moderately susceptible to susceptible. Lines ICC 1151x ILC3279, ICC 1151xIL C482, NIFA-88 and CM-72 imported from Pakistan, were also found to be resistant to DAR 71767 while Noor-91 and Paidar-91 were susceptible.

The effects of plant age and environmental conditions on disease development were investigated in a series of experiments conducted under controlled conditions in growth rooms. Seedlings of cv. Desavic were more susceptible than older plants. The optimum conditions for ascochyta blight were 20°C and a 48-96 h period of leaf wetness. The disease development was negatively influenced by interrupting the wetness period with more than 6h dryness, but less than 6h dryness lead to increased disease.

The response of five chickpea cultivars to inoculation with *A. rabiei* in the field was evaluated over three consecutive years. It was observed that the disease intensity

increased over time, especially in cv. Desavic, which was killed 7 weeks after inoculation in 1997. Disease in 1998 was not as severe as in commercial crops, probably due to unusually dry weather and weed infestation of the field trial. Disease severity in resistant and susceptible cultivars was consistent both in greenhouse and field conditions.

The host range of *A. rabiei* was tested by inoculating 29 plant species with DAR 71767 and maintaining them in the greenhouse in humid conditions for 72 h at 20 ± 5°C. The isolate was pathogenic to chickpea and four common bean varieties (*Phaseolus vulgaris* L.), Brown-boy, CH-190-7D, Cran-34 and Rain-bird, only.

The means of penetration of the chickpea host was established in histological studies using conventional light microscopy and confocal microscopy. *A. rabiei* was found to penetrate leaf tissues through guard cells, stomata, directly through cell walls and between epidermal cells. This is the first report of penetration by the pathogen through stomata.

The phytotoxins, solanapyrones A and C, were identified in culture filtrates of Australian isolates, DAR 71767, DAR 71768 and 215/91, by comparison with standard reference toxins. The level of solanopyrone C increased gradually with age of the culture. The presence and concentration of the toxins were consistent as determined by thin layer chromatography, high performance liquid chromatography and mass spectrometry. In a preliminary bioassay, leaves of cultivars Dooen and Desavic, treated with purified culture filtrate, became necrotic.

All cultures of *A. rabiei* isolated, to date, in Australia have been shown to be mating type MATI-1, by pairing with reference mating types in Italy and the USA. The teleomorph has not been observed in field material nor was it induced in infected chickpea debris nor inoculated chickpea straw incubated in conducive conditions in South Australia.

The study, therefore, clarified the confusion between phoma and ascochyta blight in Australia, and provided advance warning of this disease for the expanding Australian chickpea industry. Confirmation of ascochyta blight has allowed the implementation of appropriate disease management strategies. Resistance identified in breeding lines will be helpful to the National Chickpea Breeding Program. Other important outcomes include the recommendations that (i) cv. Desavic, currently widely grown, should not be grown where ascochyta blight is likely to be a problem as it is highly susceptible; (ii) quarantine restrictions on the import of chickpea seeds be maintained to prevent the introduction of MATI-2.

Publications and Conference Proceedings

The following publications and conference proceedings were produced during the Ph.D candidature:

1. Khan MSA, Ramsey MD, Corbiere R, Porta-Puglia A, Bouznad Z, Scott ES, 1997. Ascochyta blight of chickpea crops in Australia. *3rd International Food Legume Research Conference*, 22-26 September 1997, Adelaide, South Australia (abstract).

2. Khan, MSA, Ramsey MD, Scott ES, 1997. Evaluation of resistance to Ascochyta blight of chickpea in Australia. P.116. *Eleventh Biennial Australasian Plant Pathology Society Conference,* 29th September-2nd October, Perth, Western Australia.

3. Khan MSA, Ramsey MD, Corbiere R, Infantino A, Porta-Puglia A, Bouznad Z and Scott ES, 1999. Ascochyta blight of chickpea in Australia: identification, pathogenicity and mating type. *Plant Pathology* **48,** 230-234.

4. Khan MSA, Ramsey MD, Scott ES, 1999. Host range studies with *Ascochyta rabiei* in Australia. *Australasian Plant Pathology* **28,** 000-000 (in press).

5. Ramsey MD, Khan MSA, Hannay J, 1998. Ascochyta blight of chickpea in South Australia. *Crop Science Society of South Australia*, No.**163**.

CHAPTER 1

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important cool-season pulse crop in the world after dry peas (Robertson *et al.*, 1995). It is particularly important as a source of protein for the population of South Asia.

Two groups are recognised within cultivated chickpea. Kabuli cultivars, common in the Mediterranean region and in the Near East, are tall with white flowers and produce large, rounded seed usually pale cream in colour. Desi cultivars are relatively short, sometimes prostrate, commonly with anthocyanin pigmentation in flowers and stems, and produce small, irregularly shaped seed of various colours (Allen, 1983).

In Australia, the first experimental evaluation with chickpeas was started between 1892-1897 in New South Wales (Anon., 1892; Valder, 1893, 1896). For reasons unknown, further research was stopped until 1971, when a new program commenced at the Agricultural Research Institute, Wagga Wagga, New South Wales. Twenty five elite lines in New South Wales were selected from 142 germplasm collections based on plant type, grain yield and suitability for mechanical harvesting. Accession CPI-53007 was selected as a commercial cultivar, "Tyson", in 1978 by the Division of Tropical Crops and Pastures, CSIRO, after testing at 20 different sites in Queensland during the growing season of 1977-78 (Beech and Brinsmead, 1980). The first Australian commercial chickpea crop was grown in 1979 (Johnston *et al.*, 1992). Chickpea has fitted well into the farming systems across a broad range of environments in Australia, extending from the tropical Ord River Irrigation Area (ORIA) in WA through subtropical southern Queensland and northern New South Wales, to the Mediterranean-type environments of southern Australia.

According to FAO (1996), chickpea is cultivated on 11,099,000 hectares world-wide and its total production is nearly 8,908,000 tonnes. Australia currently produces about 250, 000 t of chickpea per annum and is the fifth largest producer and second largest exporter in the world (Siddique and Sykes, 1997). Australia is probably the only significant chickpea-producing country in the world which has all production operations, from sowing to post-harvest handling, fully mechanised (FAO, 1993). The Grains Council of Australia strategic plan estimated a potential doubling of chickpea production to about 500, 000 t by 2005, including about 90, 000 t of high quality kabuli chickpea (Anonymous, 1995).

Of several diseases affecting this crop, Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labrousse, teleomorph *Didymella rabiei* (Kovachevski) v. Arx [syn. *Mycosphaerella rabiei* (Kovachevski)], is the most serious. It is known to occur in almost all countries where chickpea is grown (Nene, 1982). Ascochyta blight was not well known in Australia until it was first confirmed in commercial chickpea crops in south-eastern Australia (Khan *et al.*, 1997a).

Epidemics of ascochyta blight of chickpea have been recorded since the early 1900s in many countries. However, we still lack answers as to why, when and where epidemics occur. Several factors are required for epidemics, including the presence of numerous susceptible plants, enough inoculum of virulent isolates of the pathogen and climatic conditions favourable for disease development over a period of time. Although a great deal is known about ascochyta blight and its control by various means, there are serious gaps in our knowledge of the different factors that affect epidemiology.

Sporadic outbreaks of disease in chickpea crops in South Australia in the 1980s and early 1990s were attributed to Phoma blight (*Phoma medicaginis* var. *pinodella*) (J. Walker and E. Punithalingam, personal communication, 1990). However, symptoms of ascochyta blight and phoma blight are similar except that pycnidia of *A. rabiei* on lesions

are typically concentrically arranged whereas those of *P. medicaginis* are not (Haware and Nene, 1981).

The studies reported here were initiated to determine the etiology of blight disease of chickpea in south eastern Australia in response to recent outbreaks in South Australia. Once the disease was confirmed as ascochyta blight, epidemiology and host-pathogen interactions were investigated.

The aims of the project were

- to study the etiology of chickpea blight in south-eastern Australia

- to evaluate the response of cultivars and germplasm of chickpea to the pathogen in the glasshouse and in the field

- to study the influence of inoculum concentration, plant age, temperature and wetness on disease development

- to investigate the possible host range of the pathogen

- to investigate the mode of infection by means of histological studies

- to study the role of toxins in pathogenesis

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

This research project began in 1995 and this chapter provides a review of the relevant literature up to that date. As a result of preliminary studies, *Ascochyta rabiei* was found for the first time in commercial crops. The focus of the study was, therefore, changed from phoma blight to ascochyta blight, as this disease is particularly destructive, is of world importance, and poses a major threat to the Australian chickpea industry which is in a stage of rapid expansion. This review presents information on ascochyta blight of chickpea, and includes comparison with phoma blight where appropriate.

2.2 Blight diseases of chickpea

Ascochyta blight, also known as chickpea blight, gram blight, ascochytosis, anthracnose, rabia or scorch of chickpea, affects all above ground-parts of the host plant. The disease has now been reported from the following countries: Afghanistan, Algeria, Australia, Bangladesh, Bulgaria, Canada, China, Colombia, Cyprus , Egypt, Ethiopia, France, Greece, Hungary, India, Iran, Iraq, Israel, Italy, Jordan, Kenya, Libya, Mexico, Morocco, Pakistan, Portugal, Romania, Spain, Sudan, Syria, Tanzania, Tunisia, Turkey, USA and the former USSR (Nene and Sheila, 1992; Nene *et al.*, 1996; Khan *et al.*, 1997a).

Phoma medicaginis has been reported on chickpea in India, Zambia, Canada, UK, North America and Australia (Morgan-Jones and Burch, 1987; Sutton, 1973). It is most commonly reported on lucerne (*Medicago sativa* L.), on which it may cause black stem, foot rot and leaf spots (Boerema, 1976).

Ascochyta blight can cause serious yield loss in chickpea (Benlloch, 1941; Biggs, 1944; Kaiser, 1972; Kausar, 1965; Malik and Tufail, 1984; Porta-Puglia and Crino, 1993; Radulescu *et al.*, 1971; Singh and Reddy, 1991; Zalpoor, 1963.). In Morocco the disease has caused up to 100% yield loss in wet conditions in 1929 and the whole crop may be destroyed in 3 days under optimum conditions (Labrousse, 1930; Neergard, 1977). In Azerbaijan, *A. rabiei* attacked all cultivars and yield loss of 15 to 83% occurred in conducive climatic conditions (Askerov, 1968).

The incidence and severity of ascochyta blight varies from crop to crop, year to year, and from one geographical area to another, depending on host, pathogen and environmental conditions. In wet seasons substantial yield losses are likely to occur, whilst in dry season losses will be minimal.

2.2.1 Symptoms of ascochyta blight

Primary infection from seed results in dark brown lesions at the collar region which vary in size depending on climatic conditions, and results in damping-off (Nene and Reddy, 1987). Infection by airborne inoculum results in small, necrotic specks in the young, developed leaves. Under optimum conditions, the specks rapidly enlarge and coalesce, resulting in necrosis of young leaves and shoots with numerous pycnidia in the infected area (Nene and Reddy, 1987). Necrosis progresses downwards most rapidly in susceptible cultivars and kills the whole plant. In cases of severe foliar infection, the whole plant may become dry, but with little stem infection. Under conditions adverse for disease development, the symptoms are restricted to circular spots with grey centres and brown margins (Nene and Reddy, 1987).

Symptoms include circular, brown spots on leaflets and pods, elongated, irregular lesions on stems and petioles and stem breakage at the point of infection. Pycnidia on the lesions are often concentrically arranged. The fungus penetrates the pod wall and

infects the seed. Infected seeds may have irregular patches of brown discolouration (Nene and Reddy, 1987).

It can be difficult to identify the disease in the field if conditions remain dry for several weeks because infected tissue rapidly dies, withers or breaks off, and is masked by sprouting of shoots from the base.

2.2.2 Symptoms of phoma blight

In comparison, symptoms of phoma blight of chickpea include irregular, light brown lesions on the leaves, stems and pod, surrounded by margins with dark, minutes, submerged pycnidia, irregularly scattered. The fungus can penetrate the pod and infect developing seeds. Leaves fall from badly infected plants (Haware and Nene, 1981; Lamb and Poddar, 1987).

Haware and Nene (1981) reported that the symptoms they observed resembled those caused by *A. rabiei*, and Khune and Kapoor (1980) suggested that *A. rabiei* be called *Phoma rabiei* on the basis of similar symptoms produced on chickpea and aspect of pycnidiospores.

2.3 The pathogens

2.3.1 *Ascochyta rabiei*

2.3.1.1 The anamorph

The taxonomic history of *A. rabiei* has proved to be quite controversial. The causal fungus of ascochyta blight of chickpea was first named *Zythia rabiei* by Passerini in 1867, based on unicellular and hyaline pycnidiospores (Khune and Kapoor, 1980). Subsequent researchers disagreed with Passerini's findings: Comes (1891) identified the fungus as *Ascochyta pisi* Lib., and Prillieux and Delacroix (1893) named it *Phyllosticta cicerina* (Khune and Kapoor, 1980). Trotter (1918), after studying Saccardo's material, concluded that the fungus was not a species of *Ascochyta* and then proposed the name *Phyllosticta rabiei* (Pass.). Later, Labrousse (1931a) suggested that the fungus should be called *Ascochyta rabiei* because 2-4% of conidia recovered from inoculated plants were 2-celled. However, Luthra and Bedi (1932) and Aujla (1960) used the name *Phyllosticta rabiei* and Khune and Kapoor (1980) suggested that the fungus should be named *Phoma rabiei* (Pass.) because *Phoma* species can have 5% of the pycnidiospores 2-celled. However, *A. rabiei* (Pass.) Lab. is now accepted by the majority of researchers and by the International Mycological Institute (IMI).

A. rabiei is characterised by pycnidia, produced on infected tissues and on artificial media (Sattar, 1934). The pycnidia, which are visible as dark-brown, pin-head-like structures in infected plant tissues, are immersed, amphigenous, spherical to subglobose, and vary from 65-245 µm in size (Sattar, 1934). The pycnidial wall is composed of 1 to 2 layers of elongated pseudo-parenchymatous cells and the ostiole is 30-40 µm wide. Pycnidiospores (conidia) are hyaline, oval to oblong, straight or slightly curved at one or both ends, non or one septate, constricted at the septum when bi-celled, rounded at both ends, 3.5 x 10-16 µm and formed on hyaline, ampulliform phialides (Haware *et al.*, 1986).

Colonies on artificial media are flat, submerged, with sparse mycelium, white at first, becoming dark and fumaeceous on oat-meal agar (OMA), while on potato dextrose agar (PDA) at 20-25°C they are creamy to pinkish at first, darkening with time. Pycnidia are formed within 4-5 days and appear in concentric rings (Nene, 1982; Nene, 1984).

According to Bedi and Aujla (1970), pycnidia developed best at pH 7.6 to 8.6 at 20°C on double strength Richard's medium. Kaiser (1973) reported that maximum spore production occurred on 8% chickpea seed meal agar (CSMA), while mycelial growth was greatest on CSMA or OMA at 15-20°C. Under continuous light, mycelial growth and conidial production increased but zonation occurred in alternating light and dark periods. At the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Nene (1984) confirmed these observations. In contrast, Chauhan and Sinha (1973) found reduced sporulation on infected plants in a glasshouse under continuous light. These discrepancies regarding the effect of light on mycelial growth and sporulation, suggest the need for further experimental work.

A number of studies have demonstrated that the optimum temperature for growth, pycnidial production and spore germination is around 20°C (Bedi and Aujla, 1970; Chauhan and Sinha, 1973; Kaiser, 1973; Maden *et al.*, 1975; Zachos *et. al.*, 1963). Temperatures below 10°C and above 30°C are unfavourable to the fungus (Chauhan and Sinha, 1973; Kaiser, 1973; Luthra and Bedi, 1932). Maden *et al.* (1975) reported that pycnidia did not form at 4°C nor at 28°C and above, and that the colonies were pinkish-brown with zonation and maximum pycnidial formation in near UV light but light pink, fluffy, without zones and pycnidia in darkness.

2.3.1.2 The teleomorph

Didymella rabiei (Kovachevski) von Arx (syn. *Mycosphaerella rabiei* (Pass.) Kovachevski) is the teleomorph of *A. rabiei.* In 1936, Kovachevski (1936) was the first

to observe the sexual stage on infected chickpea debris that had over-wintered on the soil in southern Bulgaria, and named it *Mycosphaerella rabiei* Kovachevski. Subsequently, *M. rabiei* was reported on over-wintered infected chickpea residues in the field in the former USSR (Gorlenko and Bushkova, 1958), Greece (Zachos *et al.*, 1963), Hungary (Kovics *et al.*, 1986), USA (Kaiser and Hannan, 1987), Spain (Jimenez-Diaz *et al.*, 1987) and Syria (Haware, 1987). Subsequently, Trapero-Casas and Kaiser (1992) identified the pathogen as *D. rabiei* rather than *M. rabiei*, based on morphological characteristics of pseudothecia, asci and ascospores. They were the first researchers to induce pseudothecia *in vitro* on naturally infected chickpea straw. The straw was incubated in moist conditions at 5-10°, and development and maturation of pseudothecia occurred within 8 weeks at 8°C. The fungus was heterothallic and the isolates tested were assigned to two mating type groups, MATI-1 and MATI-2, respectively (Trapero-Casas and Kaiser, 1992). The presence of the teleomorph is likely to lead to increased genetic variability in the pathogen and this has implications for disease control.

Subsequently, Kaiser (1995) incubated naturally infected chickpea debris from different countries at optimal conditions and found fertile pseudothecia developed on infected debris from Algeria, Pakistan, Portugal, Spain, Syria, Tunisia, Turkey and USA. These studies showed that *D. rabiei* may be present in other countries where it was not previously documented. It is more likely that *D. rabiei* will be found in other countries as overwintered samples of infected chickpea debris are examined for the teleomorph.

The ascomata (pseudothecia), which are produced only on chickpea debris, are difficult to distinguish from the pycnidia solely by their appearance. They are dark brown or black, globose or depressed globose, with a hardly perceptible beak and ostiole, 76.25-152.5 x 120.75-250.12 µm. Asci are hyaline, cylindrical-clavate, more or less curved, pedicellate and 48.8-70.15 x 9.15-13.7 µm in size. The ascospores (8 in each ascus) are hyaline, monostichous or hardly distichous, ovoid, divided into two unequal cells,

strongly constricted at the septum and measuring 12.5-18.9 x 6.71-7.62 µm (Kovachevski, 1936).

As the anamorph only has been found in Australia to date, therefore, in this thesis the name *A. rabiei* will be used except where reference is made to the teleomorph.

2.3.2 *Phoma medicaginis*

Phoma medicaginis Malbr. and Roum. belongs to the subdivision Deuteromycotina (now The Mitosporic Fungi; Hawksworth *et al.*, 1995), class Coelomycetes, order Sphaeropsidales (Sutton, 1973). Morgan-Jones and Burch (1987) list the following as synonyms: *Phoma herbarum* Westend forma *medicaginis* Westend (1862); *Ascochyta imperfecta* Peck (1912); *Phoma cuscuta* Negru and Verona (1966).

Morgan-Jones and Burch (1987) described *P. medicaginis* as follows: "Colonies on potato dextrose agar (PDA) generally uniform in appearance, occasionally sectoring, olive grey to dark olive grey. Mycelium composed of hyphae of two types: hyaline, smooth, branched, septate, 2-3 µm wide; and pale yellow brown, smooth, branched, 5-6 µm wide. The latter hyphae are sometimes aggregated into strands, as in immersed mycelium when grown on MEA, and individual cells may become slightly inflated, to 8 µm wide, with age. In older colonies on PDA, pycnidia are partly embedded in a thick matrix composed of intertwining, darker brown, thick-walled hyphae giving the colony surface a crusty, carbonaceous appearance. Pycnidia produced on PDA are gregarious, frequently confluent but maintaining discrete venters, more or less globose, superficial or partly immersed, without a well defined ostiole, black at base and sides, pale above, pseudo-parenchymatous, 120-280 µm in diameter. Pycnidial wall 20-30 µm thick, made up of four to five layers of cells. Outer pigmented portion composed of two irregular layers of ellipsoid to subglobose, inflated, loosely organised, brown, thick-walled, 8-16 x 7-11 µm cells. Conidia enteroblastic, hyaline, smooth, oblong to short cylindrical,

obtuse at each end, unicellular or occasionally becoming one-septate, especially with age, guttulate, 7-1 x 2-3.5 µm. Conidia masses whitish to pale pink in colour. Chlamydospores are rarely produced in young cultures, but are common in one month-old colonies on PDA, mostly solitary, intercalary or terminal, subglobose to globose, smooth, thick-walled, brown, 7-14 µm in diameter."

Mmbaga (1993) suggested that *P. medicaginis* closely resembles to *A. rabiei*, and that its taxonomic status needs to be clarified.

2.4 Disease cycles

2.4.1 Ascochyta blight

The spread of ascochyta blight is attributed to pycnidiospores produced at sites of primary infection, either by means of crop debris or infected seed (Fig. 2.1). Subsequent inoculum dispersal, penetration and infection of plant tissues results in disease.

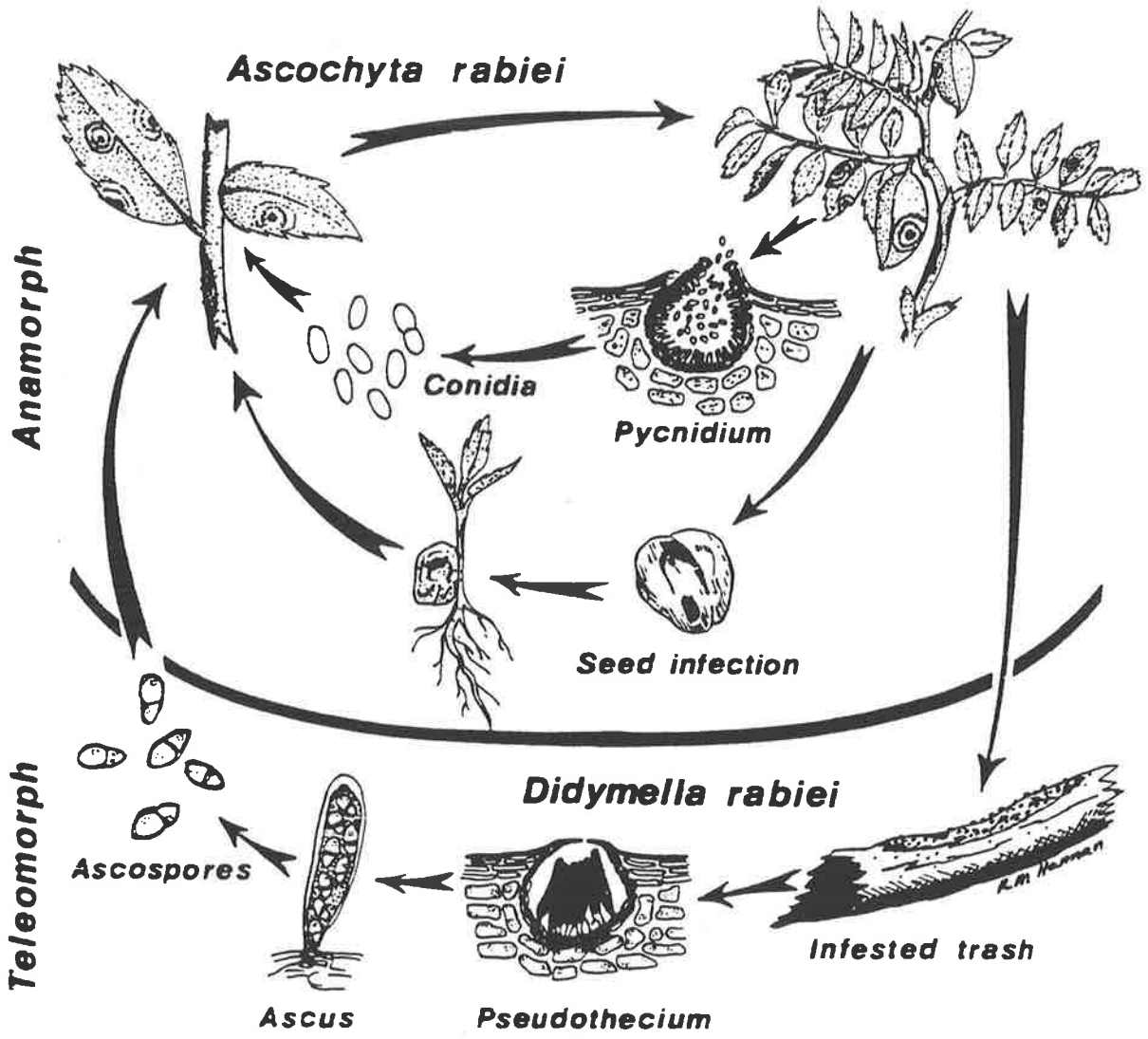

Figure 2.1. Disease cycle of ascochyta blight of chickpea (Kaiser, 1992)

2.4.2 Phoma blight

The pathogen becomes established in or in chickpea crops from inoculum surviving in infected seed or crop residues (Lamb and Poddar, 1987). Infection can occur at any stage of plant growth provided conditions are favourable. Little is known about the disease cycle on chickpea other than that moisture is essential for infection to occur. During wet weather, the disease may spread, especially when spores are carried onto neighbouring plants by wind and rain splash.

2.5 Epidemiology

Epidemiology is the science of disease in populations (Vanderplank, 1968), involving studies of the temporal and spatial changes that occur during epidemics of plant diseases caused by populations of pathogens in populations of plants (Kranz, 1974). An epidemic occurs when there is a change in disease intensity in a host population over time and space. Host-pathogen interactions are often environmentally dependent, so epidemiology deals with the effects of the biotic and abiotic environments (Campbell and Madden, 1990).

Gaumann (1950) specified the conditions that must occur simultaneously if an epidemic is to develop: "...On the part of host, an abundant supply of susceptible individuals produced by (a) an accumulation of susceptible individuals...; (b) heightened disease proneness of the hosts...; (c) the presence of appropriate alternate hosts...; on the part of pathogen, the possession of high infective capacity, conditioned by (d) the presence of an aggressive pathogen...; (e) high reproductive capacity...; (f) efficient dispersal...; (g) unexacting growth requirements...; on the part of environment, (h) optimal weather growth conditions for the development of the pathogen..." (Campbell and Madden, 1990).

Much work has been done on ascochyta blight but still there are serious gaps in the knowledge of the different factors that affect epidemiology, and further research is needed.

2.5.1 Survival of *A. rabiei*

In common with most foliar pathogens, *A. rabiei* survives in infected crops debris and seeds (Nene, 1982), and the same is likely to be true to *P. medicaginis.* These materials act as a reservoir of primary inoculum which can cause infection in favourable conditions. More work on the survival of the pathogens is required to improve understanding of the disease cycle and epidemiology.

2.5.1.1 Infected seed

Infected seed is an important means of survival for *A. rabiei* and allows dissemination from one geographical area to another. It plays an important role in the epidemiology of the disease, ensuring a random distribution of the pathogen in a field, which provides many primary infection-courts from which the pathogen can spread and produce secondary infection. Butler (1918) was probably the first scientist to report the infection of chickpea seed by *A. rabiei*, and that pathogens were transmitted from infected seed during germination. Luthra and Bedi (1932) reported that *P. rabiei* penetrated from the ovary wall into the testa at its contact point and finally colonised the cotyledons. According to Dey and Singh (1994), *A. rabiei* naturally occurs both externally and internally in seed and external infection is dominant, the pathogen being located in or on the seed coat and occasionally penetrating the cotyledons and embryo. Internally and externally seed-borne inocula were found to be equally responsible for the transmission of disease and the disease was transmitted to the aerial parts in a non-systemic manner (Dey and Singh, 1994).

Similarly, *P. medicaginis* has been isolated from samples of chickpea seed which were collected from Iran, Pakistan, Sudan and Syria (Haware *et al.*, 1986). Bretag and Mebalds (1987) isolated various fungi, including *P. medicaginis,* from chickpea seeds in Victoria; the incidence of *P. medicaginis* was 0.5 to 6.8% on different chickpea lines. It is not clear whether *P. medicaginis* survives in the seed coat or in the cotyledons of chickpea.

2.5.1.2 Infected Crop Debris

Researchers in various countries have stressed the importance of infected crop debris in the survival of *A. rabiei* from one growing season to another (Askerov, 1968; Kaiser, 1973; Khachatryan, 1962; Kovachevski, 1936; Anonymous, 1973; Lukashevich, 1958; Luthra *et al.*, 1935; Navas-Cortes *et al.*, 1995; Weltzien and Kaack, 1984; Zachos *et al.*, 1963).

Lukashevich (1958), in the former USSR, found that *A. rabiei* grew saprophytically on dead, infected chickpea plant parts and in the subsequent spring, saprophytic activity on this material increased manyfold. Similarly, Luthra *et al.* (1935), Trapero-Casas *et al.* (1988) and Zachos *et al.* (1963) found that the pathogen colonised chickpea stubble after harvest. Luthra *et al.* (1935) found that *A. rabiei* remained alive for more than 2 years in the infected tissues, but did not survive more than 1 month if the infected debris was buried 5 cm deep in moist soil.

Kaiser (1973) confirmed that the fungus survived for more than 2 years in naturally infected tissues at 10-35°C and 0-30% relative humidity (RH) at the soil surface but rapidly lost viability at 65-100% RH and at 10-40 cm deep in soil. Subsequently, Kaiser *et al.* (1987) studied the survival of the anamorph and teleomorph stages of the pathogen in chickpea in field soil, in a weather station shelter at 4-6°C with RH 30%-40%. Only brief details were published but it appeared that conidia lost viability after 10 and 15

weeks in infected stem tissues and pods, respectively, when buried in soil, but remained viable for 57 and 81 weeks, respectively, when placed on the soil surface. Some conidia in the infected tissues remained viable after 120 weeks at 4-6°C. Discharge of viable ascospores from pseudothecia ceased after 8 weeks if buried in the soil and after 27 weeks in infected tissues placed on the soil surface.

Recently, in Spain, Navas-Cortes *et al.* (1995) observed that *D. rabiei* grew saprophytically on infected chickpea tissues lying on the soil surface and remained alive for at least 2 years but lost viability within 2-5 months if the infested debris was buried in the soil. Weltzien and Kaack (1984) also found that infested plant debris is an important soil-borne form of inoculum. However, in Syria it has been reported that the pathogen survived for only 8 months (ICARDA, 1993).

The survival of *A. rabiei* in crop debris in hot dry Australian summers has not been studied. There are no reports in the literature of survival of *P. medicaginis* in chickpea crop debris.

2.5.2 Dissemination of inoculum

2.5.2.1 Conidia

Mature conidia of *A. rabiei* ooze from pycnidia in a gelatinous matrix under wet conditions. The matrix dissolves to release the spores, which are then washed or splashed to other plants or scattered in droplets of driving rain. In dry conditions, the extruded conidia dry as hard masses on the infected tissues and, subsequently, are splash-dispersed, over short distances only (Kaiser, 1992). Zachos *et al.* (1963) had previously reported that the disease developed in circles by means of rain-splashed conidia but wind-driven rain spread the disease in the direction of the wind. Also, Sattar (1933) and Luthra *et al.* (1935) found that infected tissues could be blown by wind for

hundreds of metres to act as a source of secondary infection when detached from infected plants by heavy rains and wind. Pycnidia of *P. medicaginis* are formed in lesions, produced on all above-ground parts of the plant, and conidia are dispersed primarily by wind and rain (Lamb and Poddar, 1987), but little else has been reported.

2.5.2.2 Ascospores

The teleomorph contributes to long distance dissemination of the pathogen. The ascospores are forcibly discharged into air through the opening of the pseudothecium in wet conditions (Kaiser, 1987). More than 70% of the ascospores were discharged from mature pseudothecia on naturally infected chickpea debris during a 2-h wet period at 15-25°C (Trapero-Casas and Kaiser, 1987). It has been reported that airborne ascospores served as primary inoculum to establish new infections in chickpea fields that were located 10-15 km from the nearest infected fields (Trapero-Casas and Kaiser, 1992).

2.5.3 Spore germination and infection

2.5.3.1 *A. rabiei*

2.5.3.1.1 Effect of interaction of temperature and light on A. rabiei

Sattar (1933) showed that when chickpea seeds were smeared with conidia of *A. rabiei* and then incubated in batches at 25°C, 30°C and 35°C for 5 months, 50% of conidia germinated at 25°C and 30°C while only 5% germinated after incubation at 35°C. In comparison, Kaiser (1973) demonstrated that optimum production of pycnidia on dried chickpea stem pieces occurred over the range from 10-30°C, with the optimum temperature of 20°C. Pycnidia matured in 46 h in continuous light, 50 h in alternating light and dark, and 68 h in continuous darkness at 20°C.

2.5.3.2 Effect of temperature and leaf wetness on ascochyta blight

Khachatryan (1962) found that RH of over 60%, with 350-400 mm rainfall during the fallow season and an average daily temperature ≥ 15°C, were the ideal conditions for disease development in Armenia. Chauhan and Sinha (1973), in a glasshouse study, demonstrated that 85-98% RH and 20°C for at least 46 hours were optimum for disease development, and there was a 6 day incubation period. It has been demonstrated that a minimum of 6 h wetness at 9-27°C is required for disease development in chickpea, but more than 10 h of wetness at these temperatures is required for severe disease, moreover, there was no infection of chickpea plants below 6°C nor above 30°C (Weltzien and Kaack, 1984).

Similarly, Trapero-Casas and Kaiser (1992) reported that the optimum temperature for infection was 20°C and severe disease development required a leaf wetness period of 17 h.

Relatively little information is available on the effects of temperature and leaf wetness period on disease development, so further research is needed to understand these aspects of epidemiology under Australian conditions.

2.5.3.3 Effect of plant age on ascochyta blight

Sattar (1933) studied the effect of plant age on disease development and found the plant to be more susceptible at pod formation than at the seedling stage. The period of greatest susceptibility coincided with maximum secretion of malic acid from glandular hairs on the leaf, which was assumed to favour the pathogen. These results were also confirmed by Reddy and Singh (1984) and, later, by Singh and Reddy (1993), but contradicted by Hafiz (1952), who claimed that resistant cultivars secreted more malic acid than did

susceptible cultivars. However, Trapero-Casas and Kaiser (1992), using controlled conditions, did not find any significant difference in disease severity between plants inoculated at 2 and 8 weeks old.

It is not clear whether the apparent greater susceptibility of chickpea plants to ascochyta blight at maturity is due to the age of the plant or favourable environmental conditions for disease development. Further research on the effect of plant age on disease development is needed to determine if it is a significant factor.

2.5.3.2 *P. medicaginis*

2.5.3.2.1 Effect of conidial matrix on P. medicaginis

The conidia of *P. medicaginis* are released from the pycnidium with an associated matrix (Chung and Wilcoxson, 1969). Renfro and Wilcoxson (1963) reported that the crowded conidia did not germinate due to a substance produced by the conidia themselves or the matrix in which the conidia were carried from the pycnidium. The conidial matrix contains substances that both inhibit and stimulate germination of conidia. These substances help in survival during stress conditions e.g. low humidity and high temperature (Louis and Cooke, 1985).

2.5.3.2.2 Effect of temperature on P. medicaginis

Spores of *P. medicaginis* germinate over a wide range of temperatures provided there is adequate moisture (Lamb and Poddar, 1987). The optimum temperature range for germination is 20 to 25°C, but most spores are able to germinate at 5 to 35°C (Jones and Vaughan, 1921; Sattar, 1934). In general, infection can occur at temperatures ranging from 5 to 27°C, and high humidity is necessary for germination of the spores (Brewer, 1960; Mead, 1963; Barbetti, 1991).

2.6 Host range

2.6.1 A. rabiei

Numerous studies have reported that chickpea is the only host of *A. rabiei* (Gorlenko and Bushkova, 1958; Khachatryan 1962; Nene, 1980; Sprague, 1930; Tripathi *et al.*, 1987). Sprague (1930) failed to infect several plant species, including lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.) and French bean (*Phaseolus vulgaris* L.), and Zachos *et al.* (1963) failed to infect lentil, pea and vetch with *A. rabiei* during their host range studies. Similarly, Tripathi *et al.* (1987) inoculated 40 species of crop plants and weeds with an isolate of *A. rabiei* from Pantnagar, India but only chickpea was infected. However, some Syrian isolates were able to infect cowpea and common bean in greenhouse inoculation studies at ICARDA, in Syria, and produced only small, restricted, necrotic lesions without pycnidia (Nene and Reddy 1987). Similarly, Kaiser (1973) reported that an Iranian isolate of the fungus could infect cowpea and common bean, giving rise to small, reddish brown spots on the stems, petioles and leaves of common bean, but the lesions did not increase in size. However, in a subsequent experiment, alfalfa (*Medicago sativa* L.) and white sweet clover (*Melilotus alba* L.) were infected by an isolate of *A. rabiei* obtained from Idaho, USA, and pycnidia developed in necrotic tissues of both species (Kaiser, 1991).

The above-mentioned literature has revealed that, in terms of host range, there is some variation among isolates from different countries. Therefore, it is important to test Australian isolates of *A. rabiei* on possible alternative hosts in Australian conditions. These studies would be helpful to understand the epidemiology of ascochyta blight.

2.6.2 Phoma blight

Phoma medicaginis and *P. medicaginis* var *pinodella* are reported to infect *Brassica oleracea, Cicer arietinum, Glycine soja, Lathyrus odoratus, Medicago sativa, Solanum tuberosum, Lolium perenne, Phaseolus vulgaris* and *Curcuma longa* (Sutton, 1980; Iqbal and Kang, 1989; Simay, 1989; Bisht and Nath, 1991; Dhyani *et al.*, 1989).

2.7 Host-pathogen interactions

There are reports in the literature that spores of *Phoma medicaginis, P. medicaginis* var. *pinodella, Mycosphaerella pinodes, Ascochyta pisi, Ascochyta rabiei* and *Botrytis cinerea* produce germ tubes which are able to penetrate the cuticle directly or through stomata (Ludwing, 1928; Brewer and MacNeil, 1953; Blackeman, 1969; Punithalingam and Holliday, 1972a, b; Rijkenberg *et al.*, 1980; Höhl *et al.*, 1990; Angelini *et al.*, 1993; Dey and Singh, 1994). Pandy *et al.* (1987) studied, by light microscopy, the process of infection and histological changes in susceptible genotypes of chickpea infected by *A. rabiei*. Germ tubes from conidia penetrated the stem tissues at the juncture of two epidermal cells and form subepidermal aggregates until the fourth day after inoculation. On the sixth day, yellowing and necrosis of host tissues coincided with formation of mature pycnidia. There was extensive damage to cell walls of parenchymatous cortical and pith tissues in advance of invading hyphae, indicating involvement of cell wall degrading enzymes. Information on the invasion of chickpea tissues by *P. medicaginis*, and associated histological changes, is lacking.

2.8 Phytotoxins

Production of phytotoxins has been implicated in disease and symptom expression in plants infected by a number of *Phoma* and *Ascochyta* pathogens, including *P. medicaginis, P. herbarum, A. fabae, A. pisi, A. rabiei* and *A. imperfecta* (Oku and

Nakanishi, 1963; Stoessl, 1981). Solanapyrones A, B and C have been identified in the culture filtrates and spore germination fluids of *A. rabiei* (Alam *et al.*, 1989; Hohl *et al.*, 1991; Kaur, 1995). The same phytotoxins were also found previously in culture filtrates of *Alternaria solani,* which causes early blight of potato and tomato (Matern *et al.*, 1978; Ichihara *et al.*, 1983). It has been observed that isolates of *A. rabiei* differed in their ability to synthesize the solanapyrones A, B and C and production was influenced by the age of the culture and composition of the culture medium (Alam *et al.*, 1989; Chen and Strange, 1991; Hohl *et al.*, 1991; Strange and Alam, 1992; Latif *et al.*, 1993; Kaur, 1995; Porta-Puglia *et al.*, 1997). Previous studies have reported that isolates of *A. rabiei* from different countries differ in the production of solanapyrones A, B and C. Therefore, it is important to investigate toxigenicity and solanapyrone production in Australian *A. rabiei* isolates.

2.9 Control measures

2.9.1 Cultural practices

Sattar (1933) stressed the need for improved sanitation practices to reduce the risk of ascochyta blight disease, by removal and destruction of the diseased crop debris, rotation of the crop with non-host crops and the adoption of deep sowing which prevents the emergence of infected seedlings. In addition, Luthra *et al.* (1935) found that inter-cropping chickpea with non-hosts plants such as wheat, barley and mustard reduced the spread of the disease. Crop rotation was found to be very effective in reducing the level of primary inoculum, as *A. rabiei* has a very narrow host range (Nene, 1982). There was a negative correlation between inter-row spacing and disease development (Reddy and Singh 1980). Lukashevich (1958), during a study in the Ukraine, found that adopting late and deep sowing (3 cm) practices and the application of potassium fertiliser (45 kg/ha) before sowing reduced the severity of ascochyta blight.

Similarly, sanitation has been recommended for the control of phoma blight (Lamb and Poddar, 1987).

2.9.2 Chemical control

2.9.2.1 Seed treatment

Treating infected seed is recommended to reduce this primary source of inoculum (Grewal, 1982). Seed dressing applied to tolerant or resistant cultivars would help to reduce the level of inoculum and subsequently disease development when climatic conditions favour disease development.

Many seed dressing fungicides have been reported to control seed-borne infection of chickpea by *A. rabiei.* Benlate, Daconil, Topsin-M, Tecto-60, Thiabendazole and Calixin were very effective in inhibiting growth of *A. rabiei* (Ilyas and Bashir, 1983; Hussain and Malik, 1989). Calixin, alone or in combination with Benlate, was reported to be effective for the eradication of seed-borne infection (Reddy, 1980; Bhatti *et al.*, 1984). Thiabendazole has been found to be more effective and safer than Calixin when applied at a rate of 3g/kg seed, with no adverse effect on germination (Reddy and Kabbabeh, 1984).

Kaiser and Muehlbauer (1988) reported that treating seed with effective fungicides would help to reduce the disease economically and allow the free movement of seed internationally without any fear of introducing the disease into new areas. Screening of systemic fungicides registered in Australia for seed treatment should, therefore, be undertaken.

2.9.2.2 Foliar treatment

The foliar application of fungicides, including Bordeaux mixture (Kovachevski, 1936), wettable sulphur (Lukashevich, 1958), zineb (Solel and Kostrinski, 1964), ferbam (Puerta Romero, 1964), maneb (Retig and Tobolsky, 1967), captan (Vir and Grewal, 1974), Daconil (Nycirek *et al.*, 1977), chlorothalonil (Bashir *et al.*, 1987) carbendazim and thiabendazole (Kader *et al.*, 1990) prochloraz and mancozeb (Morjane *et al.*, 1993), has been reported to minimise disease severity. However, foliar sprays are generally ineffective and uneconomical in epiphytotic conditions as a minimum of four to six sprays is required to control the disease in a susceptible genotype (Nene, 1982; Reddy and Singh, 1983; Nene and Sheila, 1992). When the disease appears in an epidemic form it is very difficult to meet the application schedule and it is clear that the available foliar fungicides have very limited scope for chickpea industry.

2.9.3 Breeding for resistance

2.9.3.1 Races of *A. rabiei*

The widespread damage in recent years to chickpea genotypes released as resistant/tolerant to ascochyta blight suggests the occurrence of different races of fungus. However, the first indication of races in *A. rabiei* was when the resistant Indian chickpea cultivar, C 12/34, became susceptible in 1963 (Nene and Reddy, 1987). Bedi and Aujala (1969) reported that several races occurred in the Indian state of Punjab. Vir and Grewal (1974) identified races 1 and 2 and one biotype of race-2 in India by using five differential cultivars, and this was later confirmed using three differential cultivars (Grewal, 1981). Qureshi and Alam (1984) found five races of *A. rabiei* in Pakistan, while Reddy *et al.* (1984) reported that some resistant chickpea genotypes developed in Syria were susceptible in Pakistan, suggesting the existence of different races of *A. rabiei* in Pakistan and Syria. However, Luthra *et al.* (1938), Arif and Jabbar (1965),

Anonymous (1987) and Gowen (1983), found no evidence for the existence of races, although they did find great variability in the aggressiveness of the isolates tested and also variation in the size of pycnidia, colony growth rate and sporulation *in vitro*.

Research in Italy, involving 50 isolates from different locations, showed significant variability, suggesting race specialisation. Six pathogenic groups or races were identified by inoculation of a set of six differential cultivars. All the differentials in Syria, Lebanon and Italy were susceptible to race 6, suggesting that there is a common race in these three countries (Crinò *et al.*, 1985; Porta-Puglia *et al.*, 1985, 1986, 1987; Porta-Puglia, 1990).

The pathogen appears to be quite variable, but the basis for this is unknown. It is important to determine its variability under Australian conditions.

2.9.3.2 Screening techniques

Numerous techniques have been adopted by various researchers to evaluate chickpea germplasm for response to *A. rabiei*. Satisfactory evaluation of material was achieved by the introduction of chopped, infected chickpea debris into the field (Labrousse, 1931b; Luthra *et al.*, 1938; Sattar and Hafiz, 1951; Vedysheva, 1966a). However, sowing a susceptible line after every two to four test lines and then inoculating the susceptible line proved to be an efficient technique for the large scale evaluation of breeding material (Lal and Amin, 1992).

Similarly, in the greenhouse, methods for inoculating material grown in pots or trays have been standardised but some researchers inoculate plants at the seedling and others at the podding stage. There is a need to further standardise the inoculation technique to enable research workers to conduct internationally comparable screening experiments.

2.9.3.3 Disease rating scales

Many rating scales have been used by various researchers for assessing chickpea material in glasshouse and field conditions, but there is no universally accepted disease assessment scale. Rating scales with their specifications (field and glasshouse) are summarised as follows:

Vir and Grewal (1974) used a 5-point scale based on severity of stem and foliar infection, for glasshouse and field screening, whereas Morrall and McKenzie (1974) used a 6-point scale based mainly on foliar infection, for field screening. Reddy and Nene (1979) suggested a 9-point scale for glasshouse screening. Later, Singh *et al.* (1981) suggested a scale with five defined categories of severity, for the evaluation of large-scale breeding programs in field conditions. Subsequently, Reddy and Singh (1984), Reddy *et al.* (1984) and Gowen *et al.* (1989) modified previous screening systems and developed 9-class scale for glasshouse and field screening and these scales have been accepted by the majority of researchers.

Undoubtedly, the above schemes have provided valuable information, but one standard international rating scale should be adopted to help to achieve uniform results wherever chickpea is grown.

2.9.3.4 Sources of resistance

Sources of resistance to ascochyta blight have been reported in the literature (Table 2.1). Chickpea breeding programs at ICRISAT and ICARDA have screened huge germplasm collections (Table 2.1) and have supplied resistant lines to national programs through the Chickpea International Ascochyta Blight Nursery (CIABN) program for further screening against local isolates of *A. rabiei* in chickpea growing-countries. In general, it has been observed that the frequency of resistance is higher in the kabuli genotypes than

in the desi types, based on studies of the inheritance of resistance to ascochyta blight (Tewari and Pandey, 1986; Verma *et al.*, 1987; Singh *et al.*, 1992). Singh *et al.* (1992 identified 12 kabuli and 3 desi types as resistant from a total of 15,310 accessions, among 5107 kabuli and 10,203 desi types during screening of the world germplasm collection in ICRISAT and ICARDA.

Singh *et al.* (1984) evaluated 112 CIABN chickpea lines in Algeria, Greece, India, Jordan, Morocco, Pakistan, Spain, Syria, Tunisia and Turkey. Four lines, ILC 72, ILC 191, ILC 3279 and ILC 3856, were found to be resistant to ascochyta blight in eight of these 11 countries. In addition, Reddy and Singh (1985) reported that chickpea lines ILC 191, ILC 194, ILC 200, ILC 202, ILC 2548, ILC 2956, ILC 3279, ILC 340, ICC 3996, ICC 4107 and ICC 3375 were resistant to four isolates of *A. rabiei* in a multi-location trial in Syria and Lebanon.

2.10 Conclusion

While there is considerable information on the epidemiology of ascochyta blight of chickpea in other countries, significant gaps in our knowledge include the host range of *A. rabiei*, the importance of infected seed in the disease cycle, the world-wide distribution of the teleomorph. Furthermore, the lack of uniform rating scale for the identification of sources of resistance on an international scale, has hampered chickpea breeding efforts. Prior to 1995, very little was known about ascochyta blight in Australian conditions. When the disease was identified in commercial crops in this country, there was a need to understand aspects of the epidemiology of ascochyta blight in Australia.

Table. 2.1: Studies undertaken between 1931 and 1995 to identify sources of resistance in *Cicer arietinum* L. to *Ascochyta rabiei*

Researcher	Year	Country[a]	No. of lines screened	No. of resistant lines
Labrousse	1931a	Morocco and/or France	36	3
Labrousse	1931b	Morocco and/or France	167	11
Pavlova	1935	USSR	-	6
Luthra *et al.*	1938	Indian Punjab	187	4
Luthra *et al.*	1941	Indian Punjab	392	3
Luthra *et al.*	1943	Indian Punjab	-	1
Padwick	1948	-	-	2
Ahmed *et al.*	1949	Pakistan	-	2
Hafiz	1952	Pakistan	-	3
Enken	1954	USSR	-	2
Aziz and Kainth	1960	Pakistan	700	1
Bushkova	1960	USSR	273	1
Bedi and Athwal	1962	India	-	1
Puerto-Romero	1964	Spain	-	4
Solel and Kostrinski	1964	-	-	1
Vedysheva	1965	USSR	184	4
Vedysheva	1966a	USSR	352	17
Vedysheva	1966b	USSR	58	2
Aujla and Bedi	1967	India	189	11
Scharif *et al.*	1967	Iran	-	2
Kojnov and Redkin	1970	Bulgaria	-	2
Redkov	1970	Bulgaria	-	1.5%
Kaiser	1972	Iran	-	1
Sandhu	1972	India	600	11
Sohoo and Singh	1972	India	-	1
Vedysheva	1972	USSR	584	17
Zhelokov	1973	USSR	-	2
Golubev	1974	USSR	500	4
Grewal and Vir	1974	India	-	2
Khico	1974	USSR	200	4
Ramanujam	1974	India	-	1
Korsakov	1975	USSR	-	5

Table 2.1 continued

Researcher	Year	Country[a]	No. of lines screened	No. of resistant lines
Eser	1976	Turkey	-	1
ICRISAT	1976	India	-	7
ICRISAT	1977	India	1200	40
ICRISAT	1978	India	2000	5
ICRISAT	1982	India	-	60
Iqbal *et al.*	1989	Pakistan	759	1
Iqbal *et al.*	1994	Pakistan	467	7
Redkov	1976	Bulgaria	50	3
Geneva and Matsov	1977	Bulgaria	220	48
Religh and Lehrer	1977	-	-	2
Singh	1978	India	262	4
Okhovat	1979	Iran	729	1
Bejiga	1980	Ethiopia	1086	2
Haq *et al.*	1981	Pakistan	208	2
Singh *et al.*	1981	Syria	9385	57
Pandey *et al.*	1982	India	76	2
Gaur *et al.*	1983	India	47	25
Jalali *et al.*	1983	India	150	7
Okhovat	1983	Turkey	5000	36
Singh *et al.*	1984	Syria	6005 Desi	655
Reddy *et al.*	1984	Syria	9574 Desi	6
Malik	1986	Pakistan	4000	34
Verma *et al.*	1987	India	1258 Desi	12
Verma *et al.*	1987	India	174 Kabuli	1
Singh *et al.*	1992	Syria	5107 Kabuli	12
Singh *et al.*	1992	Syria	10203 Desi	3
Singh and Reddy	1993	Syria	19000	5

[a]Country in which screening tests were done;
- unknown

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Chickpea material

Seeds of chickpea cultivars/lines used in these studies were obtained from Primary Industries and Resources, South Australia; the Australian Temperate Field Crops Collection, Agriculture Victoria, Horsham; New South Wales Agriculture; and the Pakistan Agricultural Research Council (PARC), Islamabad, Pakistan. Seed from Pakistan was imported in accordance with AQIS permit No. 008640 and then multiplied in a quarantined glasshouse at Waite Campus. All plant material used in this study are listed in appendix 1, with type (desi or kabuli) and country of origin noted.

3.2 Maintenance of chickpea plants in the glasshouse

Seeds were surface sterilised with domestic bleach solution (0.1% available chlorine) for 2 minutes and sown in 10-cm diameter plastic pots containing pasteurised University of California (UC) potting mixture (Baker, 1957). No additional nutrients were added. Pots were watered from the top prior to inoculation and watered from the base following inoculation. Each pot contained three chickpea seedlings unless stated otherwise. Except where mentioned, plants were grown in a greenhouse at $20 \pm 2^{\circ}C$ in natural light for 14 days before inoculation.

3.3 Fungal isolates

3.3.1 Sources of isolates

Fungi were isolated from infected chickpea material collected from commercial crops during disease surveys in South Australia and Victoria, and samples of diseased plant material were provided by plant pathologists and agronomists in New South Wales and Queensland. The last two digits of the isolate code represent the year in which they were collected.

3.3.2 Isolation and identification of fungi

Diseased stem tissues were cut into 2-3-mm^2 pieces, surface sterilised with domestic bleach solution (0.1% available chlorine) for 1-2 minutes and washed three times with sterile distilled water (SDW). The stem pieces were plated on 1/4 strength potato dextrose agar (1/4 PDA; Oxoid) and incubated for 7 days at room temperature (approx. 23°C) under alternating 12h near-UV light (Philips TLD 18W/08) and 12h darkness. Pycnidial fungi were tentatively identified according to the keys of Sutton (1980) and Punithalingam and Holliday (1972).

3.3.3 Storage of fungi

Single spore derived-cultures of selected isolates were established on 1/4 PDA, and incubated as above. Plugs (5 mm diam.) were cut with a sterile cork borer from the edges of actively growing colonies and placed in 1mL SDW in Nunc Cryo Tube™ vials (Nalge Nunc International, Denmark), five plugs per vial. These vials were stored at 4°C in the dark. When required, isolates were grown on about 20 mL 1/4 PDA in 90 mm diameter plastic Petri dishes (Disposable Products, South Australia) as described in section 3.3.2.

3.4 Preparation of inoculum

To prepare inoculum, each isolate was subcultured onto PDA plates and incubated for 21 days as described in section 3.3.2. The cultures were then flooded with SDW for 30 minutes, then the surface of the culture was gently rubbed with a sterile glass rod to dislodge the conidia. The resulting spore suspension was filtered through four layers of sterile cheese cloth and adjusted to the desired concentration using a haemocytometer. Tween-20 was added to give a concentration of 0.25% v/v.

3.5 Foliar inoculation

Except where mentioned, 15 days after sowing, plants were sprayed to run-off with spore suspension (5 x 10^5 conidia per mL). Control plants were treated with SDW. Control and inoculated plants were kept in different incubation cabinets in the glasshouse at 20 ± 2°C and misted for 1 minute at 7 am, 10 am, 1 pm, 4 pm, 7 pm and 10 pm for 72 h to provide relative humidity close to saturation. The incubation cabinet consisted of a clear polyethylene tent with two misters at the top. Misters were connected to Nylex™ garden hoses. After incubation, plants were removed from the cabinets, kept in the glasshouse as described in section 3.2 and watered on alternate days. There were four replicate pots of three seedlings per treatment, and disease on vegetative parts was assessed 14 days after inoculation unless stated otherwise. The three seedlings in each pot were assessed as one unit.

3.6 Assessment of disease severity

Except where stated otherwise, the severity of ascochyta blight was assessed using the rating scale of Gowen *et al.* (1989). In addition, categories were designated as highly resistant to highly susceptible.

% infection	Symptoms	Designation
0-10	No infection - small lesions	Highly resistant
11-20	Some stem lesions - minor stem breakage in upper foliage	Highly resistant
21-30	1-2 branches broken - several girdling stem lesions low down on some branches	Resistant
31-40	Large basal stem lesions or several branches broken near to main stem	Moderately resistant
41-50	Half foliage dead or partly severed	Moderately resistant/ Moderately susceptible
51-60	> Half foliage dead or dying, young shoots still actively growing from base	Moderately susceptible
61-70	Most foliage dead - some healthy stem tissue with lateral buds	Susceptible
71-80	Most foliage dead, no healthy lateral buds in leaf axils	Susceptible
81-99	Most foliage dead, decreasing areas of living stem tissue	Highly susceptible
100	Plants completely dead.	Highly susceptible

The scale was modified into a 1-10 point scale to facilitate the rating of field trials, as follows:

% infection (Gowen *et al.*, 1989)	Equivalent 1-10 point scale	Designation
0-10	1 - <2	Highly resistant
11-20	2 - <3	Highly resistant
21-30	3 - <4	Resistant
31-40	4 - <5	Moderately resistant
41-50	5 - <6	Moderately resistant/ Moderately susceptible
51-60	6 - <7	Moderately susceptible
61-70	7 - <8	Susceptible
71-80	8 - <9	Susceptible
81-99	9 - <10	Highly susceptible
100	10	Highly susceptible

3.7 Data analysis

Data obtained from experiments were subjected to analysis of variance (ANOVA) using the SuperANOVA computer program unless stated otherwise. Where data were subjected to an analysis of variance and when F values indicated significant differences, mean separation was based on the least significant difference at a 5 % level of probability (P=0.05) (SuperANOVA, 1984)

CHAPTER 4

ETIOLOGY OF CHICKPEA BLIGHT

4.1 INTRODUCTION

The chickpea (*Cicer arietinum* L.) ranks among the world's three most important food legumes with an annual production of about 9 Mt from about 11 Mha (FAO, 1996). The major production regions lie in the Indian subcontinent, western Asia, North America, southern Europe and, more recently, Australia, and their contribution to production is 82%, 6%, 4%, 7%, and 1%, respectively (Knight, 1991).

Chickpea production, especially in the Indian subcontinent, may be reduced by disease. Many fungal and viral diseases have been reported on chickpea, but only a few, such as ascochyta blight, caused by *Ascochyta rabiei*, and phoma blight, caused by *Phoma medicaginis*, have the ability to cause considerable damage to chickpea crops (Alam *et al.*, 1989; Haware and Nene, 1981; Kaiser and Muehlbauer, 1988; Lamb and Poddar, 1987; Nene and Reddy, 1987)

The disease has been recorded in 35 countries (Nene *et al.*, 1996; Anonymous, 1986), however, the inclusion of Australia was subsequently revised as the record was not properly validated (Anonymous, 1991). Early records for Australia were based on reports from New South Wales (Cother, 1977) and Victoria (Bretag, 1982) but the isolates concerned (IMI 200962, 282936 and 282937) were subsequently determined as *Ascochyta pinodes* and *Phoma medicaginis* var. *pinodella* (J. Walker, New South Wales Agriculture and E. Punithalingam, IMI, 1990, personal communication; Chandrasheker and Culvenor, 1993). Ascochyta blight on chickpea occurred in evaluation trials in South Australia in 1973 (DAR 72373) and was eradicated (R. Knight and M. Carter,

personal communication 1996). The disease had not been observed since and, prior to the present study, had never been recorded in commercial crops in Australia. Therefore, quarantine restrictions were maintained to prevent the introduction of *A. rabiei* on chickpea seed.

Even though phoma blight has been recorded sporadically on chickpea in Australia since the 1980s, little research has been done on this disease, especially with regard to inoculation techniques. Therefore, the studies reported in this chapter were initiated with the following aims :

i) to develop a reliable method for inoculation of *P. medicaginis* on to chickpea under controlled conditions;
ii) to determine the etiology of the phoma-like blight of chickpea;
iii) to determine what plant parts were susceptible to the disease

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Four chickpea cultivars: two desi (Dooen and Tyson) and two kabuli (Garnet and Kaniva) types were used in the first three experiments to establish methods for inoculation. Desavic (desi) was used in the experiment to test pathogenicity. Seeds were provided by the Department of Primary Industry and Resources, South Australia. They were surface sterilised with domestic bleach solution (0.1% available chlorine) for 2 minutes.

4.2.2 Production of inoculum of *P. medicaginis*

Two cultures previously identified as *Phoma medicaginis*, 132/91 and 165/91, isolated from chickpea were kindly provided by Mark Ramsey, South Australian Research and Development Institute (SARDI), Waite Campus. These isolates were randomly selected from his collection and had been stored in sterilised water. Single spore derived-cultures were established on 1/4 PDA and in potato dextrose broth (PDB, Difco), and incubated without shaking at room temperature (approx. 23°C) for 21 days under alternating 12h near-UV light (Philips TLD 18W/08) and 12h darkness.

4.2.2.1 Suspension of conidia and mycelium

A homogeneous mixture of conidia and mycelium was made by blending cultures aseptically in PDB in an electric blender for 30 seconds and adjusting the concentration to $5x10^5$ conidia per mL. Hyphal fragments were not quantified.

4.2.2.2 Suspension of conidia

Conidia were harvested from PDA cultures by adding SDW for 30 minutes and gently scraping the surface with a sterile glass rod. After sieving through four layers of sterilised muslin cloth, suspensions of $5x10^5$ conidia per mL were prepared and Tween-20 was added as described in section 3.4.

4.2.3 Inoculation of chickpea with *P. medicaginis*

4.2.3.1 Inoculation of chickpea plants

Chickpea seedlings were grown in the glasshouse as described in section 3.2. Fifteen days after sowing, plants were sprayed to run-off with a conidial suspension or with a suspension of conidia and mycelia. Control plants were treated with SDW. Control and

inoculated plants were kept covered with polyethylene bags for 72 h in the glasshouse at 20 ± 2°C. The bags were then removed and the plants watered from below on alternate days.

Disease on foliar parts was assessed 14 days after inoculation using a rating scale based on that of Reddy and Singh (1984) as follows: 1 = no infection; 2 = highly resistant (1-5% of plant blighted); 3 = resistant (6-10%); 4 = moderately resistant (11-15%); 5 = intermediate (16-40%); 6 = moderately susceptible (41-50%); 7 = susceptible (51-75%); 8 = highly susceptible (76-100%); 9 = plant killed. Disease symptoms on the collar region were scored by use of a modified version of Key No. 2.1.1 (James, 1971) as follows: 0 = no infection, 1= ≤ 1%, 2 = > 1-5%, 3 = > 6-25%, 4 = 26-75%, 5 = > 75% infection on the collar region.

4.2.3.2 Inoculation of chickpea seed

Surface-sterilised seeds (see section 4.2.1) were soaked in a conidial suspension ($5x10^5$ conidia per mL) for 5 minutes, while control seeds were soaked in SDW. After drying on sterile filter papers, seeds were sown into pasteurised UC potting mixture and maintained in the glasshouse, as described in section 3.2, for 21 days. Plants were then gently removed from the pots, the roots were washed and disease symptoms on the collar region were scored as described in section 4.2.3.1. Plant growth was measured by recording fresh and dry weights (g) of shoots and roots combined. Dry weights were determined after plant material had been oven-dried at 80°C for 24h. There were four replicate pots of three seedlings per treatment.

4.2.4 Isolation of pathogens from diseased chickpea

Plants with brown to black lesions on above-ground parts, especially at the stem base, and plants with withered stems were collected from mature commercial crops in the mid-North of South Australia in 1995. Fungi were isolated and identified as described in

section 3.3.2. Cultures were stored in SDW as described in section 3.3.3 and grown on 1/4 PDA as required.

4.2.5. Pathogenicity testing

Single spore-derived cultures of nine *Phoma*-like isolates, four of which were isolated in this study and five isolated in 1991 (listed in Fig. 4.4) were grown on 1/4 PDA for 21 days. Conidial suspensions were prepared as described in section 3.4.

Seedlings of cv. Desavic were grown in the glasshouse as described in section 3.2. Seedlings were inoculated and disease was assessed as described in section 4.2.3.1. There were four replicate pots of three seedlings per treatment

4.2.6 Identification of the pathogens

The morphological characteristics of all the *Phoma*-like isolates used in pathogenicity tests were determined in South Australia. The identity of the two most aggressive isolates, 392/95 and 435/95 was determined using morphological criteria. These two isolates were then subjected to analysis of mating type, pathogenicity and isozymes by A. Porta-Puglia and A. Infantino, Istituto Sperimentale per la Patologia Vegetale (ISPAVE), Rome, Italy and of randomly amplified polymorphic DNA (RAPD) by R. Corbiere and Z. Bouznad, Institut National Agronomique (INRA), Angers, France. Later, identifications, were confirmed by M. Priest and the isolates were accessioned as DAR 71767 and DAR 71768 at the Agricultural Scientific Collections Unit, New South Wales Agriculture, Australia. Subsequently, these and additional isolates selected from those used in pathogenicity tests in Australia were sent to W. J. Kaiser, USA, to confirm their identity using mating type analysis (Kaiser, 1997).

4.2.7 Seed testing

Due to the serious threat that ascochyta blight poses to the Australian chickpea industry, the Grains Research and Development Corporation of Australia sponsored seed testing by SARDI personnel prior to sowing in 1996, to determine the prevalence of *A. rabiei* in seeds. Fifty-nine seed samples were received from growers throughout Australia. Most samples were harvested in 1995 and one had been stored since in 1992. Seeds, 400 per sample, were surface sterilised with domestic bleach solution (0.1% available chlorine) for 2 minutes, and 10 seeds were placed on approx. 20 mL PDA in each 9-cm Petri dish (International Seed Testing Association, 1993). They were placed in an incubator at 20°C with alternating cycles of 12 h light (6x30W fluorescent tubes) and 12 h darkness (Neergaard, 1977). The above procedure was conducted by C. Wilmshurst, SARDI.

As part of the present study, fungi growing from the seeds were identified after 7 days, according to descriptions by Sutton (1980), Punithalingam and Holliday (1972c), Haware *et al.* (1986) and by comparison with known cultures of *P. medicaginis* and *A. rabiei.*

4.3 RESULTS

4.3.1 Spraying foliage with suspensions of conidia and mycelium of *P. medicaginis*

The response of four chickpea cultivars, Dooen, Tyson, Garnet and Kaniva, to inoculation with *P. medicaginis* isolates 132/91 and 165/91 is presented in Fig 4.1. The control plants remained healthy. Inoculated seedlings developed light brown lesions with dark margins on all foliar parts. On the leaves the lesions were surrounded by a yellow halo. Irregular, dark, minute, submerged pycnidia developed in the infected area and older blighted leaves abscised.

The difference between the control and inoculated treatments was highly significant ($P<0.001$). There was no significant difference observed in the severity of disease on the foliar parts of the four cultivars, nor did the isolates differ in pathogenicity. In general, the disease severity on foliar parts was slightly greater in the kabuli types than in the desi types, and the interaction between cultivar types and isolates was significant ($P<0.05$).

Light brown lesions with dark margins developed on the collar regions and they were more elongated than were lesions on leaves. Isolate 165/91 caused more severe disease on the collar region than did isolate 132/91 ($p<0.001$). When the data for both isolates were combined Garnet was less severely diseased than were the other three cultivars ($p<0.05$). Disease on the collar region was more severe on the desi types than on the kabuli types tested ($p<0.001$).

4.3.2 Spraying foliage with conidial suspension of *P. medicaginis*

Foliar symptoms similar to those described in section 4.3.1 developed on all inoculated plants but not on the control plants (Fig. 4.2). Isolate 132/91 was more aggressive than 165/91 in terms of producing foliar symptoms on all four chickpea cultivars ($P<0.01$). In general, the kabuli types were more susceptible than the desi types and the interaction between isolate and cultivars was highly significant ($P<0.001$).

4.3.3 Inoculation of seed with *P. medicaginis* isolates

Control plants remained healthy. All inoculated plants developed lesions on the collar region (Fig. 4.3). The interaction between isolate and cultivar was significant ($P<0.05$). Isolate 165/91 caused more disease on Dooen than did isolate 132/91 ($P<0.05$),

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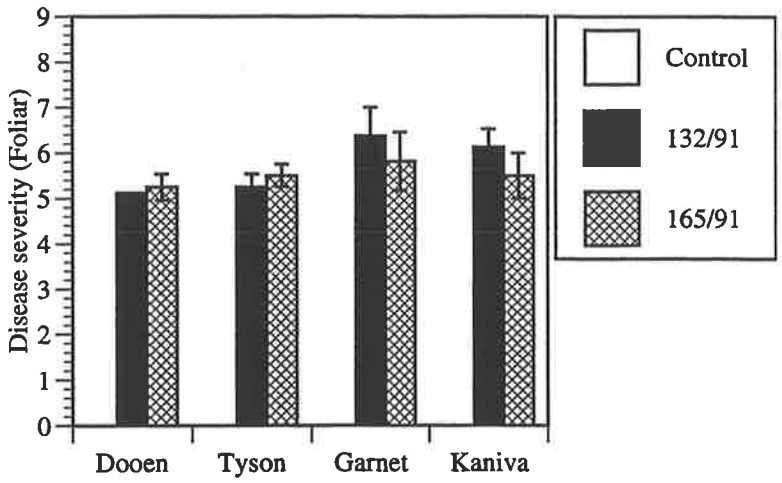
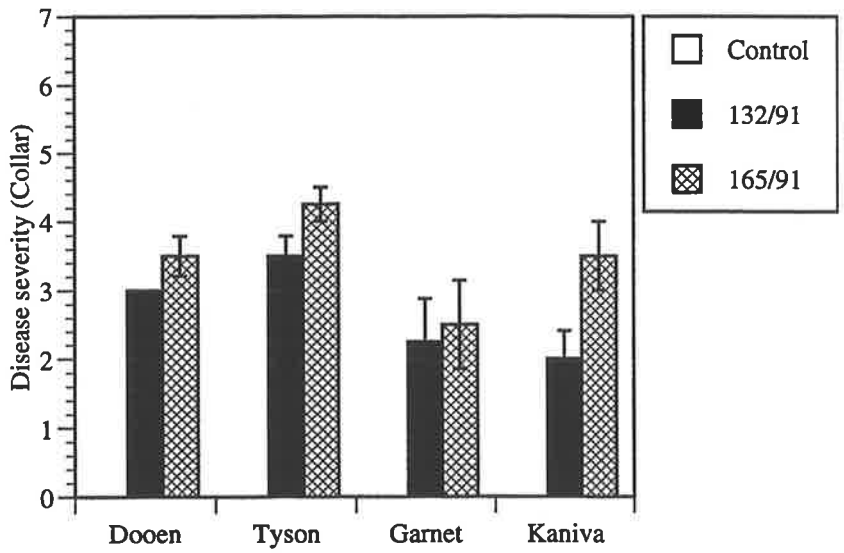
Figure 4.1 Effect of two isolates of *Phoma medicaginis* on foliar and collar disease severity of four chickpea cultivars inoculated by spraying with suspensions of mycelium and conidia. Severity of disease on seedlings was recorded 14 days after inoculation. A 1-9 scale based on Reddy and Singh (1984) was used to assess disease on foliar parts and the modified scale of James (1971) was used for the collar region (see section 4.2.3.1). There were four replicate pots each containing three seedlings per treatment; bars represent LSD at 5%. Dooen and Tyson are desi types; Garnet and Kaniva are kabuli types.

A)


Disease severity (Foliar)
0
1
2
3
4
5
6
7
8
9
Dooen
Tyson
Garnet
Kaniva
Control
132/91
165/91

B)


Disease severity (Collar)
0
1
2
3
4
5
6
7
Dooen
Tyson
Garnet
Kaniva
Control
132/91
165/91

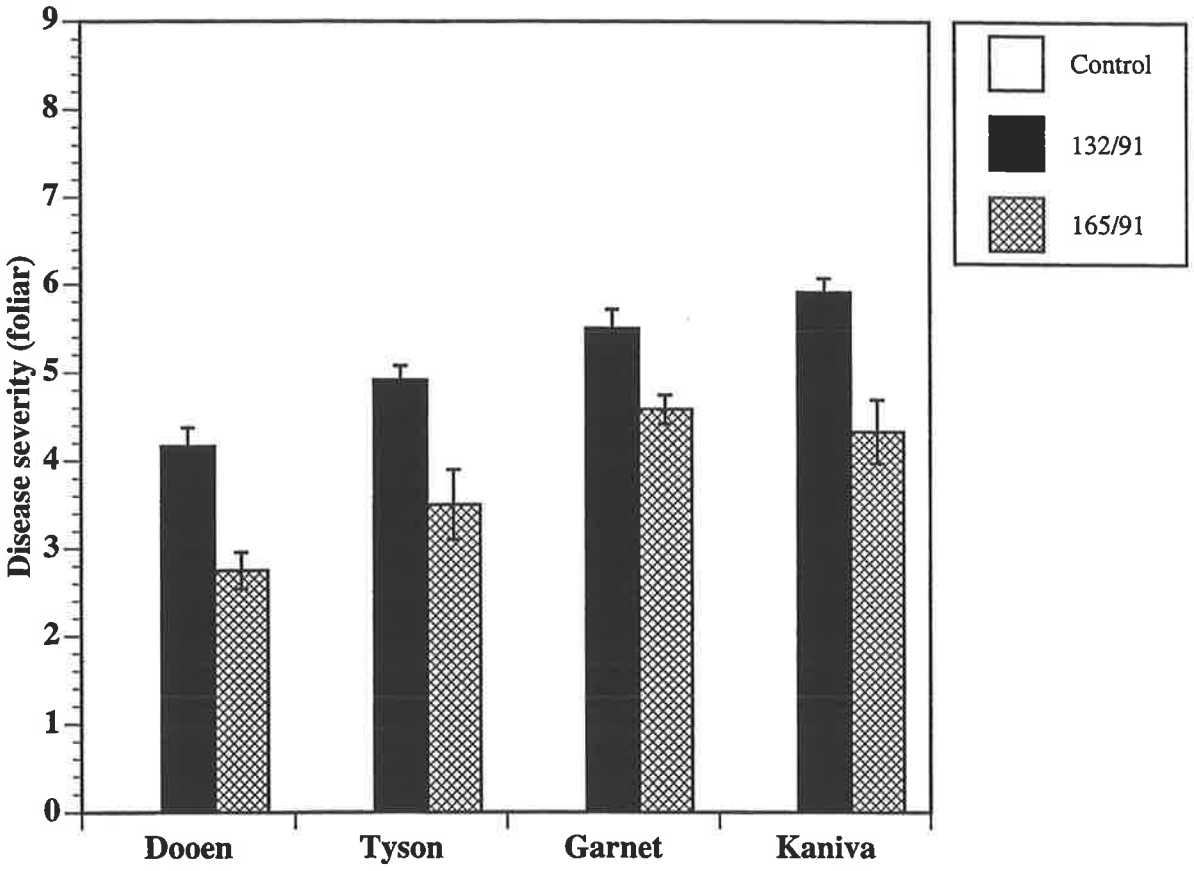


Figure 4.2 Disease reaction on seedlings of four chickpea cultivars: Dooen and Tyson (desi), Garnet and Kaniva (kabuli), 14 days after inoculation of seedling foliage with conidial suspensions of two isolates of *Phoma medicaginis*. Disease severity was assessed using a 1-9 scale based on Reddy and Singh (1984) (see section 4.2.3.1). There were four replicate pots each containing three seedlings per treatment; bars represent LSD at 5%.

however, the isolates did not differ in the severity of disease on Tyson and Garnet and the difference on Kaniva was slight (Figure 4.3 A).

Inoculation with *P. medicaginis* isolates 132/91 and 165/91 significantly reduced the fresh weight and dry weight of plants of all four chickpea cultivars compared to the uninoculated control and this difference was more obvious in the kabuli than in the desi types (Fig 4.3 B and C). In general, growth of kabuli types was greater than desi types and this was reflected in the fresh and dry weights of the control (Fig. 4.3 B and C), resulting in a highly significant interaction between cultivar and isolates ($P<0.001$).

4.3.4 Isolation of pathogens from diseased chickpea

Five samples were examined and the more than 85% of the pathogens isolated from diseased chickpea were pycnidial *Phoma/Ascochyta*-like fungi but other fungi were isolated sporadically, including *Stemphylium, Rhizoctonia, Alternaria* and *Fusarium* species.

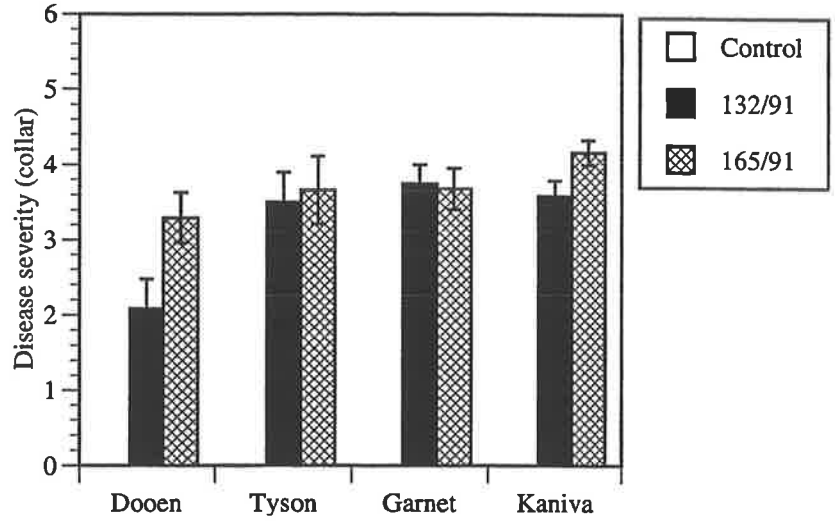
4.3.5 Pathogenicity testing

Three groups of isolates were identified based on relative disease severity on cv. Desavic (Fig. 4.4). Isolates 214/91, 218/91, 171/91, 389/95, 215/91 and 390/95 were weakly pathogenic, causing mean disease severity of 1-2, isolate 132/91 was intermediate and isolates DAR 71768 and DAR 71767 (formerly 435/95 and 392/95) were very pathogenic with mean disease severity of 6.5 and 8, respectively. Chickpea plants inoculated with isolates in the first two groups developed dark-brown to black lesions, without pycnidia, which were more severe at the base of the seedling. DAR 71767 and 71768 produced symptoms typical of ascochyta blight on all the above-ground parts. Initially, tan to brown-black lesions surrounded by a yellow halo developed on leaves which quickly became blighted and abscised. On stems and petioles, lesions were dark

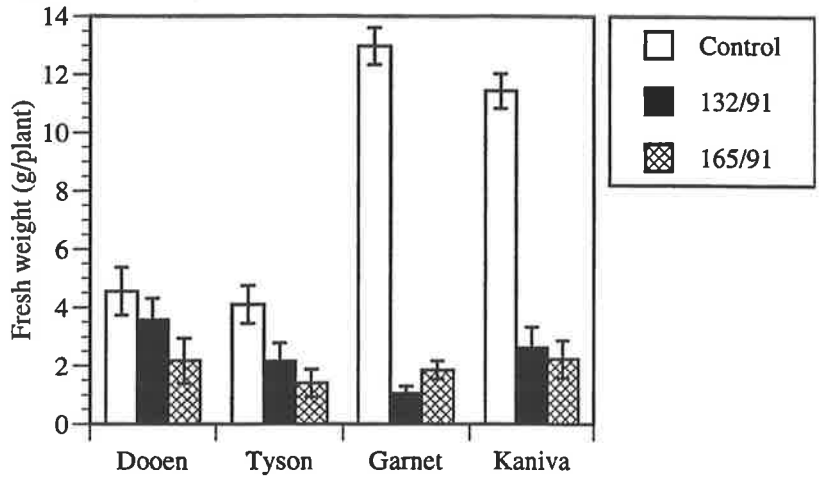
facing page

Figure 4.3 (A, B and C) Effect of two isolates of *Phoma medicaginis* on disease severity on the collar region, fresh weight and dry weight of chickpea cultivars inoculated by soaking seed in conidial suspension. Disease severity was assessed 21 days after inoculation using a 1-5 scale based on James (1971) (see section 4.2.3.1). There were four replicate pots each containing three seedlings per treatment; bars represent LSD at 5%. Dooen and Tyson are desi types; Garnet and Kaniva are kabuli types.

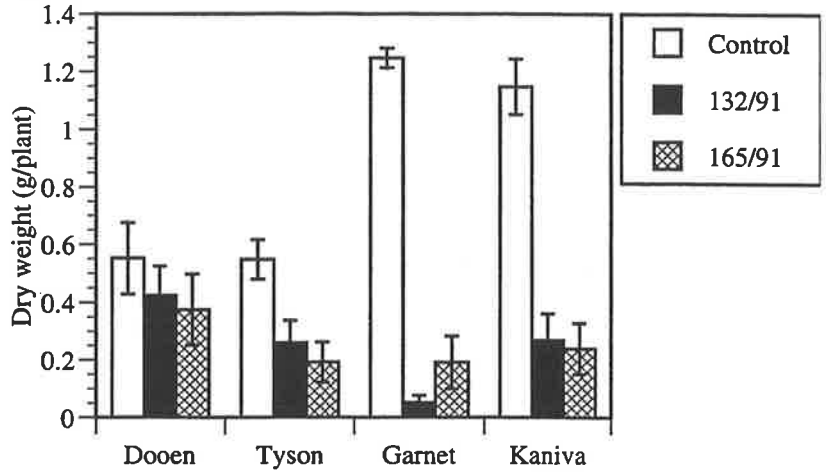
A)


Disease severity (collar)
0
1
2
3
4
5
6
Dooen
Tyson
Garnet
Kaniva
Control
132/91
165/91

B)


Fresh weight (g/plant)
0
2
4
6
8
10
12
14
Dooen
Tyson
Garnet
Kaniva
Control
132/91
165/91

C)


Dry weight (g/plant)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Dooen
Tyson
Garnet
Kaniva
Control
132/91
165/91

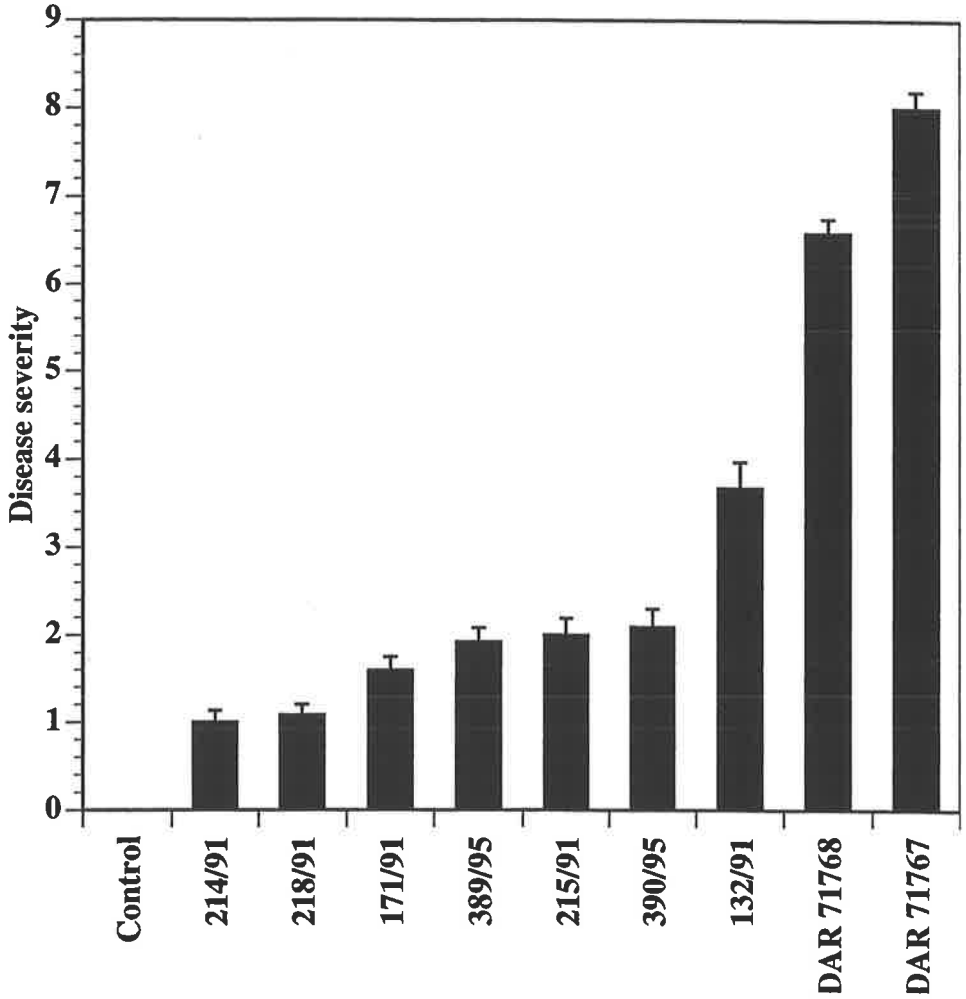


Figure 4.4 Severity of disease on seedlings of chickpea cv. Desavic 14 days after inoculation with *Ascochyta* and *Phoma*-like isolates obtained from chickpea in Australia. Disease severity was assessed using a 1-9 scale based on Reddy and Singh (1984) (see section 4.2.3.1.). There were four replicate pots each containing three seedlings per treatment; bars represent LSD at 5%.

brown to black, bearing pycnidia; stems and branches were completely girdled by the lesions and finally broke.

4.3.6 Identification of the pathogens

Colonies of DAR 71767 and DAR 71768 on 1/4 PDA were initially white, becoming dark brown to black and abundant pycnidia developed after 3-4 days. Conidial ooze was cream-pink to light tan. Conidia were straight to slightly bent at one or both ends, hyaline, occasionally 2-celled, round at both ends and 10.0-17.5 x 3.1-5.0 µm. Chlamydospore formation was not observed. These characteristics suggested that DAR 71767 and DAR 71768 were *A. rabiei*. Isolates 389/95 and 390/95 were identified as *P. medicaginis* based on septate hyphae, initially hyaline later becoming dark brown, hyaline, unicellular conidia and chlamydospores which were formed in the older cultures. Of the other isolates, 214/91, 218/91, 171/91 and 132/91 were subsequently identified as *P. medicaginis*, and 215/91 as *A. rabiei* by W.J. Kaiser based on morphological characteristics.

4.3.7 Seed testing

A. rabiei was identified in one sample of seed (0.25% of seed infected) which was harvested in South Australia in 1992, a growing season with wet conditions during pod formation.

4.4 DISCUSSION

As the disease of interest had earlier been identified as phoma blight, preliminary experiments were undertaken to confirm the pathogenicity of the *P. medicaginis* cultures isolated in 1991 and to evaluate methods for inoculation. Chickpea plants which were sprayed with homogenised liquid cultures comprising growth medium, mycelia and

conidia developed symptoms on both the foliar parts and collar regions. Similarly, plants sprayed with suspensions of conidia dislodged from cultures on agar developed symptoms on all the foliar parts and plants which were inoculated by soaking seed in conidial suspension developed disease on the collar region. Disease developed regardless of inoculation technique, however, conidial suspension can be quantified and is easy to prepare and apply so this method was used in subsequent experiments.

A variety of pathogens was isolated from the diseased chickpea plants collected from the mid-North area of South Australia in 1995, with *Ascochyta*- and *Phoma*-like isolates being predominant. Two *A. rabiei* isolates, DAR 71767 and DAR 71768, were very aggressive, with mean disease severity of 8 and 6.5, respectively. The symptoms caused by DAR 71767 and DAR 71768 were typical of ascochyta blight. This identification was confirmed by tests conducted by collaborators in Italy and France (Khan *et al.*, 1999) and subsequently reinforced by W.J. Kaiser. The weakly pathogenic isolates, with the exception of 215/91, were found to be *P. medicaginis*. Subsequently, 215/91 was identified as *A. rabiei* by W.J. Kaiser (personal communication, 1998). The intermediate isolate, 132/91, was identified as *P. medicaginis*.

Ascochyta blight was positively identified for the first time in commercial chickpea crops in Australia. The disease had previously been confused with, and identified as, phoma blight because the diagnostic symptoms of ascochyta blight, principally concentric rings of pycnidia on pods (Haware and Nene, 1981), had not been observed. Also, there was a history of confusion over the identity of the pathogen (Cother, 1977; Bretag, 1982). A contributing factor to this confusion is the fact that *P. medicaginis* var. *pinodella* and *M. pinodes* are both commonly found on chickpeas in Australia and cause black stem lesions, particularly at the stem base. Clear differentiation of *A. rabiei* and *P. medicaginis* var. *pinodella* is now possible using RAPD analysis (Bouznad *et al.*, 1996). Infantino *et al.* (1997) also showed that the pathogens could be differentiated using

isozymes. The banding pattern of Australian isolates of *A. rabiei* was identical to that of Italian isolates of *A. rabiei* for all enzyme systems screened.

The pathogen has been identified in a seed-lots from Australian crops and has been present in South Australian crops since at least 1992 (Khan *et al.*, 1999). In addition, the identification of isolate 215/91 as *A. rabiei* confirms that the pathogen was present in commercial crops in 1991.

Infection of seed adversely affects germination and leads to seedling infection (Maden *et al.* 1975; Tripathi *et al.* 1987; Dey and Singh, 1994), and results in local and international spread of the disease (Kaiser, 1984). Infected seed was responsible for the introduction of the pathogen into Iran in 1968 (Kaiser, 1972), Canada in 1974 (Morrall and McKenzie, 1974), and the United States from 1983 (Derie *et al.*, 1985; Kaiser and Muehlbauer, 1984; Guzman *et al.*, 1995). *A. rabiei* probably entered Australia in the same way.

It appears that *A. rabiei* is surviving at low levels in seed and/or in infected crop residues. The finding of this major pathogen of chickpea in commercial crops in Australia has important implications for the industry, its viability and its expansion. In view of the threat posed by *A. rabiei* to the Australian chickpea industry, it is important to understand the epidemiology of the disease in Australian conditions and to develop control measures.

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The studies reported in this chapter were initiated to identify possible sources of resistance in chickpea germplasm including major Australian cultivars, breeding lines from the National Chickpea Breeding Program; and breeding lines and cultivars imported from Pakistan. The experiments were conducted in glasshouse, "outdoor" and field conditions.

5.2 MATERIALS AND METHODS

5.2.1 Glasshouse trials

Chickpea genotypes were grown as described in section 3.2, with three seedlings per pot. Isolates DAR 71767 and DAR 71768 were subcultured onto PDA plates and incubated for 21 days as described in section 3.3.2, and spore suspensions were prepared as described in section 3.4. For the first experiment in the glasshouse, inoculum of the two isolates was prepared separately whereas for the subsequent experiments, conidial suspensions of the two isolates ($5x10^5$ conidia per mL) were combined 1:1 (vol:vol). Four glasshouse trials were conducted over the period February 1996 - May 1998, and cv. Desavic was included in each. Plants were inoculated as described in section 3.5, and controls were treated with SDW. A randomised block design (RBD) with four replicate pots per treatment was used in each experiment. Symptoms were assessed 21 days later using the scale of Gowen *et al.* (1989), as described in section 3.6.

5.2.2 Outdoor trial

Thirty chickpea genotypes were grown in the glasshouse as described in section 3.2, one seed per pot. Pots were placed in plastic trays placed outdoors, and four seedlings per genotype were inoculated with a mixed conidial suspension ($5x10^5$conidia per mL) as described in section 3.5. Controls, consisting of four seedlings per genotype, were

treated with SDW. Plants grew under natural light and rainfall with supplementary watering from sprinklers twice a day. Individual plants were assessed for symptoms 4 weeks after inoculation using the scale of Gowen *et al.* (1989).

5.2.3. Field trial

The trial was sown in the field on June 18, 1998 in a birdproof enclosure at Waite Campus, in single row plots (4 m long) using a randomised complete block design with four replications. Inter- and intra-row spacing were 25 and 10 cm respectively. About 40 seeds were sown per test line in a single 4-m row. A row of the blight-susceptible cv. Desavic was sown as a buffer for the disease between replications. There were 14 rows in each replication. A further 17 chickpea genotypes obtained from Agriculture Victoria, Horsham, were included but with one replication only due to shortage of seed. Desavic plants in pots, inoculated 4 weeks previously with mixed conidial suspension of DAR 71767 and DAR 71768 in the glasshouse (see sections 3.4 and 3.5), were placed in the buffer rows at 1 m intervals on 13 August 1998. Rainfall was supplemented by irrigation from sprinklers for 30 minutes per day during dry periods to increase relative humidity to > 60%.

All the cultivars and breeding lines were rated once at maturity on 19 and 20 October, 1998 when plants in the buffer rows were almost dead. Disease was assessed using the 1-10 point scale modified from that of Gowen *et al.* (1989), as described in section 3.6. One score was assigned to all of the plants in each row of each replicate of approx. 40 plants.

5.3 RESULTS

5.3.1 Glasshouse trials

In the first glasshouse trial, 11 chickpea cultivars and four breeding lines were inoculated with DAR 71767 and DAR 71768 separately (Fig 5.1). Uninoculated plants remained healthy. The ANOVA showed highly significant differences ($P<0.001$) among both chickpea genotypes and isolates. The interaction of genotypes and isolates was also highly significant ($P<0.001$). Dooen, Tyson, CTS 11308, Kaniva and Semsen, with mean disease scores of 12 to 42, were resistant to moderately resistant to both isolates, whereas Barwon, Opal and Garnet were moderately resistant to DAR 71767 and moderately susceptible to DAR 71768. Barwon, Desavic, Norwin, Narayen, CPI 56288, ICC 14307 and T 1587, with mean disease scores of 48 to 78, were moderately susceptible to highly susceptible.

In the second glasshouse trial, 27 genotypes, comprising 11 cultivars and 16 breeding lines, were inoculated with a mixture of conidia of DAR 71767 and DAR 71768 (Fig. 5.2). Uninoculated plants remained healthy. Differences among both chickpea genotypes and isolates were highly significant ($P<0.001$), as was the interaction between genotypes and isolates ($P<0.001$). Dooen, Tyson, and WAD O32 were moderately resistant, while the remainder of the cultivars were moderately susceptible to highly susceptible.

Seeds of 10 chickpea genotypes, obtained from Agriculture Western Australia, were sown for evaluation in the third glasshouse trial. Seed of genotypes ICCV 9207, ICCV 92219, ICCV 95911 and ICCV 96702 failed to germinate. The response of the remaining six lines and of the known susceptible genotypes Desavic and ICC 14307 to inoculation with a mixture of conidia of DAR 71767 and DAR 71768, is shown in Fig. 5.3. Uninoculated plants remained healthy. ICCV 96703 was moderately resistant,

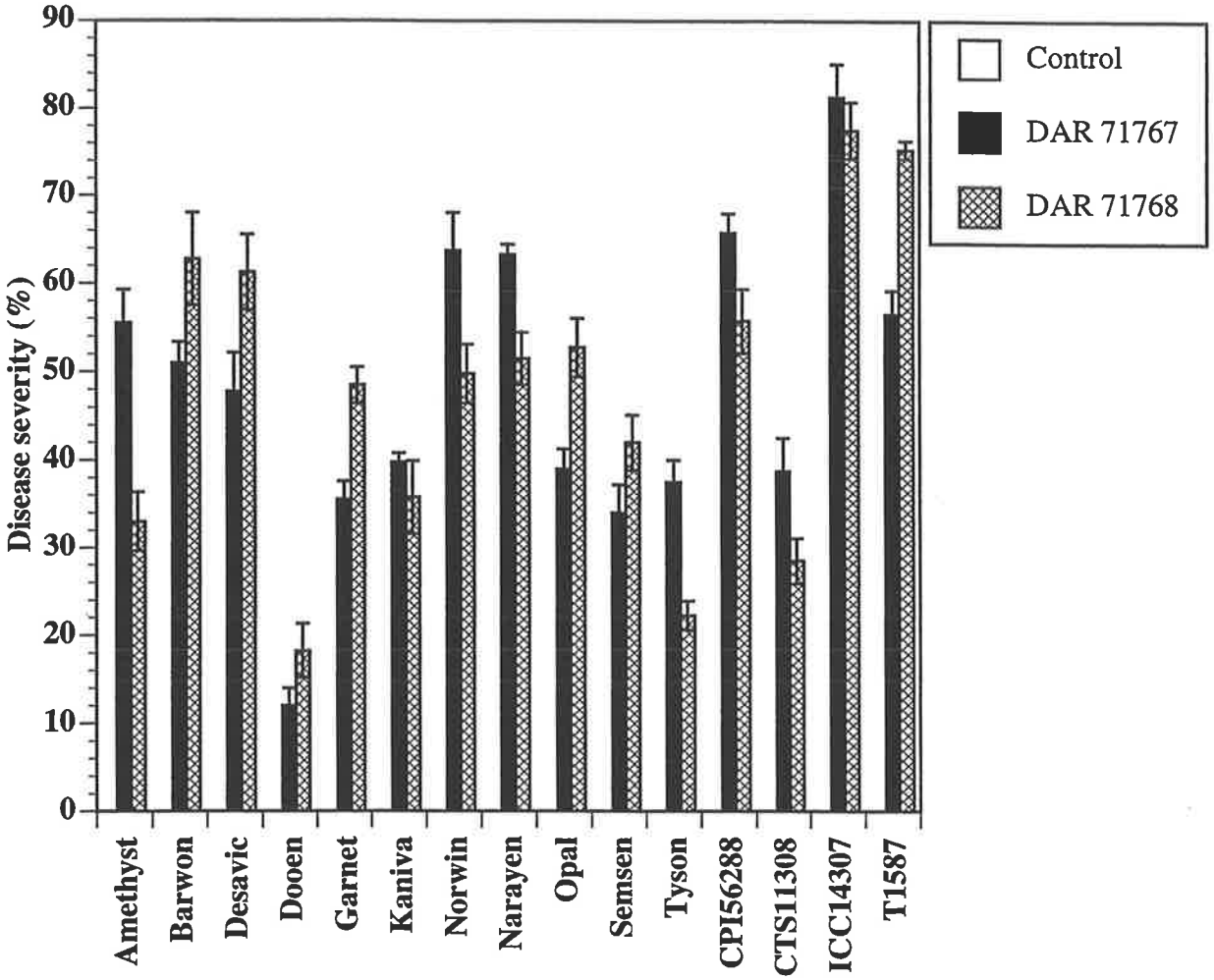


Fig 5.1 Response of 15 chickpea genotypes to inoculation with a conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768, separately, in the glasshouse. Symptoms were assessed after 21 weeks using the scale of Gowen *et al.* (1989) (see section 3.6). The vertical bars represent standard errors based on four replicates.

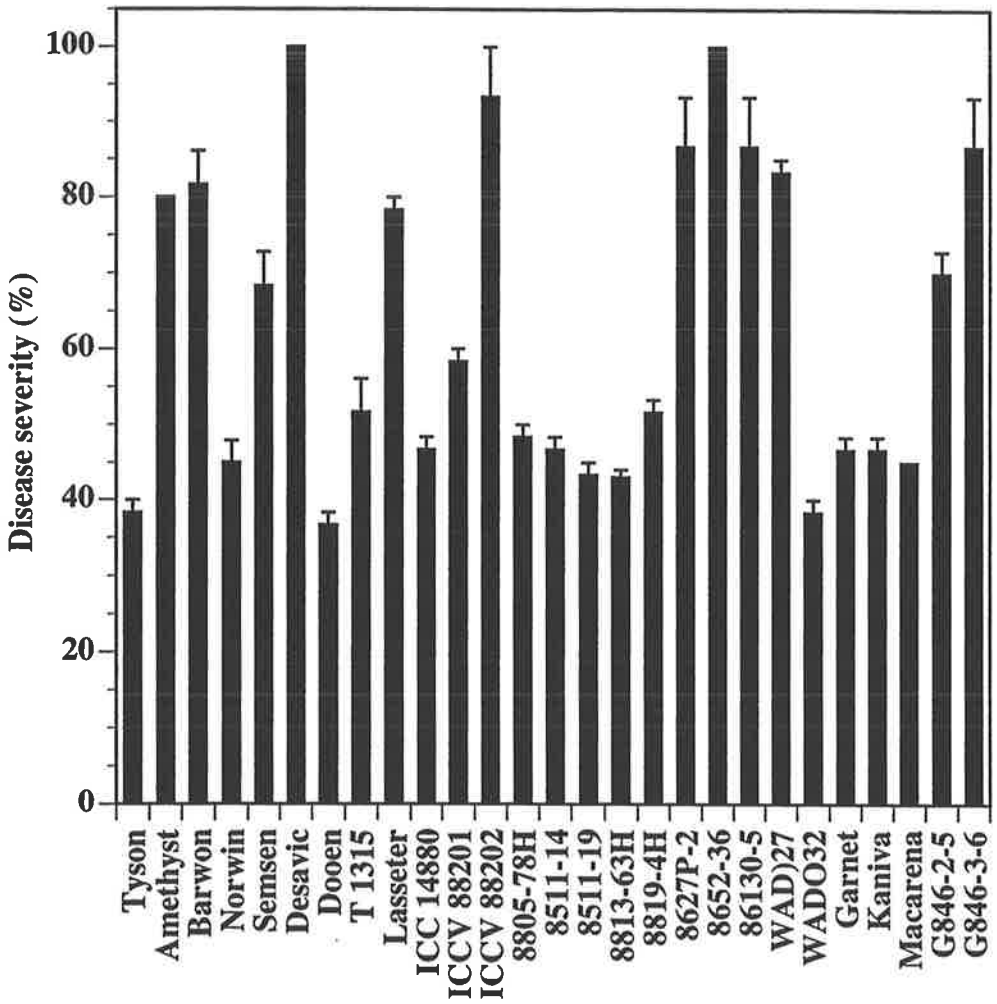


Figure 5.2 Response of 27 chickpea genotypes to inoculation with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768 in the glasshouse. Symptoms were assessed after 21 weeks using the scale of Gowen *et al.* (1989) (see section 3.6). The vertical bars represent standard errors based on four replicates.

ICCV 96704, ICCV 96701, ICCV 96705 and ICCV 96706 were moderately susceptible while ICCV 95906 was highly susceptible.

The response of the 10 imported Pakistani resistant lines to inoculation with a mixture of DAR 71767 and DAR 71768 in the fourth glasshouse trial is shown in Fig. 5.4. Uninoculated controls remained healthy, while mean disease severity on Dooen (resistant) and Desavic (susceptible) was 42 and 95%, respectively. ANOVA showed highly significant differences ($P<0.001$) among both chickpea genotypes and isolates. ICC 1151xILC 482, ICC 1151x ILC 3279, NIFA-88, NEC-138x CM 72, CM-72, CM-88 and C-44, with mean disease severity of 28 to 48%, were classified as resistant to moderately resistant whereas Paidar-91, Punjab-91 and Noor-91, with mean disease severity of 59 to 62 %, were classified as moderately susceptible to highly susceptible (Figure 5.4)

5.3.2 Outdoor trial

Plants inoculated with a mixed suspension of DAR 71767 and DAR 71768 at the end of August, 1996 and placed outdoors at the Waite Campus, were exposed to conditions highly favourable for development of ascochyta blight. Uninoculated controls remained healthy. Among the inoculated plants, there were highly significant differences ($P<0.001$) among both chickpea genotypes and isolates (Figure 5.5). Only ICC 03996, with mean disease severity of 2 was highly resistant, ATC 41933 and ICC 04958 with mean disease severity 20 and 22 were resistant and the remainder of the genotypes were moderately susceptible to highly susceptible.

5.3.2 Field trial

In the field trial, the first symptoms of disease appeared in the Desavic buffer lines 1 week after the first rain event on 13 and 14 August 1998 (see appendix 16), and were

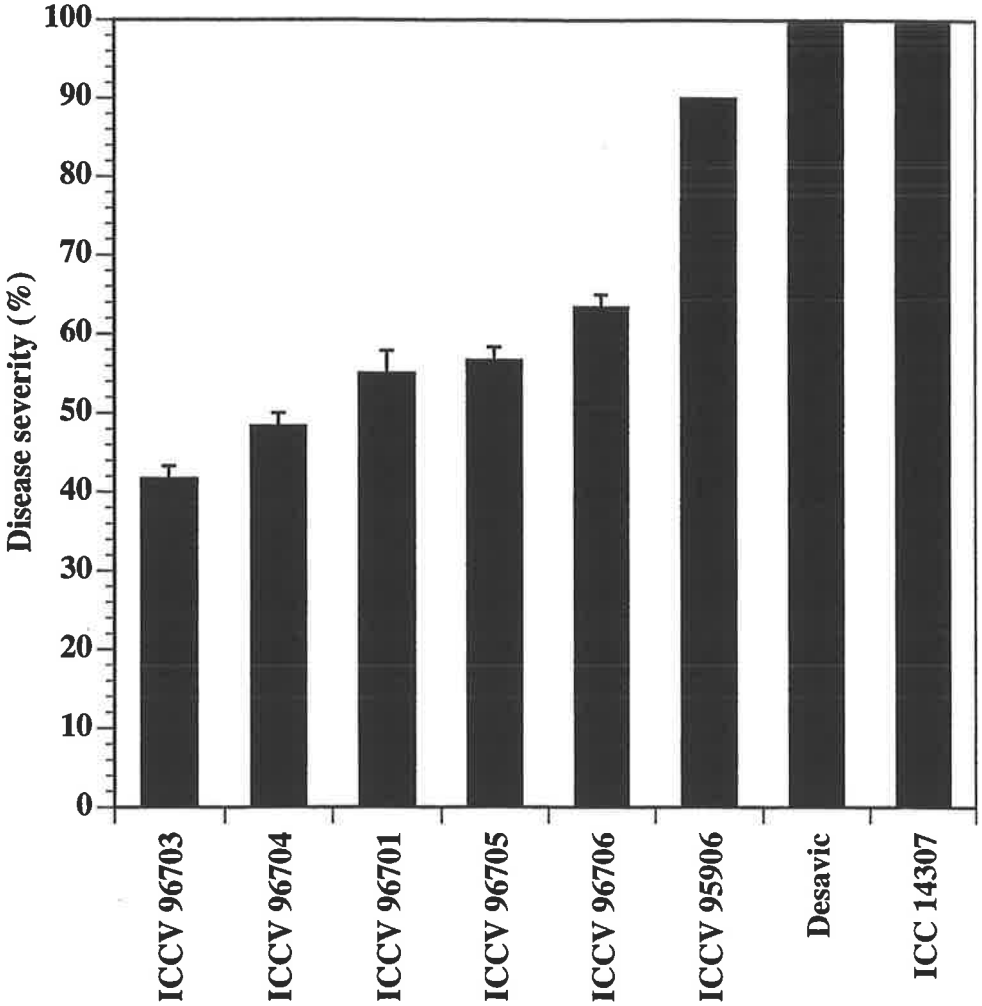


Figure 5.3 Response of six Western Australian chickpea genotypes to inoculation with a mixed conidial suspension of *A. rabiei* DAR 71767 and DAR 71768 in the third glasshouse trial. Two known susceptible genotypes, Desavic and ICC 14307, were included for comparison. Symptoms were assessed after 21 days using the scale of Gowen *et al.* (1989) (see section 3.6). The vertical bars represent standard errors based on four replicates.

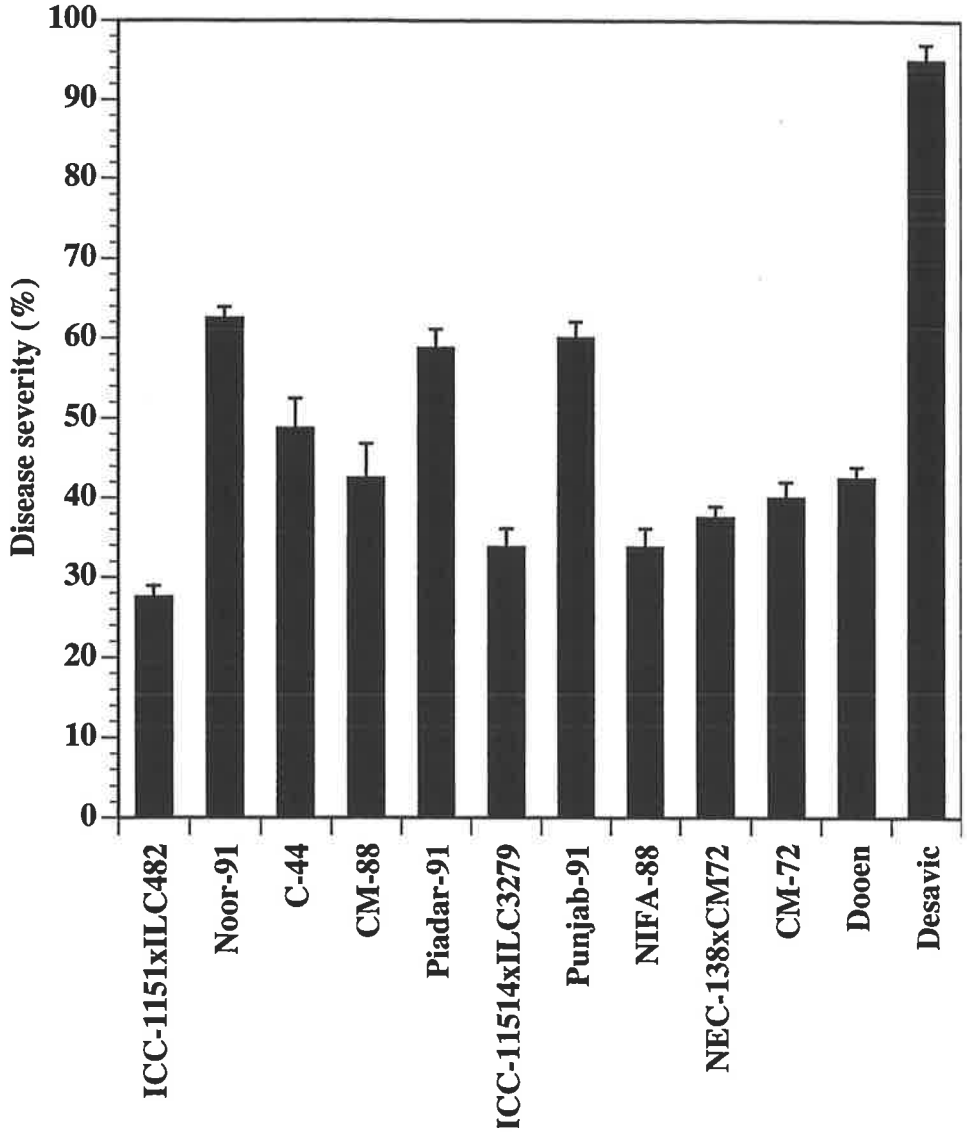


Figure 5.4 Response of 10 Pakistani chickpea genotypes (Noor-91 was a kabuli and the rest were desi types) and two Australian desi cultivars Dooen (resistant) and Desavic (susceptible) to inoculation with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768 in the fourth glasshouse trial. Symptoms were assessed after 21 days using the scale of Gowen *et al.* (1989) (see section 3.6). The vertical bars represent standard errors based on four replicates.

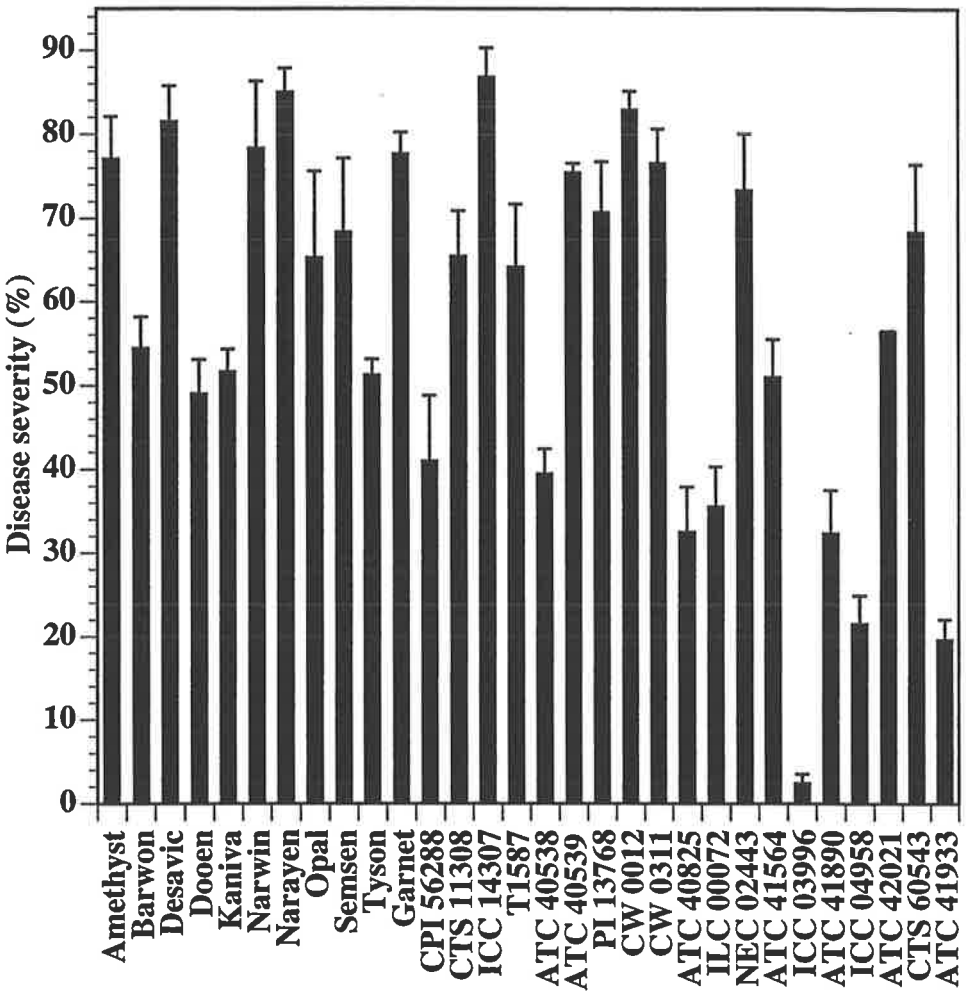


Fig 5.5 Response of 30 chickpea genotypes to inoculation with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768 in the outdoor trial. Symptoms were assessed after 4 weeks using the scale of Gowen *et al.* (1989) (see section 3.6). The vertical bars represent standard errors based on four replicates.

Table 5.1.A. Reaction of chickpea genotypes 8 weeks after inoculation with *A. rabiei* isolates DAR 71767 and DAR 71768 in field conditions (birdproof enclosure) at the Waite Campus.

Mean Rating	Cultivar reaction[a]
1 - <2	ICC 3996
2 - <3	-
3 - <4	Tyson, 8511-14, 8511-19, FLIP 85.58, ILC 3279
4 - <5	Damla, Dooen, ICCV 88201, 8523.1, 92.193.1.7, WACPE 2003
5 - <6	ATC 1890, 8810-2, 8813-74H, 8813-63H, 92.194.1.9, G 846-3-6, WACPE 2004, 8506-05, 8829-17, Heera
6 - <7	8813-31H, 92.186.2.10, Amethyst, FLIP 8685C, G846-3-13, Kaniva, WACPE 2016, 86.059.3.2, 8616.2H, 8905-14N, 92.194.1.14, Sona, WACPE 2014, WACPE 2021, 92.185.1.1, Barwon, WACPE 2012, WACPE 2019, Whitey, 8809-19H, Bumper, ILC 482, Sanford
7 - <8	8825-20H, 8825-59H, 8903P-03B, 92.187.1.8, Spanish White, 365.117, 86130-05, 8627P-02, 8801-35, WACPE 2011, WACPE 2017, 8673.3, 86.085.54, 8813-113H, 8931-52Q, Garnet, ICCV 93928, T 1069, T 1822, WACPE 2013, WAD 032, 8801-92, 8819-04H, 8931-6Q, 92.194.1.11, WACPE 2001
8 - <9	G 846-3-4B, ILC 6055, 8652.36, 8518-48, 90140-46Q, 92.186.1.5, 92.187.1.1, Gully, ILC 1463, Blanco Lechoso, 8623.5, 8820-118H, Desavic, ILC 482.205, 8502-39, 8513-10, 8914-65Q
9 -<10	G 846-1-1B, T 1239, 8806-33H, 94-105, ICCV 92504, Lasseter, ICC 4958

[a]Mean of four replicate rows, each comprising approx. 40 plants. Disease was assessed, in August 1998, using the modified scale of Gowen *et al.* (1989), in which percentages were converted to a 1-10 point scale (see section 3.6).

Table 5.1.B. Reaction of chickpea genotypes 8 weeks after inoculation with *A. rabiei* DAR 71767 and DAR 71768 in field conditions (birdproof enclosure) at the Waite Campus.

Mean Rating	Cultivar reaction[a]
1 - <2	-
2 - V3	Stepnoj 1, ATC 41843, Kassab, VYR 32
3 - <4	Aydin, Militrenskij 4, ICC 01903
4 - <5	Rizki, TS 1502
5 - <6	ILC 202
6 - <7	-
7 - <8	-
8 - <9	Sanford, ILC 482
9 -<10	Desavic, Menemen 92, Atalaya, ICC 11551, ILC 00191

[a]**Mean of single replicate row, each comprising approx. 40 plants. Disease was assessed, in August 1998, using the modified scale of Gowen *et al.* (1989), in which percentages were converted to a 1-10 point scale (see section 3.6).**

most obvious in plants adjacent to the inoculum source. With the second rain event from 20 to 27 August 1998 (see appendix 16) the disease spread throughout the trial site. When disease was assessed at plant maturity (20 October 1998), plants in the buffer rows were almost dead.

The data presented in Tables 5.1A (95 genotypes) and 5.1B (17 genotypes) represent the means of four and one replication, respectively. Only one genotype, ICC 3996, was highly resistant (mean disease severity of 1). Eleven genotypes: Tyson, 8511-19, FLIP 85.58, ILC 3279, Damla, Dooen, ICCV 88201, 8523.1, 92.193.1.7 and WACPE 2003 (disease severity of 3-<5) were resistant to moderately resistant, a further 11 genotypes were moderately resistant/moderately susceptible (disease severity of 5-<6), 23 were moderately susceptible (disease severity of 6-<7) and remaining 50 genotypes were highly susceptible (disease severity of 7-10) (Table 5.1A).

Among the 17 genotypes of the Agriculture Victoria collection, seven: Stepnoj 1, ATC 41843, Kassab, VY 32, Aydin, Militernskij 4 and ICC 01903 were highly resistant to resistant (mean disease severity of 2-<4) while Rizki, TS 1502 and ILC 202 were intermediate (mean disease severity of 4-<6) and Sanford, ILC 482, Desavic, Menemen 92, Atalaya, ICC 11551 and ILC 00191 were highly susceptible (Table 5.1B).

5.4 DISCUSSION

Although the results can not be compared statistically, the response of Amethyst, Desavic, Dooen and Kaniva inoculated with a mixture of conidia of DAR 71767 and DAR 71768 and placed in glasshouse and outdoors was generally similar. However, symptoms on Barwon appeared to be less severe on plants maintained outdoors than on those in the glasshouse. There was considerable variation in the resistance of chickpea genotypes, including commercial cultivars, however, no line was immune. Desavic, a major cultivar in southern Australia, was highly susceptible and Dooen was moderately

resistant to ascochyta blight in all glasshouse, outdoor and field trials. Of the commonly available cultivars, Dooen, Kaniva, Tyson were moderately resistant as was the breeding line WAD 032.

Disease severity on Desavic was lower in the first glasshouse than in the subsequent three glasshouse trials, with mean disease score of 48 (for DAR 71767) to 61 (for DAR 71768) compared to mean disease scores of 95-100% in glasshouse trials 2-4. The same was true for Amethyst, Barwon and Dooen in the first glasshouse trial compared to glasshouse trial 2. The difference in disease severity could be due to a synergistic effect of the mixed inoculum, as the inoculation was done separately with DAR 71767 and DAR 71768 in the first glasshouse trial and in all the subsequent trials with a mixture of DAR 71767 and DAR 71768.

Of the genotypes imported from Pakistan, ICC 1151xILC 482, ICC 1151x ILC 3279, NIFA-88, NEC-138x CM72, CM-72, CM-88 and C-44 were found to be resistant to DAR 71767 and DAR 71768. These results are consistent with their response to Pakistani isolates, as reported by Haq and Hassan (1980), Malik and Bashir (1984) and Mitsueda *et al.* (1997). As a result of this work, these genotypes have been incorporated into the National Chickpea Breeding Program for use in breeding for resistance to ascochyta blight.

Among the breeding lines, ICC 03996, a desi line known to be resistant to ascochyta blight and that originated from ICRISAT, was highly resistant in both the outdoor and field trials. This is consistent with its response in a trial conducted in Italy by Porta-Puglia (1992). ILC 3279, 8511-14, 8511-19, FLIP 85.58, ILC 3279, Damla, Dooen, ICCV 88201, 8523.1, 92.193.1.7 and WACPE 2003 were found to be resistant in field conditions. In field conditions, the disease appeared first in the buffer rows (Desavic) especially around the infected pots and then spread to the test lines, presumably by rain-

splash, whereas in the glasshouse and outdoor trials the disease appeared within 4-5 days of inoculation. Generally, the disease was slightly higher on the extreme rows of the trial which might be attributed to the proximity of the irrigation sprinklers. Apart from the number of replicate rows, all the conditions were similar in both trials (four replicates and one replicate, respectively). The mean ratings for Sanford and Desavic were 6 to <7 and 8 to <9 in the first trial but the same cultivars showed disease levels of 8 to <9 and 9-10 in the single replicate trial. The difference could be due to proximity to the sprinklers, and shows the need for adequate replication in a trial of this nature.

Among the genotypes common to glasshouse, outdoor and field, the response to inoculation with DAR 71767 and DAR 71768 was generally similar. The reaction of ILC 3279 to inoculation with the Australian isolates DAR 71767 and DAR 71768 was similar to that reported in Syria, Lebanon, Turkey, Algeria and Pakistan to local isolates of *A. rabiei*. During screening of chickpea for resistance to ascochyta blight Singh *et al.* (1984) inoculated the trial with infested debris, sowed the susceptible line ILC 1929 after every two test entries and also inoculated by spraying with conidia when levels of infection were insufficient.

Based on the results obtained in this study, Desavic, the most susceptible of the cultivars tested and the most commonly grown in Australia, cannot be recommended for cultivation in areas where ascochyta blight is likely to occur. Moderately resistant cultivars, such as Dooen, Kaniva, Tyson and the breeding line WAD 032 should perform better than Desavic in such areas.

Lines ICC 3996 and 8511-14, identified as resistant to DAR 71767 and DAR 71768 in this study, were used as parents in crosses conducted by plant breeders at Tamworth, New South Wales, as part of the National Chickpea Breeding Program. Progeny resulting from these crosses are shortly to be released as resistant cultivars for use in the Australian chickpea industry.

CHAPTER 6

EPIDEMIOLOGICAL STUDIES OF ASCOCHYTA BLIGHT OF CHICKPEA IN GLASSHOUSE AND FIELD CONDITIONS

6.1 INTRODUCTION

Ascochyta blight is considered to be an important disease of chickpea grown in regions located between 30 to 45 °N and 27 to 37 °S latitudes (Corbin, 1975; Reddy and Sing, 1990). The crop yield may be affected by a wide range of photoperiod and hydro-thermal regimes but most of the fluctuations in productivity are due to ascochyta blight, which can devastate chickpea crops when cool and wet weather occurs during the growing season (Saxena and Singh, 1984; Nene and Reddy, 1987; Jhorar *et al.*, 1998). Under conducive climatic conditions in the Palouse region of eastern Washington and northern Idaho, USA, the disease can cause an annual economic loss of over US $1 million (Kaiser and Muehlbauer, 1988).

The disease and the causal fungus are affected by the weather at all physiological stages up to maturity (Singh and Sharma, 1998). Epidemics are favoured by temperatures of 9-24 °C, more than 60% relative humidity, wetness periods of 10 h or more and windy conditions (Nene, 1982; Weltzien and Kaack, 1984; Trapero-Casas and Kaiser, 1992; Reddy and Singh, 1990b; Jhorar *et al.*, 1998). These requirements were established by research in the northern hemisphere, using local cultivars and isolates of *A. rabiei.*

However, there are gaps which exist in the knowledge of epidemiology of ascochyta blight, especially to the southern hemisphere conditions where the disease recently been identified and where different cultivars are grown. Therefore, the studies reported in this

chapter were undertaken to determine the following, with special reference to Australian conditions:

(i) the influence of inoculum concentration on disease development;

(ii) the effect of plant age on disease development;

(iii) the effect of different temperature and wetness period, and interrupted wetness period on disease development;

(iv) the influence of environmental factors on disease development in the field

6.2 MATERIALS AND METHODS

6.2.1 Glasshouse trials

Seedlings of five Australian chickpea cultivars (Barwon, Desavic, Dooen, Kaniva and Tyson) and one breeding line (ICC 14307) were grown in the glasshouse as described in section 3.2. There were four replicate plots, each containing three seedlings, per treatment, and the seedlings were inoculated 15 days after sowing, as described in section 3.5, unless stated otherwise. Symptoms were assessed 14 days after inoculation using the scale (0-100%) of Gowen *et al.* (1989), as described in section 3.6, unless stated otherwise.

6.2.1.1 Effect of inoculum concentration on disease development

Conidial suspensions of $5x10^4$, $5x10^5$, $1x10^6$, $1x10^7$ conidia per mL of *A. rabiei* isolates DAR 71767 and DAR 71768 were prepared as described in section 4.2.2.2. Equivalent suspensions of the two isolates were combined 1:1 (vol:vol) and Tween-20 was added as described in section 3.4. Seedlings of Barwon, Desavic, Dooen and Tyson were inoculated and incubated in a humid chamber for 72 h as described in section

3.5. The control plants were treated with SDW and incubated in a separate humid cabinet, otherwise the conditions were the same.

6.2.1.2 Effect of plant age on disease development

The effects of growth stage on infection and disease development were examined in the glasshouse. Seeds of Desavic and ICC 14307 were sown at 2-week intervals for 12 weeks. The purpose of the staggered sowing dates was to provide plants, at the time of inoculation, at different growth stages to avoid any variation in the inoculum and glasshouse conditions. Plants were inoculated with a combined suspension (1:1) of DAR 71767 and DAR 71768, $5x10^5$ conidia per mL, and incubated as described in section 3.5. The control plants were treated with SDW.

6.2.1.3 Effect of temperature and wetness period on disease development

Seedlings of Desavic were inoculated with combined conidial suspension ($5x10^5$ conidia per mL) of *A. rabiei* DAR 71767 and DAR 71768 (1:1). Control plants were treated with SDW. Immediately after inoculation, control and inoculated plants were placed in growth rooms maintained at 10, 15, 20, 25 and 30 °C and covered with polyethylene bags for 72 h. The photoperiod was adjusted to 14 h light (140-160 $\mu E/m^2/s$) and 10 h dark. The bags were then removed and the plants watered from below on alternate days.

6.2.1.4 Effect of interrupted wetness periods on disease development

Seedlings of Desavic were inoculated as described in section 6.2.1.3, and then subjected to one of three wet-dry treatments: 1) a continuous wetness period of 0, 6, 12, 24, 48, 72 and 96 h; 2) a dry period of 6-, 12-, 24-, 48-, 72- and 96-h, followed by a 48-h

wetness period; and 3) a 6-h wetness period followed by a dry period of 6, 12, 24, 48, 72 and 96 h followed by a 48-h wetness period. During the wetness period, plants were covered with polyethylene bags in a growth room at 20 °C as described in section 6.2.1.3.

6.2.2 Field trials

Field trials were sown in the birdproof enclosure at the Waite Campus, the University of Adelaide, in three consecutive years, and comprised five cultivars, Barwon, Desavic, Dooen, Kaniva and Tyson. The trials were sown on 13 June 1996, 18 June 1997 and 10 June 1998, in three replicates of six 3-m long rows of each cultivar. Six rows of Desavic (buffer) separated each replicate. The plot size was 4.5 m^2, the inter- and intra-row spacings were 25 and 10 cm, respectively, and the distance between adjacent replications was 1.2 m. A thermohygrograph (Microtech, Model-01, Aurther Bailey Pty. Ltd., Japan) was placed in the middle of the trial to record temperature and relative humidity, and rainfall data were obtained from the nearby Waite Campus meteorological station. Daily weather data are recorded in appendices 2-19.

Residues of Desavic plants in pots, inoculated 4 weeks previously with mixed conidial suspension of DAR 71767 and DAR 71768 in the glasshouse (see sections 3.4 and 3.5) were placed in the centre of the buffer rows at 1 m intervals on 26 July 1996, 30 July 1997 and 3 August 1998, respectively. Ten individual plants were randomly selected in each plot, tagged with pink plastic tape and disease on these plants was assessed using the scale (0-100%) of Gowen *et al.* (1989), as described in section 3.6. Disease severity was assessed at 1-week intervals for 12 weeks, beginning 20, 22 and 19 days after the inoculum was introduced in 1996, 1997 and 1998, respectively.

6.3 RESULTS

6.3.1 Glasshouse trials

6.3.1.1 Effect of inoculum concentration on disease development

A positive correlation was observed between disease severity and inoculum concentration in the range $5x10^4$ to $1x10^7$ conidia per mL (Fig 6.1). The control plants remained healthy. Disease symptoms were generally least severe on Dooen plants, with mean disease severity of ≤ 50% at all inoculum concentrations tested. Barwon, Kaniva and Tyson showed mean disease severity of 40, 27 and 20%, respectively, when inoculated with $1x10^4$ conidia per mL and 84, 71 and 59%, respectively, when inoculated with $1x10^7$ conidia per mL. Desavic showed mean disease severity of 64% when inoculated with $1x10^4$ conidia per mL and plants were completely dead 14 days after inoculation with $1x10^7$ conidia per mL.

6.3.1.2 Effect of plant age on disease development

Desavic and ICC 14307 were tested at different physiological stages from 2 to 12 weeks after sowing, representing the seedling to the pod stage. Disease was more severe on 2-week-old seedlings of both genotypes than it was on older plants ($P<0.01$) (Fig 6.2). Initially white spots appeared on the leaves and light green lesions on the stems and petioles of the younger plants and eventually the stems and petioles were girdled and broke. The older plants showed pale yellowish discolouration of the entire foliage and leaf abscission was more severe compared with 2 to 4 week-old seedlings. Symptoms on the stems of older plants were less severe compared to young plants. The control plants of all ages remained healthy.

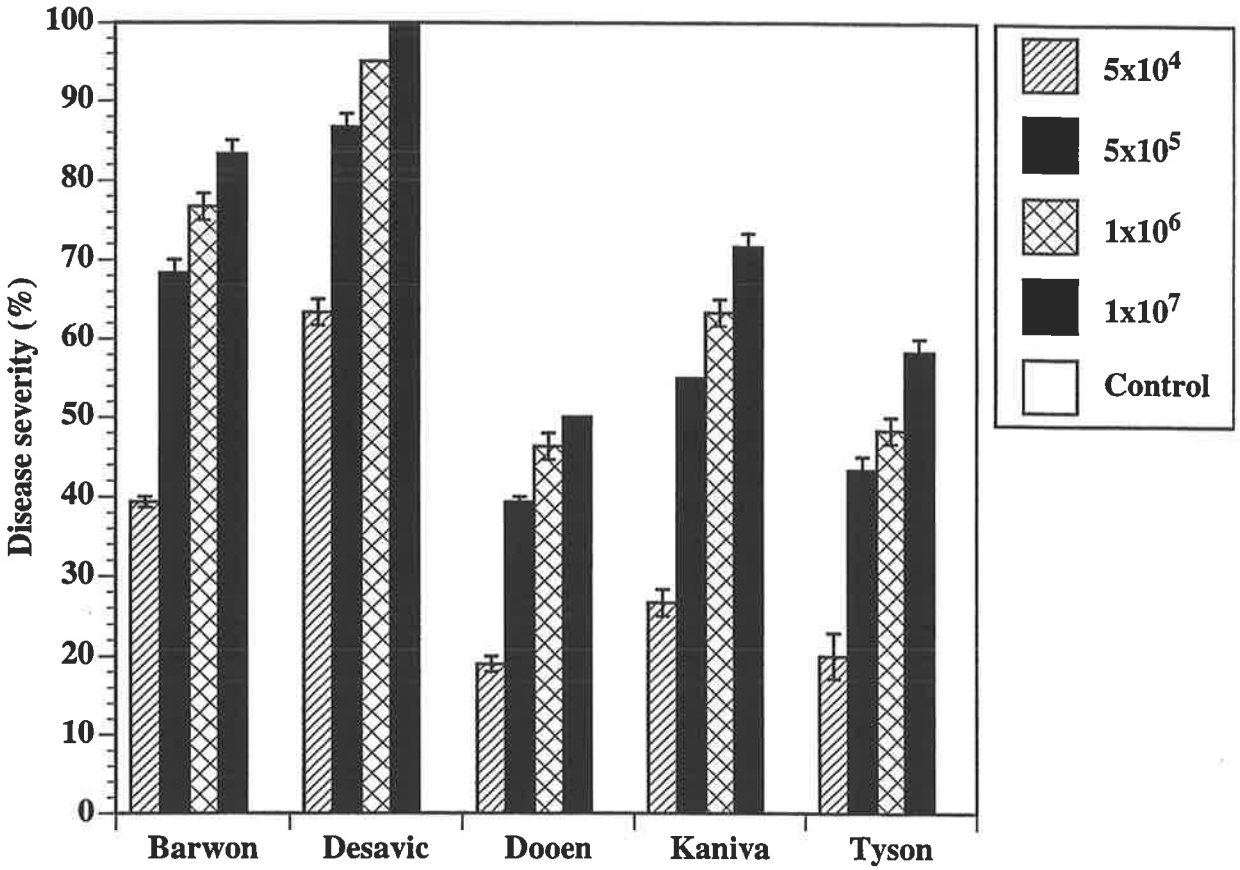


Fig 6.1 Effect of inoculum concentration ($5x10^4$, $5x10^5$, $1x10^6$ and $1x10^7$ conidia per mL) of mixed conidial suspension of *A. rabiei* DAR 71767 and DAR 71768 on disease severity on five chickpea cultivars. Symptoms were assessed 14 days after inoculation using the scale of Gowen *et al.* (1989) as described in section 3.6. The bars represent means and standard errors based on four replicates.

6.3.1.3 Effect of temperature and wetness periods on disease development

Disease severity was significantly affected by temperature, wetness period and their interactions ($P<0.001$). A period of less than 3 h wetness following inoculation resulted in few symptoms of disease (Fig 6.3). In general, when the wetness period exceeded 3h, there was a proportional increase in disease severity as the duration of the wetness period increased. Disease severity increased with increasing temperature to a maximum at 20 °C and decreased again at 25 and 30 °C. Disease was significantly more severe on plants exposed to a 96-h wetness period compared to those exposed to a 24-h wetness period at 20 °C ($P< 0.01$), however, no significant difference was observed between plants exposed to 72 and 96 h wetness periods at 20 °C. Similarly, differences in disease severity in plants exposed to 24 to 96 h wetness periods at 30 °C were not significant.

6.3.1.4 Effect of interrupted wetness periods on disease development

Disease severity increased in response to increasing the duration of the wetness period from 0, 6, 12, 24, 48, 72 to 96 h, to a maximum of 90% following the 96-h wetness period (Table 6.1). When inoculation was followed immediately by a dry period of 6 h and a subsequent 48-h-wetness period, disease severity was 73% and decreased with increasing duration of dry period, to a minimum of 20% when the dry period was 96 h. The figure of 73% for disease severity when inoculation was followed by 6-h dry period followed by a 48-h wetness period did not differ significantly from the disease severity (74%) observed when inoculation was followed immediately by a 48-h-wetness period. Similarly, disease severity was 73% when inoculation was followed by a 6-h wetness period, then 6-h dry period followed by 48 h of wetness. Again, disease severity

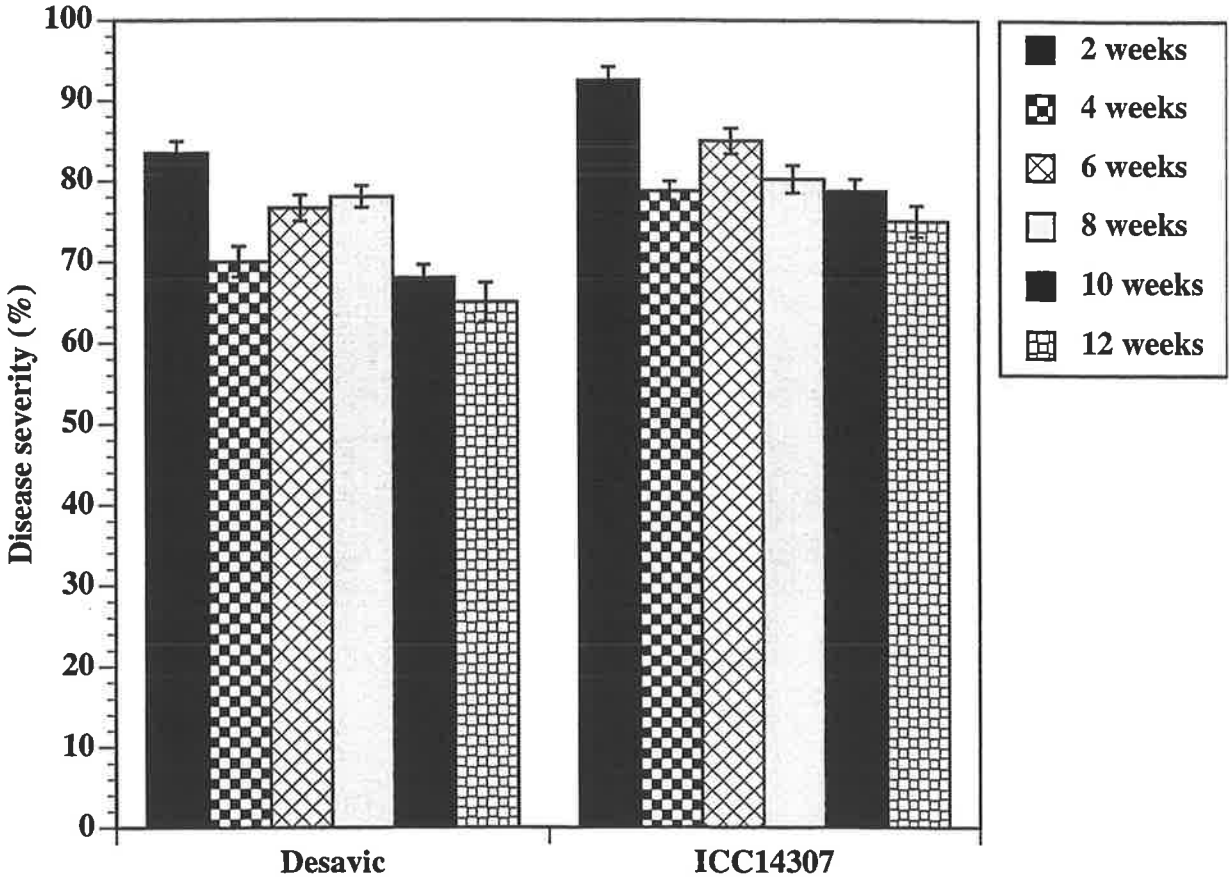


Fig 6.2 Effects of plant age, at the time of inoculation, on disease development in two susceptible chickpea genotypes ICC 14307 and Desavic, inoculated with mixed conidial suspension of *A. rabiei* DAR 71767 and DAR 71768 ($5x10^5$ conidia/ mL). Symptoms were assessed 14 days after inoculation using the scale of Gowen *et al.* (1989) as described in section 3.6. The bars represent means and standard errors based on four replicates.

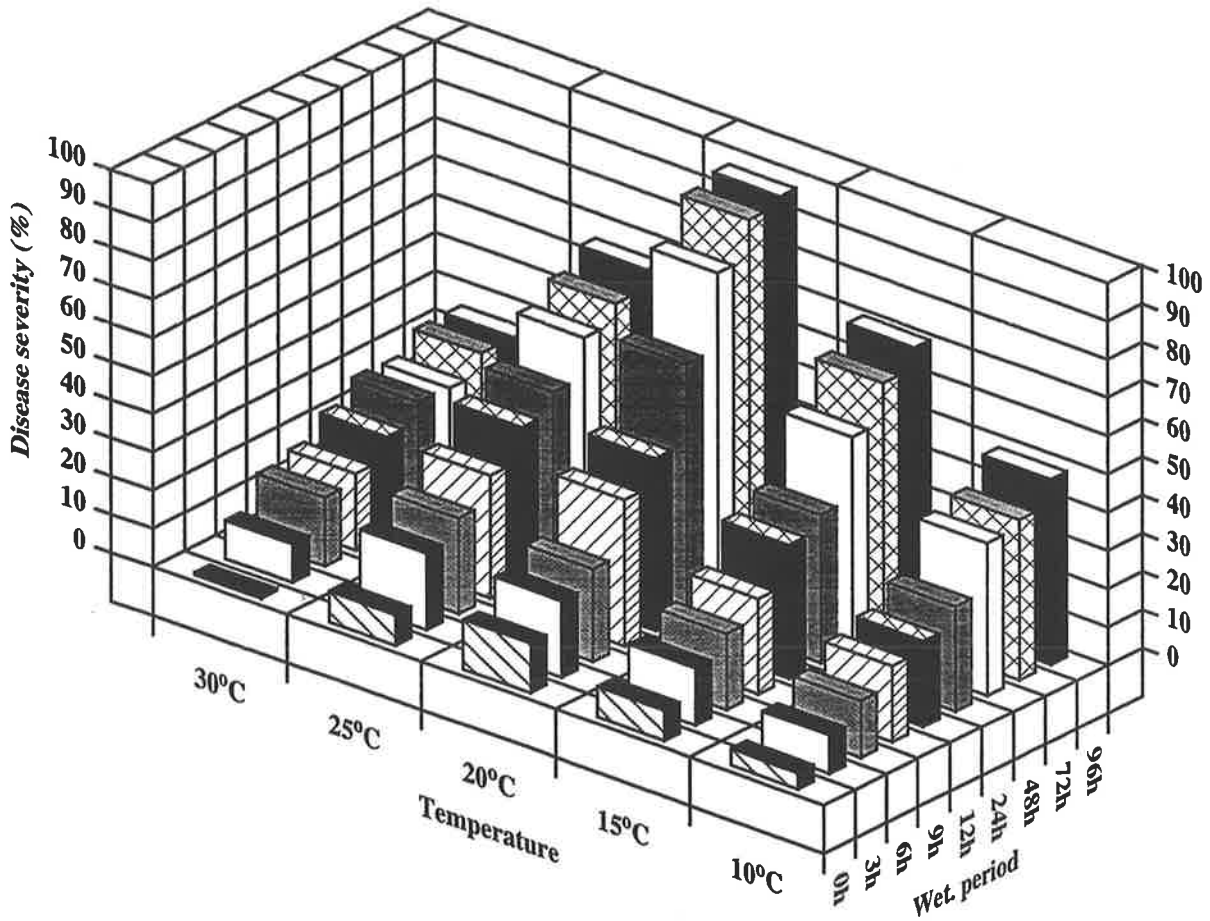


Fig 6.3 Effect of temperature and wetness period on disease development on Desavic seedlings inoculated, 2 weeks after sowing, with mixed conidial suspension of *A. rabiei* DAR 71767 and DAR 71768 ($5x10^5$ conidia/ mL). Symptoms were assessed 14 days after inoculation using the scale of Gowen *et al.* (1989) as described in section 3.6. Each bar represents the mean percent disease severity based on four replicates.

Table 6.1 Effect of interrupted wetness period on disease severity in chickpea inoculated with *A. rabiei* [a]

Post inoculation period (h)			
Wet. period	**Dry**	**Wet. period**	**Disease severity (%)[b]**
0	....	....	10
6	....	....	12
12	....	....	20
24	....	....	40
48	....	....	74
72	....	....	81
96	....	....	90
....	6	48	73
....	12	48	64
....	24	48	46
....	48	48	35
....	72	48	25
....	96	48	20
6	6	48	72
6	12	48	65
6	24	48	50
6	48	48	42
6	72	48	30
6	96	48	25

[a] Fifteen days after sowing, seedlings of cv. Desavic were inoculated with mixed conidial suspension of *A. rabiei* DAR 71767 and DAR 71768 ($5x10^5$ conidia/ mL) and incubated at 20 °C.

[b] Symptoms were assessed 14 days after inoculation, using the scale of Gowen *et al.* (1989) as described in section 3.6.

decreased from 73% to 25% as the length of the dry period interrupting the wetness period increased from 6 h to 96 h (Table 6.1).

6.3.2 Field trials

1996: Symptoms of ascochyta blight were first observed in the buffer rows 10 days after the inoculum was introduced and pycnidia were observed on the lesions 3 days later. At this stage, the disease was restricted to plants around the inoculum source (pots containing infected seedlings) and was not observed in the test plots. With the first event of rainfall (29 July 1996) the disease spread from the buffer rows to the test plots (Fig. 6.4). Overall, among the five chickpea cultivars tested, disease severity increased over time. Disease progress curves for the five cultivars showed that disease increased from week 1 to week 11, when disease approached 100% in Desavic (Fig. 6.4). Similar disease progress curves were observed for all five cultivars, with Desavic being the most severely affected (Fig 6.4).

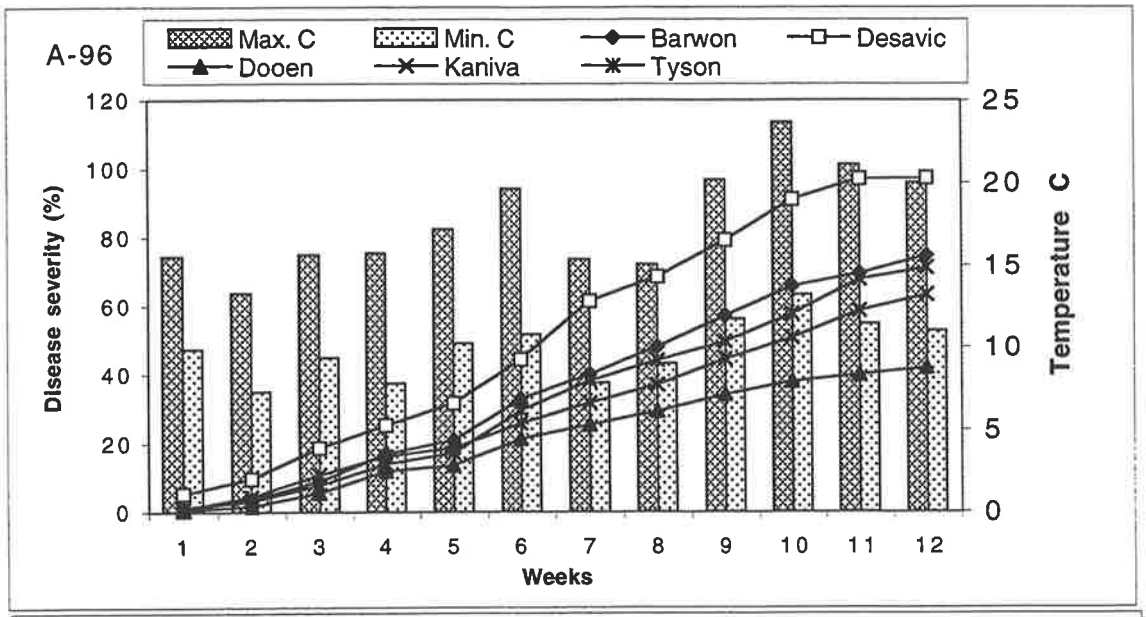
1997: Disease symptoms were first observed in the buffer rows, and were more severe than in 1996. Rainfall on 7 August 1997 (1 week after placing the inoculum in the buffer rows) was 31 mm and, in the subsequent days, falls of 11, 4, 1, 14 and 18 mm, respectively, were recorded (see appendix 10). Rainfall of 13 mm occurred during week 12. Disease severity on Desavic increased sharply from 55% in week 5 to 100% in week 7 (Fig. 6.5). Disease severity increased fairly steadily on Barwon from weeks 2-6 and on Kaniva, Tyson and Dooen from weeks 2-7. Barwon, Dooen, Kaniva and Tyson plants had mean disease severities of 49, 16, 38 and 25 %, respectively, at week 5 and increasing to 73, 36, 67 and 48 %, respectively, on week 7 (Fig. 6.5)

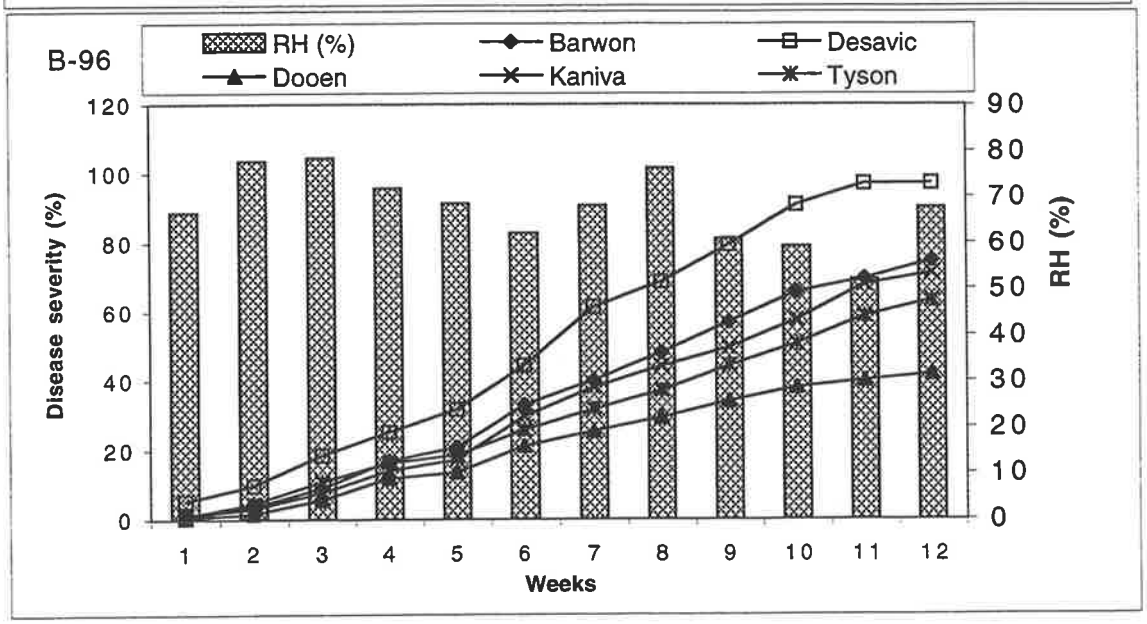
Fig 6.4 Development of ascochyta blight on five chickpea cultivars (Barwon, Desavic, Dooen, Kaniva and Tyson) sown in a bird-proof enclosure at the Waite Campus on 13 June 1996. The test cultivars were inoculated by placing diseased plants (Desavic) among the buffer rows on 26 July 1996. Disease progress was recorded from 14 August 1996 (week 1) to 30 October 1996 (week 12) on ten randomly selected plants of each cultivar in each of three replicate plots, using the scale of Gowen *et al.* (1989) as described in section 3.6. Data points are means of 30 replicate plants.

Fig. 6.4a Disease progress curve in relation to temperature (average maxima and minima for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.4b Disease progress curve in relation to relative humidity (RH, average for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.4c Disease progress curve in relation to rainfall (average for the week), as measured by the meteorological station at the Waite Campus.


A-96
Max. C
Min. C
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Temperature C
Weeks


B-96
RH (%)
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
RH (%)
Weeks

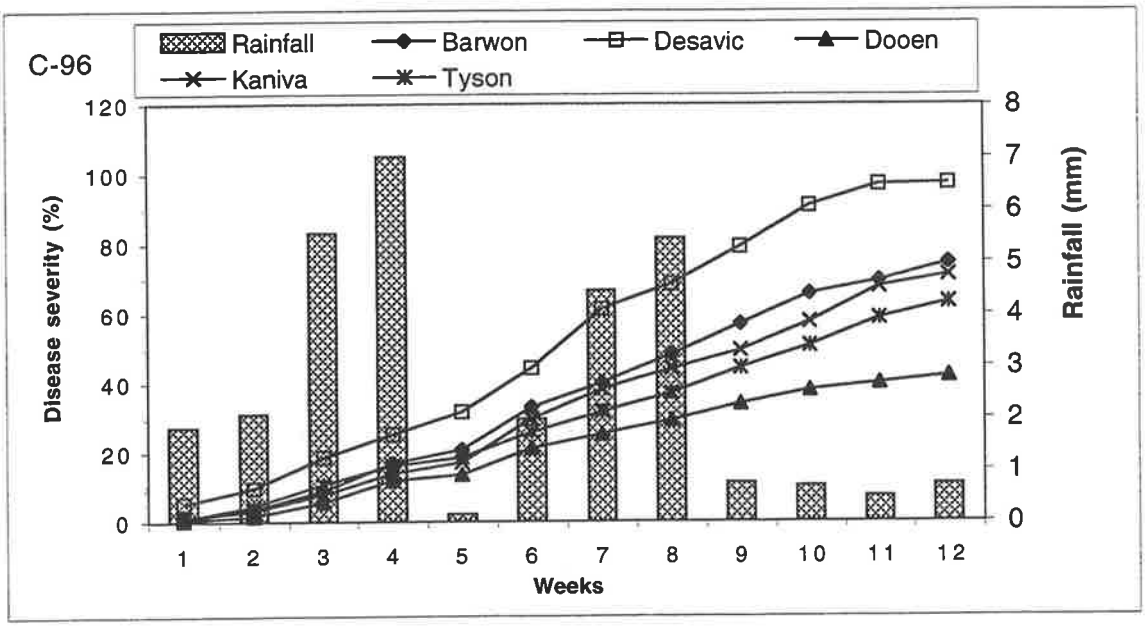
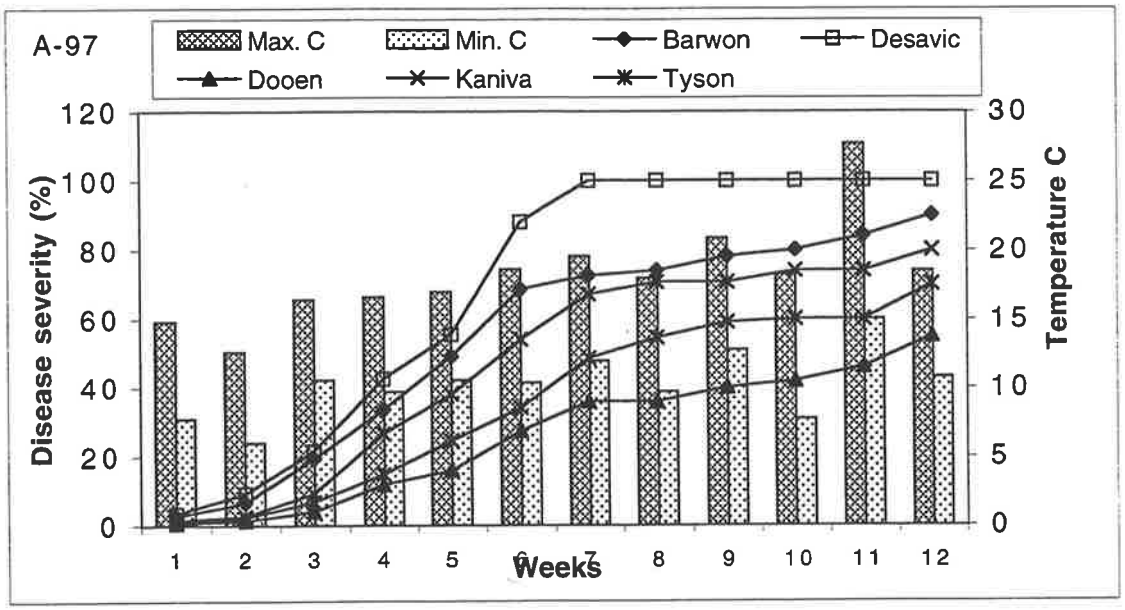

C-96
Rainfall
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Rainfall (mm)
Weeks

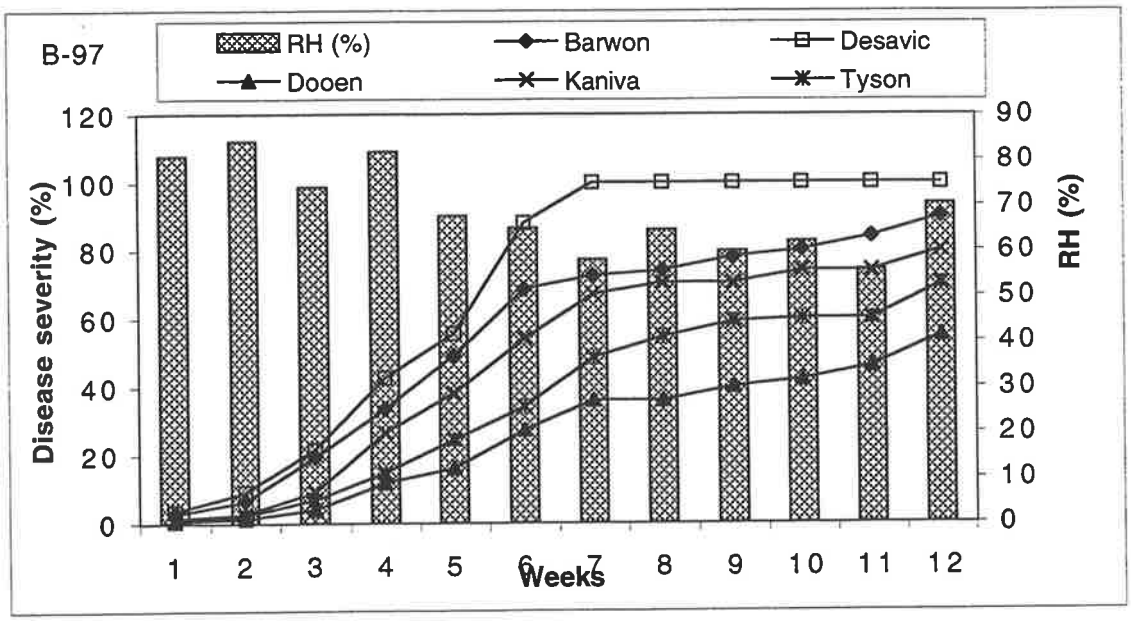
Fig 6.5 Development of ascochyta blight on five chickpea cultivars (Barwon, Desavic, Dooen, Kaniva and Tyson) sown in a bird-proof enclosure at the Waite Campus on 18 June 1997. The test cultivars were inoculated by placing diseased plants (Desavic) among the buffer rows on 30 July 1997. Disease progress was recorded from 20 August 1997 (week 1) to 5 November 1997 (week 12) on ten randomly selected plants of each cultivar in each of three replicate plots, using the scale of Gowen *et al.* (1989) as described in section 3.6. Data points are means of 30 replicate plants.

Fig. 6.5a Disease progress curve in relation to temperature (average maxima and minima for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.5b Disease progress curve in relation to relative humidity (RH, average for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.5c Disease progress curve in relation to rainfall (average for the week), as measured by the meteorological station at the Waite Campus.


A-97
Max. C
Min. C
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Temperature C
Weeks


B-97
RH (%)
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
RH (%)
Weeks

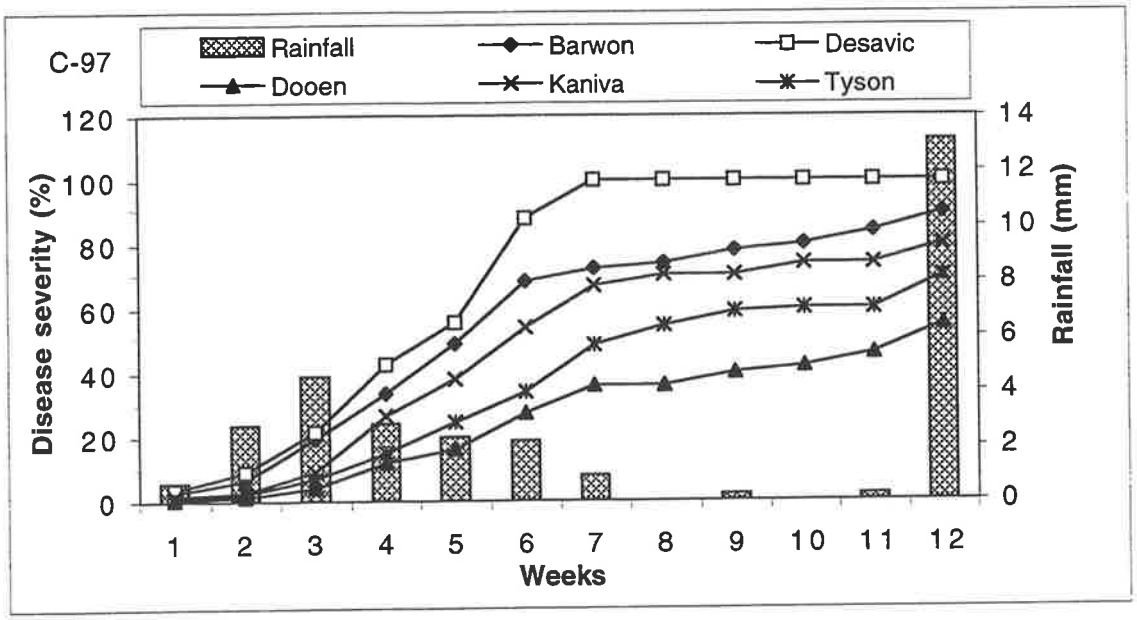
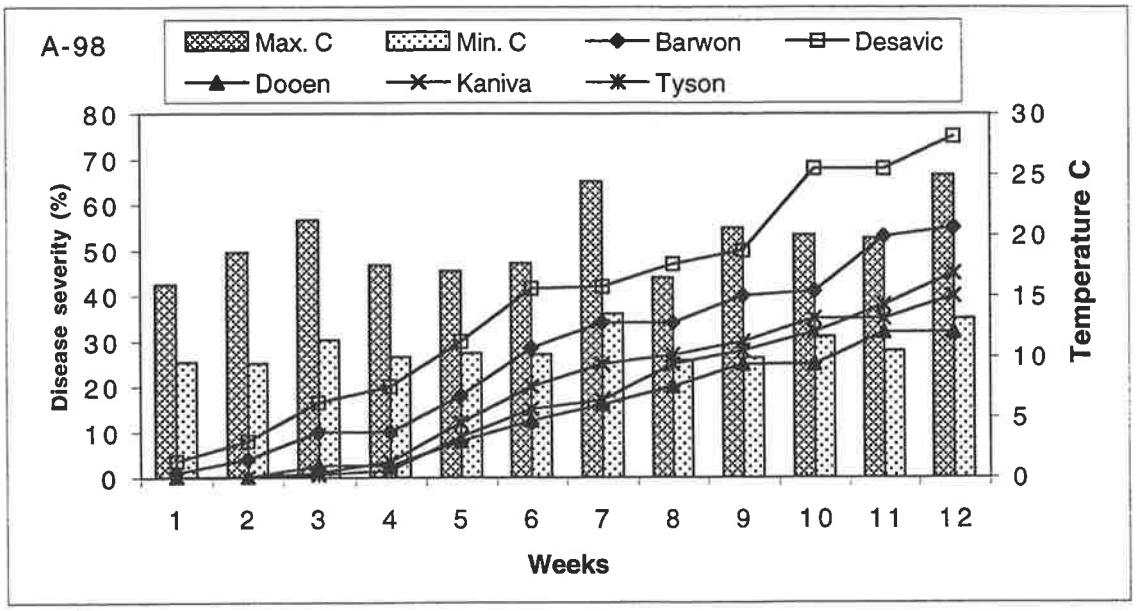

C-97
Rainfall
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Rainfall (mm)
Weeks

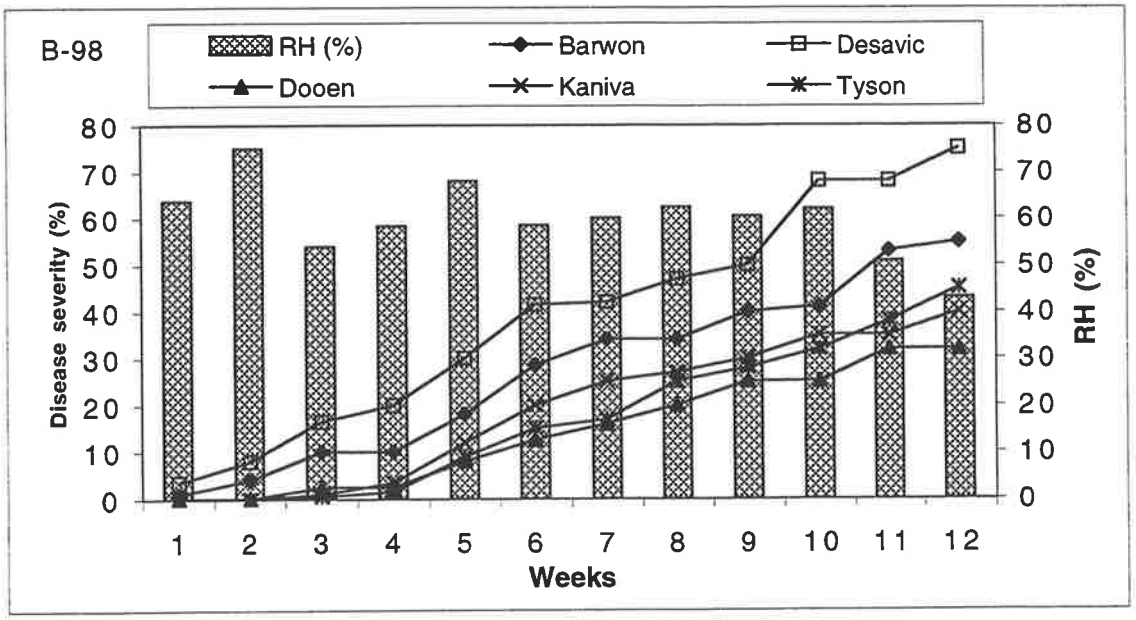
Fig 6.6 Development of ascochyta blight on five chickpea cultivar (Barwon, Desavic, Dooen, Kaniva and Tyson) sown in a bird-proof enclosure at the Waite Campus on 10 June 1998. The test cultivars were inoculated by placing diseased plants (Desavic) among the buffer rows on 3 August 1998. Disease progress was recorded from 21 August 1996 (week 1) to 6 November 1998 (week 12) on ten randomly selected plants of each cultivar in each of three replicate plots, using the scale of Gowen *et al.* (1989) as described in section 3.6. Data points are means of 30 replicate plants.

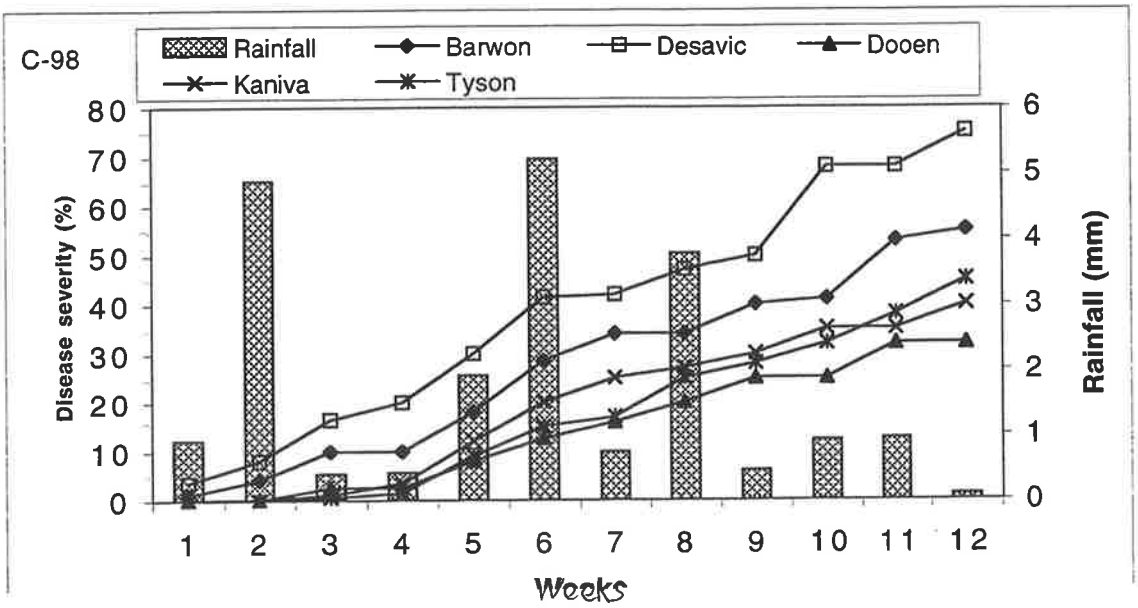
Fig. 6.6a Disease progress curve in relation to temperature (average maxima and minima for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.6b Disease progress curve in relation to relative humidity (RH, average for the week), as measured by a thermohygrograph placed in the centre of the trial.

Fig. 6.6c Disease progress curve in relation to rainfall (average for the week), as measured by the meteorological station at the Waite Campus.


A-98
Max. C
Min. C
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Temperature C
Weeks


B-98
RH (%)
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
RH (%)
Weeks


C-98
Rainfall
Barwon
Desavic
Dooen
Kaniva
Tyson
Disease severity (%)
Rainfall (mm)
Weeks

1998: Disease progress curves increased fairly steadily from week 1 to week 12 (Fig. 6.6). In cv. Desavic the maximum disease severity, at week 12, was 75%, and the corresponding figures for Barwon, Dooen, Kaniva and Tyson were 55, 32, 40 and 45%, respectively (Fig. 6.6).

6.4 DISCUSSION

Inoculation experiments in controlled conditions showed that disease severity can vary according to the concentration of the inoculum, the age of the plant at the time of inoculation, temperature and duration of leaf wetness following inoculation, and the duration of interruptions of the wetness period. The results support the hypothesis that within the range tested ($5x10^4$ to $1x10^7$ conidia per mL) increasing inoculum concentration will lead to increased disease severity, but this effect depends on the susceptibility of the chickpea cultivar and other environmental conditions. It has been reported that inoculum concentration has an important impact in the screening of chickpea germplasm for disease resistance (Nene *et al.*, 1981; Nene and Reddy, 1987; Del Serrone *et al.* 1987). In the present study, an inoculum concentration of $5x10^5$ conidia per mL was considered optimal for inoculation experiments because the response of the highly susceptible cv. Desavic did not differ significantly at concentrations between $5x10^5$ and $1x10^7$ conidia per mL. This concentration has been reported previously as appropriate for artificial inoculation in the glasshouse and in field conditions (Del Serrone *et al.*, 1987; Anonymous, 1989; Trapero-Casas and Kaiser, 1992).

It has been reported that plant age is an important factor affecting disease development and susceptibility to ascochyta blight on different crops (Sattar, 1933; Hafiz, 1952; Puerta Romero, 1964; Del Serrone *et al.* 1987; Nene and Reddy, 1987; Bretag, 1991; Pedersen and Morrall, 1994). Sattar (1933) , Reddy and Singh (1984) and Singh and Reddy (1993) reported that older plants were more susceptible than seedlings. In the present study, 2-week-old seedlings were found to be slightly more susceptible than

older plants, in agreement with the findings of Hafiz (1952) and Trapero-Casas and Kaiser (1992). Similarly, Pedersen and Morrall (1994) reported that disease severity in lentil plants inoculated with *A. lentis* at the podding stage showed lower disease severity than plants inoculated at the seedling stage. These authors suggested that the resistance was related to the age of the tissue and that newly developed leaves were more susceptible to the pathogen than older leaves. A similar phenomenon has been reported in rice blast disease (Roumen *et al.*, 1992). Likewise, in chickpea, the presence of young leaves may make the seedling stage more susceptible to *A. rabiei* than the older plants.

Temperature and moisture are important factors determining disease severity. While slight symptoms of ascochyta blight did develop in inoculated plants incubated at 10-25° C without a wetness period, exposure to wet conditions for more than 3 to 6 h promoted disease development. In the present study, the optimum conditions for ascochyta blight on cv. Desavic were 20 °C and a 48-72 h wetness period, and disease severity was less at temperatures above or below 20 °C. These results are in agreement with those reported by Luthra *et al.* (1935), Weltzein and Kaack (1984), Nene and Reddy (1987), and Trapero-Casas and Kaiser (1992), and contradict those of Chauhan and Sinha (1973), who reported that there was no infection at 10 °C and 30 °C and a minimum 60-h wetness period was required for blight disease development at the optimum temperature of 20 °C. These authors found a wetness period of at least 144 h was conducive for disease development. In addition to temperature and wetness periods, the chickpea cultivar, microclimate and the isolates of *A. rabiei* affect disease development. It has been reported that 24 h was the minimum wetness period required to produce 100% disease in a susceptible cultivar whereas the same level of disease in the resistant cultivar needed a 96 h wetness period (Hafiz, 1952; Anonymous, 1989).

The results regarding the effect of interrupted wetness period on disease development in the present study are in partial agreement with those of Trapero-Casas and Kaiser

(1992), who found that, for 2-week-old seedlings, approx. 20°C and inoculum of $2x10^5$ conidia per mL were optimal for disease development in controlled environmental conditions, and that increasing the wetness period above 6 h resulted in increased disease severity. In the present study, a few infections developed on inoculated plants not exposed to a wetness period. A dry period of 6 h immediately after inoculation or after 6-h of wetness had no effect on disease severity, as disease following 48 h of wetness was the same if no dry period was imposed. However, dry periods of ≥ 12 h did reduce disease severity when imposed either immediately after inoculation or after a 6-h wetness period. Disease severity decreased as the length of the dry period increased. However, disease severity of 20-25% after 96-h dry periods demonstrated that ascochyta blight can develop in alternating wet and dry conditions, as are likely to occur in the field.

In the field experiment in 1996, disease severity increased gradually during the first 4 weeks of assessment (i.e. from 3 to 7 weeks after placing the inoculum in the buffer rows). The average maximum weekly temperatures recorded during the trial, ranging from 12-24 °C, were close to the optimum observed in trials in controlled conditions and rainfall and relative humidity were adequate for disease. These results are in agreement to those obtained in field conditions by Weltzein and Kaack (1984).

In 1997, disease severity increased more sharply with time than in 1996, especially on the highly susceptible Desavic. The trial was planted 5 days later than in 1996, which may have contributed to the increased disease severity compared to 1996, however, temperatures were similar to those recorded in 1996. Heavy rain one week after the inoculum was introduced probably promoted the rapid spread of ascochyta blight from the buffer rows to the trial plots, facilitating sporulation of *A. rabiei* and the dispersal of conidia, hence increasing the amount of inoculum available to the trial plots.

In 1998, disease severity increased fairly steadily over the 12-week period, but was low compared to 1996 and 1997. The inoculum was introduced 11-12 days later, relative to

sowing, in 1998 than in 1996 and 1997. Temperatures were similar to those recorded in 1996 and 1997. Rainfall in the weeks after placing the inoculum in the buffer rows was lower in 1998 than in the previous two years, and this may have restricted the spread of the disease from the source to the buffer rows and then into the test plots. Also, in 1998, the site was heavily infested by wild mustard, and these weeds may have impeded the dispersal of inoculum to the trial plots.

It was observed that disease on the susceptible Desavic was severe in each of the three trials, ranging from 75 to 100% and that in none of the 3 years did Desavic produce any harvestable chickpeas. Disease on Barwon, Kaniva and Tyson was intermediate in all years, ranging from 55 to 90, 40 to 80 and 45 to 70%, respectively. Dooen was the least diseased in all 3 years, with maximum disease severity 32% in the drier years (1996, 98) and 55% in the wetter year (1997).

Direct comparison between the inoculation studies in controlled conditions and in the field reported here are confounded by the fact that multiple infection cycles are likely to occur in the field whereas only one infection cycle occurs in controlled conditions. However, this study showed that susceptible cultivars are likely to suffer considerable disease in most years provided inoculum is present.

CHAPTER 7

HOST RANGE STUDIES

7.1 INTRODUCTION

There are contradictory reports on the host range of *A. rabiei* (see section 2.6.1). Sprague (1930), Nene (1980) and Tripathi *et al.* (1987) reported that *A. rabiei* infected only chickpea, whereas Kaiser (1973) and Nene and Reddy (1987) described infection, but with atypical symptoms, on cowpea and common bean. However, Kaiser (1991) later reported the development of pycnidia of *A. rabiei* in necrotic tissues of alfalfa and white sweet clover inoculated with *A. rabiei* in glasshouse studies.

Until recently, the host range of *A. rabiei* under field conditions was thought to be restricted to chickpea. However, Kaiser (1992) found that the leaves of nine plant species, including alfalfa (*Medicago sativa* L.), henbit (*Lamium amplexicaula* L.), pea (*Pisum sativum* L.) and black nightshade (*Solanum nigrum* L.) were infected with *A. rabiei* in the field in Idaho, USA. This means that the pathogen has the capability to colonise several plant species other than chickpea and to remain alive on them. As *A. rabiei* has only recently been recorded in commercial crops in Australia, nothing is known about the host range of Australian isolates of the pathogen. The studies reported in this chapter were initiated to explore the possibility of multiplication and survival of *A. rabiei* on plant species other than chickpea.

7.2 MATERIALS AND METHODS

7.2.1 Plant production

Twenty nine plant species were tested as possible hosts for *A. rabiei* (Table 1). Plants were grown in 10-cm plastic pots containing pasteurised University of California (UC) potting mixture (Baker, 1957) in a glasshouse at 20 ± 2°C. There were four seedlings per pot for large seeded species and 10 seedlings per pot for small seeded species.

7.2.2 Inoculation with *A. rabiei*

A suspension of conidia of isolate DAR 71767 was prepared from 15-day-old cultures grown on 1/4 PDA as described in section 3.4. The suspension was adjusted to $5x10^5$ conidia/mL and 0.25% (v/v) Tween-20 was added. Inoculum was misted until run-off onto 2-week-old plants of each species. Control plants were treated with SDW. Inoculated and control plants were maintained at 20 ± 5°C in a humidity chamber for 72 h following inoculation then returned to the same glasshouse. There were four replicate pots per treatment. Disease symptoms were evaluated 21 days later.

7.2.3 Re-isolation of the pathogen

Plant tissues with symptoms of ascochyta blight were surface sterilised with domestic bleach solution (0.1% available chlorine) for 1 minute, rinsed three times with SDW, dried on sterile filter papers, placed on 2% water agar (Difco) in Petri dishes and incubated as described in section 3.3.2. Tips of hyphae growing from symptomatic tissue were transferred to 1/4 PDA. Resulting cultures were compared with the original isolate for colony morphology, growth rate, microscopic appearance and pathogenicity to chickpea, as above.

7.3 RESULTS

A. rabiei caused symptoms of disease only on chickpea (Table 1, Fig. 1A) and four of the six cultivars of common bean (*Phaseolus vulgaris* L.), Brown-boy, CH-190-7D, Cran-34 and Rain-bird. Symptoms appeared on chickpea after 4 to 5 days and on common bean after 7 to 8 days. The first symptoms observed on common bean were small, tan to reddish-brown scattered lesions on the leaves and petioles (Fig. 1B). With time, lesions enlarged, coalesced and bore pycnidia, necrosis appeared at the leaf margins, and leaves finally abscised (Fig. 2). Symptoms on Cran-34 were more severe than were those on the other susceptible common bean cultivars. *A. rabiei* was re-isolated from the infected tissues of all plants sampled and was identical with the parent culture. Cultures re-isolated from common bean were pathogenic to chickpea and caused symptoms typical of ascochyta blight.

7.4 DISCUSSION

The observation that DAR 71767 infected and caused disease of common bean, contrasts with previous reports that chickpea is the only host of *A. rabiei* (Nene, 1980) but supports the finding of a more extended host range by Kaiser (1973, 1991). Previously, Sprague (1930) failed to infect common bean (French bean) with an isolate from Bulgaria. In common with Sprague (1930), however, the lentil and pea cultivars used in this study were not infected.

In the present study, the pathogen produced spreading lesions and pycnidia on four varieties of common bean, in contrast to the report by Nene and Reddy (1987), who obtained only limited lesions without pycnidia, and Kaiser (1973) who obtained small lesions on this plant species.

A. rabiei DAR 71767 produced pycnidia on the four varieties of common bean infected and, therefore, is capable of producing additional inoculum on this host. It may also survive on this host in the absence of chickpea. The observation that common bean is an alternative host presents a minor threat to chickpea production in most regions of Australia but could be important in areas where these crops are grown together. Further studies, involving more isolates and a wide range of plant species and varieties, are required to provide additional information on host range and thereby identify appropriate crops suitable for use in rotation with chickpea.

Table 7.1. Infection of plant species tested as possible hosts of *A. rabiei*

Botanical name	Common name	Cultivar	Disease
Brassicaceae			
Brassica napus L.	Canola	Westar	–
Graminae			
Hordeum vulgare L.	Barley	Schooner	–
Oryza sativa L.	Rice	Amaroo	–
X.triticosecale Wittmack	Triticale	Treat	–
Triticum aestivum L.	Wheat	Tatiara	–
Zea mays L.	Maize	Unknown	–
Leguminosae			
Arachis hypogea L.	Peanut	Unknown	–
Cicer arietinum L.	Chickpea	Desavic	+
Lathyrus cicera L.	Grasspea	Unknown	–
Lathyrus sativus L.	Grasspea	Unknown	–
Lens culinaris Medik.	Lentil	Invincible	–
Lupinus angustifolius L.	Lupin	Unknown	–
Medicago polymorpha L.	Medic	Santiago	–
Phaseolus vulgaris L.	Common bean	Actolac	–
"	"	Black-jack	–
"	"	Brown-boy	+
"	"	CH-190-7D	+
"	"	Cran-34	+
"	"	Rain-bird	+
Pisum sativum L.	Pea	Laura	–
Trifolium hirtum L.	Clover	Haykon	–

Table 7.1 Continued

Botanical name	Common name	Cultivar	Disease
Leguminosae			
Trifolium subterraneum L.	Sub clover	Clare	_
Vigna angularis (Willd.) Weight	Adzuki bean	Unknown	_
Vicia faba L.	Faba bean	Icarus	_
Vicia sativa L.	Vetch	Unknown	_
Vigna sinensis (L.) Hassk.	Cowpea	Unknown	_
Umbelliferae			
Anethum graveolens L.	Dill	Unknown	_
Apium graveolens L.	Celery	Unknown	_
Coriandrum sativum L.	Coriander	Unknown	_
Cuminum cyminum L.	Cumin	Unknown	_
Daucus carota L.	Carrot	Unknown	_
Foeniculum vulgare Mill.	Fennel	Unknown	_
Petroselinum crispum (Miller) Hill.	Parsley	Unknown	_
Pimpinella anisum L.	Aniseed	Unknown	_

facing page

Figure 7.1. Symptoms of ascochyta infection on chickpea and common bean Cran-34. **A** Chickpea, 10 days after inoculation, showing lesions with pycnidia (arrow). **B** Cran-34, 10 days after inoculation, showing lesions with pycnidia (arrow).


A


B

facing page

Figure 7.2. Cran-34, 21 days after inoculation, showing lesions (arrow) and collapse of tissue on infected leaves.

CHAPTER 8

MODE OF INFECTION OF CHICKPEA BY AUSTRALIAN ISOLATES OF *ASCOCHYTA RABIEI*

8.1 INTRODUCTION

To initiate disease a fungus must breach the physical and chemical barriers of the host plant to gain access to host nutrients, and subsequently establish a parasitic relationship. Penetration may be direct through the plant surface, through natural openings or through wounds caused by physical or chemical means, the most important being penetration through stomata (Lucas, 1998).

There appears to be some variability in the processes of infection of chickpea by *A. rabiei* and subsequent development of ascochyta blight, based on reports of research conducted in Germany, India and Italy (Pandey *et al.*, 1987; Hohl *et al.*, 1990; Angelini *et al.*, 1993). Pandey *et al.* (1987) reported that the pathogen invaded between epidermal cells and between the stomatal guard and subsidiary cells of stem tissues. Subsequently, in Germany, Hohl *et al.* (1990) and, in Italy, Angelini *et al.* (1993) reported that the pathogen penetrated directly through the cuticle and between the epidermal cells, and not through stomata. In contrast, *A. pisi* penetrates pea through stomata as well as through the epidermis (Heath and Wood, 1969). Hohl *et al.* (1990) and Angelini *et al.* (1993) used local isolates of *A. rabiei* and examined both resistant and susceptible cultivars, whereas Pandey *et al.* (1987) used a susceptible cultivar only for their studies.

In view of the variation reported in the infection process, the early stages of the interaction between chickpea and Australian isolates of *A. rabiei* were investigated by confocal microscopy and light microscopy, in order to determine the mode of infection.

8.2 MATERIALS AND METHODS

8.2.1 Plant inoculation

Plants of Desavic (susceptible) and Dooen (moderately resistant) were inoculated with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768 as described in section 3.5. and kept in the glasshouse as described in section 3.2. Leaves were harvested 12 to 96 h after inoculation and prepared for microscopic examination.

8.2.2 Solutions for staining and clearing leaves

Leaf clearing solution

Acetic acid	500 mL
95% Ethanol	500 mL

Acid fuchsin

Lactic acid (Sigma)	875 mL
Glycerol	63 mL
ddH2O	63 mL
Acid fuchsin (Sigma)	0.1g

Stored at room temperature (approx. 23°C) in the dark.

8.2.3 Clearing and staining of inoculated chickpea leaves

Inoculated leaves were boiled for 3 minutes in leaf clearing solution to remove the chlorophyll. Cleared leaves were stained in acid fuchsin, a fluorescent histochemical stain, for 3 h then placed in glycerol for 1 h on an orbital shaker to remove the stain from the plant material. Stained leaves were then mounted in 50% glycerol on microscope slides and the cover slips sealed with clear nail polish. These were observed using light and confocal microscopy.

8.2.4 Preliminary light microscopic examination of cleared and stained leaves

Inoculated leaves harvested 12, 24, 30 and 36 h after inoculation of both Dooen and Desavic, and stained with acid fuchsin were observed for spore germination, germ tube development and formation of appressoria by using a Leitz Orthoplan light microscope.

8.2.5 Confocal microscopic examination of intact leaves

Leaf samples harvested 36, 48, 72 and 96 h after inoculation and stained with acid fuchsin, as described above, were analysed using the Confocal Laser Imaging System Bio-Rad MRC-1000 attached to a Nikon Diaphot-300 inverted microscope utilising Krypton/Argon laser. A x40 water immersion lens (Numerical Aperture = 1.15) was used. Plant tissue was visualised with a blue excitation filter (488/10 nm) and a green emission filter (522/32) was used. The fungi were visualised with a yellow excitation filter (568/10 nm) and a red emission filter (605/32 nm). The 3D reconstruction images of fungi on and in plant tissue were created using Image Volume software (Minnesota Datametrics Corporation) and a Silicon Graphics computer, under the direction of Dr. P. Kolesik, manager, Confocal Facility, Department of Horticulture, Viticulture and Oenology, The University of Adelaide.

8.2.6 Light microscopic examination of leaf sections

8.2.6.1 Glycol-methacrylate embedding and sectioning

Inoculated leaves were harvested 36, 48, 72 and 96 h after inoculation and cut into slices of approx. 1mm wide at right angles to the mid-rib. Leaf slices were dehydrated through an alcohol series: methoxy ethanol, ethanol, propanol and butanol, then infiltrated for 2 h in 1:1 butanol:glycol methacrylate (GMA, 2-hydroxyethyl methacrylate, Sigma H-8633). The GMA monomer was prepared by mixing 93 mL GMA with 7 mL polyethylene glycol 400 and 0.6g benzoyl peroxide at room temperature for 2 h (O'Brien and McCully, 1981). Infiltration was followed by two successive changes of 100% GMA, each for 48 h, then the leaf material was embedded in gelatine capsules (No. 2 or No. 00, Park-Davis, Sydney) in GMA. The GMA was polymerised at 60°C for 48 h (O'Brien and McCully, 1981).

GMA blocks were filed back to remove excess embedding material and serial transverse sections (TS), 4 µm thick, were made on a Reichert Jung 2050 Supercut Microtome using glass knives. Sections were collected with forceps and needles, floated in a water droplet on a microscope slide and dried overnight at 40°C to 60°C.

8.2.6.2 PAS-TBO staining

Sections were routinely stained with periodic acid-Schiff's reagent (PAS) and counterstained with toluidine blue O (TBO) as described by McCully and O'Brien (1981). This combination is a good general stain for plant tissues, staining polysaccharides, polysulphates, polycarboxylates and pectic acid red or pink, while polyphenols and lignin are stained green, blue or an aqua colour. Sections on slides were submerged in a saturated solution of 2,4-dinitrophenylhydrazide for 30 minutes in 1% periodic acid (BDH Laboratory Supplies) then rinsed with running water for 5

minutes. The sections were exposed to commercial Schiff's reagent (BDH Laboratory Supplies) for 1 h to stain the tissues. The slides were submerged in three successive changes of metabisulphite solution, each for 2 to 3 minutes. Following a brief rinse in RO water for 2-3 minutes, 0.05% Toluidine Blue O (Aldrich) in benzoate buffer pH 4.5 was used to stain the sections for 5 minutes. Excess TBO was removed by rinsing in running water until the embedding material surrounding the sections was clear. Dried slides were mounted using Micromount mounting medium (Surgipath) and a glass cover slip. Sections were viewed using a Leitz Orthoplan microscope and photographed using black and white film (TMX 100/36).

8.3 RESULTS

8.3.1 Preliminary light microscopic examination of cleared and stained leaves

Germination of conidia was observed on the leaf surface between 12 to 24 h after inoculation. Subsequently, germ tube development, appressorium formation and penetration of hyphae through and between the epidermal cells, through guard cells and stomata were observed between 30 to 36 h. Some conidia produced more than one germ tube. No differences were observed with respect to germination of conidia of DAR 71767 and DAR 71768, development of hyphae, formation of appressoria on, and penetration of, Desavic and Dooen.

8.3.2 Confocal microscopic examination of intact leaves

In cleared leaf material stained with acid fuchsin, hyphae of *A. rabiei* were red in colour and the host tissues were green. There were no differences with respect to germination of conidia of DAR 71767 and DAR 71768, development of hyphae, formation of appressoria on, and penetration of, Desavic and Dooen. Therefore, the images presented in Figures 8.1.1 to Fig. 8.1.6 were obtained from the susceptible cultivar, Desavic, and are optical sections progressing from the surface of the leaf (A) to tissue with the leaf (B, C, D).

Leaf material examined 36 h after inoculation (Fig. 8.1.1 A) shows conidia with germ tubes and appressoria on the leaf surface. The optical section at 3 µm below the surface shows that the hypha has penetrated through the guard cell (Fig. 8.1.1 B) and is still visible 10 µm below the surface (Fig. 8.1.1 C). Penetration through a stoma is shown in Fig. 8.1.2 A, an image taken 48 h after inoculation, and subsequent growth of the hypha up to 17 µm below the epidermis is shown in Fig. 8.1.2 B-D. In Fig. 8.1.1.3 A, obtained 96 h after inoculation, two hyphae (a and b) are shown to have penetrated between the epidermal cells and direct through the epidermal cells, respectively. The same hyphae are visible up to 41 µm below the surface (Fig. 8.1.1.3 B-D). In Fig. 8.1.1.4 A, obtained 96 h after inoculation two hyphae (a and b) are shown to have penetrated between the epidermal cells and through stoma, respectively. The same hyphae are visible up to 18 µm below the surface (Fig. 8.1.1.4 B-D). Figure 8.2 shows a 3-D rotation of the optical sections shown in Fig. 8.1.4 and was produced using a silicon graphics computer. This image highlights the penetration of a stoma and direct intercellular penetration of the epidermis.

8.3.4 Light microscopic examination of whole leaves

Representative transverse sections through infected leaves of the susceptible cultivar Desavic 96 h after inoculation show hyphae which have penetrated directly through the cuticle and stoma (Fig. 8.3 A), hyphae which have penetrated between epidermal cells, and colonisation within the mesophyll tissue (Fig. 8.3 A and B).

8.4 DISCUSSION

The response of Desavic and Dooen, the susceptible and moderately resistant cultivars, observed by conventional light and confocal microscopy, was similar with respect to germination of conidia, germ tube development and penetration during the early stages of infection of chickpea by *A. rabiei* isolates DAR 71767 and DAR 71768. There was good agreement between observations made using conventional light microscopy and confocal microscopy, but the latter provided more detailed information. Acid fuchsin was found to be an appropriate stain for observing interactions between chickpea tissues and *A. rabiei*, and allowed clear visualisation by confocal microscopy to 50 µm below the surface. However, when observations were attempted beyond 50 µm below the surface, the host tissues lost contrast, in that the green colour was no longer visible.

Germination of conidia was first observed 12 to 24 h after inoculation, which agrees with the results of Pandey *et al.* (1987). Penetration occurred between 30 to 36 h after inoculation, observations which contradict those of Pandey *et al.* (1987), who observed penetration after 24 hours. This apparent delay may reflect differences between the isolates, host cultivars and environmental conditions used in the two studies.

The hyphae developed appressoria at their tips, in agreement with the observation by Hohl *et al.* (1990) but contradicting that of Pandey *et al.* (1987), who found that there was no formation of appressoria. In the present study it was observed that penetration

occurred direct through epidermal cells, between the epidermal cells, between the guard cells and through the stomata. While previous studies have demonstrated that penetration occurred directly through the cuticle (Hohl *et al.*, 1990) and epidermal cells, between the epidermal cells (Angelini *et al.*, 1993) and through the juncture of stomatal guard and subsidiary cells (Pandey *et al.*, 1987), this is the first report of penetration through stomata. However, *A. pisi* is known to penetrate the stomata of peas (Heath and Wood, 1969). The present study, therefore, has shown that *A. rabiei* can penetrate all surface structures of the chickpea leaf.

Interestingly, the early stages of infection, at least in the first 96 h after inoculation, appeared to be similar in cv. Desavic and cv. Dooen. These results are in agreement with the findings of Angelini *et al.* (1991), who found germination of conidia, development of hyphae and formation of appressoria were similar in both resistant (Sultano) and susceptible (Calia) chickpea cultivars. Evidently, the resistance expressed by Dooen is not based on restricting penetration and early invasion by the pathogen. Rather resistance may be due to the production of phytoalexins and phytotoxins by the plants and the pathogen, respectively.

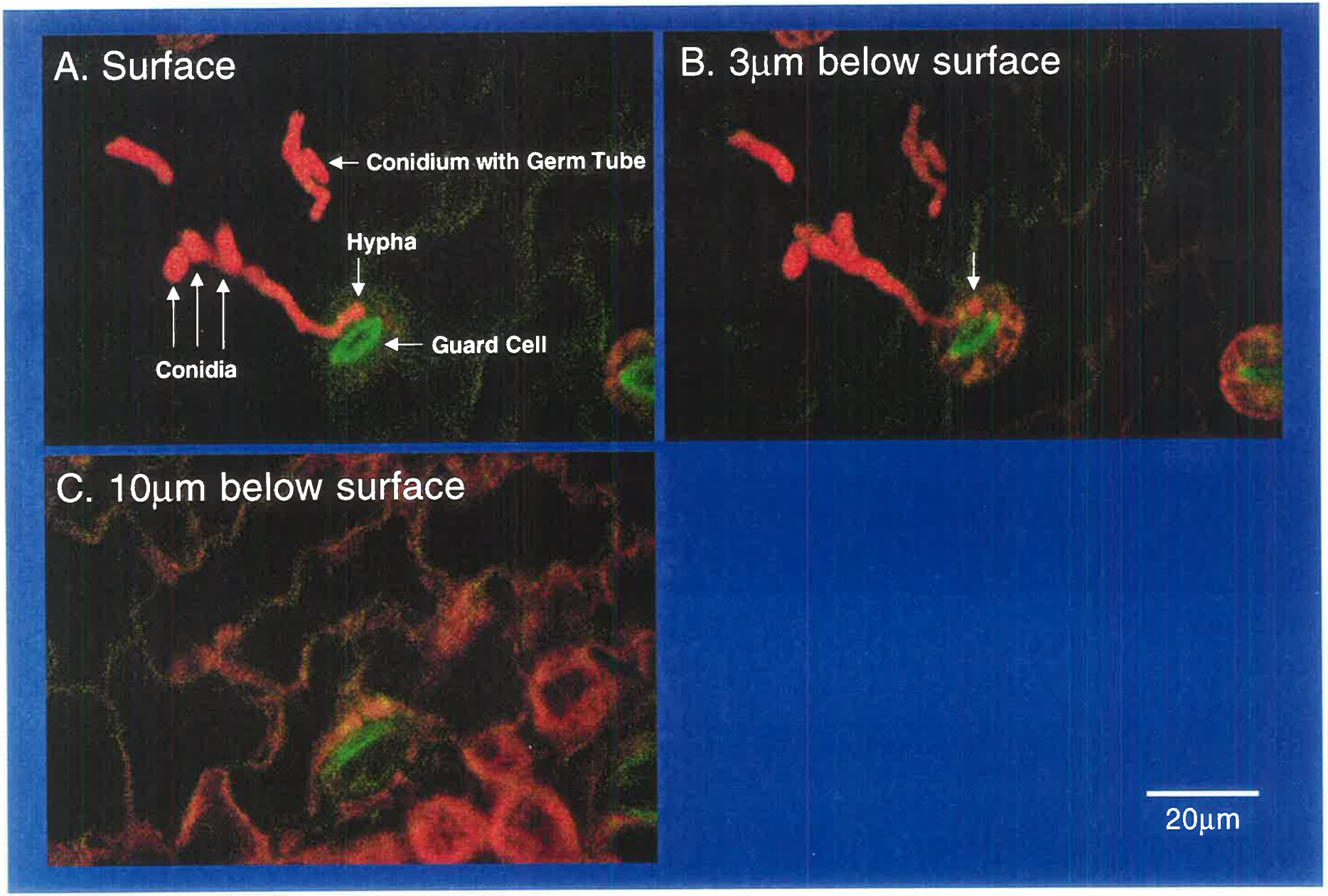
Figure 8.1 Early stages of infection of chickpea cv Desavic by *A. rabiei*, viewed by confocal microscopy. Fifteen-day-old seedlings were inoculated with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768. Leaves were sampled 36, 48, 72 and 96 h after inoculation, cleared and stained with acid fuchsin; hyphae and plant tissues are red and green, respectively.

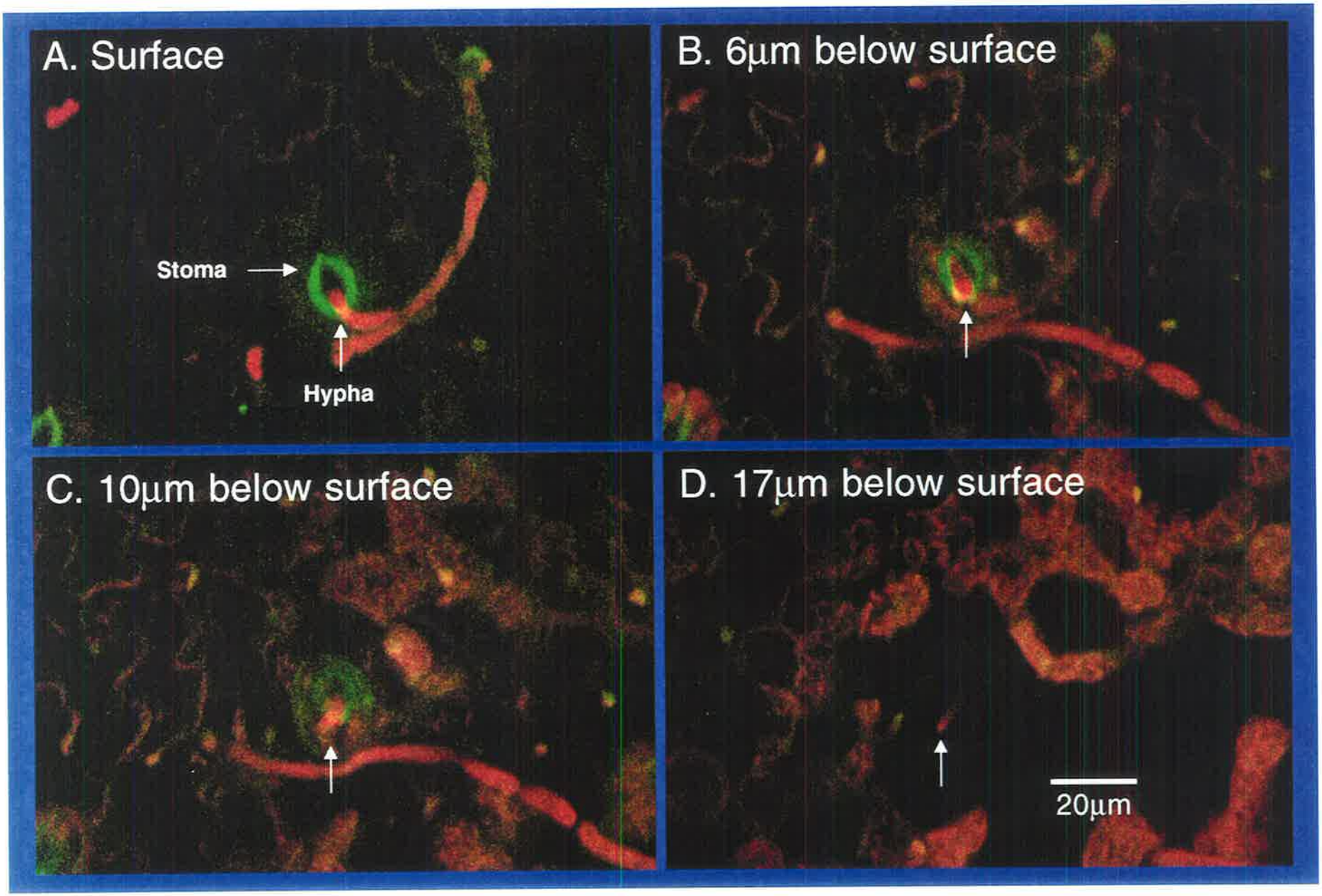
Figure 8.1.1 Confocal images at 36 h after inoculation. A to C are optical sections from the surface to 10 µm below. Germinated conidia and a hypha penetrating through stomatal guard cell are seen on the surface (A) (Fig. 8.1.1A). Penetration of the guard cell is shown in Fig. 8.1.1.B. The hyphae can be seen up to 10 µm beneath the leaf surface (Fig. 8.1.1 C). Bar = 20 µm.

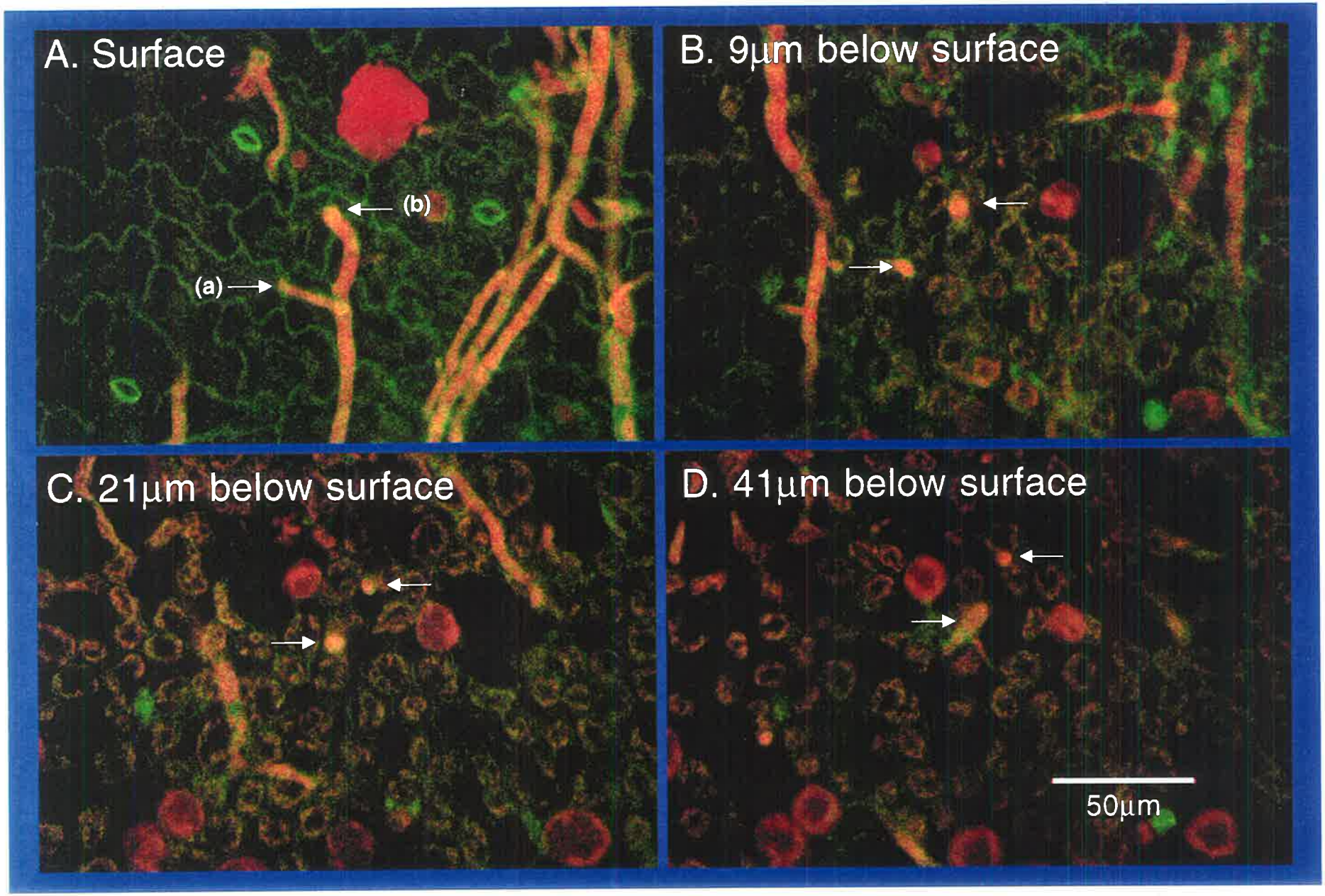
Figure 8.1.2 Confocal images at 48 h after inoculation. A to D are optical sections from the surface to 17 µm below. Hyphae penetrating through stoma and between the epidermal cells are shown (Fig. 8.1.2A). Hyphae can be seen up to 17 µm beneath the leaf surface (Figs. 8.1.2B-D). Bar = 20 µm.

Figure 8.1.3 Confocal images at 96 after inoculation. A to D are optical sections from the surface to 41 µm below. Hyphae (a and b) penetrating direct through an epidermal cell and between the epidermal cells (Fig. 8.1.3 A), and up to 41 µm beneath the leaf surface (Fig. 8.1.3 B-D). Bar = 50 µm.

Figure 8.1.4 Confocal images at 96 h after inoculation. A to D are optical sections from the surface to 18 µm below. Hyphae (a and b) are shown penetrating between the epidermal cells and stoma (Fig. 8.1.4 A), and can be seen up to 18 µm beneath the leaf surface (Fig. 8.1.4 B-D). Bar = 20 µm.


A. Surface
Conidium with Germ Tube
Hypha
Guard Cell
Conidia
B. 3µm below surface
C. 10µm below surface
20µm


A. Surface
Stoma
Hypha
B. 6µm below surface
C. 10µm below surface
D. 17µm below surface
20µm


A. Surface
(b)
(a)
B. 9µm below surface
C. 21µm below surface
D. 41µm below surface
50µm

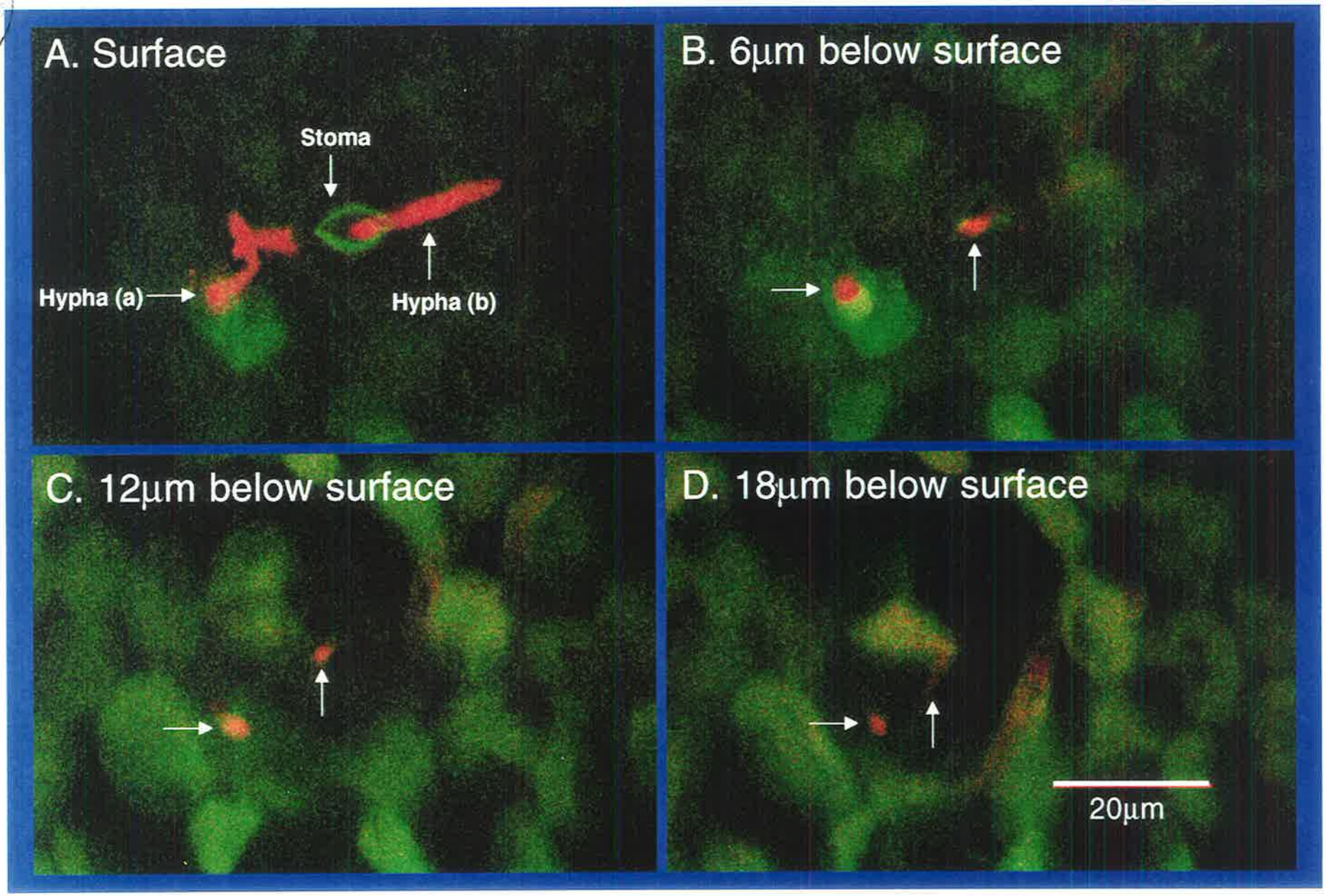

A. Surface
Stoma
Hypha (a)
Hypha (b)
B. 6µm below surface
C. 12µm below surface
D. 18µm below surface
20µm

Figure 8.2 3-D rotation of optical sections shown in Fig. 8.1.4 constructed using a silicon graphics computer. Hyphae = red; plant tissue = green. Image shows hypha (a) penetrating between the epidermal cells and hypha (b) penetrating through a stoma. Bar = 10 μm.

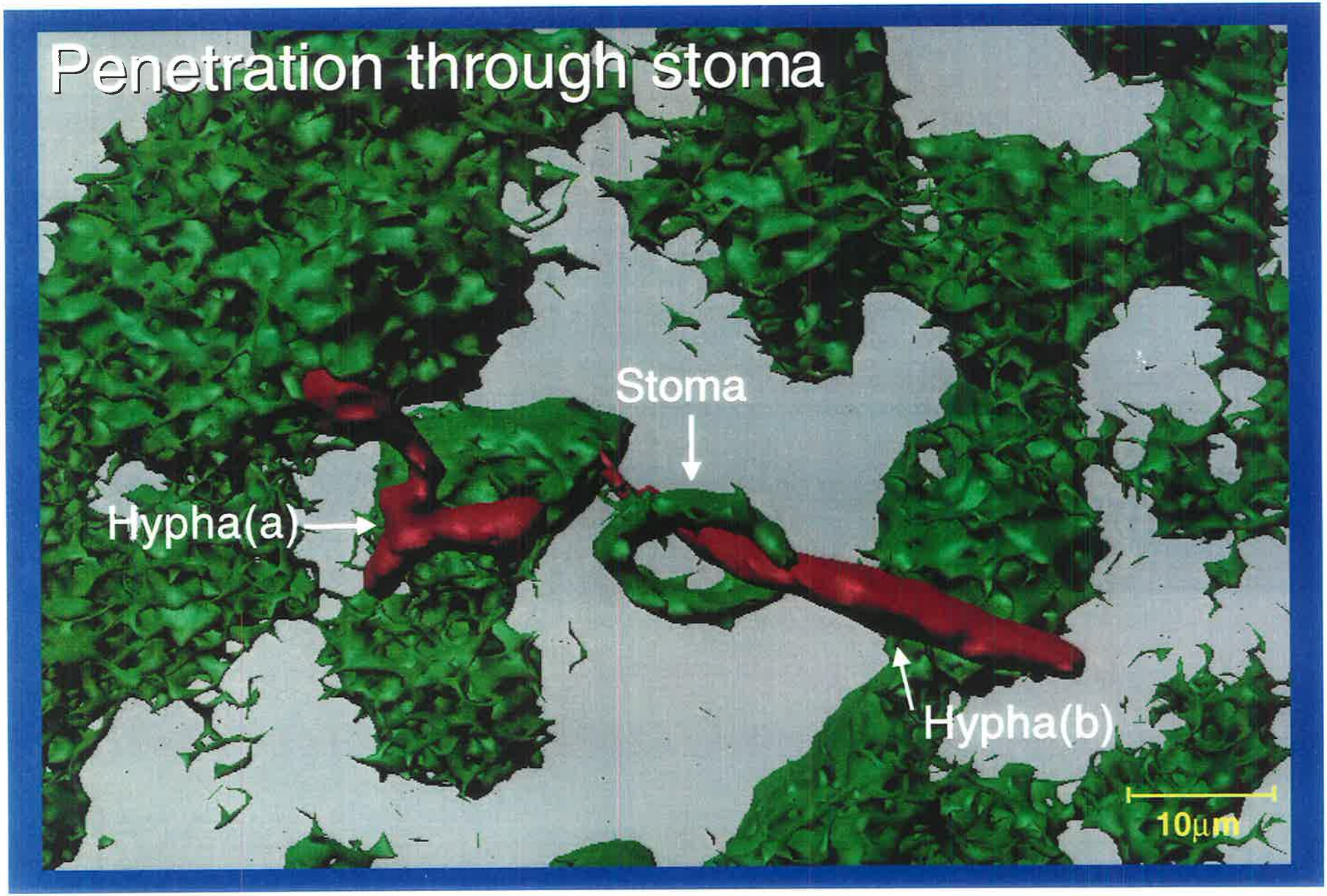
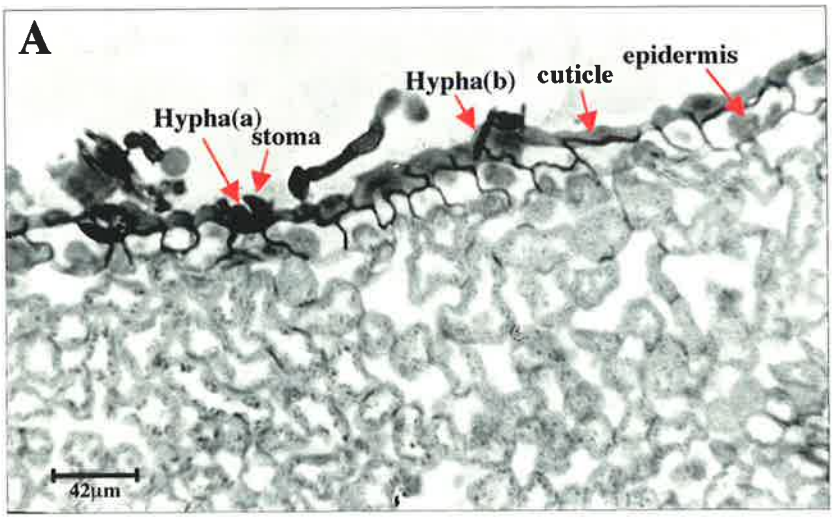
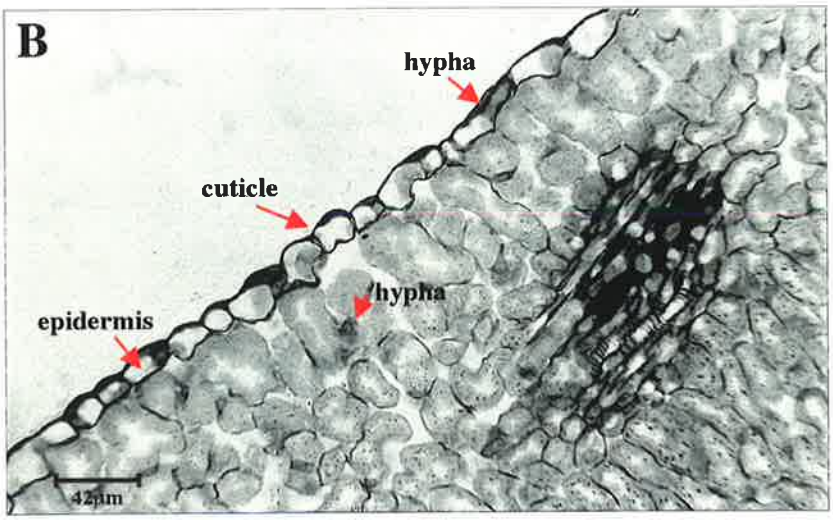
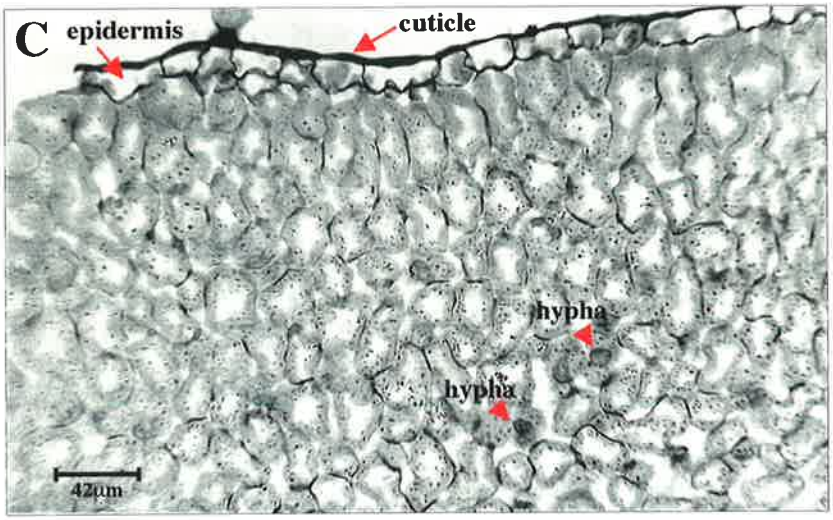

Penetration through stoma
Stoma
Hypha(a)
Hypha(b)
10μm

Figure 8.3 Early stages of infection of chickpea cv Desavic by *A. rabiei*, viewed by light microscopy. Fifteen-day-old seedlings were inoculated with a mixed conidial suspension of *A. rabiei* isolates DAR 71767 and DAR 71768. Leaves were sampled 36 and 96 h after inoculation. Leaf pieces were fixed, dehydrated, embedded in GMA and polymerised at 60°C. Transverse sections (4 μm) were cut then stained with PAS/TBO before mounting, and were viewed using a Leitz Orthoplan light microscope. Hyphae (a) penetrating through a stoma and (b) penetrating on epidermal cell 36 h after inoculation are shown in A: Hyphal growth between the epidermal cells (arrowheads) and within the mesophyll (arrows) 96 h after inoculation is shown in B and C. Bar = 42μm.


A
epidermis
Hypha(b)
cuticle
Hypha(a)
stoma
42µm


B
hypha
cuticle
hypha
epidermis
42µm


C
epidermis
cuticle
hypha
hypha
42µm

CHAPTER 9

OCCURRENCE OF SOLANAPYRONE PHYTOTOXINS IN CULTURES OF AUSTRALIAN ISOLATES OF *A. RABIEI*

9.1 INTRODUCTION

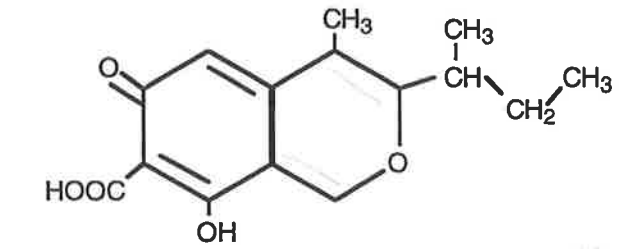
Many micro-organisms produce secondary metabolites which may be toxic to humans, animals, plants and other microbes. Examples of important fungal secondary metabolites include the aflatoxins, penicillins and many other antibiotics. Hutchinson (1913) first proposed a toxigenic hypothesis for fungal plant disease, and plant pathology research on phytotoxins from culture filtrates of fungal pathogens has recently been reviewed by Kohmoto (1992). Among the known secondary metabolites, some are responsible for symptom development and others appear to have no role in pathogenesis.

In 1964, Pringle and Scheffer proposed the concept of host-specific or host-selective toxins (HST), which has become a landmark for subsequent phytotoxin studies in plant pathology. Subsequently, microbial toxins have been the subject for many investigations concerning their role in disease development, in particular, whether the toxic metabolites are required by the causal organism to invade the host tissues and finally cause the disease or whether they enhance the aggressiveness of the invading organism (Scheffer, 1983). Pathogens having a wide host range produce non-host selective toxins (Scheffer, 1983), while pathogens with a selective or limited host range produce host-selective toxins (Chelkowski, 1995; Hesseltine *et al.*, 1971; Kohmoto, 1992; Nishimura and Kohmoto, 1983; Scheffer, 1983; Shotwell and Ellis, 1976). Correlations between sensitivity to the toxin and varietal disease reaction, led to discovery of the toxin's role in the host-specificity of the pathogen and also the expression of disease symptoms in the host (Scheffer and Livingston, 1984). In some instances, the toxin may suppress the

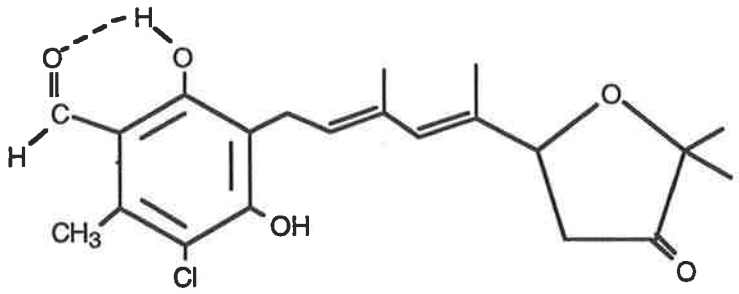
host defence mechanisms (Vidhyasekaran *et al.*, 1992). Most of the *Ascochyta* species synthesise toxins which are host-selective, such as ascochitine, ascochlorin, ascofuranone, decumbin, epoxydon and epoxydon monoacetate, produced by *Ascochyta fabae* Speg., *A. pisi* Lib., *A. viciae, A. imperfecta* Peck. and *A. chrysanthemi*, respectively (Fig. 9.1; Oku and Nakanishi, 1963; Bertini, 1957; Tamura *et al.*, 1968; Sasaki *et al.*, 1972; Suzuki *et al.*, 1970; Assante *et al.*, 1981).

Ascochyta blight causes severe damage to the host plant wherever the chickpea is grown (Nene, 1982). Extensive cellular disintegration takes place in advance of the invading fungal hyphae, thereby inducing necrotic symptoms in leaves. These observations suggest that fungal toxins are involved in the onset of pathogenesis (Pandey *et al.*, 1987; Hohl *et al.*, 1990). The phytotoxins which have been found in culture filtrates and spore germination fluids of *A. rabiei* have been identified as solanapyrones A, B and C; their molecular structures are shown in Fig. 9.2 (Alam *et al.*, 1989; Hohl *et al.*, 1991; Kaur, 1995; Latif *et al.*, 1993).

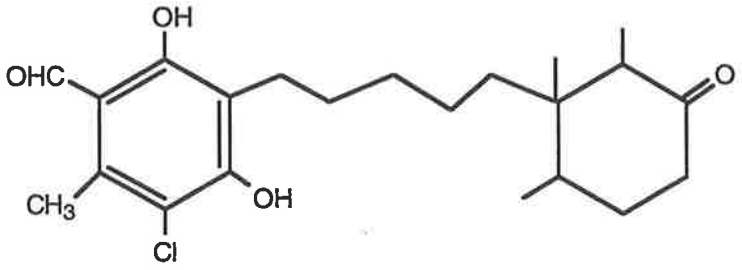
The pathogenic capacity of many fungi is strictly correlated with their ability to produce phytotoxins affecting only the host genotypes that are susceptible to the pathogens (Yoder, 1980; Strobel, 1982). Keeping in mind that *A. rabiei* isolates consistently differ in their ability to form the solanapyrone toxins A, B and C (Alam *et al.*, 1989; Porta-Puglia *et al.*, 1997), the present studies were undertaken with the objective of examining whether three Australian isolates, DAR 71767, DAR 71768 and 215/91 (two aggressive and one intermediate), produced the same three solanapyrone toxins.



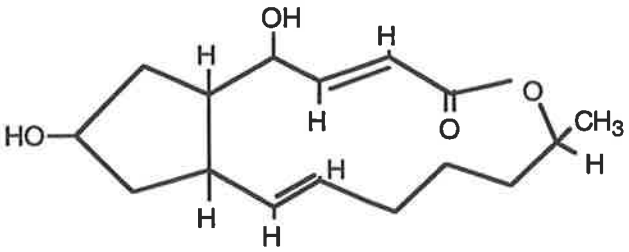
Ascochitine
A. pisi
A. fabae



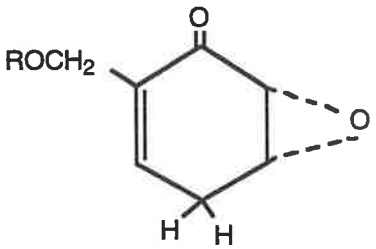
Ascofuranone
A. viciae



Ascochlorin
A. viciae

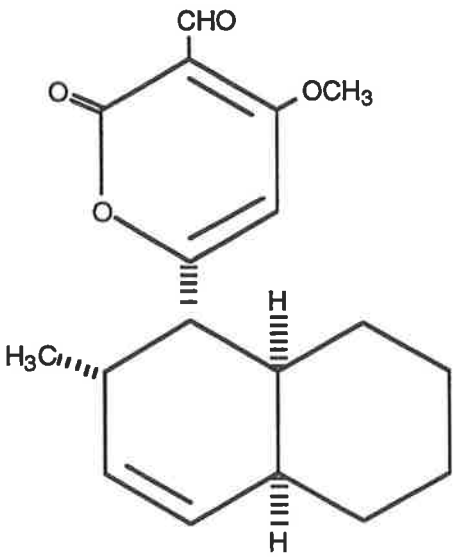


Decumbin
A. imperfecta

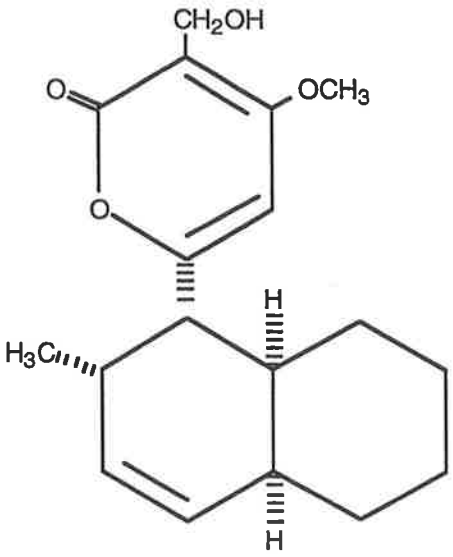


R= H (+)-Epoxydon
R= Ac its monoacetate
A. chrysanthemi

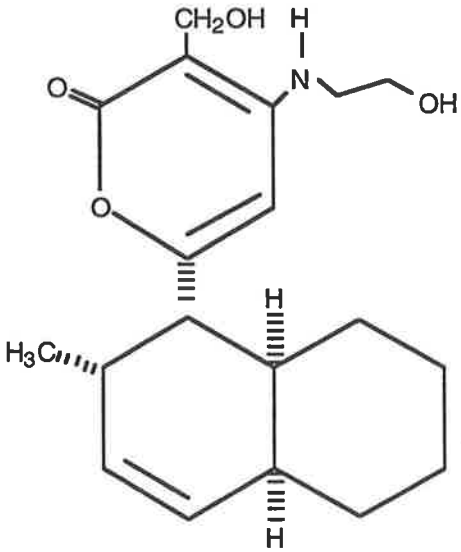
Fig. 9.1 Structures of toxins produced by *Ascochyta* species



Solanapyrone A ($C_{18}H_{22}O_4$)



Solanapyrone B ($C_{18}H_{24}O_4$)



Solanapyrone C ($C_{19}H_{25}NO_4$)

Fig. 9.2 Structures of solanapyrones A, B and C, produced by *A. rabiei*

9.2 MATERIALS AND METHODS

9.2.1 Culture of *A. rabiei*

Two aggressive isolates (DAR 71767 and DAR 71768) and one intermediate isolate (215/91) of *A. rabiei* were used for the detection and isolation of the host-selective solanapyrones. The isolates were maintained on 1/4 PDA as previously described (section 3.3.2), and grown in Czapek Dox liquid medium (Oxoid) supplemented with chickpea seed extract to promote toxin production (Alam *et al.*, 1989). Chickpea extract was prepared by rinsing 60g of seeds of cv. Desavic (susceptible to *A. rabiei*) with distilled water and boiling them for 30 minutes in 500 mL distilled water. After removal of the seed, Czapek Dox liquid medium was added to make the volume up to 1L. The medium was dispensed into 250 mL conical flasks, 100 mL per flask, autoclaved and inoculated with 1mL of spore suspension ($5x10^5$ conidia per mL) prepared as described in section 3.4. The flasks were incubated without shaking at 20°C in a lighted incubator as described in section 4.2.7. In a preliminary experiment, three flasks of DAR 71767 were prepared and incubated at 20°C in a lighted incubator, as described above, for 14 days. In subsequent experiments, cultures of all three isolates were incubated for 21 days prior to harvesting of the culture filtrate.

9.2.2 Reference samples

Purified samples of solanapyrones A, B and C, obtained from *A. rabiei*, were kindly provided by Dr. R.N. Strange, Department of Biology, University College London, U.K. The samples were used as reference standards in analysis of toxic compounds in culture filtrates of Australian isolates of *A. rabiei*.

9.2.3 Extraction and separation of toxic compounds

Preliminary filtration was done by passing each liquid culture, separately, through four layers of sterile muslin cloth followed by filtration through Whatman No. 541 filter paper. The culture filtrates were then serially extracted with chloroform according to Kaur (1995). Extraction was repeated three times for each replicate and then extracts were pooled for each isolate. The pooled extracts of three replicates were concentrated to dryness in a rotary vacuum evaporator at 40°C and re-dissolved in 10 mL ethanol (Kaur (1995). The extracts were separated by thin layer chromatography (TLC). The reference standards and the extracts obtained from culture filtrates of each isolate, in ethanol, were separated as spots or bands on 20x20 cm TLC-Silica Gel F_{254} (Merk KGaA, Germany) plates and developed in the solvent system: n-Hexane : Ethyl acetate 75: 25 (v/v).

The TLC plates were developed repeatedly (three to six times) to achieve clear separations of UV-absorbing components. The absorbance, or fluorescence, of UV-absorbing bands was visualised using a portable UV-lamp at 254 nm or 366 nm wavelength.

9.2.3.1 Purification of solanapyrone A

Because the reference standard of solanapyrone A was found to be impure, solanapyrone A was purified by preparative TLC. This confirmed that the spot with the major intensity (Fig. 9.3, 18.5 mm) exhibited the expected molecular ion (302 m/z) for the solanapyrone A mass spectrum.

9.2.4 Detection and characterisation of the chemical structure-activity relationships

The biological activity exhibited by the partially purified extracts of 21-day-old cultures of *A. rabiei* was correlated with the presence of solanapyrones in isolates DAR 71767, DAR 71768 and 215/91 as determined by high performance liquid chromatography (HPLC).

9.2.4.1 Leaf bioassay

Detached leaves of 15-day-old plants of cultivars Dooen (resistant) and Desavic (susceptible), of the same age, were slightly scratched on the surface with the sharp edge of a glass capillary. Wounded leaves were placed in Petri dishes on three layers of moist, sterilised filter paper. A drop of partially purified extract of isolate DAR 71767 was applied to each wound while the control leaves were treated with SDW. After 24 h at room temperature (approx. 23°C), the necrotic area was measured as described by Kohmoto (1992).

9.2.4.2 HPLC

Reference standards and purified extracts were analysed by Mr. Jelle Lhanstein (Nucleic Acid and Protein Chemistry Unit, Department of Plant Science, University of Adelaide, using reversed phase HPLC under the following conditions:

Instrument	Hewlett-Packard Series II 1090 Liquid Chromatograph
Column	Vydac C18 protein, 250 x 4.6 mm with guard cartridge
Eluents	A) Aqueous 20% Tetrahydrofuran, 20% Methanol
	B) Aqueous 90% Acetonitrile
Flow rate	0.6 mL/minute

Gradient	Time (min.)	0	8	20	21	24	25	30
	% solvent	2	2	36	100	100	2	2
Temperature	40 °C							
Detection	Absorbance at 310 nm							
Spectra	Peak spectra between 220 and 400 nm were collected							

TLC provides for UV absorbing components such as the solanapyrones, partition chromatography using thin layers of fluorescent silica gel and is a single quantitative method for monitoring the presence or absence of the biologically active components. It is a complementary technique to the quantitative HPLC procedure, but because multiple samples can be examined simultaneously it provides a rapid screening procedure for selecting suitable samples for the quantitative HPLC procedure. HPLC is a "single run" procedure and, as with all such procedures, there is a certain amount of run-to-run variation in retention times. By using TLC in conjunction with HPLC it can be readily seen whether two samples with slightly different retention times by HPLC are indistinguishable or not by TLC.

9.2.5 Mass Spectroscopy (MS)

Partially purified extracts from 21-day-old cultures of DAR 71767, DAR 71768 and 215/91 plus standards (see above) were submitted for mass spectroscopy at the Australian Wine Research Institute, Waite Campus. Samples were examined with a Finnegan Triple Stage Quadrupole Mass Spectrometer using a heated direct insertion probe and ionised using electron impact in the positive ion mode.

9.3 RESULTS

9.3.1 TLC

No toxin was detected in 14-day old culture filtrate, therefore in subsequent experiments 21-day-old cultures were used. The relative TLC mobilities after repeated elution (6x) for the three standard samples of solanapyrones A, B and C and also the three Australian isolates (DAR 71767, DAR 71768 and 215/91) are shown in Fig. 9.3.

9.3.2 HPLC

The reverse phase HPLC retention characteristics of solanapyrones A, B and C in the reference standards and extracts from 21-day-old cultures of Australian *A. rabiei* isolates DAR 71767, DAR 71768 and 215/91 are shown in Fig. 9.4-9.7 a, b and are summarised in Table 9.1

The reference standard for solanapyrone A contained two major components (Fig. 9.4a). The UV maxima for the diode array spectra (Fig. 9.4b) for both components (retention times 11.13 and 13.05 minutes). Purification of the major, and slowest moving, component by preparative TLC yielded a sample with a molecular ion $M^{+\cdot}$ of 302.1 m/z, which is in accordance with the expected mass for solanapyrone A (reference).

The HPLC traces for the homogeneous solanapyrone B and C standards, and the extract of 21-day-old cultures of DAR 71767, are shown sequentially in Fig. 9.5a. The major component in the extract from DAR 71767 at 13.665 minutes in the solanapyrone region (11-14 minutes) was in accord with the presence of solanapyrone C (13.664 minutes). This was also confirmed by the presence of a strong $M^{+\cdot}$ of 331.1 m/z in the mass spectrum, as well as the TLC data (Fig. 9.3) and the characteristic solanapyrone C

UV data (Fig. 9.5b). There was no evidence for either solanapyrone A or B in this extract of the isolate DAR 71767.

The HPLC trace for an extract of the filtrate from 21-day-old cultures of isolate DAR 71768 is shown in Fig. 9.6a. The peak at 12.694 minutes exhibits a UV spectrum (λmax 232.0 and 325.8 nm) consistent with that of solanapyrone A. The peak at 14.2 minutes with bands at 239.8, 283.6 and 317.2 nm in the UV spectra corresponds to solanapyrone C (Fig 9.4b). These data for the presence of solanapyrones A and C are supported by ions at 302.1 and 331.1 in the mass spectra and by the TLC profile (Fig 9.3).

Fig. 9.7a shows the much weaker response scale for the weakly pathogenic 215/91 isolate. On the UV spectra (Fig. 9.7b), only the peak at 12.809 minutes was consistent with the presence of the second component (13.05 minutes) in the reference solanapyrone A (Fig. 9.4a). There was a weakly detectable spot in the TLC corresponding to solanapyrone C, but the weak shoulder at the putative retention time of 13.2 minutes for solanapyrone C was too small for diode array data to be collected.

9.3.3 Leaf bioassay

Necrotic symptoms were observed at and around the points of application of partially purified culture filtrate on treated detached leaves, while the controls treated with SDW remained healthy. Necrosis was more severe on leaves of cv. Desavic than on Dooen. The necrotic symptoms developed within 24 h in cv. Desavic, and covered the whole leaf, while in cv. Dooen the necrotic lesions developed after 36 h and covered only a portion of the leaf.

9.3.4 Mass spectrometry

Figures 9.8a - 9.15a contain the raw data of direct insertion probe mass spectra for the following samples; crude solanapyrone A, Fig. 9.8a and 8b; TLC purified solanapyrone A, Fig. 9.9a and 9b; pure solanapyrone B, Fig. 9.10a and 10b; pure solanapyrone C, Fig. 9.11a and 11b; extracts from 14-day-old culture of DAR 71767, Fig. 9.12a, and b; extracts from 21-day-old cultures of DAR 71767, Fig. 9.13a, b, and c; extracts from 21-day-old cultures of DAR 71768, Fig. 9.14a, b, and c; 21-day-old cultures of 215/91, Fig. 9.15a, and b.

In each case, the first figure, for example Fig. 9.8a, represents the trace for the reconstructed ion current (RIC) for the heated sample on the probe. It is presented as a percentage of the strongest total ion scan which is set to 100%. The subsequent figures, lettered b, c, etc. represent the individual mass spectra (usually "background corrected" for weak scans) for a specified range of scans.

The direct insertion probe is temperature programmed so that later scan numbers represent higher desorption temperatures. For crude solanapyrone A (Fig. 9.8a and b), no satisfactory mass spectra were obtained, because the more volatile degradation product apparently obscured the weaker solanapyrone A spectrum. However, the TLC-purified solanapyrone A (Fig. 9.9a and b), obtained from the crude sample showed a strong molecular ion ($M^{+\cdot}$) at 302 m/z. Likewise the homogenous solanapyrone B (Fig. 9.10a, b and c) gave an intense $M^{+\cdot}$ ion at 304 m/z and the homogenous solanapyrone C (Fig. 9.11a and b) also gave a very strong $M^{+\cdot}$ ion at 331.1 m/z. All these molecular ions are in accord with the formulae for solanapyrone A, B and C as shown in Fig 9.2.

These reference spectra were then used to identify $M^{+\cdot}$ and fragment ions in the crude extracts from *A. rabiei* isolates DAR 71767, DAR 71768 and 215/91. Fig. 9.12a and b and associated mass spectra for the 14-day-old cultures of DAR 71767 provided no

evidence for any significant ions which could be associated with the solanapyrones A, B and C. In contrast, Fig. 9.13c and d from the reconstructed ion current (RIC) trace for the extract from a 21-day-old culture of DAR 71767 show clear evidence for the presence of solanapyrone C, $M^{+\cdot}$ at 331.1 m/z. Fig. 9.14a shows the RIC trace for an extract of 21-day-old cultures of DAR 71768. The corresponding mass spectra (Fig. 9.14d and e) show good evidence supported by fragment ions for the presence of solanapyrone A (Fig. 9.14d) and solanapyrone C (Fig. 9.14e).

Fig. 9.15 a shows a series of single ion traces (304, 331, 345 m/z) as well as the RIC for an extract of 21-day-old cultures of 215/91. Fig. 9.15d, in addition to unrelated ions, shows evidence for the $M^{+\cdot}$ ion at 331.1 m/z and fragment ions at 303, 259.1 and 182 m/z consistent with the presence of solanapyrone C. Fig. 9.15b shows no ions characteristic of the reference solanapyrones A, B and C and Fig. 9.15c shows only two ions at 331.1 and 303 m/z that may be related to the desorption of a small amount of the solanapyrone C present (Fig. 9.15d).

The results for all these data are tabulated for comparative purposes in Table 9.1

Figure 9.3 TLC trace showing separations for reference solanapyrones A, B and C (two spots) as well as extracts of 21-day-old culture filtrate for isolates DAR 71767, DAR 71768 and 215/91 using multiple elution (6x) in Hexane:Ethyl acetate (75:25, v/v). Sample A_1 (D) was a purified component of Sample A, which is below the level of detection.

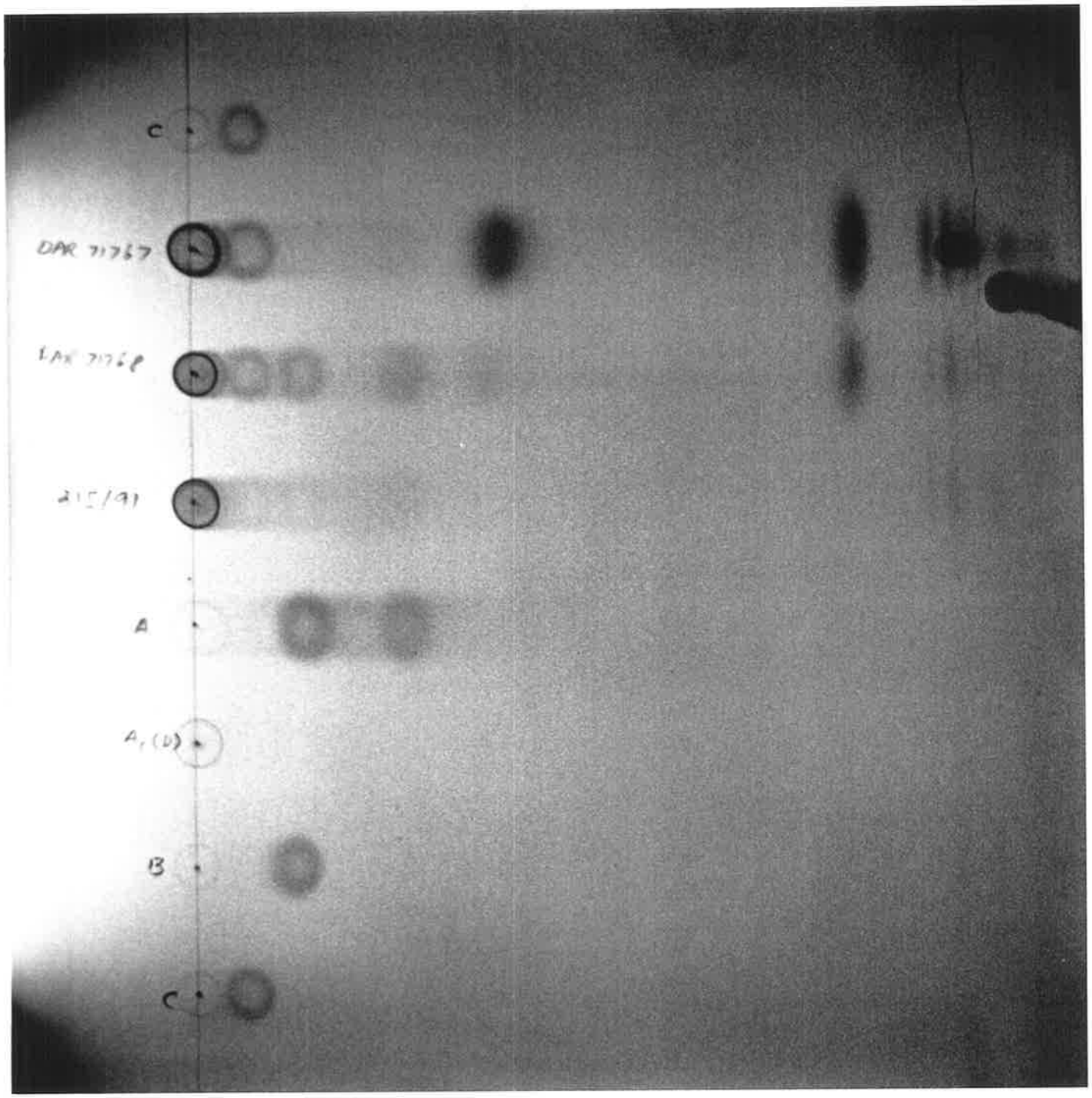

C
DAR 71767
DAR 71768
215/91
A
A1 (D)
B
C

Figure 9.4a HPLC trace of solanapyrone A (reference standard). The significant data from this trace are summarised in Table 9.1. Diode array UV data for two selected peaks are presented in Fig. 9.4b.

Current Chromatogram(s)

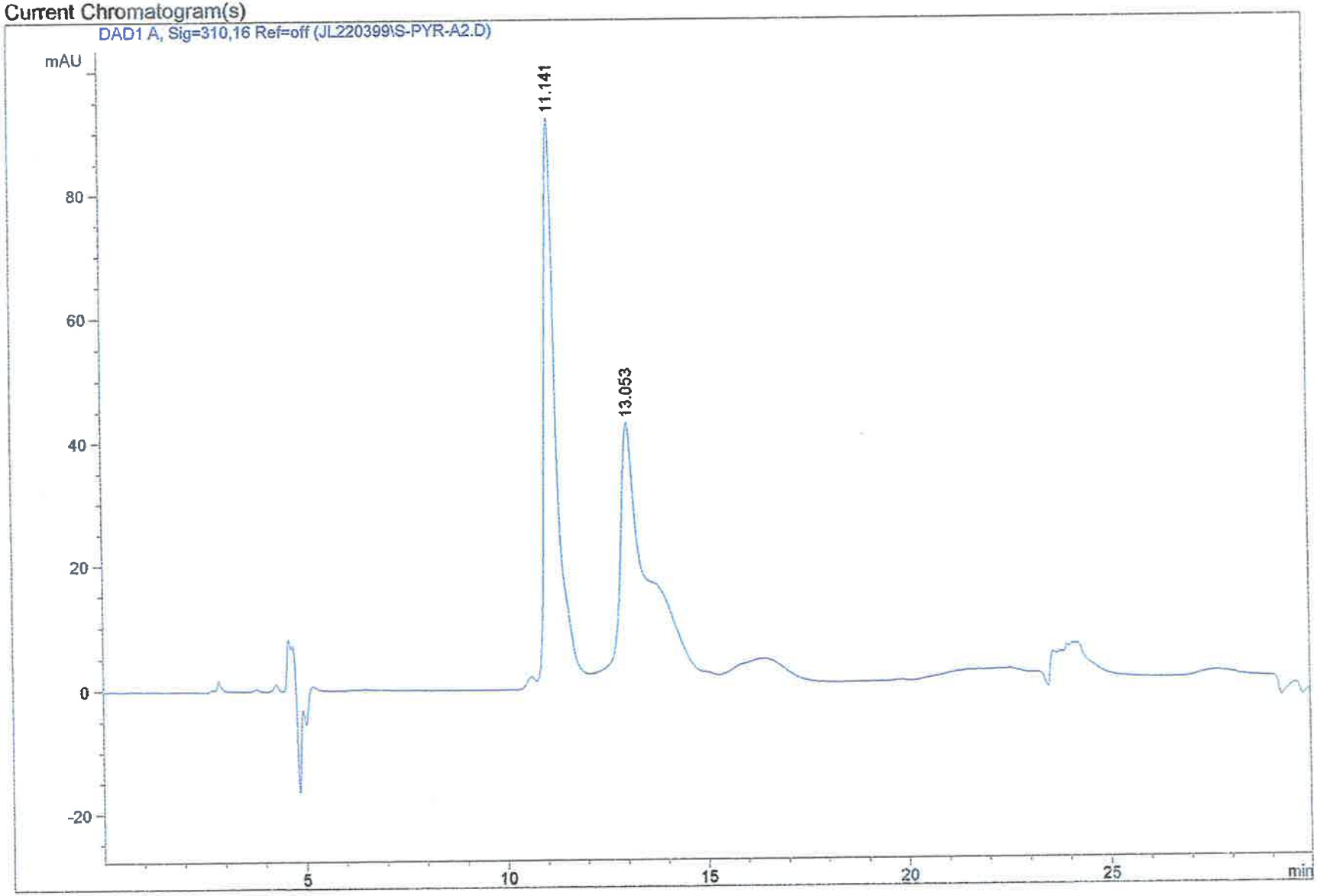


Figure 9.4b Diode array UV spectra for solanapyrone A peaks at 13.05 and 11.13 minutes (Fig. 9.4.a). Blue trace represent the peak retention time 13.05 minutes and the red trace equals the major component at 11.14 minutes which corresponds to pure solanapyrone A, as determined by Mass Spectrometry. The blue trace has the same chromophore as the peak of solanapyrone A 11.13 minutes but appears to be a degradation product of higher mass due to storage. The significant data from these traces are summarised in Table 9.1.

UV Apex spectrum of Peak 11.141 of S-PYR-A2.D

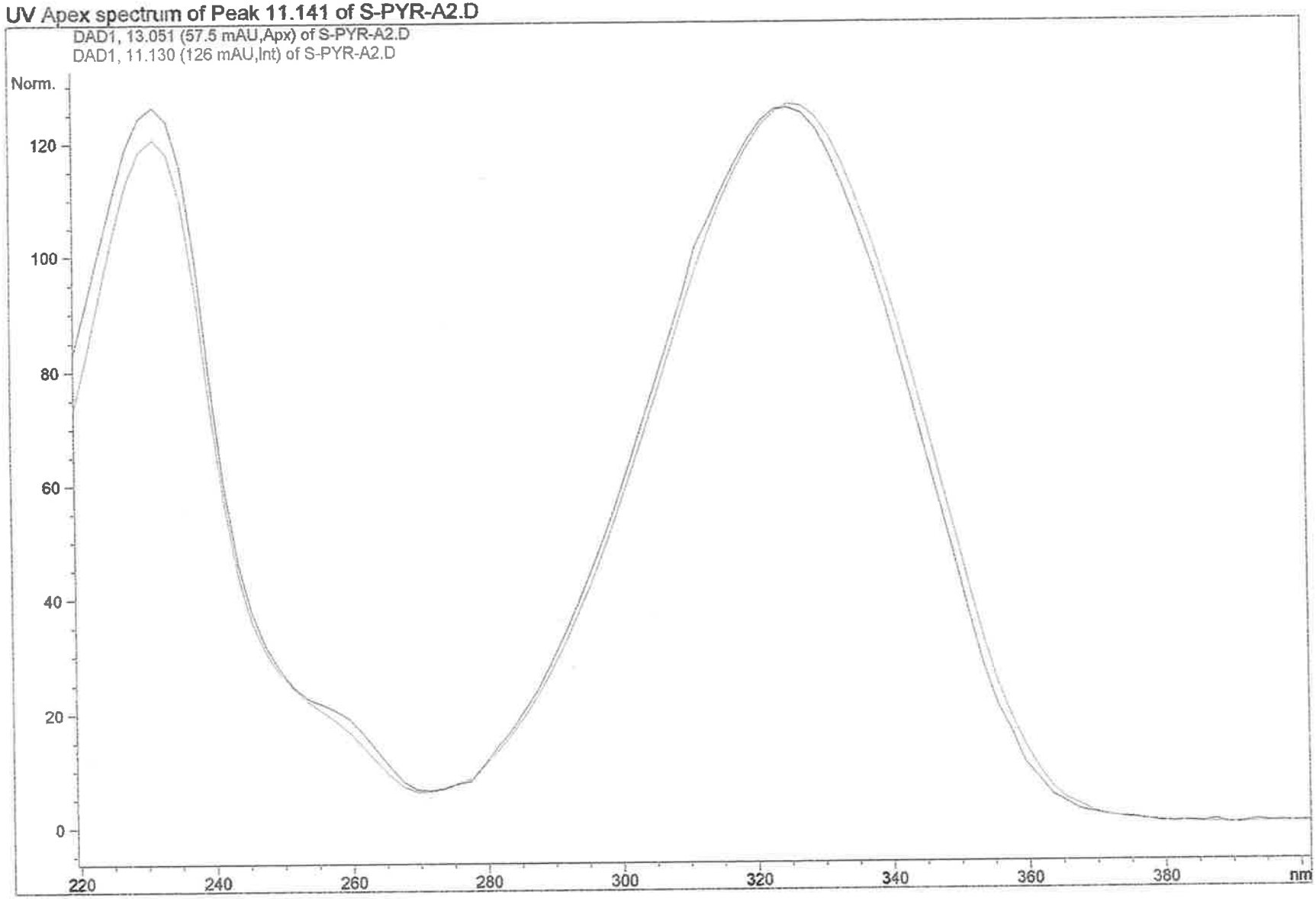


Figure 9.5a HPLC traces of solanapyrones B and C and DAR 71767. In descending sequence, (i) the peak retention time 11.764 minutes in the first trace represents pure solanapyrone B, (ii) pure solanapyrone C with retention time 13.664 minutes and (iii) *A. rabiei* isolate DAR 71767 extract from 21-day-old culture shows a peak with retention time 13.665 minutes corresponding to solanapyrone C. Other peaks have not been identified. The significant data from these traces are summarised in Table 9.1.

Current Chromatogram(s)

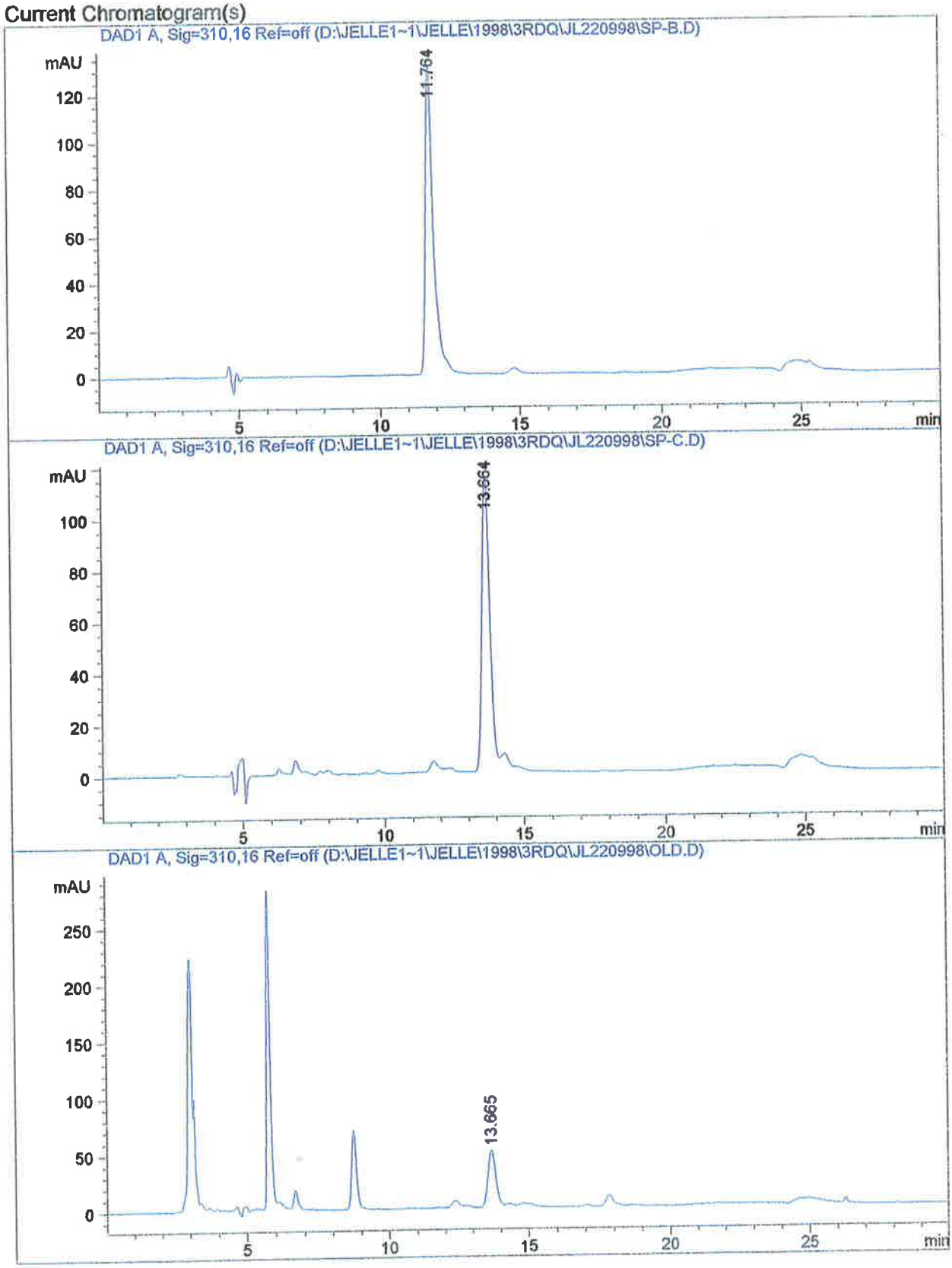


Figure 9.5b Diode array UV spectra for relevant peaks on the preceding HPLC traces (Fig 9.5a). The green trace of solanapyrone B with retention time 11.763 minutes is clearly distinguishable from solanapyrone C (red trace). The red trace of pure solanapyrone C with retention time 13.662 minutes and blue trace from isolate DAR 71767 of *A. rabiei* extracted from 21-day-old culture with retention time 13.666 minutes are superimposable. The significant data from these traces are summarised in Table 9.1.

DAD1, 11.763 (149 mAU,Apx) of SP-B.D

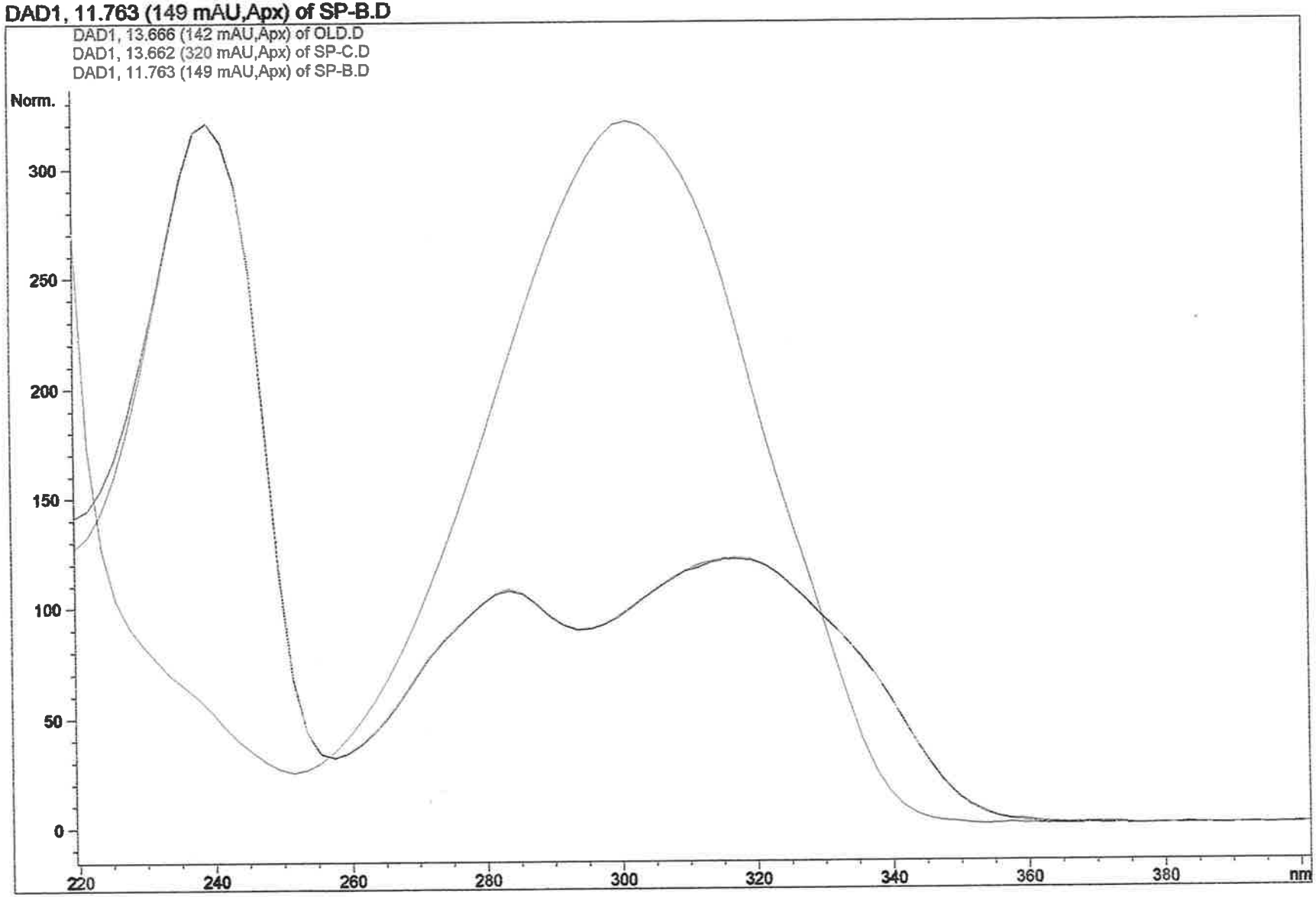


Figure 9.6a HPLC trace for *A. rabiei* isolate DAR 71768 extracted from 21-day-old culture. The trace peak at 12.695 minutes corresponds, in retention time, approximately to solanapyrone A, likewise the peak at 14.202 minutes corresponds approximately to solanapyrone C. The assignments were confirmed by diode array spectra (Fig 9.5b). All the significant data from these traces are summarised in table 9.1.

Current Chromatogram(s)

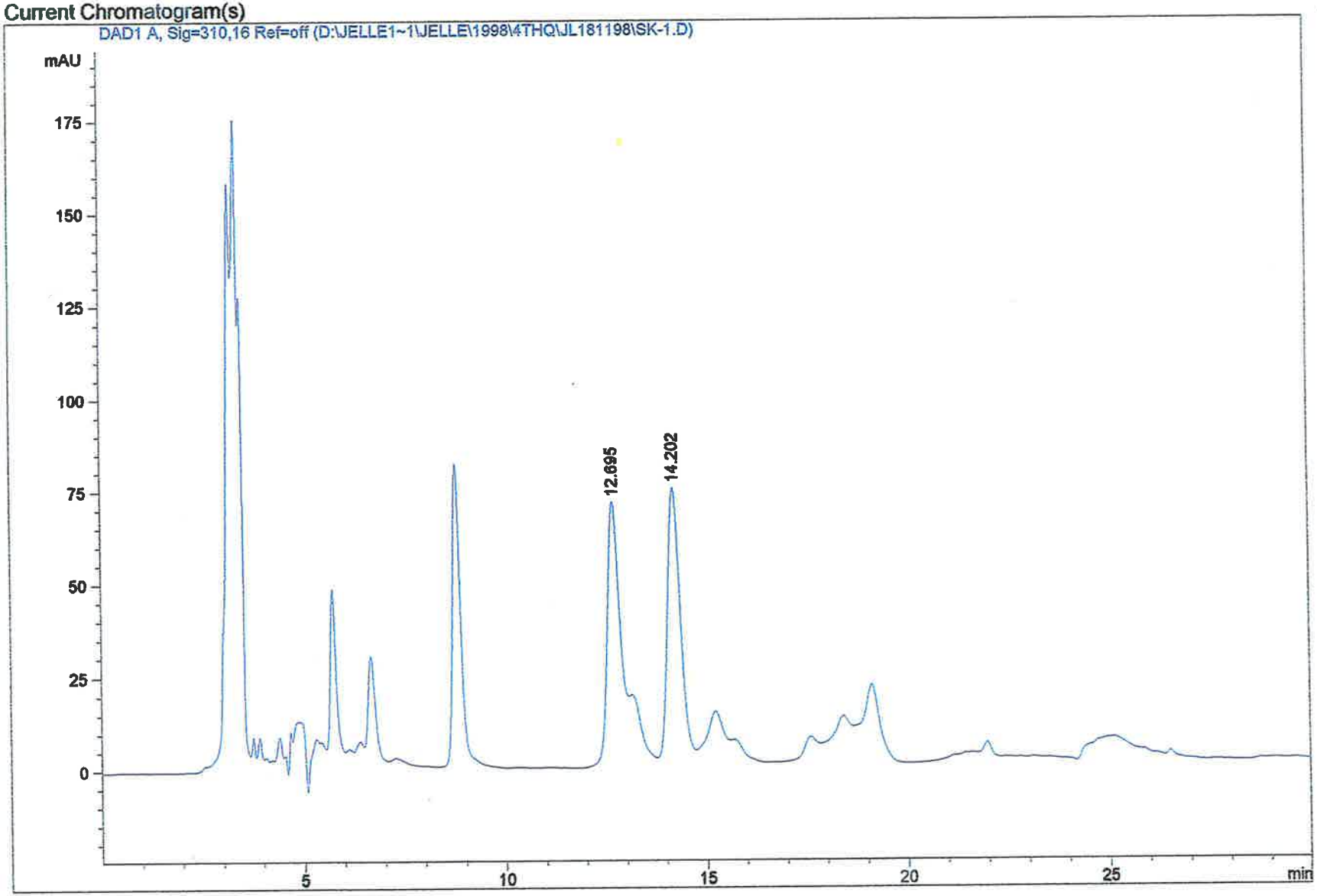


Figure 9.6b Diode array UV spectra for *A. rabiei* DAR 71768 peaks at 12.694 and 14.200 minutes in Fig 9.6a. The blue trace with retention time 14.200 corresponds with the trace for solanapyrone C retention time (13.665 minutes) in Fig. 9.4b likewise the spectrum at 12.694 minutes is consistent with solanapyrone A trace in retention time 11.14 minutes in Fig. 9.4b.

DAD1, 12.694 (90.7 mAU,Apx) of SK-1.D

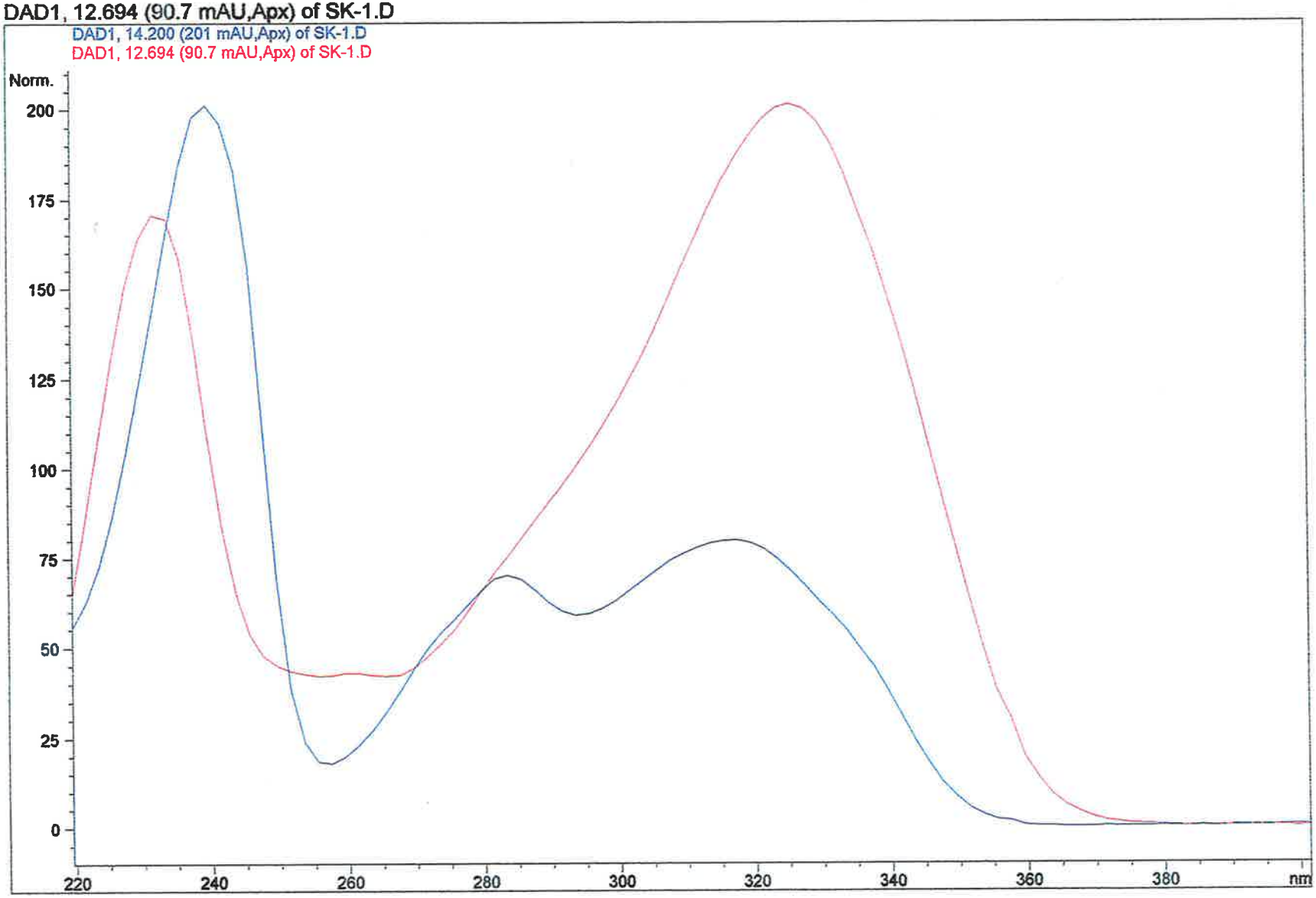


Figure 9.7a HPLC trace for *A. rabiei* isolate 215/91 extract from 21-day-old and is noteworthy for the weaker response on the Y-axis compared to 9.3a and 9.4a. Only peaks at 15.8, 12.809 and the shoulder near 12.89 minutes provided diode array spectra. The significant data from this trace are summarised in Table 9.1, and suggest the presence of a retarded solanapyrone A peak at 12.809 minutes.

Current Chromatogram(s)

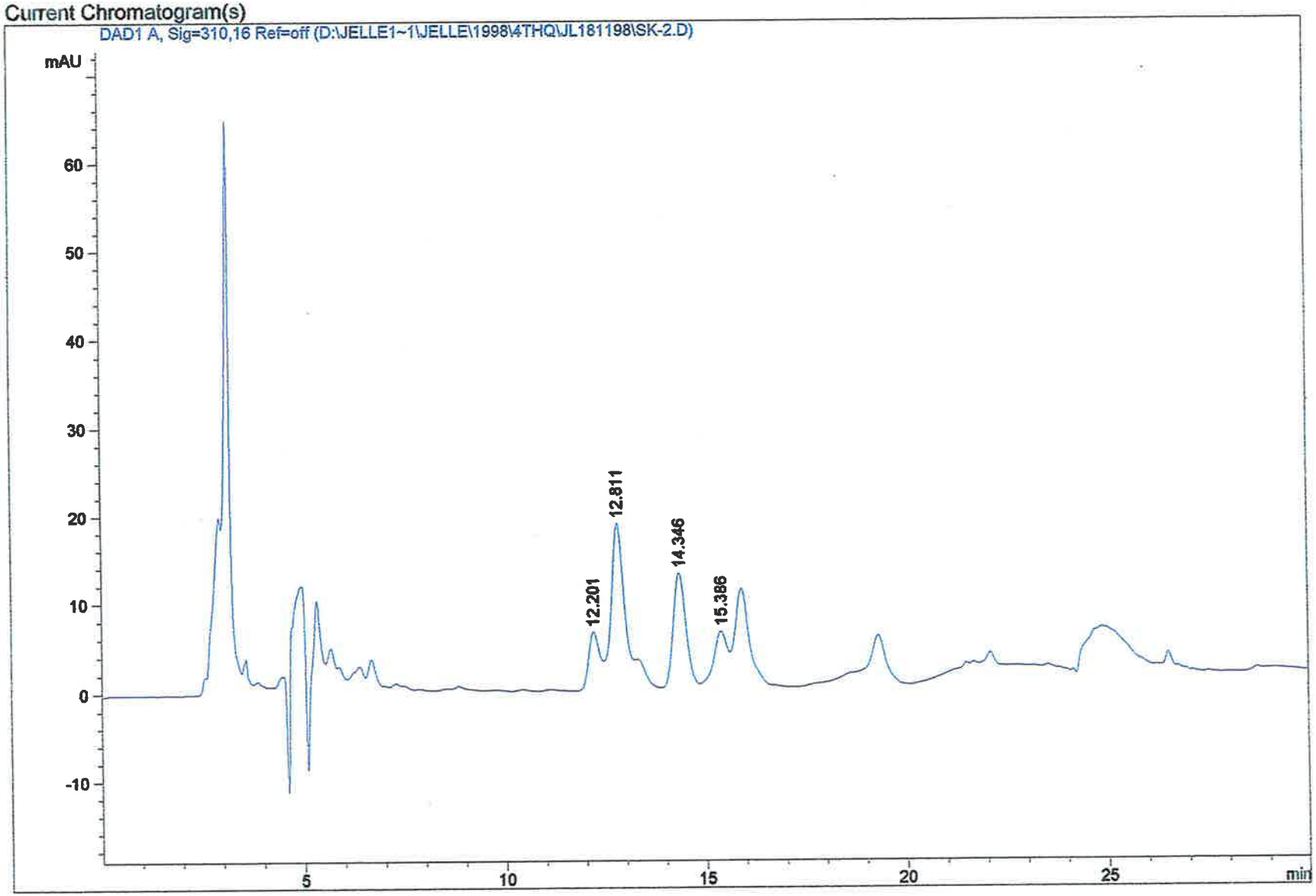


Figure 9.7b Diode array UV spectra for *A. rabiei* 215/91 peaks at 12.8861, 12.809, 15.783 minutes in Fig 9.7a (shoulder at 13.2 and peaks at 14.346 and 15.386 minutes did not provide spectra). The purple line at 12.809 is consistent with the spectrum for solanapyrone A. The green trace for the component 12.89 has the same chromophore and the red trace 15.783 cannot be assigned.

DAD1, 12.809 (35.3 mAU,Apx) of SK-2.D

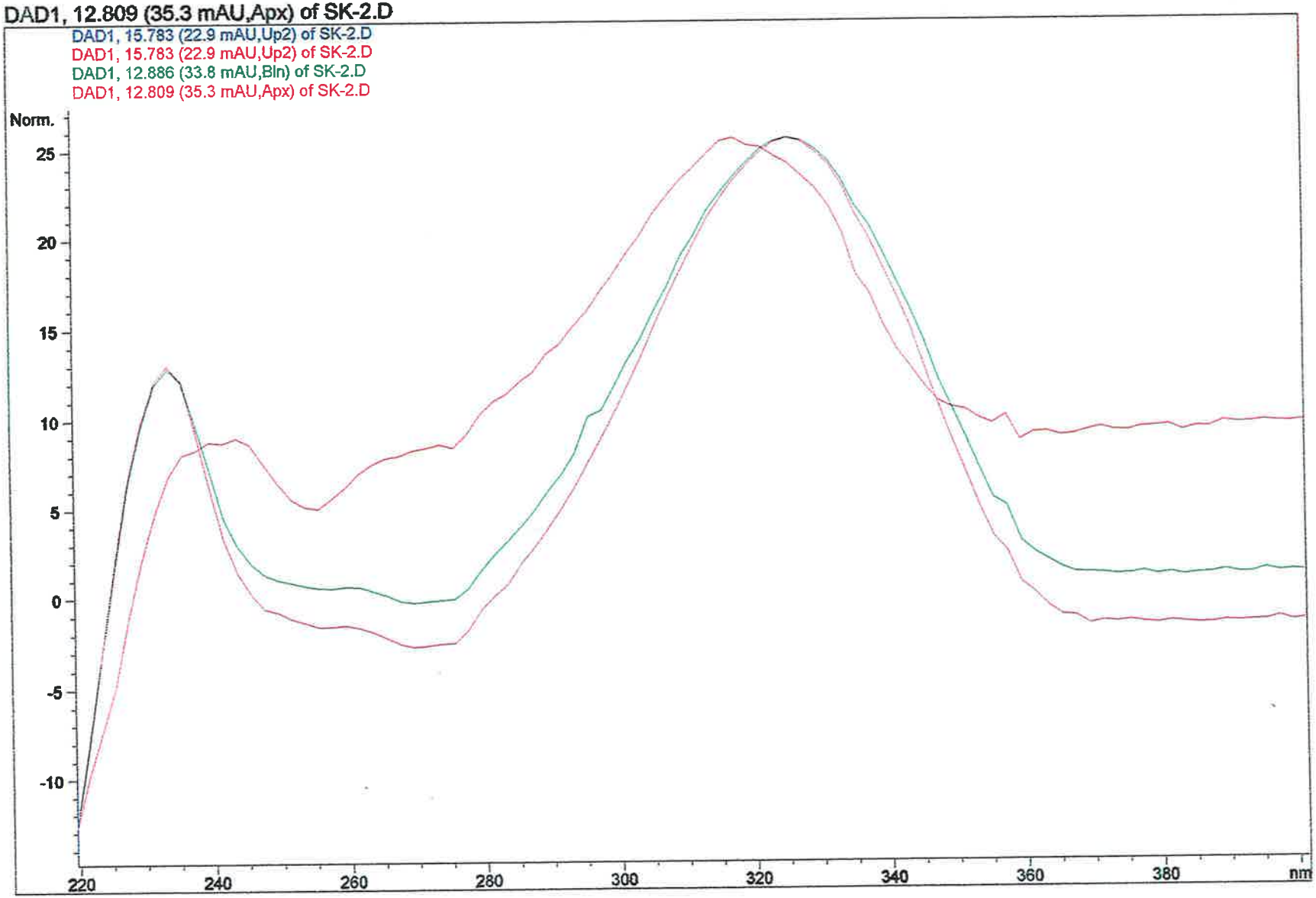
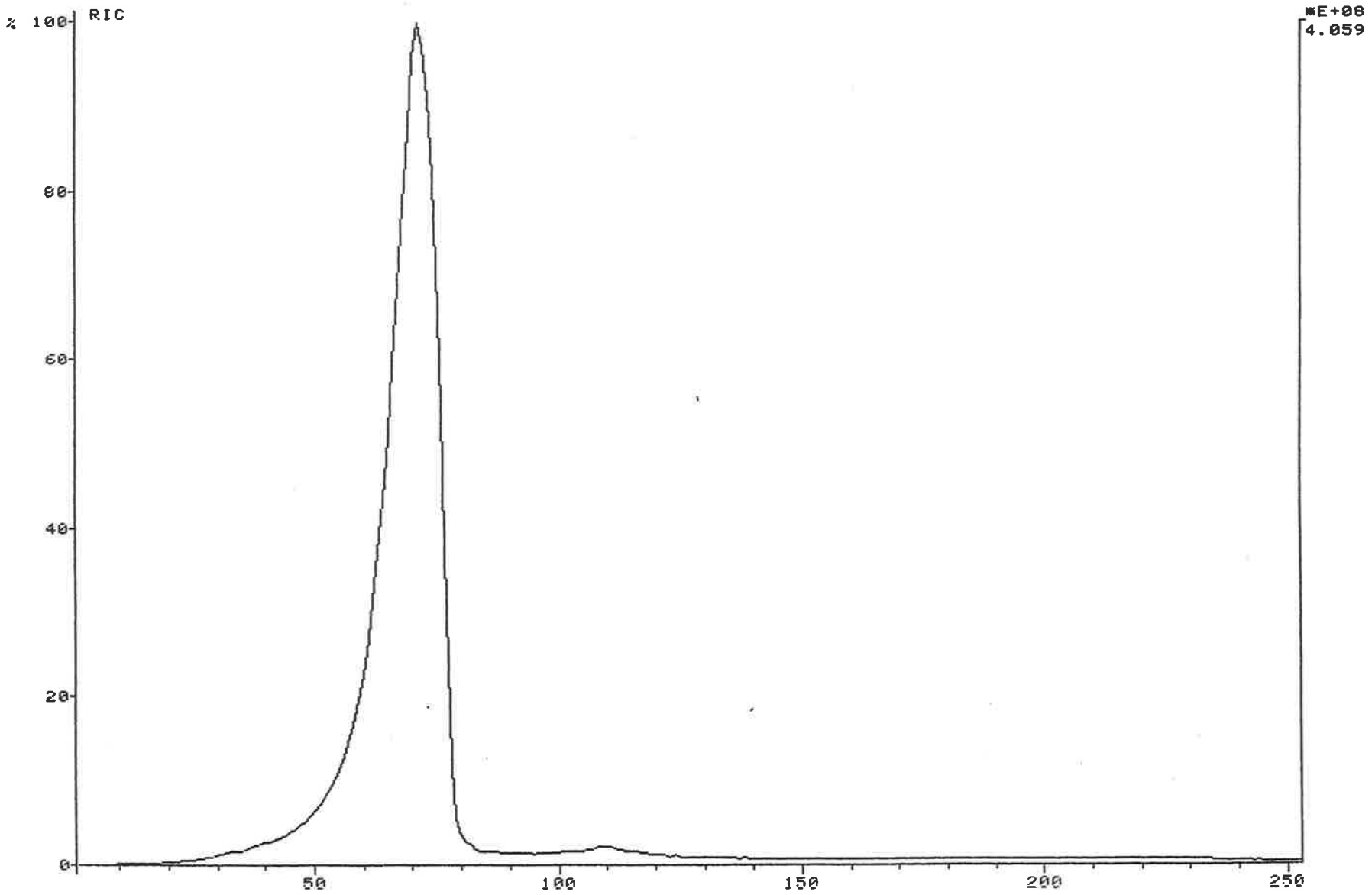


Figure 9.8 Mass spectrometry of reference standard, solanapyrone A.

Figure 9.8a Represents the reconstructed ion current (RIC) as the temperature programmed probe gradually desorbs the volatile constituents of the crude solanapyrone A sample. The volatilisation of the sample is essentially complete by scan #130. The major component (s) desorb in scans 50-80. A minor component desorbs in scans 100-120. The major volatile component did not provide evidence for the presence of solanapyrone A. Subsequently preparative TLC provided a satisfactory trace (See Figs 9.9 a and b).

Figure 9.8b The early portion of the RIC trace of crude solanapyrone A is not consistent with an $M^{+\cdot}$ ion at 302 for solanapyrone A. The bulk of this spectrum indicates loss of an ·OH radical from weak $M^{+\cdot}$ at 362 to yield a series of fragment ions differing in mass by 14 m/z units (-CH_2-). For the mass spectral data purified for solanapyrone A isolated by TLC from this sample see Fig. 9 9a and b.

```
CHRO: YH980C01B ver 2 on UIC 7 11     30-SEP-98 Elapse: 00:00:13.2    9
Samp: SAMPLE-A                                  Start : 03:22:35   253
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI                                      Inlet :
Peak: 1000.00 mmu     Label wndw: 1 > 253       Masses: 50 > 500
Area: 0, 4.00         Baseline  : 0, 3          Label : 0, 40.00
```



SPEC: YH980C01B ver 2 on UIC 7 11 30-SEP-98 DERIVED SPECTRUM 9
Samp: SAMPLE-A Start : 03:22:35 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 331.2 Inten : 25069352 Masses: 50 > 500
Norm: 331.2 RIC : 296757056 # peaks: 454
Peak: 1000.00 mmu
Data: + 52 > 58 - 33 > 27

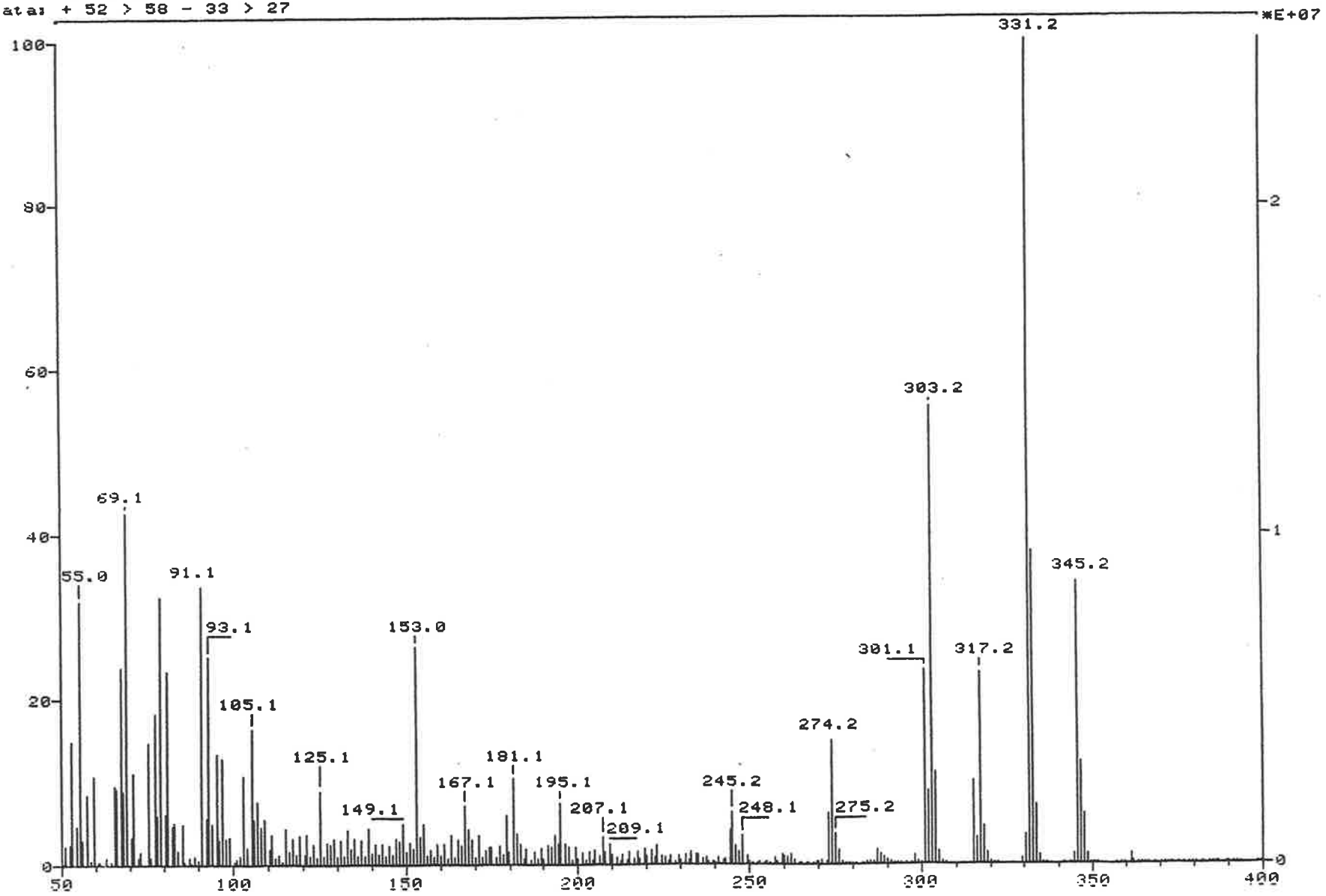


Figure 9.9a RIC trace for purified (TLC) solanapyrone A. The major peak of desorbed ions at 60-70 scans and is consistent with the presence of solanapyrone A (Fig 9.9b). Scans 70-250 provided no useful information.

CHRO: YH980C01D ver 2 on UIC 7 11 1-OCT-98 Elapse: 00:00:09.5 9
Samp: SAMPLE-ℓD Start : 16:05:00 266
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Peak: 1000.00 mmu Label wndw: 1 > 266 Masses: 50 > 500
Area: 0, 4.00 Baseline : 0, 3 Label : 0, 40.00

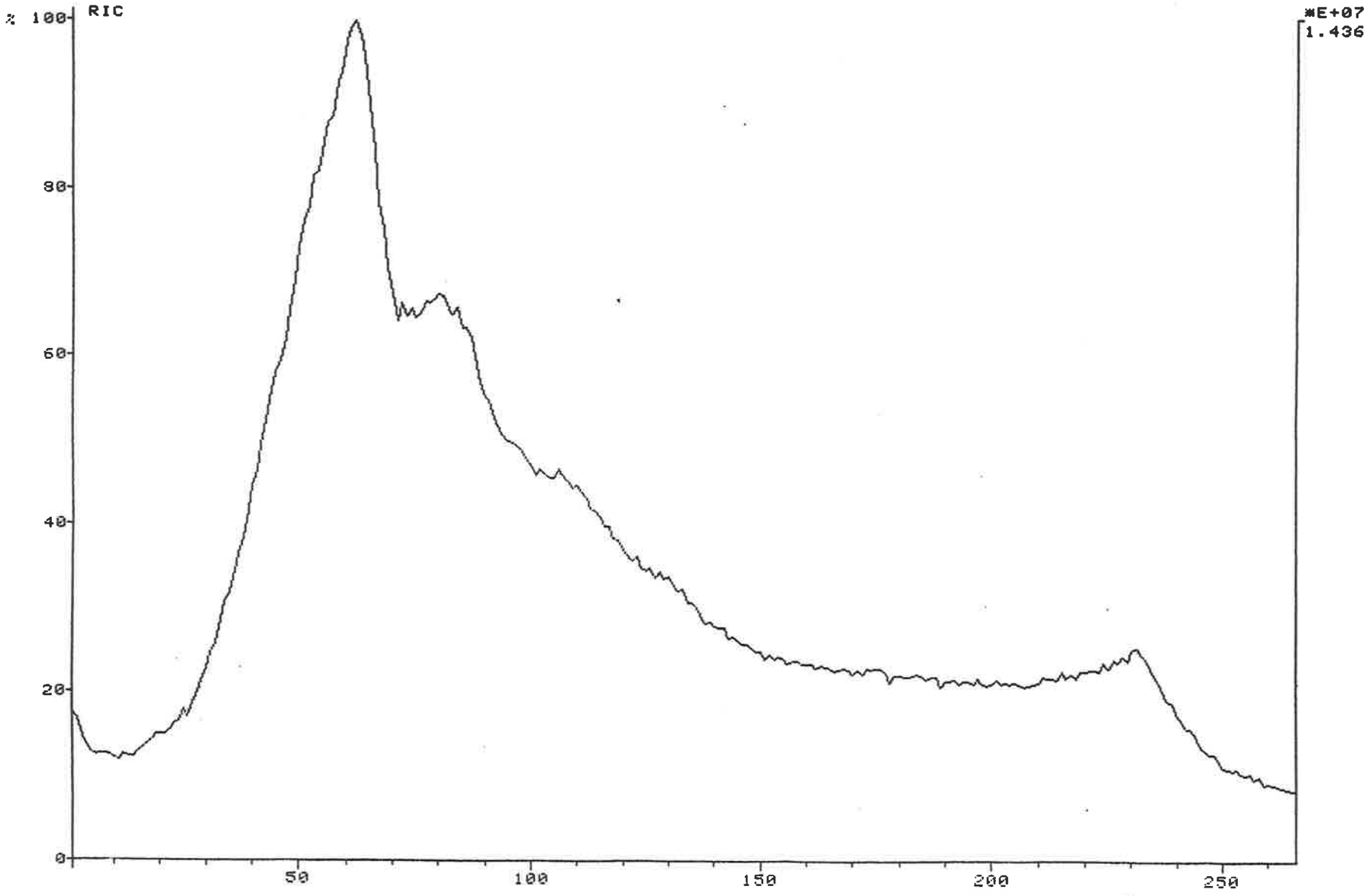


Figure 9.9b The mass spectrum from peak scans 61-62 (Fig. 9.9a) of the purified solanapyrone A exhibits the expected molecular ion $M^{+\cdot}$ at 302.1, in addition to a strong fragment ion at 274.2 corresponding to loss of 28m/z (-CO).

SPEC: YH980C01D ver 2 on UIC 7 11 1-OCT-98 DERIVED SPECTRUM 9
Samp: SAMPLE-D Start : 16:05:00 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 69.1 Inten : 1616066 Masses: 50 > 500
Norm: 69.1 RIC : 25100056 # peaks: 423
Peak: 1000.00 mmu
Data: + 61 > 62 - 17 > 16

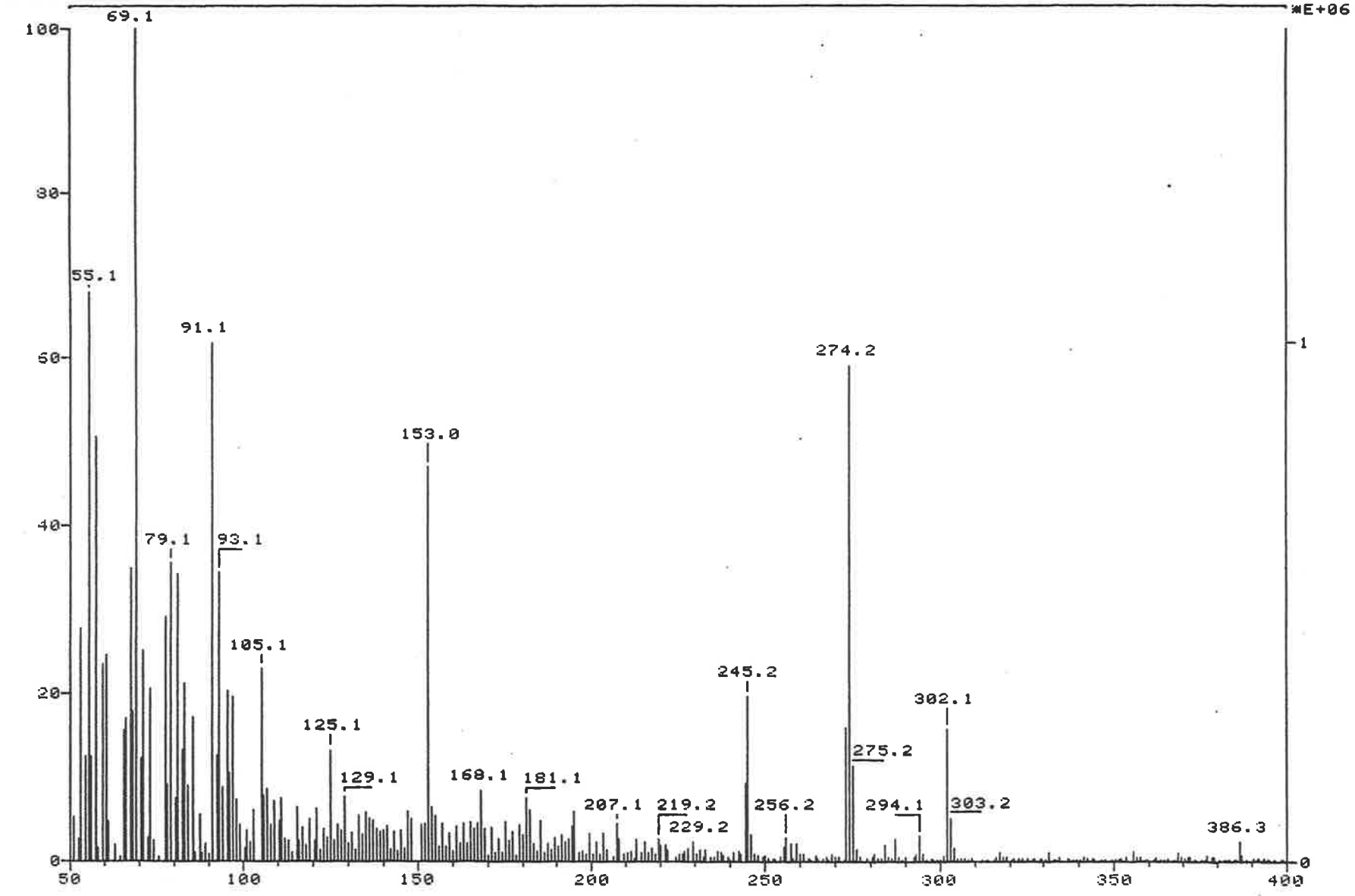


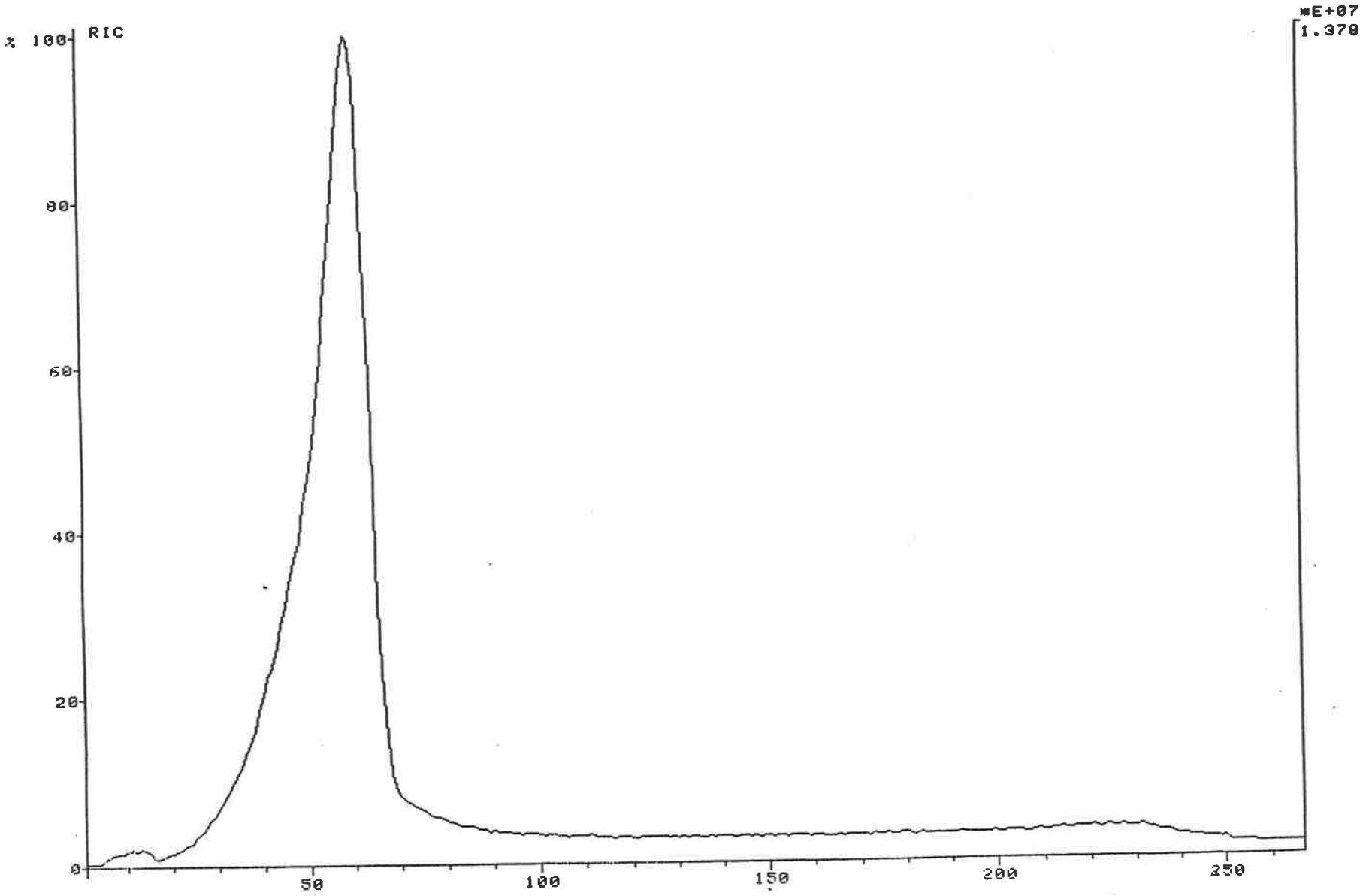
Figure 9.10 Mass spectrometry of reference standard, solanapyrone B

Figure 9.10a RIC trace for homogenous solanapyrone B. The peak of desorbed ions at 30-60 scans is consistent with solanapyrone B (see Fig. 9.10b and c).

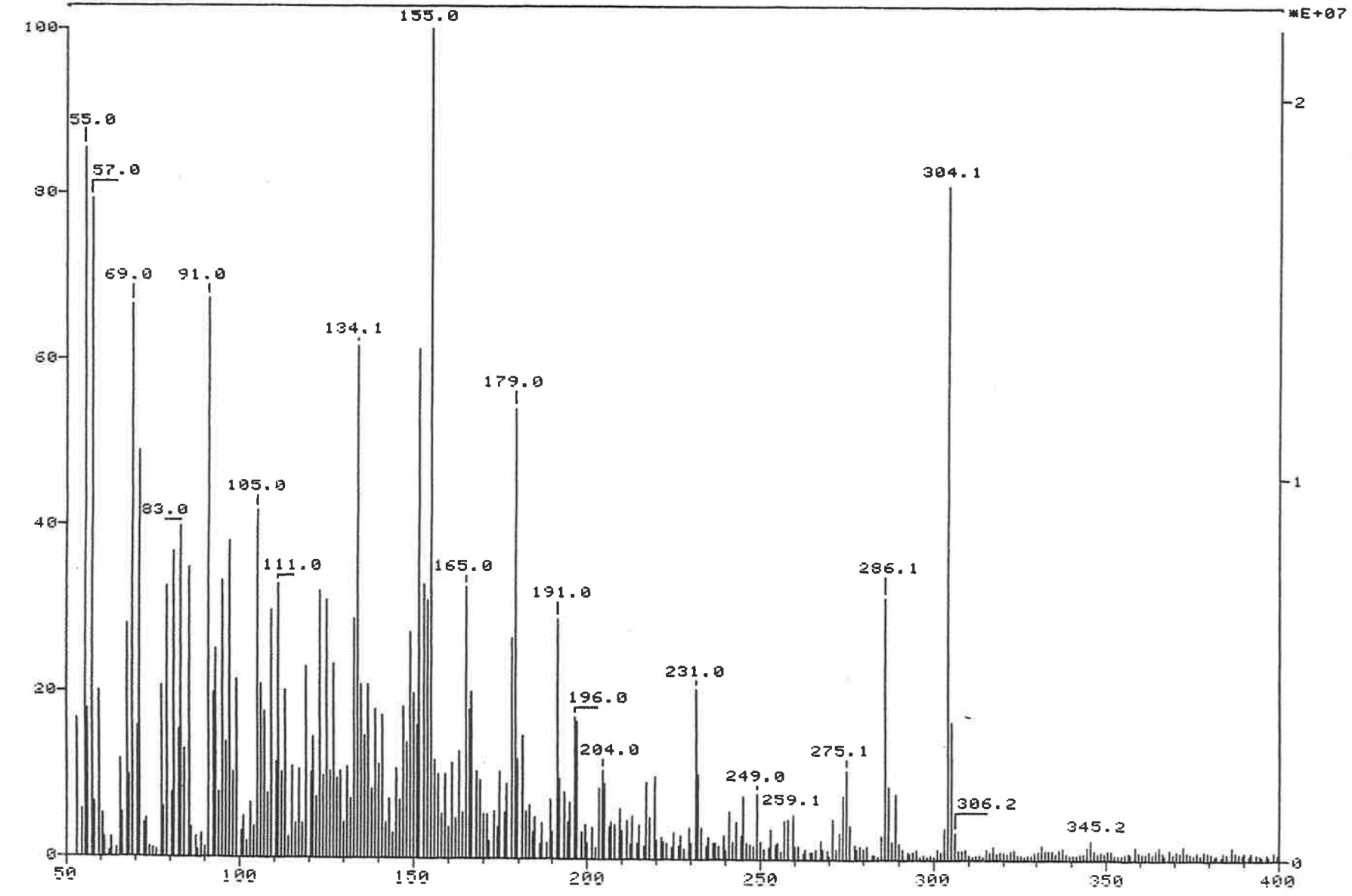
Figure 9.10b The peak in the region at 31-38 scans exhibits an intense molecular ion $M^{+\cdot}$ at 304m/z for reference standard solanapyrone B.

Figure 9.10c The later scans 57-59 of Fig. 9.10a are also consistent with the expected $M^{+\cdot}$ ion for solanapyrone B and are indicative of a homogeneous desorption for all scans 30-60.

CHRO: YH980C01C ver 1 on UIC 7 11 1-OCT-98 Elapse: 00:00:13.7 9
Samp: SAMPLE-B Start : 15:33:59 267
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR Inlet :
Oper: YOJI Masses: 50 > 500
Peak: 1000.00 mmu Label wndw: 1 > 267 Label : 0, 40.00
Area: 0, 4.00 Baseline : 0, 3



SPEC: GB98NO24B ver 1 on UIC 3 1 14-NOV-98 DERIVED SPECTRUM 9
Samp: REFERENCE B Start : 20:35:52 10
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE Inlet :
Base: 155.0 Inten : 21822930 Masses: 50 > 650
Norm: 155.0 RIC : 635906944 # peaks: 525
Peak: 1000.00 mmu
Data: + 31 > 38 - 17 > 10



SPEC: YH980C01C ver 1 on UIC 7 11 1-OCT-98 DERIVED SPECTRUM 9
Samp: SAMPLE-B Start : 15:33:59 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 91.1 Inten : 1701403 Masses: 50 > 500
Norm: 91.1 RIC : 38666108 # peaks: 436
Peak: 1000.00 mmu
Data: + 57 > 59 - 20 > 19

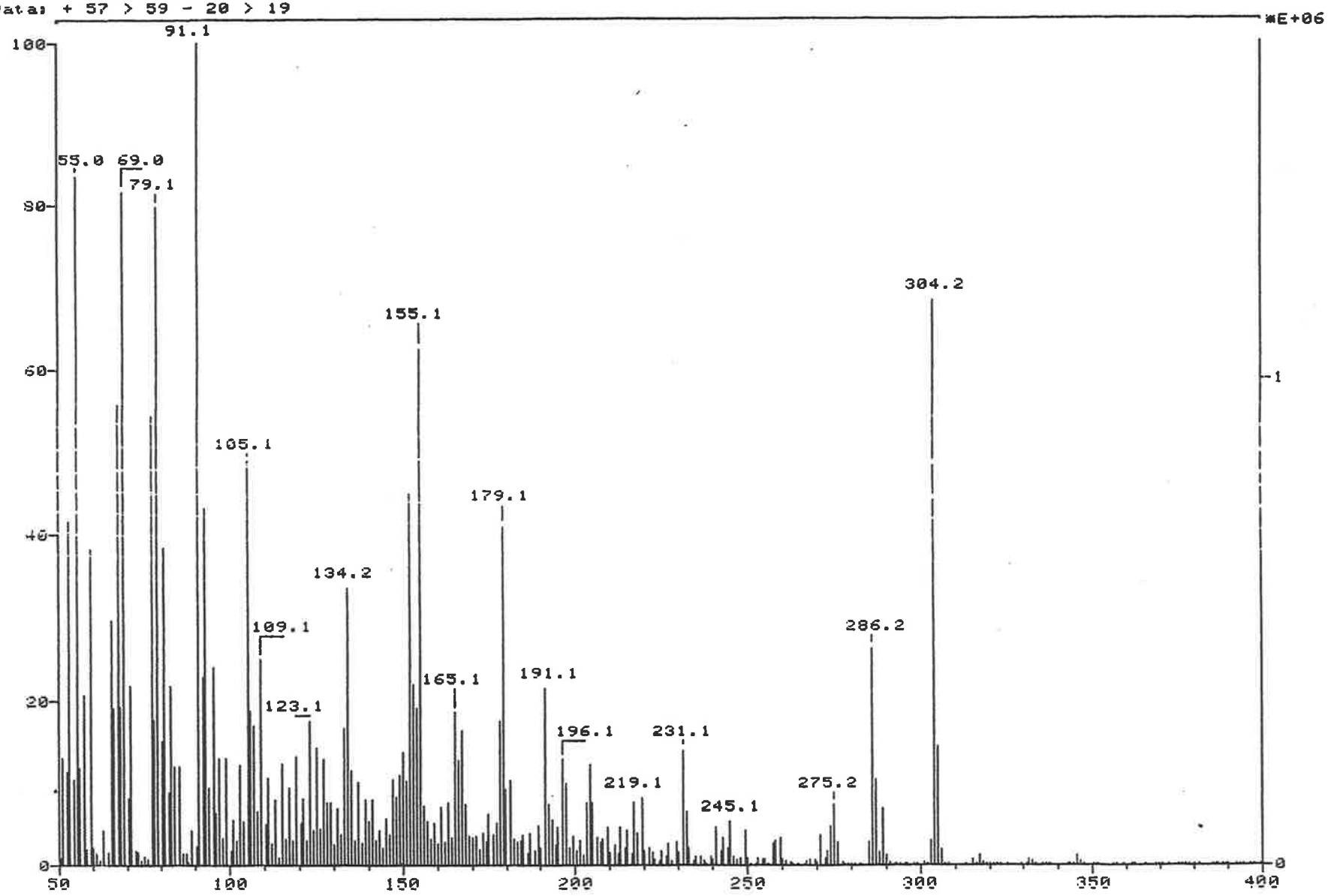
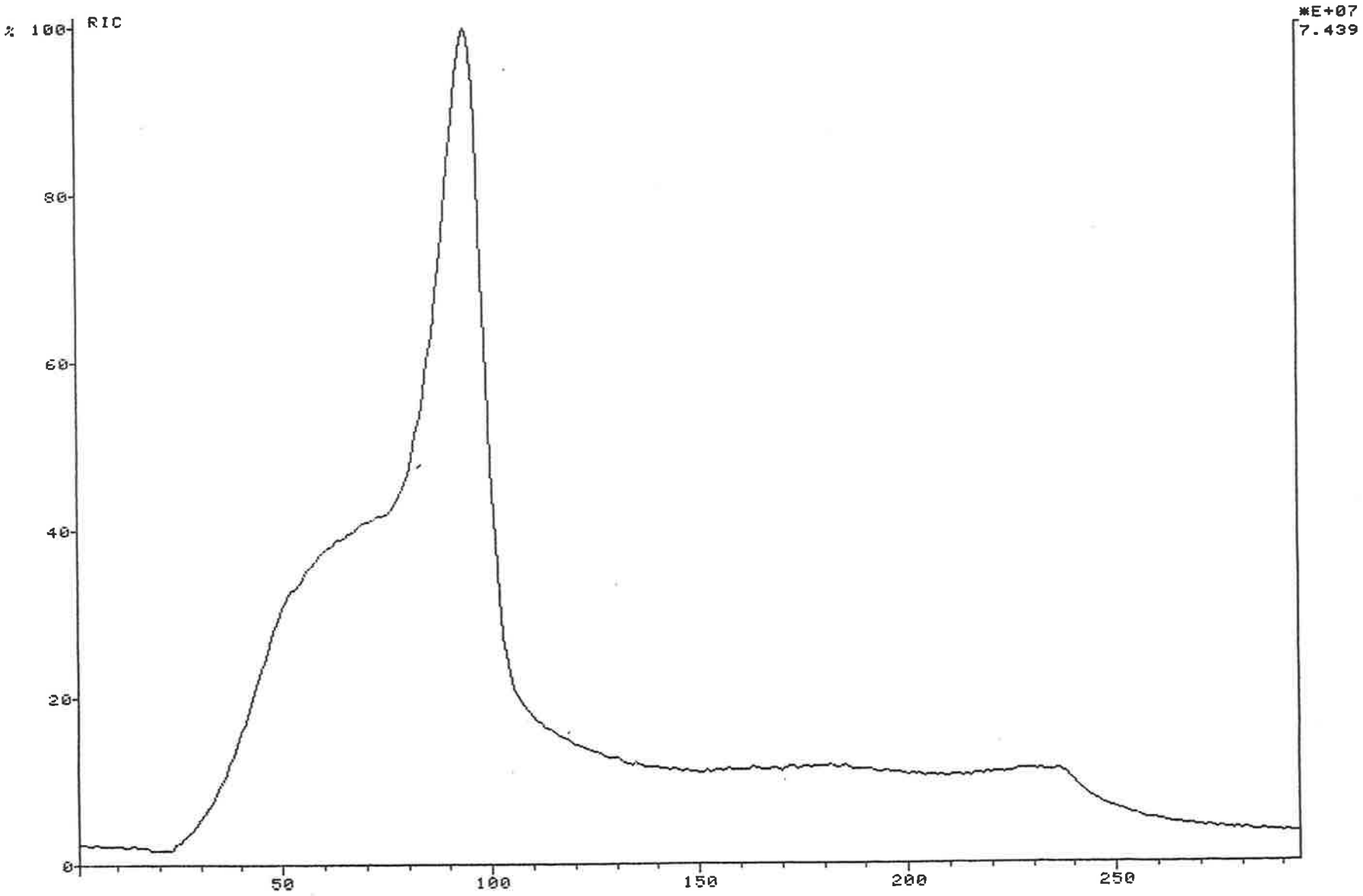


Figure 9.11 Mass spectrometry of reference standard, solanapyrone C

Figure 9.11a RIC trace for a homogenous sample of solanapyrone C. The major peak of desorbed ions at 90-100 scans is consistent with solanapyrone C (see Fig. 9. 11b).

Figure 9.11b The mass spectrum for scans 90-97 (Fig 11a) shows a very strong base peak molecular radical ion $M^{+\cdot}$ at 331.1 which is consistent with the presence of solanapyrone C.

CHRO: GB98N024F ver 1 on UIC 3 1 15-NOV-98 Elapse: 00:00:09.8 9
Samp: reference c Start : 11:14:43 294
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE Inlet :
Peak: 1000.00 mmu Label wndw: 1 > 294 Masses: 50 > 650
Area: 0, 4.00 Baseline : 0, 3 Label : 0, 40.00



SPEC: GB99N024F ver 1 on UIC 3 1 15-NOV-99 DERIVED SPECTRUM 9
Samp: reference c Start : 11:14:43 10
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE Inlet :
Base: 331.1 Inten : 20554192 Masses: 50 > 650
Norm: 331.1 RIC : 338156832 # peaks: 425
Peak: 1000.00 mmu
Data: + 90 > 97 - 62 > 69

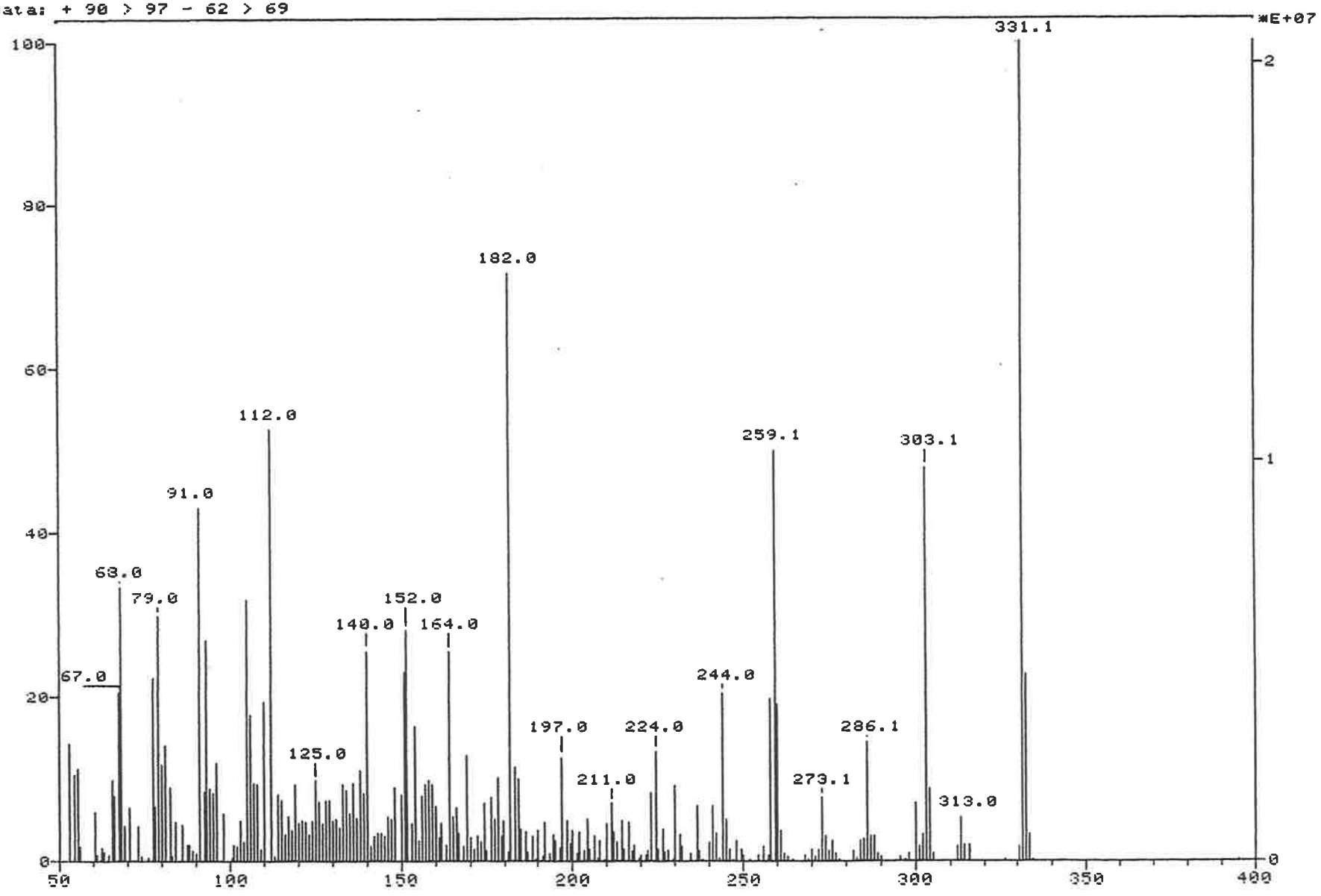


Figure 9.12a RIC trace for the extract of 14-day-old culture of DAR 71767. None of the peaks from scans 13-20, 38-40, 57-59 or 85-91 provided any evidence for $M^{+\cdot}$ ions of solanapyrones A, B or C. Only the mass spectrum for the scans 13-20 is shown in Fig. 9.12b as one example of negative data.

CHRO: YH980C01F ver 1 on UIC 7 11 1-OCT-98 Elapse: 00:00:13.9 9
Samp: NEW Start : 16:15:45 285
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Peak: 1000.00 mmu Label wndw: 1 > 285 Masses: 50 > 500
Area: 0, 4.00 Baseline : 0, 3 Label : 0, 40.00

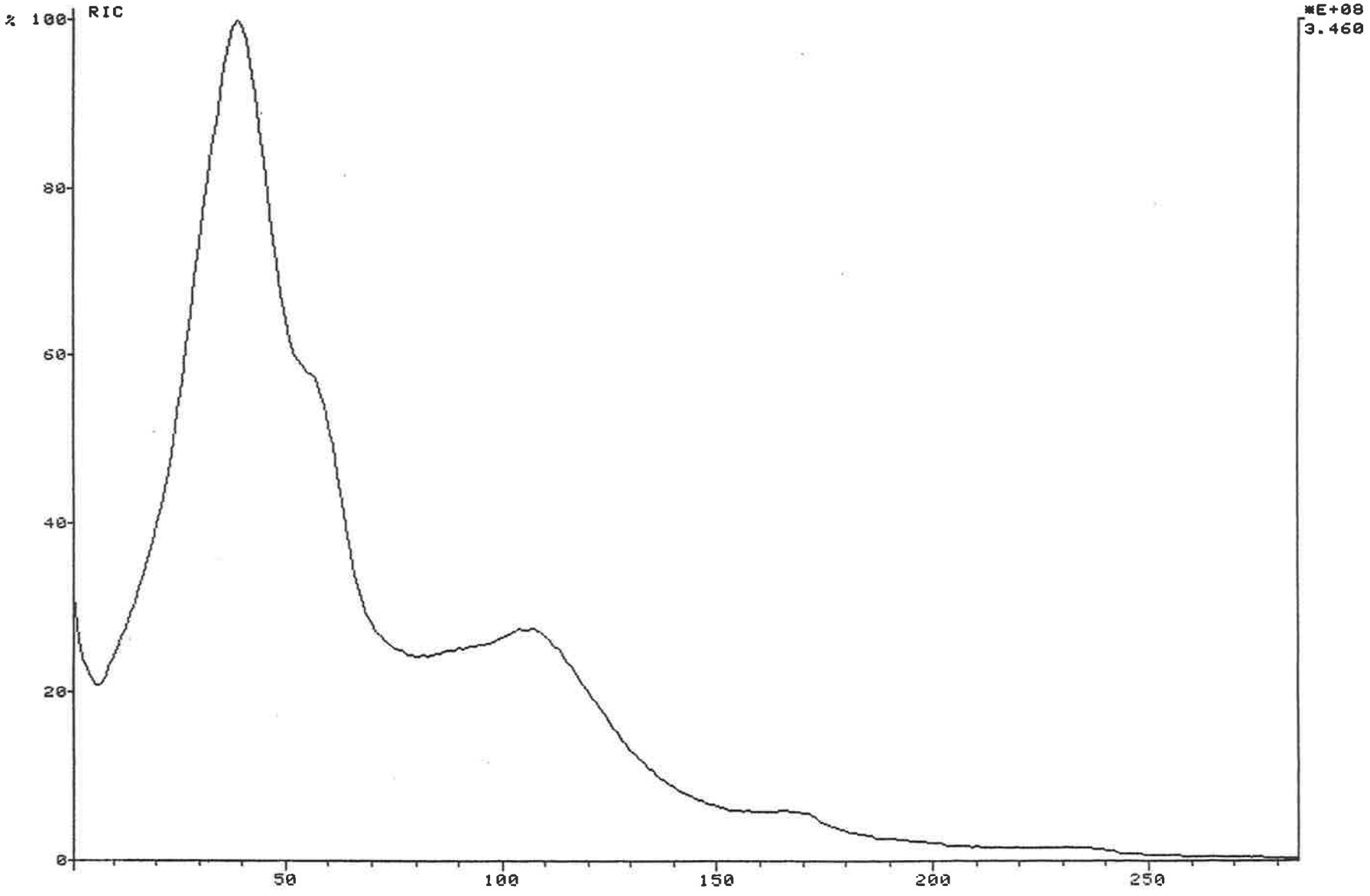


Figure 9.12b Mass spectrum of scans 13-20 from an extract from a 14-day-old culture of DAR 71767 scans at 13-20. No evidence for strong $M^{+\cdot}$ ions at 302, 304, 331m/z, in this extract.

SPEC: YH980C01F ver 1 on UIC 7 11 1-OCT-98 DERIVED SPECTRUM 9
Samp: NEW Start : 16:15:45 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 88.0 Inten : 101262776 S Masses: 50 > 500
Norm: 88.0 RIC : 923237568 # peaks: 448
Peak: 1000.00 mmu
Data: + 13 > 20

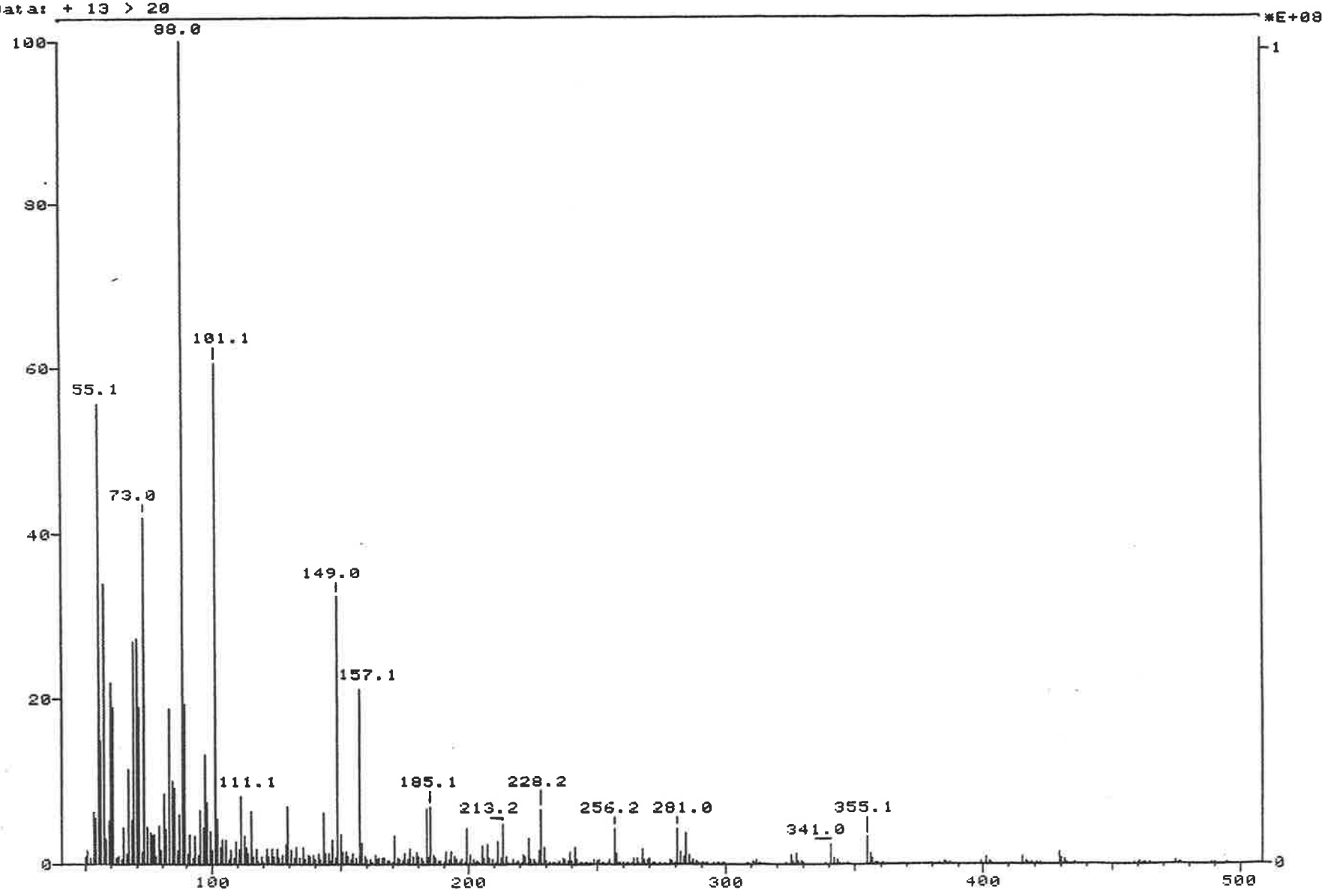
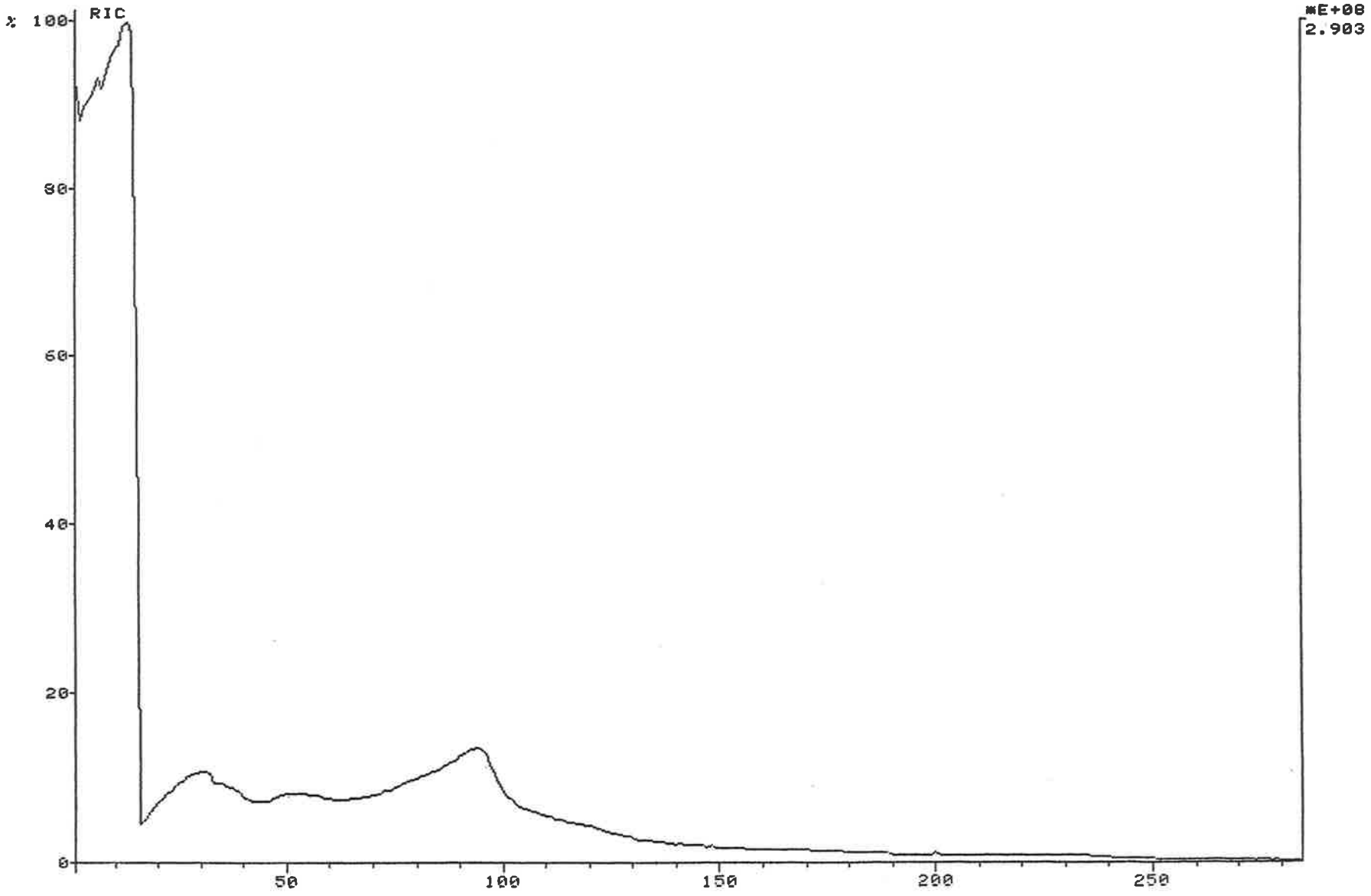


Figure 9.13 Mass spectrometry of extracts from 21-day-old cultures of DAR 71767.

Figure 9.13a RIC trace scans 87-93 (Fig. 9.13b) and 94-96 (Fig. 9.13c) are consistent with the presence of solanapyrone C. Scans 48-50 and 107-114 (data not shown) provided no evidence for $M^{+\cdot}$ ions of solanapyrones A, B and C (302, 304 or 331).

Figure 9.13b The scan range (87-93) shows a clear $M^{+\cdot}$ ion at 331.2 and supporting fragment ions at 303.2, 259.2, 244.1, 182, 140.1 and 112.1 which are characteristic of solanapyrone C (see Fig. 9.11b).

```
CHRO: YH980C01H ver 1 on UIC 7 11      1-OCT-98 Elapse: 00:00:13.3   9
Samp: OLD                                       Start : 16:31:39   285
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI                                      Inlet :
Peak: 1000.00 mmu     Label wndw: 1 > 285       Masses: 50 > 500
Area: 0, 4.00         Baseline  : 0, 3          Label : 0, 40.00
```



SPEC: YH980C01H ver 1 on UIC 7 11 1-OCT-98 DERIVED SPECTRUM 9
Samp: OLD Start : 16:31:39 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 91.1 Inten : 12081849 Masses: 50 > 500
Norm: 91.1 RIC : 250286432 # peaks: 464
Peak: 1000.00 mmu
Data: + 87 > 93

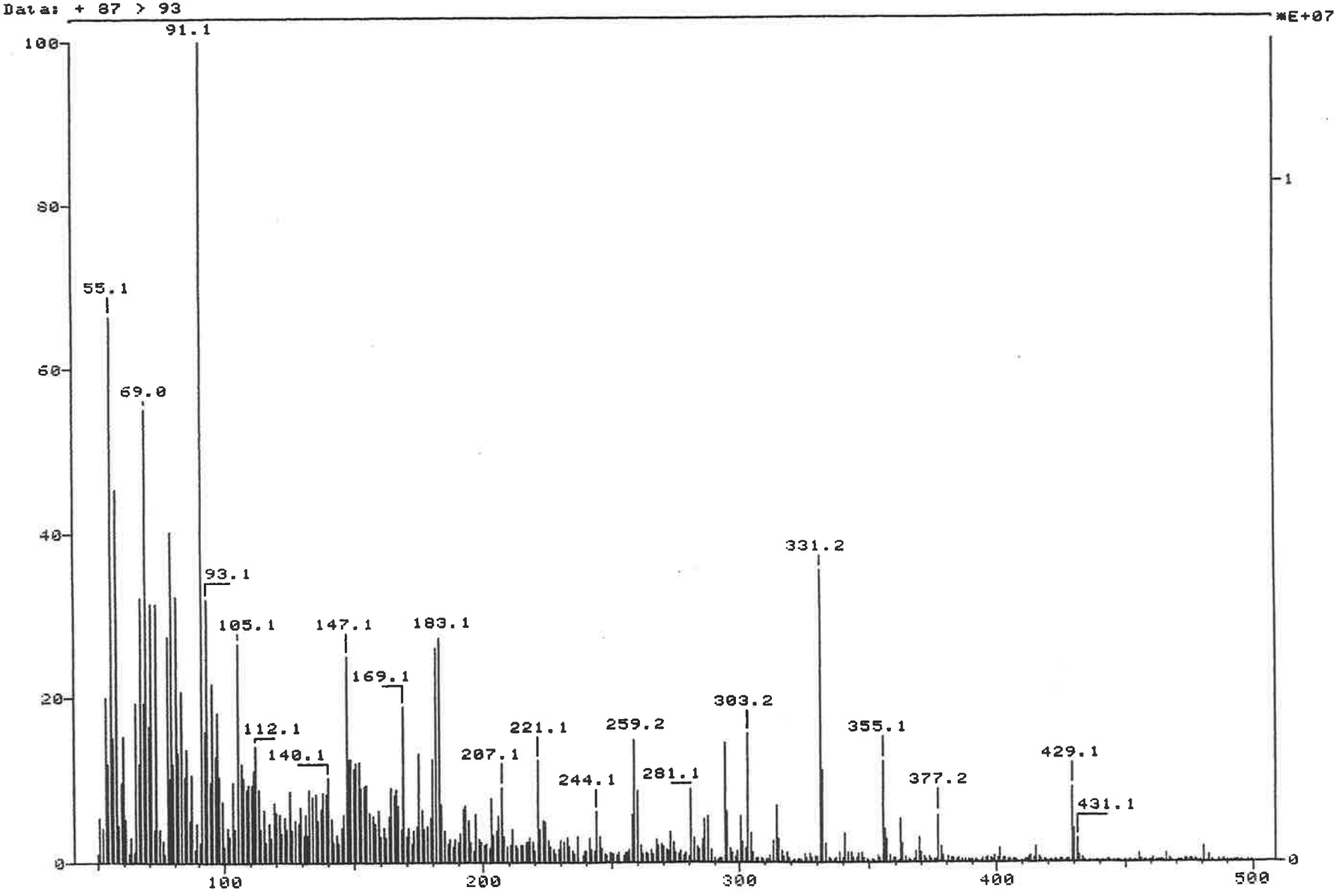


Figure 9.13c As in the case of Fig. 14c, scans 94-96 also show the characteristic ions $M^{+\cdot}$ 331.2 and supporting fragment ions at 303.2, 259.2, 244.1, 182, 140.1 and 112.1 characteristic of solanapyrone C (see Fig. 9.11b).

SPEC: YH980C01H ver 1 on UIC 7 11 1-OCT-98 DERIVED SPECTRUM 9
Samp: OLD Start : 16:31:39 10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI Inlet :
Base: 91.1 Inten : 7360918 Masses: 50 > 500
Norm: 91.1 RIC : 113608960 # peaks: 458
Peak: 1000.00 mmu
Data: + 94 > 96

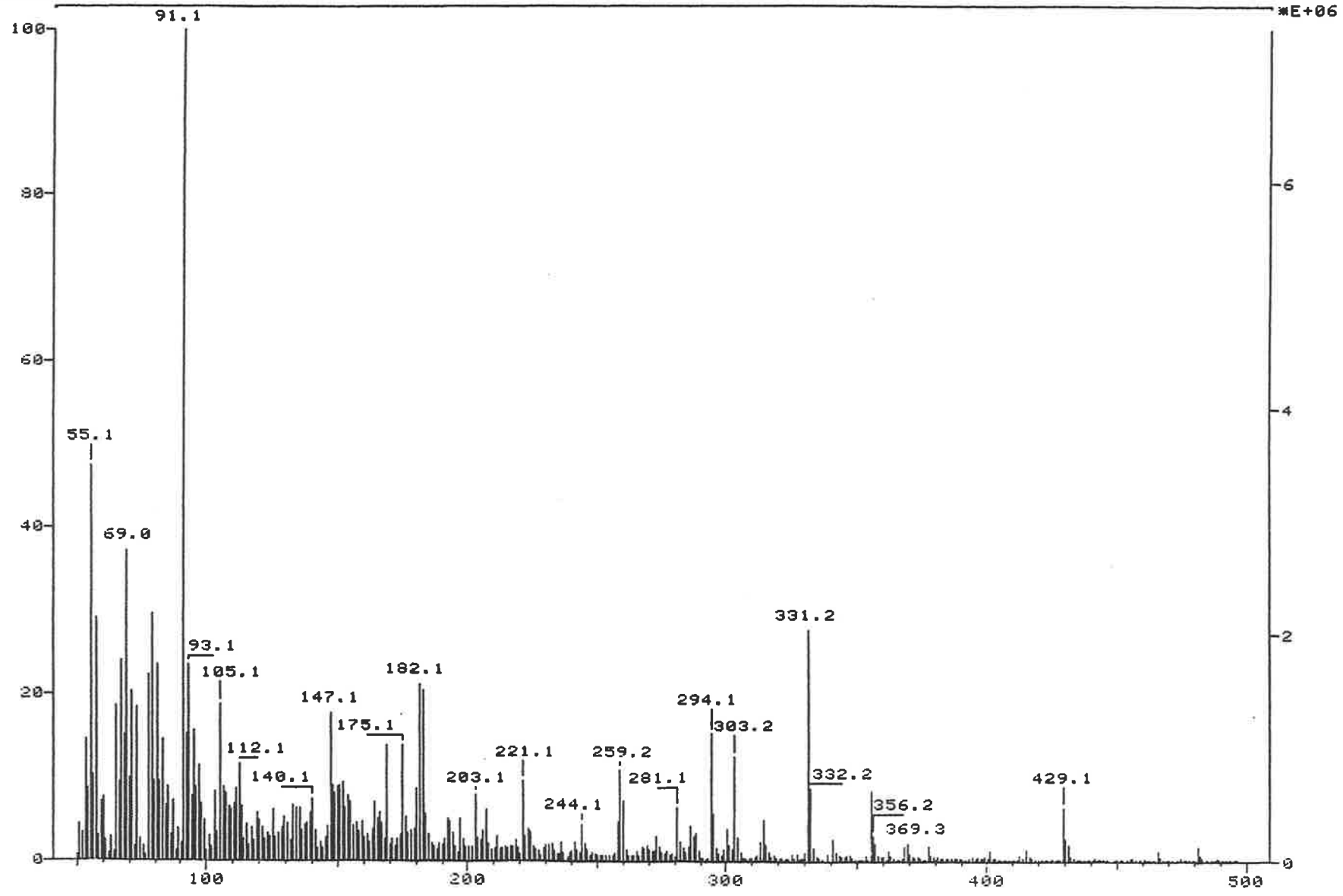


Figure 9.14a RIC trace for the extract of a 21-day-old culture of DAR 71768. Only scans 69-76 and 90-97 for the minor peaks (Fig. 9.14b and 9.14c) provided evidence for solanapyrone A and C, respectively. Scans 47-54 and 52-59 (data not shown) showed no major $M^{+\cdot}$ ions corresponding to solanapyrone A, B and C.

```
CHRO: GB98NO24D ver 1 on UIC 3 1      14-NOV-98 Elapse: 00:00:09.2    9
Samp:    I                                      Start : 21:15:07   295
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE                                     Inlet :
Peak: 1000.00 mmu     Label wndw: 1 > 295       Masses: 50 > 650
Area: 0, 4.00         Baseline  : 0, 3          Label : 0, 40.00
```

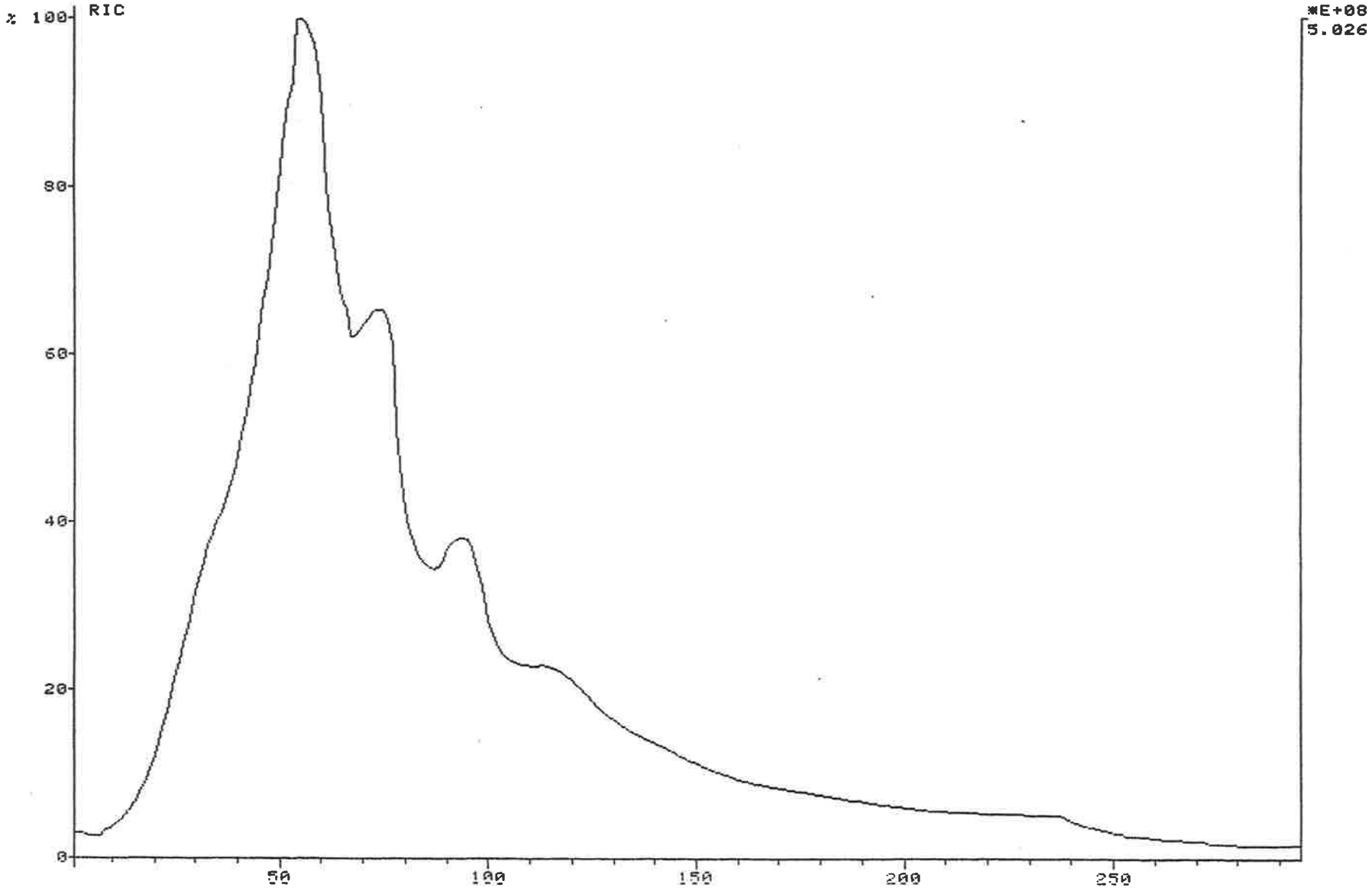


Figure 9.14b Scans 69-76 for 21-day-old culture of DAR 71768, showing clear evidence for solanapyrone A $M^{+\cdot}$ ions at 302.1 and fragments ions 274.1, 245.1, 181 and 153 (see also Fig. 9.9b).

SPEC: GB98NO24D ver 1 on UIC 3 1 14-NOV-98 DERIVED SPECTRUM 9
Samp: I Start : 21:15:07 10
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE Inlet :
Base: 55.0 Inten : 88028312 S Masses: 50 > 650
Norm: 55.0 RIC : 2287707648 # peaks: 563
Peak: 1000.00 mmu
Data: + 69 > 76 - 162 > 168

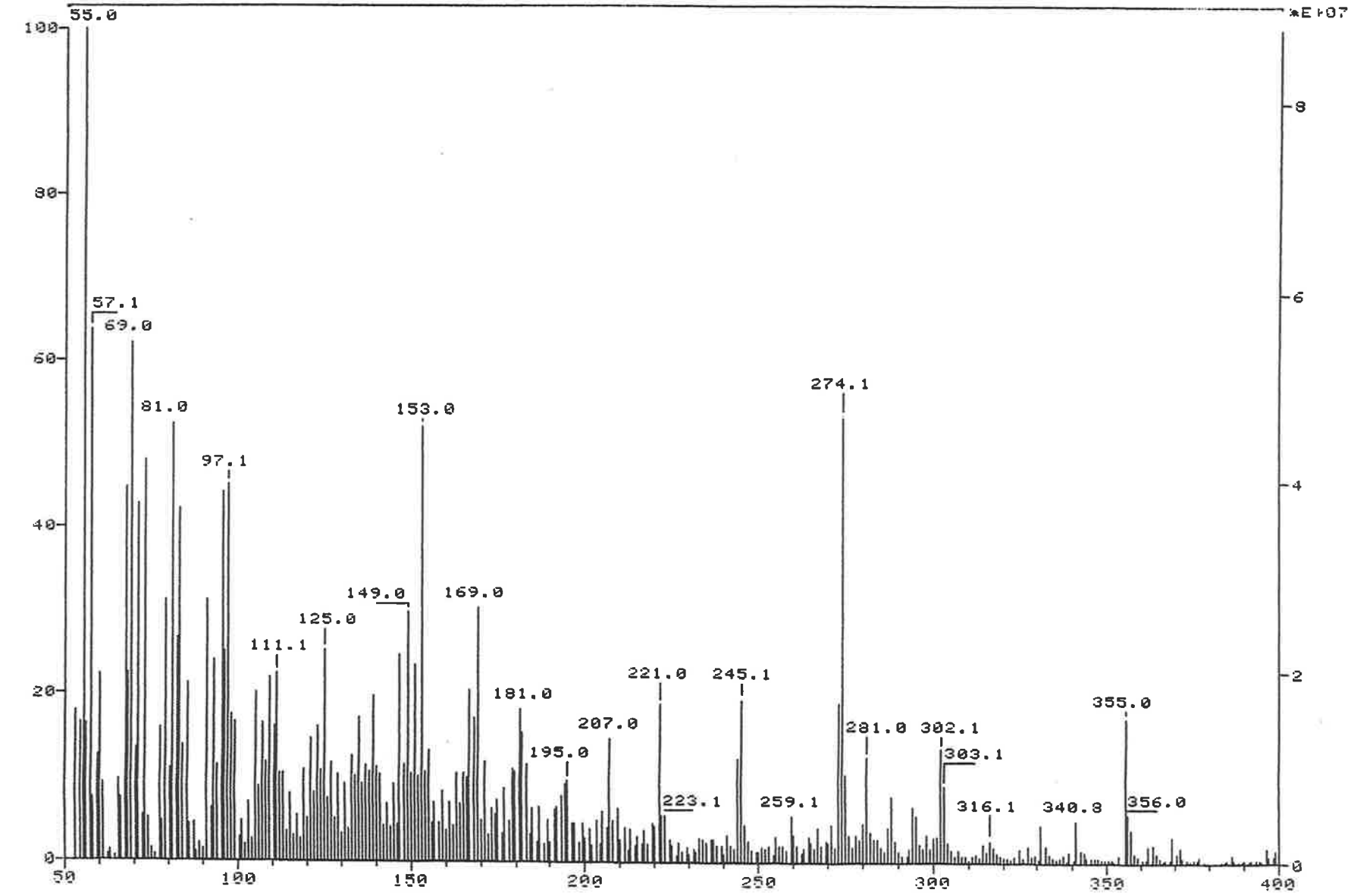


Figure 9.14c Scans 90-97 for an extract of a 21-day-old culture of DAR 71768, These scans provide further evidence for the presence of solanapyrone C (see Fig. 9.11b) with an $M^{+\cdot}$ ion at 331.1m/z and fragment ions 303.1, 259.1, 244.1, 224, 182, 112 and 91.

SPEC: GB98NO24D ver 1 on UIC 3 1 14-NOV-98 DERIVED SPECTRUM 9
Samp: I Start : 21:15:07 10
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE Inlet :
Base: 91.0 Inten : 53167616 Masses: 50 > 650
Norm: 91.0 RIC : 1079464320 # peaks: 540
Peak: 1000.00 mmu
Data: + 90 > 97 - 154 > 162

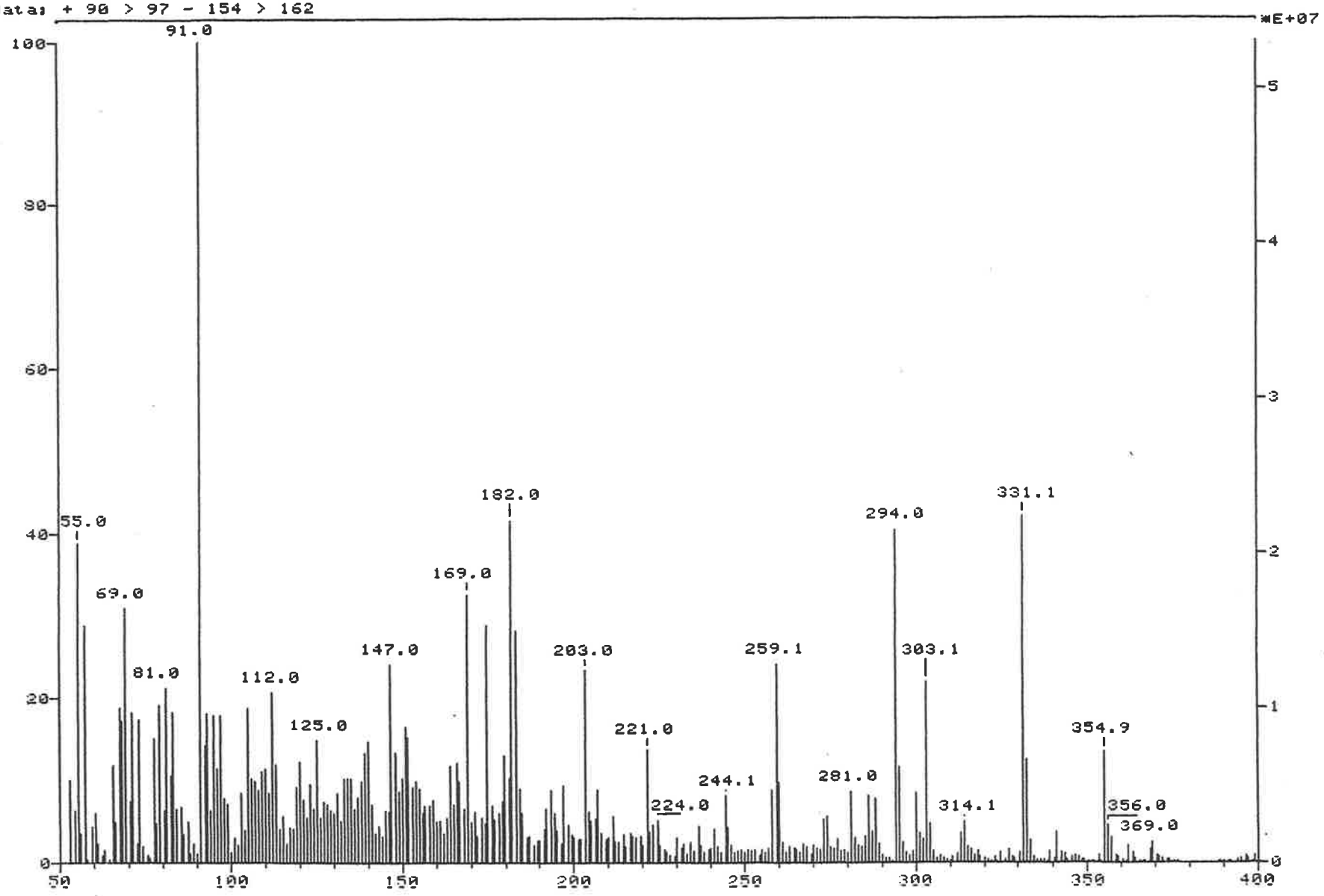


Figure 9.15a Single ion (304, 331, 345 m/z) traces and a reconstructed (total) ion current trace for an extract of 21-day-old culture of 215/91. The single ion (331m/z) trace and Fig. 9.15b indicate the presence of solanapyrone C in scans 75-81. Other scans 39-46, 53-57 (data not shown) were uninformative.

```
CHRO: GB98NO24E ver 1 on UIC 3 1      14-NOV-98 Elapse: 00:00:09.9    9
Samp:  SAMPLE II                              Start : 21:26:44    294
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP LR
Oper: GAYLE                                   Inlet :
Peak: 1000.00 mmu     Label wndw: 1 > 294     Masses: 50 > 650
Area: 0, 4.00         Baseline  : 0, 3        Label : 0, 40.00
```

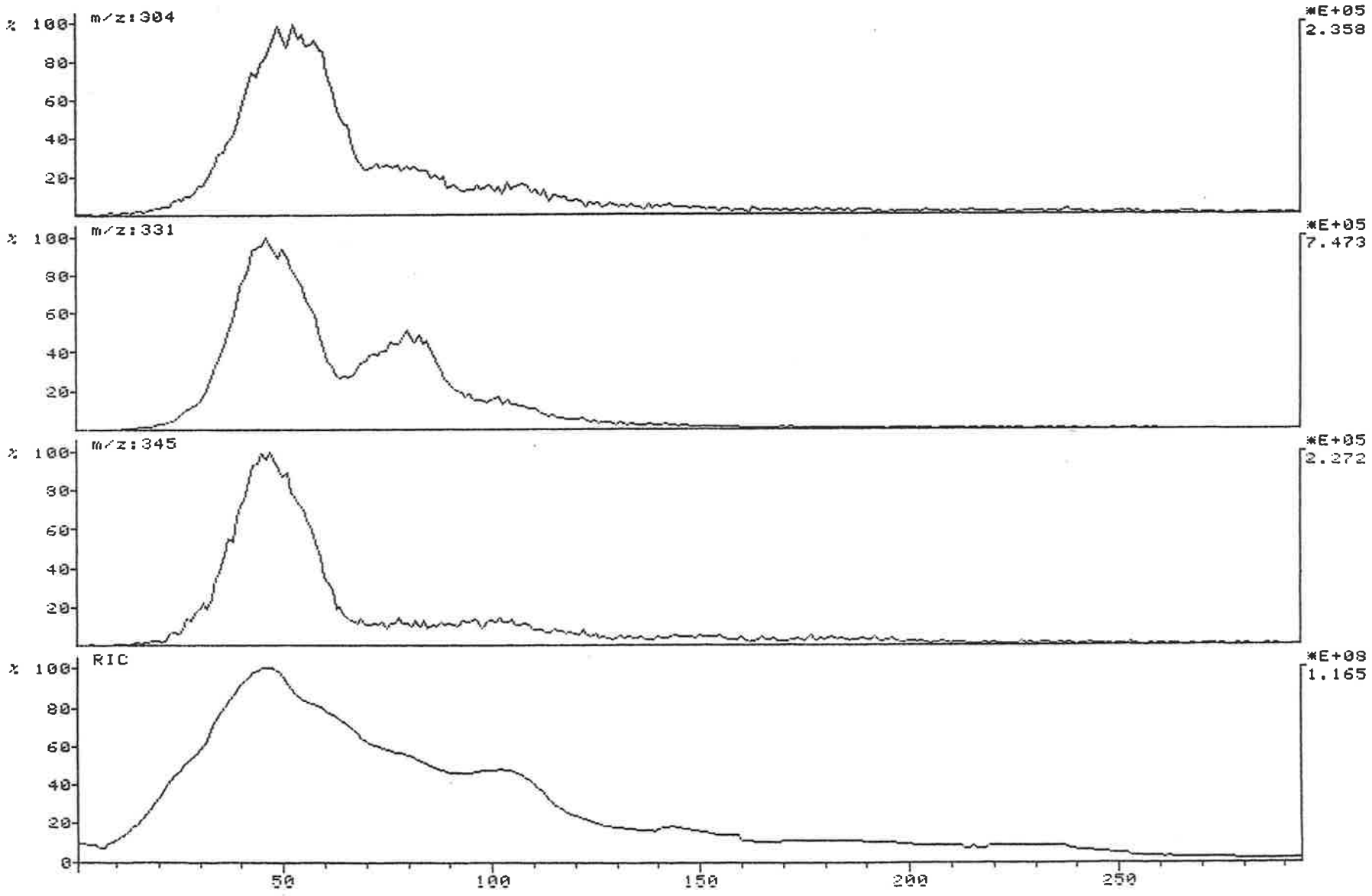


Figure 9.15b In addition to unrelated ions, the scans 75-81 show a strong $M^{+\cdot}$ ion at 331.1 with fragment ions 303, 259 and 182 consistent with presence of solanapyrone C (see Fig. 9.4b).

SPEC: GB98NO24E ver 1 on UIC 3 1 14-NOV-98 DERIVED SPECTRUM 9
Samp: SAMPLE II Start : 21:26:44 10
Comm: DIRECT INSERTION, SHAHID/MAX, CROP PROTESTIO
Mode: EI +Q3MS LMR UP,LR
Oper: GAYLE Inlet :
Base: 73.0 Inten : 6798635 Masses: 50 > 650
Norm: 73.0 RIC : 171343808 # peaks: 465
Peak: 1000.00 mmu
Data: + 75 > 81 - 110 > 117

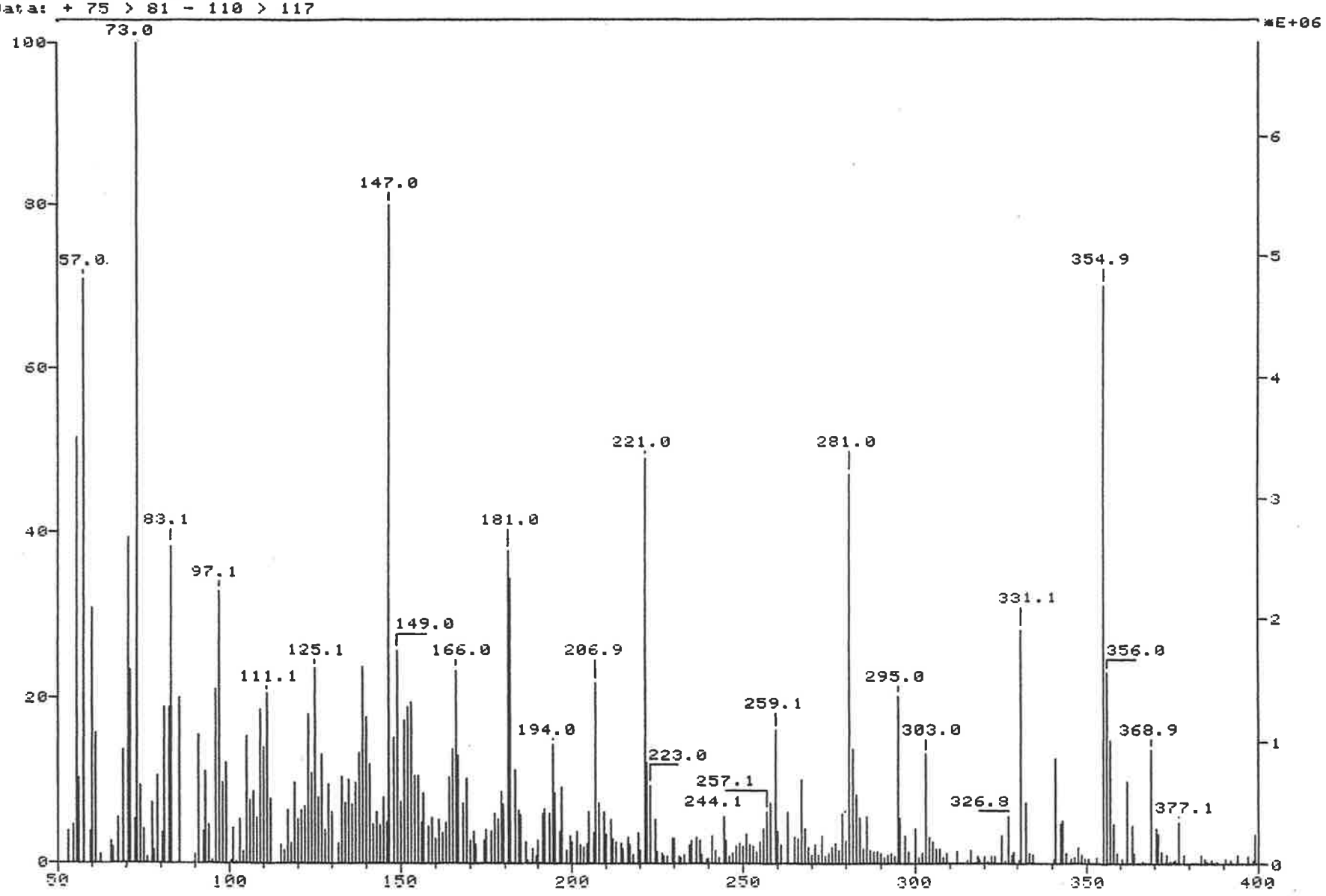


Table 9.1 Summary of data for solanapyrones in reference standards and partially purified extracts of cultures of *A. rabiei* isolates DAR 71767, DAR 71768 and 215/91

Sample	HPLC Rtn Time (Min)	UV Max. (nm) Diode array	UV, Lit. Ichihara *et al.*, 1983[1]	$M^{+\cdot}$, $Fg^{+\cdot}$,[2]	$M^{+\cdot}$ Calc. [3]	TLC (mm) from origin	Assignts. Solanapyrone[4]
Solanopyrone A1	11.13 (major)	231.1, 325.8	232,327	302.1	302	19.5	A1 Std (major)
Solanopyrone A2	13.05 (minor)	231.1, 325.0	232,327	303.2, 317.2 331.2, 345	331	37.5	A2 Std (minor)
Solanopyrone B	11.76	209, 302.5	303	304.1	304	17.5	B (std)
Solanopyrone C	13.66	239.8, 283.6, 317.2	238, 282, 320	331.2	331	9	C (std)
DAR 71767 (21-day-old culture)	13.67	239.8, 283.6, 317.2	238, 282, 320	331.2	331	9.5	C (strong)
DAR 71767 (14-day-old culture)	-	-	-	No ions corresponding to A, B, or C	-	No spots corresponding to A2, B, C	-
DAR 71768 (21-day-old culture) Peak at 14.2 has UV of Solanopyrone C and masses in MS	12.69 14.20	232.0, 325.8 239.8, 283.6, 317.2	232, 327 238, 282, 326	302.1 303.1, 331	302 331	18.5 9.5	A (med) C (wk)
215/91 (21-day-old culture)	12.81 13.2(sh)	223.7, 325.8 - -	232, 327 238, 282, 320	302.1, 303.1 331.1	302 331	18 (vwk) 9 (vwk)	A (vwk) C(?)(vwk)

[1] Corresponding UV max. diode array reported by Ichihara et al. (1983) for solanapyrones of *Alternaria solani*

[2] $M^{+\cdot}$ = Molecular radial ions $Fg^{+\cdot}$ = Fragment ions

[3] $M^{+\cdot}$ Calc. = Molar mass calculated as the sum of the atomic masses in the corresponding formulae Fig 9.2

[4] Assignment of solanapyrone; std= reference standards; med = medium; wk = weak; vwk = very weak

9.4 DISCUSSION

Preliminary experiments were undertaken with *A. rabiei* isolate DAR 71767 in order to optimise the conditions. No production of solanapyrone was detectable in filtrate obtained from 14-day-old cultures, therefore filtrates from 21-day-old cultures were used in subsequent experiments involving three isolates.

There is good agreement between observed and expected values for both standards solanapyrones and the putative assignments of solanapyrones in extracts of DAR 71767, DAR 71768 and 215/91, which is shown in Table 9.1. Alam *et al.* (1989) and Chen and Strange (1994) also observed the production of solanapyrones A and C among most of their isolates of *A. rabiei.* In contrast, Hohl *et al.* (1991) and Chen and Strange (1991) observed the presence of solanapyrones A, B and C and concluded that the components produced were dependent on the basal medium used. In the work of Hohl *et al.* (1991), solanapyrone B was found to be the major toxin in the nine isolates which were examined. Kaur (1995) observed only solanapyrone A in the spore germination fluid of *A. rabiei.* Because of the close structural relationship (see Fig. 9.2) between solanapyrone A (the aldehyde) and solanapyrone B (the primary alcohol), which is the reduction product of A, it is probably not unexpected that the composition of the medium and, consequently, its redox status is important to the observed levels of solanapyrone B. The observation of Kaur (1995) that solanapyrone A can be produced in the absence of solanapyrone B may indicate that solanapyrone A is the immediate precursor for solanapyrone B.

There was clear evidence for the presence of solanapyrone C in extracts from 21-day-old cultures of both aggressive Australian isolates (DAR 71767 and DAR 71768) as shown by the data summarised in Table 9.1. In addition DAR 71768 showed the presence of solanapyrone A. Only a small amount of solanapyrone A was detectable in the intermediate isolate (215/91), and the HPLC and TLC data also suggested a trace amount

of solanapyrone C, but no diode array data were obtained to confirm this. No evidence was obtained for the presence of solanapyrone B in any of the extracts. Initially, the detection of solanapyrone A was confounded by a reference sample which contained two peaks in the HPLC traces and two spots on the TLC plates. This could have arisen by gradual deterioration of the sample in storage. Both components had the same chromophore, but only the major component exhibited a molecular ion at 302 m/z corresponding to solanapyrone A.

Culture filtrates of the aggressive Australian *A. rabiei* isolates, DAR 71767 and DAR 71768, therefore, showed the presence of solanapyrone C in the former and solanapyrone A plus C in the latter. However, in culture filtrates of the less aggressive isolate, 215/91, only trace amounts of solanapyrone A and, possibly, solanapyrone C were observed. These data are in accord with the work of Chen and Strange (1991). There was a notable absence of solanapyrone B in all three Australian isolates. It has been reported that solanapyrone A and C from *A. rabiei* were active individually and had an additive effect in combination (Alam *et al.*, 1989). Likewise, Ichihara *et al.* (1983) found that solanapyrone A and C produced by *Alternaria solani*, were active only in combination. Both DAR 71767 and DAR 71768 caused severe disease in spite of the observation here that the former produced only solanapyrone C. However, it is naturally that disease severity in chickpea plants inoculated with both isolates in combination was greater than in plants inoculated with single isolates in the glasshouse studies reported in chapter 5. The reason for trace production of solanapyrones in 215/91 might be the age of the isolate, which had been obtained in 1991 and stored in SDW.

In the current work, only isolate DAR 71767, which produced only solanapyrone C *in vitro,* was tested for biological activity towards resistant (Dooen) and susceptible (Desavic) chickpea cultivars. From these limited data and assuming that toxin production *in vivo* minimics the solanapyrone toxin *in vitro*, it seems reasonable to suggest that the

resistance and susceptibility towards DAR 71767 has evolved towards the solanapyrone C molecule.

It is known that the growth medium influences the nature of the solanapyrones produced (Chen and Strange, 1991), therefore it would be of interest in future studies to examine whether the incorporation of an extract from the resistant chickpea (Dooen) gave rise to the same solanapyrone profiles as the medium used here which employed an extract of the susceptible chickpea (Desavic). Further studies involving more isolates and a wider range of conditions for *in vitro* culture and for bioassays, would contribute to our understanding of toxin production in *A. rabiei*.

CHAPTER 10

MATING TYPE STUDIES

10.1 INTRODUCTION

A. rabiei is heterothallic, with a bipolar and biallelic mating system. Sexual reproduction, therefore, requires contact between two compatible mating types, designated MATI-1 and MATI-2, and results in the formation of pseudothecia (Wilson and Kaiser, 1995).

The teleomorph plays an important role in the life cycle of a pathogen, as it contributes to increased variability in the population and provides additional means of survival and dissemination of inoculum. Pseudothecia are considered important in the epidemiology and control of ascochyta blight in Bulgaria, Greece, Spain, Syria, Turkey and USA (Kaiser and Kusmenoglu, 1997). Kaiser (1997) induced the development of pseudothecia in naturally infected chickpea debris obtained from the countries listed previously and from Algeria, Canada, Iran, Morocco, Pakistan, Portugal and Tunisia, and suggested that *D. rabiei* is present in more countries than previously reported. In addition, both mating types have been recorded in infected chickpea seed and residues obtained from France and Italy (Kaiser, 1997).

The release of ascospores coincides with the susceptible vegetative growth stages of the chickpea crop, providing primary inoculum for epidemics in the Palouse region of the Pacific Northwest, USA (Trapero-Casas and Kaiser, 1992). A similar situation has been reported in southern Spain, where maximum disease coincided with the release of large quantities of ascospores during rainy days (Trapero-Casas *et al.*, 1996). Trapero-Casas

and Kaiser (1992) have estimated that approx. 15,000 ascospores were produced per square mm of severely infested chickpea debris in the Palouse region.

Ascospores of *D. rabiei* may be spread over long distances by wind, whereas the conidia are dispersed over relatively short distances by rain-splash and wind-borne rain (Kaiser and Muehlbauer, 1988; Trapero-Casas and Kaiser, 1992; Trapero-Casas *et al.*, 1996). Kaiser (1997) described the importance of the presence or absence of the teleomorphic state in outbreaks of ascochyta blight in the Pacific Northwest, USA and California, USA, respectively. Long-distance dissemination of ascospores provided primary inoculum for establishing new disease foci in chickpea fields located at distances of up to 15 km from the nearest blighted fields and played a major role in a blight epidemic in the Pacific Northwest in 1987 (Trapero-Casas and Kaiser, 1992). In comparison, seed infected with *A. rabiei* MATI-2 only is the primary source of inoculum in California, where disease outbreaks are more localised (Kaiser, 1995).

The teleomorph has not been observed in Australia since the first record of ascochyta blight in commercial crops in South Australia in 1995. The distribution of the disease prior to 1998 was consistent with the existence of the anamorph only. The disease was sporadic in 1997, however, it appeared in epidemic form in 1998, giving rise to concern that the teleomorph may be present in the field. Ascochyta blight devastated some crops which had been established from apparently disease-free seed treated with fungicide at the time of sowing. Therefore, the objective of the studies presented in this chapter was to determine whether both mating types of the pathogen were present in material obtained from severely affected crops in Australia, and whether the teleomorph could be induced in naturally infected or inoculated chickpea tissues.

10.2 MATERIALS AND METHODS

10.2.1 Mating type and mycelial intercompatibility studies of *A. rabiei* in Australia

All isolates used in studies of pseudothecium development, except DAR 71767 and DAR 71768, were collected from commercial crops severely affected by ascochyta blight during the growing season of 1998. Sixteen isolates of *A. rabiei* obtained from crops surveyed in 1998 (Table 10.1), were paired *in vitro* with DAR 71767 and DAR 71768, both of which had previously been identified as MATI-1 (see section 4.2.6). Isolates of MATI-2 were not available in Australia.

Cultures were grown on PDA and conidial suspensions ($5x10^5$ conidia/mL) prepared as described in section 3.4. Equal volumes (10 mL) of each test isolate and either DAR 71767 or DAR 71768, were mixed in sterile McCartney bottles. Dried chickpea stem pieces (6 cm long), collected from healthy plants grown in the field, were autoclaved twice at 121°C for 20 minutes. Sterilised stem pieces were cooled and immersed in mixed conidial suspension for 1 h. The spore suspension was then decanted and the stem pieces were allowed to drain for 15 minutes in the bottles. Four stem pieces were placed in each 9 cm diameter Petri dish containing 10 filter papers (Whatman No.1) moistened with 15 mL of SDW (Trapero-Casas and Kaiser, 1992). Each cross was represented by four replicate Petri dishes, each with four pieces of chickpea stem, and stem pieces immersed in spore suspension of each isolate separately were used as controls.

The Petri dishes were incubated for 48 h at 20°C and then at 10°C for 10-12 weeks. SDW, 10 mL, was added weekly to the filter papers in the Petri dishes to maintain a high moisture level. Observations for pseudothecium development were made using a stereoscopic microscope 4 weeks after inoculation and at weekly intervals for 12 weeks.

Each of the 16 isolates was paired with DAR 71767 and DAR 71768 on PDA plates to determine mycelial compatibility reactions. Plugs, 5 mm, in diameter, were placed 5 cm apart on the agar. Plates were sealed with parafilm (American National Can, Neenah, WI) and incubated as described above.

Table 10.1 Origin of isolates crossed with DAR 71767 and DAR 71768 (MATI-1) *in vitro*.

Isolate	Locality	Collection Date	Cultivar
81-2/98	Owen (SA)	10-9-98	Semsen
81-3/98	"	"	"
81-7/98	"	"	"
81-8/98	"	"	"
81-9/98	"	"	"
81-10/98	"	"	"
81-12/98	"	"	"
81-13/98	"	"	"
133/98	Goondiwindi (QLD)	8-9-98	Unknown (desi)
134/98	Wagga Wagga (NSW)	9-9-98	Unknown (desi)
147/98	Bethal-1(SA)	10-9-98	Heera
148/98	Tanunda (SA)	"	Dooen
149/98	Bethal-2(SA)	"	Heera
151/98	Bethal-3(SA)	"	Heera
152/98	Bethal-4 (SA)	"	Lasseter
153/98	Salter Spring (SA)	"	Kaniva

10.2.2 Incubation of diseased residues

As *A. rabiei* was positively identified in a seed sample harvested in 1992, diseased residues were collected from straw bales of that same crop. After surface sterilisation as described in section 3.3.2, 80 stem pieces were incubated as described in section 10.2.1. Residues of cultivars Barwon, Desavic, Dooen, Kaniva and Tyson, which had been inoculated with isolates DAR 71767 and DAR 71768, were collected at the conclusion of

the field trial at the Waite Campus in November, 1996. Each replicate plot was represented by four replicate Petri dishes, each with four pieces of chickpea stem, for each cultivar. Diseased stem pieces (6 cm long) were surface sterilised as described in section 3.3.2 and incubated as above to promote the development of pseudothecia.

10.3 RESULTS

10.3.1 Mating type and mycelial intercompatibility studies of *A. rabiei* in Australia

None of the stem pieces inoculated with a mixture of conidia of the DAR 71767 or DAR 71768 and one of the 16 isolates obtained from severely affected commercial crops in 1998 developed pseudothecia. Only pycnidia were formed.

In mycelial compatibility tests, none of the 16 isolates was compatible with either DAR 71767 or DAR 71768. The mycelia did not intermingle, rather the colonies stopped growing at about 5 mm distant from one another. The colonies were otherwise normal and formed abundant pycnidia.

10.3.2 Incubation of diseased residues

Stem pieces collected from naturally infected chickpea straw harvested in 1992 and from plants inoculated in the bird-proof enclosure at the Waite Campus in 1996 and incubated in conducive conditions did not develop pseudothecia.

10.4 DISCUSSION

The teleomorph of *A. rabiei* was not found in naturally infected or inoculated chickpea straw incubated in conditions previously reported to be conducive to its development. It

is possible that Australian isolates of *A. rabiei* may have requirements for sexual reproduction which differ from isolates elsewhere. However, in view of the failure of isolates obtained from widely distributed sources to mate with either DAR 71767 or DAR 71768, known to be MATI-1, it is considered likely that all isolates examined to date are MATI-1. Likewise, the results of mycelial intercompatibility tests showed that none of the test isolates were compatible with DAR 71767 or DAR 71768. The absence of the teleomorph in diseased residues from the field experiment in 1996, in which five varieties were inoculated with DAR 71767 and DAR 71768, suggests that natural infection by MATI-2 genotypes did not occur on this material.

A similar situation has been reported in California, where extensive testing has revealed only isolates of MATI-2 (Kaiser, 1997). The sample size in the present study, however, was small so no conclusions can be drawn regarding the absence of mating type MATI-2 in Australia. There is a need to study large numbers of isolates from all states of Australia where ascochyta has been positively identified. Also, it would be valuable to have MATI-2 standard isolates available in Australia, under strict quarantine restrictions, for testing mating type *in vitro*. However, this was not attempted due to the possible risks posed to the Australian chickpea industry.

The failure to find *A. rabiei* MATI-2 among isolates obtained from crops affected during the ascochyta blight epidemic in south eastern Australia in 1998 suggests that infected seed, harvested from diseased crops exposed to heavy rain at the end of October 1997 (see section 6.3.2), may have provided primary inoculum for this epidemic.

CHAPTER 11

GENERAL DISCUSSION

At the initiation of this project, ascochyta blight had not been recorded in commercial chickpea crops in Australia, although there was one record in 1973 in a chickpea germplasm evaluation trial at the Waite Campus, the University of Adelaide. During pathogenicity testing of *Phoma*-like isolates from commercial chickpea crops, two were highly pathogenic and caused symptoms typical of ascochyta blight. Subsequently, these two isolates were identified as *Ascochyta rabiei* based on morphological characters, and by collaborators using RAPD-PCR in France and mating type studies in Italy. This is the first time that *A. rabiei* has been conclusively identified in commercial chickpea crops in the southern hemisphere. By the end of the 1998 growing season the disease had been positively identified in South Australia, Victoria, New South Wales and Queensland. Western Australia, a major contributor to Australian chickpea production, remains free from ascochyta blight. Therefore, it is suggested that strict quarantine should be imposed to avoid the introduction of *A. rabiei* to Western Australia.

Keeping in view the potential threat of *A. rabiei* to the Australian chickpea industry, and that resistance offers the best means of control, material from the Australian chickpea breeding programs and also material imported from Pakistan was evaluated for level of resistance to ascochyta blight. Among the commercial cultivars tested, Dooen was moderately resistant and Desavic was highly susceptible to *A. rabiei.* Breeding lines, such as ICC 03996, 8511-14, 8511-19, ILC 3279, ICC 1151xILC 3279 and ICC 1151xILC 482, identified as having resistance are now being used in the National Chickpea Breeding Program to develop ascochyta blight resistant cultivars for use in Australian conditions.

The optimal conditions for disease development, as determined by experiments conducted in controlled conditions, were 20°C and a 48-72 h wetness period following inoculation. At 10°C or 30°C a longer wetness period was required than at 20°C. Disease was more severe on the seedling stage compared to older plants. These findings were in agreement with those of Trapero-Casas and Kaiser (1992). In field trials, disease intensity increased over time, especially in cv. Desavic, which was killed 7 weeks after inoculation in 1997. The responses of the cultivars tested under controlled and field conditions were generally similar, suggesting that it should be possible to screen routinely for resistance under controlled environment conditions, thus eliminating some of the problems associated with screening for disease resistance in the field. During three consecutive years of field trials, it was observed that the disease was most severe in 1997. The weather conditions were conducive and rainfall in October, when pods were mature, resulted in severe disease, especially on Desavic. Although pods were not harvested and examined, such conditions would promote infection of seed.

Ascochyta blight was recognised in epidemic form in Australia for the first time in 1998 and was widely distributed. Based on the observations made in the course of this study, a possible explanation is as follows. Tyson (desi type) was released in 1979 as the first commercial variety of chickpea for general cultivation in Australia (Beech and Brinsmead, 1980). It was originally released as cv. C-235, and had been selected from the cross "IP58 x C 1234" made at the Indian Agricultural Research Institute on the basis of high yield and ascochyta blight resistance (Bedi and Athwal, 1962). It is suggested that earlier cultivars had some resistance to ascochyta blight, so that the disease occurred at low levels in Australian crops until the highly susceptible cv. Desavic was released in 1993. The widespread cultivation of Desavic appears to have led to significant levels of disease being observed in commercial crops. Prior to 1995/96, symptoms apparently were confused with other diseases, such as phoma blight. Ascochyta blight at first did not appear in severe form, possibly due to low levels of inoculum and dry weather. Then, the gradual build up of inoculum on cultivars such as Desavic led to widespread

disease in 1997. Heavy rainfall in October 1997 may have resulted in significant seed infection. It is common practice for growers to sow seed returned from the previous crop rather than to buy in fresh seed and this may have contributed to the epidemic of ascochyta blight in 1998.

To date, only the anamorph of the fungus has been found in Australia and all isolates tested appear to be mating type MATI-1. However, it is suggested that further detailed studies involving more isolates be conducted as the sample size in the experiments was small. In the apparent absence of the teleomorph, infected seed is considered the most likely means of survival and dissemination of *A. rabiei.* Current national quarantine restrictions on chickpea should remain to prevent accidental introduction of MATI-2 until the release of chickpea genotypes which are resistant to ascochyta blight, it is recommended that growers avoid using seed from diseased crops and apply a fungicide to seed (Khan *et al.*, 1999). Grewal (1982) found that treatment of chickpea seed with a combination of carbendazim and thiram gave complete control of both internal and external infections of *A.rabiei*, as well as *Botrytis* and *Fusarium* spp.

As *A. rabiei* apparently does not survive for more than 2 years on infected debris (Navas-Cortes *et al.*, 1995), rotation of chickpea with non-host crops would reduce the level of inoculum considerably. In contrast with previous reports that chickpea is the only host of *A. rabiei* (Sprague, 1930; Khachatryan, 1962; Zachos *et al.*, 1963; Tripathi *et al.*, 1987), this study has identified four common bean varieties as hosts. This finding has implications for areas where the two crops may be grown in rotation. Further work is required, involving more isolates and a wider range of plant species, to find out if there are any other alternative hosts. This would indicate crops which could safely be used in rotation with chickpeas in Australia.

Breeding for resistance to ascochyta blight would be enhanced by further information on the interactions between the host and the pathogen. This study has provided evidence

that *A. rabiei* can penetrate the plant via stomata as well as by the previously documented routes of direct penetration of the epidermis and the stomatal guard cells (Pandey *et al.*, 1987; Hohl *et al.*, 1990). Penetration and the early stages of infection were similar in a moderately resistant and a susceptible cultivar, suggesting that resistance does not involve reduced invasion, at least in the first 96 h after inoculation. Rather, the response of the host tissue to toxins produced by *A. rabiei* may be involved in resistance.

Pathogenicity is directly correlated with the production of vivotoxins by several fungi (Luke and Wheeler, 1955; Strobel, 1974; Kohmoto *et al.*, 1979; Marcinkowska *et al.*, 1982), including *A. rabiei* (Chen and Strange, 1991). Solanapyrone C was found in culture filtrates of DAR 71767, solanapyrones A and C in DAR 71768 and only traces of solanapyrone A and C in the less aggressive isolates, 215/91. The low level of solanapyrones in the culture filtrate of isolate 215/91 could be due to the age of the culture, which had been stored in SDW since 1991. It has also been reported that solanapyrone production may be affected by fungal strain and the cultural conditions (Chen and Strange, 1991; Kaur, 1995). Further studies are required to examine toxin production by Australian isolates of *A. rabiei* to elucidate the mechanism of action of solanapyrones in pathogenesis, and to explore the suggestion that resistance in the host may be related to response to solanapyrones.

In conclusion, this study has clarified the confusion between phoma and ascochyta blight of chickpea in Australia, and has provided advance warning of ascochyta blight disease for the expanding Australian chickpea industry. Confirmation that the disease is caused by *A. rabiei* will allow the implementation of appropriate management strategies. Resistance identified in breeding lines has advanced the National Chickpea Breeding Program and it is hoped that resistant cultivars will be released for cultivation in areas of high risk. Other important outcomes include the recommendations that (i) cv. Desavic, currently widely grown, should not be grown where ascochyta blight is likely to be a problem as it is highly susceptible; (ii) quarantine restrictions on the importation of

chickpea seeds be maintained to prevent the introduction of MATI-2; (iii) national quarantine should be strictly inforced to prevent the introduction of *A. rabiei* to Western Australia, which could provide a source of disease-free seed for cultivation Australia-wide.

APPENDICES

Appendix 1: Type and origin of chickpea genotypes used in the study

No	Genotype	Type (Desi/Kabuli)	Origin (Country)
1	Amethyst	Desi	Australia
2	Barwon	Desi	Australia
3	Desavic	Desi	India
4	Dooen	Desi	Australia
5	Garnet	Kabuli	Turkey
6	Kaniva	Kabuli	Spain
7	Narwin	Desi	Australia
8	Narayen	Kabuli	USSR
9	Opal	Kabuli	USSR
10	Semsen	Desi	Unknown
11	Tyson	Desi	India
12	CPI 56288	Desi	Iran
13	CTS 11308	Desi	India
14	ICC 14307	Desi	India
15	T 1587 = ICC 8217	Desi	India
16	Lasseter	Desi	Iran
17	ICCV 88201	Desi	India
18	ICCV 88202	Desi	India
19	8805-78H	Desi	Australia
20	8819-4H	Desi	Australia
21	8511-14	Desi	Australia
22	8511-19	Desi	Australia
23	8813-63H (Jimbour)	Desi	Australia
24	8627P-2	Desi	Australia
25	8652-36	Desi	Australia
26	86130-5	Desi	Australia
27	WAD 27	Desi	Australia
28	WAD 032	Desi	Australia
29	Macarena	Kabuli	Mexico
30	G846-2-5	Kabuli	Australia

31	G846-3-6	Kabuli	Australia
32	ATC 40538 = CPI 56288	Desi	Iran
33	PI 13768	Desi	India
34	CW 0012	Desi	Unknown
35	CW 0311	Desi	Unknown
36	ATC 40825 = ILC 3279	Kabuli	USSR
37	ILC 00072	Kabuli	Unknown
38	NEC 02443 = ICC 8269	Desi	Turkey
39	ATC 41564 = CPI 56288	Desi	Iran
40	ICC 03996	Desi	Iran
41	ATC 41890 = ILC 3279	Kabuli	USSR
42	ICC 04958	Desi	India
43	ATC 42021 = CTS 11308	Desi	India
44	CTS 60543 = ATC 42022	Desi	India
45	ATC 41933 = ILC 3279	Kabuli	USSR
46	ICC 3996	Desi	Iran
47	FLIP85.58	Kabuli	Syria
48	ILC 3279	Kabuli	USSR
49	8523.1	Desi	Australia
50	ATC41890 = 1LC 3279	Kabuli	Australia
51	8813-31H	Desi	Australia
52	92.193.1.7	Kabuli	Australia
53	WACPE 2003	Desi	Australia
54	WACPE 2012	Desi	Australia
55	WACPE 2016	Desi	Australia
56	8813-74H	Desi	Australia
57	8616.2H	Desi	Australia
58	Damla	Kabuli	Syria
59	8810-2	Desi	Australia
60	WACPE 2017	Desi	Australia
61	WACPE 2021	Desi	Australia
62	8506-05	Desi	Australia
63	8813-113H	Desi	Australia
64	8829-17	Desi	Australia
65	FLIP 8685C	Kabuli	Syria
66	Sona	Desi	India

67	WACPE 2004	Desi	Australia
68	92.194.1.11	Kabuli	Australia
69	Heera	Desi	India
70	WACPE 2011	Desi	Australia
71	WACPE 2014	Desi	Australia
72	8903P-03B	Desi	Australia
73	8905-14N	Desi	Australia
74	Bumper = G5846-2-5	Kabuli	Australia
75	WACPE 2001	Desi	Australia
76	Whitey = ATC 41980	Kabuli	Israel
77	ILC 482	Kabuli	Turkey
78	Sanford	Kabuli	USA
79	T 1069	Desi	Morocco
80	T1822 = ICC 2450	Desi	Iran
81	WACPE 2019	Desi	Australia
82	8513-10	Desi	Australia
83	86.059.3.2	Desi	Australia
84	8809-19H	Desi	Australia
85	8825-59H	Desi	Australia
86	8931-52Q	Desi	Australia
87	92.185.1.1	Kabuli	Australia
88	92.186.2.10	Kabuli	Australia
89	92.187.1.8	Kabuli	Australia
90	92.194.1.14	Desi	Australia
91	ILC 482.205	Desi	Turkey
92	365.117	Desi	Australia
93	8652.36	Desi	Australia
94	8673.3	Desi	Australia
95	8801-35	Desi	Australia
96	8801-92H	Desi	Australia
97	8820-118H	Desi	Australia
98	8825-20H	Desi	Australia
99	92.186.1.5	Kabuli	Australia
100	92.194.1.9	Kabuli	Australia
101	ILC 1463	Kabuli	Afghanistan
102	WACPE 2013	Desi	Australia

103	8502-39	Desi	Australia
104	8518-48	Desi	Australia
105	86.085.54	Kabuli	Australia
106	92.187.1.1	Kabuli	Australia
107	ILC 6055	Kabuli	USA
108	8623.5	Desi	Australia
109	Blanco Lechoso	Kabuli	USA
110	8914-65Q	Desi	Australia
111	8931-6Q	Desi	Australia
112	G846-3-4B	Kabuli	Australia
113	Gully	Desi	Iran
114	T1239 = ICC2910	Desi	Iran
115	8806-33H	Desi	Australia
116	940-105	Desi	Australia
117	G 846-1-1B	Kabuli	Australia
118	ICCV 93928	Desi	India
119	90140-46Q	Desi	India
120	ICCV 92504	Desi	India
121	Spanish White	Kabuli	USA
122	G846-3-13	Kabuli	Australia
123	C-44	Desi	Pakistan
124	CM-72	Desi	Pakistan
125	CM-88	Desi	Pakistan
126	NIFA-88	Desi	Pakistan
127	Punjab-91	Desi	Pakistan
128	Paidar-91	Desi	Pakistan
129	Noor-91	Kabuli	Pakistan
130	ICC 11515xILC 482	Desi	Pakistan
131	ICC 11514xILC 3279	Desi	Pakistan
132	NEC-138-2xCM-72	Desi	Pakistan

Appendix 2: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during June 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-6-96	16.8	11.3	83	0.8
2-6-96	18.2	8.6	92	12.4
3-6-96	18.7	14.3	95	7.8
4-6-96	15.0	10.8	62	1.6
5-6-96	14.4	9.1	72	0.0
6-6-96	14.2	11.9	99	7.6
7-6-96	14.3	9.0	89	5.2
8-6-96	12.7	8.6	86	1.4
9-6-96	14.2	9.3	85	0.0
10-6-96	15.0	8.1	93	0.0
11-6-96	13.7	5.9	77	0.0
12-6-96	16.1	5.9	82	0.0
13-6-96	17.7	10.1	55	0.0
14-6-96	16.9	7.1	77	0.0
15-6-96	16.5	8.3	67	0.0
16-6-96	16.9	7.3	56	0.0
17-6-96	14.9	10.8	75	5.4
18-6-96	16.0	11.2	93	9.4
19-6-96	17.0	11.8	90	5.6
20-6-96	18.4	11.5	89	0.0
21-6-96	20.0	10.4	85	0.0
22-6-96	17.3	14.4	85	0.2
23-6-96	15.3	10.4	92	20.0
24-6-96	16.0	11.2	92	0.8
25-6-96	16.0	10.5	75	0.0
26-6-96	14.4	8.8	78	18.8
27-6-96	15.8	10.3	88	2.8
28-6-96	16.0	8.6	71	2.0
29-6-96	14.8	10.2	73	18.0
30-6-96	15.9	9.7	88	0.6

Appendix 3: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during July 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-7-96	14.6	10.0	86	0.0
2-7-96	14.8	9.2	90	4.0
3-7-96	17.1	10.0	56	0.2
4-7-96	16.1	11.3	95	10.6
5-7-96	14.1	9.0	96	12.0
6-7-96	13.1	9.2	95	3.4
7-7-96	12.5	7.0	91	2.4
8-7-96	13.6	6.0	87	0.0
9-7-96	12.9	7.5	64	0.0
10-7-96	11.6	8.7	92	3.8
11-7-96	9.8	6.6	96	12.2
12-7-96	12.6	4.6	88	2.4
13-7-96	14.0	5.6	89	2.0
14-7-96	13.5	5.8	93	0.6
15-7-96	13.7	8.7	97	1.8
16-7-96	14.0	6.4	63	0.0
17-7-96	15.4	9.4	92	7.8
18-7-96	18.6	12.9	68	0.0
19-7-96	13.7	10.5	94	12.0
20-7-96	13.5	8.0	92	9.6
21-7-96	14.1	8.8	84	0.0
22-7-96	16.2	9.3	78	1.0
23-7-96	14.7	11.8	69	0.2
24-7-96	14.1	8.8	89	2.2
25-7-96	14.5	9.5	74	0.0
26-7-96	17.3	9.6	76	0.0
27-7-96	15.5	6.5	86	0.0
28-7-96	13.6	5.6	96	0.0
29-7-96	13.7	8.2	93	6.2
30-7-96	12.8	7.6	71	1.2
31-7-98	13.6	9.4	94	13.0

Appendix 4: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during August 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-8-96	14.5	9.2	84	8.0
2-8-96	13.7	10.1	83	0.8
3-8-96	14.3	10.7	91	5.4
4-8-96	14.0	9.3	97	11.0
5-8-96	14.2	7.0	96	9.6
6-8-96	12.1	7.6	92	2.0
7-8-96	13.1	8.9	96	11.0
8-8-96	12.7	4.3	80	1.6
9-8-96	13.4	9.1	88	0.0
10-8-96	15.4	9.4	69	0.2
11-8-96	17.1	9.1	60	0.0
12-8-96	17.6	11.6	49	0.0
13-8-96	17.5	14.1	32	0.2
14-8-96	14.6	11.4	88	10.8
15-8-96	14.4	8.2	87	0.0
16-8-96	14.2	7.3	67	1.4
17-8-96	12.8	7.4	85	0.0
18-8-96	10.6	4.4	72	3.8
19-8-96	13.9	6.3	91	9.0
20-8-96	13.9	7.4	83	0.4
21-8-96	13.1	10.0	59	0.0
22-8-96	14.1	9.4	97	12.2
23-8-96	14.8	6.8	95	8.8
24-8-96	15.8	8.2	78	0.2
25-8-96	17.5	9.7	49	0.2
26-8-96	15.8	12.5	63	0.0
27-8-96	15.9	10.8	87	15.2
28-8-96	15.3	8.2	80	2.2
29-8-96	16.0	6.9	73	0.0
30-8-96	17.0	7.5	74	0.0
31-8-96	15.8	6.6	79	0.0

Appendix 5: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during September 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-9-96	16.5	6.8	60	0.0
2-9-96	13.7	8.1	76	5.8
3-9-96	15.2	7.4	83	1.2
4-9-96	15.8	11.2	58	0.0
5-9-96	15.4	10.6	82	0.6
6-9-96	18.2	9.0	57	0.0
7-9-96	18	12.2	70	0.0
8-9-96	16.5	8.9	91	0.0
9-9-96	16.0	10.0	75	0.4
10-9-96	15.1	9.3	66	0.0
11-9-96	21.0	11.4	38	0.0
12-9-96	12.6	6.0	66	4.2
13-9-96	14.4	6.4	75	7.0
14-9-96	19.4	10.8	83	2.6
15-9-96	24.9	13.8	43	0.0
16-9-96	20.2	15.2	67	0.0
17-9-96	17.6	10.2	65	0.0
18-9-96	28.1	12.9	35	0.0
19-9-96	13.6	9.9	89	9.4
20-9-96	12.4	4.9	57	2.8
21-9-96	14.5	4.2	71	3.0
22-9-96	23.3	10.6	39	0.0
23-9-96	15.4	9.6	69	9.4
24-9-96	12.8	8.8	91	6.0
25-9-96	15.3	6.8	60	0.6
26-9-96	14.5	9.4	87	2.0
27-9-96	16.4	4.2	50	3.6
28-9-96	14.4	8.9	80	1.2
29-9-96	13.9	11.4	92	11.6
30-9-96	12.9	9.4	72	14.8

Appendix 6: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during October 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-10-96	15.3	8.6	69	3.0
2-10-96	17.7	11.1	83	2.0
3-10-96	20.9	10.5	71	0.0
4-10-96	25.2	14.6	42	0.0
5-10-96	17.8	14.5	91	5.0
6-10-96	15.5	9.7	63	0.0
7-10-96	15.9	9.5	61	0.0
8-10-96	20.8	8.2	55	0.2
9-10-96	25.0	14.8	43	0.0
10-10-96	29.1	15.9	42	0.0
11-10-96	24.9	13.9	75	0.0
12-10-96	14.4	10.2	68	0.0
13-10-96	17.1	9.7	80	0.6
14-10-96	25.7	10.1	47	0.0
15-10-96	29.3	18.7	25	0.0
16-10-96	25.1	13.7	77	4.2
17-10-96	16.7	7.1	55	0.0
18-10-96	18.7	8.8	69	0.0
19-10-96	15.1	11.1	64	3.2
20-10-96	16.6	6.0	60	0.0
21-10-96	21.9	11.5	43	0.2
22-10-96	27.4	14.8	28	0.0
23-10-96	31.1	20.7	22	0.0
24-10-96	32.6	21.8	23	0.0
25-10-96	17.6	12.1	95	4.6
26-10-96	17.4	9.1	75	0.0
27-10-96	17.6	8.2	88	0.0
28-10-96	17.8	7.5	65	0.0
29-10-96	17.7	8.1	74	0.0
30-10-96	19.4	10.2	53	0.4
31-10-96	25.5	11.4	29	0.0

Appendix 7: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during November 1996.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-11-96	29.3	20.9	24	0.0
2-11-96	13.1	22.0	22	0.0
3-11-96	18.5	13.3	83	0.8
4-11-96	16.8	10.8	48	0.0
5-11-96	16.8	7.1	67	0.8
6-11-96	19.4	10.1	59	0.0
7-11-96	22.1	10.1	62	0.0
8-11-96	30.3	17.5	28	0.0
9-11-96	17.1	10.2	60	0.0
10-11-96	17.2	8.5	55	0.0
11-11-96	17.0	9.6	64	0.0
12-11-96	26.0	13.6	61	0.0
13-11-96	26.6	19.3	33	0.0
14-11-96	18.2	13.5	66	0.0
15-11-96	19.2	10.7	54	0.0
16-11-96	31.2	13.4	27	0.0
17-11-96	18.3	13.9	69	0.0
18-11-96	17.9	9.7	59	3.4
19-11-96	19.5	6.4	70	0.0
20-11-96	20.2	9.1	75	0.0
21-11-96	18.4	7.9	76	0.0
22-11-96	19.6	9.3	82	0.0
23-11-96	23.4	12.0	54	0.0
24-11-96	27.8	17.8	33	0.0
25-11-96	23.5	17.9	61	0.0
26-11-96	21.1	12.2	64	0.0
27-11-96	21.1	11.6	69	0.0
28-11-96	19.9	9.5	58	0.0
29-11-96	19.4	10.5	57	0.0
30-11-96	18.8	12.6	57	0.0

Appendix 8: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during June 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-6-97	14.8	9.4	50	0.0
2-6-97	13.3	9.6	43	0.0
3-6-97	16.2	9.3	62	0.0
4-6-97	22.1	13.0	40	0.0
5-6-97	19.1	12.7	61	1.4
6-6-97	14.7	8.9	88	8.0
7-6-97	14.6	9.1	91	0.2
8-6-97	15.6	11.5	98	11.0
9-6-97	18.3	9.6	64	0.2
10-6-97	17.7	12.1	45	0.0
11-6-97	17.0	12.0	50	0.0
12-6-97	15.7	10.9	85	2.2
13-6-97	13.2	7.9	92	15.6
14-6-97	13.9	7.8	92	1.8
15-6-97	12.7	5.5	77	0.2
16-6-97	12.8	8.8	71	0.0
17-6-97	13.2	8.0	76	0.0
18-6-97	14.4	7.6	75	0.0
19-6-97	15.6	5.1	73	0.0
20-6-97	16.1	6.2	83	0.0
21-6-97	15.0	12.1	83	0.0
22-6-97	15.0	9.6	71	3.8
23-6-97	13.1	7.5	72	0.2
24-6-97	14.2	7.9	81	0.4
25-6-97	12.1	4.5	77	0.0
26-6-97	14.5	4.9	71	0.0
27-6-97	13.8	7.5	78	0.0
28-6-97	15.4	10.0	78	0.0
29-6-97	14.7	9.5	79	0.0
30-6-97	13.9	8.6	73	0.0

Appendix 9: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during July 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-7-97	14.4	5.9	71	0.0
2-7-97	14.3	8.0	62	0.0
3-7-97	14.0	5.4	70	0.0
4-7-97	14.2	5.8	77	0.0
5-7-97	13.3	6.4	71	0.0
6-7-97	15.1	5.8	57	0.0
7-7-97	17.6	9.4	47	0.2
8-7-97	13.9	10.2	91	0.8
9-7-97	12.1	5.1	81	10.4
10-7-97	11.9	4.4	91	0.0
11-7-97	12.7	5.6	59	0.0
12-7-97	13.1	5.4	96	0.8
13-7-97	14.0	5.6	78	0.0
14-7-97	12.4	8.0	52	0.0
15-7-97	10.8	7.1	78	10.0
16-7-97	13.9	6.4	94	3.0
17-7-97	12.1	7.0	83	3.0
18-7-97	13.3	5.7	65	0.0
19-7-97	11.9	5.1	61	0.0
20-7-97	13.1	5.7	81	0.0
21-7-97	13.4	5.6	79	0.0
22-7-97	14.3	9.6	89	0.2
23-7-97	12.7	8.7	87	0.0
24-7-97	15.5	7.7	60	0.0
25-7-97	15.2	8.8	48	0.0
26-7-97	14.3	10.2	95	0.8
27-7-97	14.5	8.2	96	4.8
28-7-97	14.7	8.0	67	0.0
29-7-97	14.2	6.5	54	0.0
30-7-97	13.8	5.4	62	0.0
31-7-98	14.1	5.9	80	0.0

Appendix 10: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during August 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-8-97	13.6	5.6	73	0.0
2-8-97	13.8	4.8	78	0.0
3-8-97	13.5	6.6	70	0.0
4-8-97	16.5	6.2	71	0.0
5-8-97	17.6	8.2	40	0.0
6-8-97	15.9	9.6	58	3.0
7-8-97	12.0	6.1	97	31.6
8-8-97	12.8	7.4	87	10.6
9-8-97	15.3	9.4	100	3.3
10-8-97	18.6	11.3	60	0.8
11-8-97	13.2	10.2	99	14.0
12-8-97	13.6	8.9	100	17.8
13-8-97	13.7	9.9	97	1.6
14-8-97	15.1	9.0	97	1.6
15-8-97	18.0	9.8	60	0.8
16-8-97	16.1	10.6	57	0.8
17-8-97	14.2	7.7	84	2.2
18-8-97	13.2	5.2	93	0.0
19-8-97	12.7	5.5	93	0.0
20-8-97	14.1	6.4	81	0.0
21-8-97	14.1	4.0	73	0.0
22-8-97	14.4	6.4	82	0.0
23-8-97	10.9	7.6	73	3.2
24-8-97	10.9	4.7	76	6.0
25-8-97	11.8	4.1	95	2.0
26-8-97	12.9	7.3	90	7.6
27-8-97	13.3	7.8	99	0.6
28-8-97	18.5	7.9	71	0.6
29-8-97	19.9	11.8	66	0.0
30-8-97	17.5	11.4	48	7.4
31-8-97	15.2	11.1	96	3.0

Appendix 11: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during September 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-9-97	13.1	10.5	76	0.0
2-9-97	14.8	11.1	93	20.8
3-9-97	15.8	9.6	68	0.0
4-9-97	17.4	8.0	66	0.6
5-9-97	15.3	9.8	87	4.2
6-9-97	16.0	8.8	67	0.0
7-9-97	14.2	11.9	86	10.6
8-9-97	15.1	10.2	91	2.0
9-9-97	17.7	10.3	95	2.4
10-9-97	20.3	9.0	80	0.0
11-9-97	17.7	9.6	63	0.0
12-9-97	18.7	9.3	51	0.0
13-9-97	17.0	13.5	61	0.0
14-9-97	13.6	11.6	92	9.0
15-9-97	15.5	8.6	81	7.2
16-9-97	17.3	9.8	74	0.0
17-9-97	18.8	11.2	51	0.0
18-9-97	19.7	10.9	49	0.0
19-9-97	18.0	11.9	87	15.2
20-9-97	17.6	9.0	85	0.0
21-9-97	17.1	9.8	82	0.0
22-9-97	18.6	8.6	67	0.0
23-9-97	18.7	11.8	40	0.0
24-9-97	20.5	10.6	44	0.0
25-9-97	17.2	14.4	48	0.0
26-9-97	16.2	11.8	88	4.0
27-9-97	17.3	9.1	72	1.4
28-9-97	17.2	10.1	63	1.0
29-9-97	18.4	9.3	58	0.0
30-9-97	22.6	11.2	43	0.0

Appendix 12: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during October 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-10-97	27.7	17.8	30	0.0
2-10-97	16.9	13.5	85	0.0
3-10-97	16.2	8.8	54	0.0
4-10-97	17.6	8.7	53	0.0
5-10-97	19.6	9.3	49	0.0
6-10-97	16.6	9.5	74	0.0
7-10-97	19.9	9.8	60	0.0
8-10-97	18.8	8.2	76	0.0
9-10-97	31.0	13.3	33	0.0
10-10-97	28.0	18.9	29	0.0
11-10-97	19.1	14.2	60	0.0
12-10-97	16.6	11.3	61	0.2
13-10-97	19.3	10.6	81	0.0
14-10-97	16.2	11.5	81	0.0
15-10-97	15.6	9.4	73	1.4
16-10-97	16.8	10.1	78	0.0
17-10-97	15.6	6.6	60	0.0
18-10-97	17.6	6.5	52	0.0
19-10-97	14.1	6.4	53	0.0
20-10-97	18.7	7.5	61	0.0
21-10-97	21.8	7.6	64	0.0
22-10-97	22.9	9.5	65	0.0
23-10-97	26.5	12.0	65	0.0
24-10-97	30.2	13.7	33	0.0
25-10-97	33.3	22.8	22	0.0
26-10-97	30.7	18.3	65	0.0
27-10-97	22.6	11.7	80	0.0
28-10-97	26.6	11.3	51	0.0
29-10-97	23.6	15.1	74	1.4
30-10-97	18.0	11.9	78	3.0
31-10-97	10.9	8.6	95	57.0

Appendix 13: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite campus during November 1997.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-11-97	16.7	9.50	62	31.8
2-11-97	18.3	11.10	83	0.0
3-11-97	19.8	11.50	71	0.0
4-11-97	20.2	10.10	57	0.0
5-11-97	25.4	12.70	46	0.0
6-11-97	29.9	18.90	41	0.0
7-11-97	29.2	16.90	47	0.0
8-11-97	25.8	17.10	49	0.0
9-11-97	30.5	21.40	42	0.0
10-11-97	28.3	17.10	52	0.0
11-11-97	19.1	14.40	90	0.0
12-11-97	22.1	12.40	85	0.0
13-11-97	25.3	12.90	52	0.0
14-11-97	18.4	13.10	82	16.4
15-11-97	16.7	9.90	54	0.0
16-11-97	17.3	8.20	62	0.0
17-11-97	17.7	7.80	52	0.0
18-11-97	17.6	8.80	64	0.0
19-11-97	19.6	11.90	83	0.0
20-11-97	27.7	10.50	37	0.0
21-11-97	32.8	17.80	25	0.0
22-11-97	36.9	25.0	23	0.0
23-11-97	25.0	15.70	47	0.0
24-11-97	33.0	14.10	45	0.0
25-11-97	40.1	22.80	28	0.0
26-11-97	35.3	25.50	39	0.0
27-11-97	29.6	22.70	49	0.0
28-11-97	26.9	12.50	59	0.0
29-11-97	26.1	19.30	52	0.0
30-11-97	22.5	14.20	60	0.2

Appendix 14: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during June 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-6-98	18.2	10.2	69	0.0
2-6-98	17.7	10.4	72	0.0
3-6-98	21.8	12.3	46	0.0
4-6-98	19.9	11.2	66	0.0
5-6-98	23.7	14.6	53	0.4
6-6-98	14.8	9.6	92	22.0
7-6-98	14.5	5.8	71	1.4
8-6-98	12.3	6.1	69	0.2
9-6-98	15.2	6.9	57	0.0
10-6-98	17.6	9.6	47	0.0
11-6-98	16.6	9.5	27	0.0
12-6-98	15.5	8.5	80	22.6
13-6-98	15.0	10.6	91	0.0
14-6-98	14.6	9.7	85	0.0
15-6-98	14.5	7.9	87	1.6
16-6-98	13.4	6.8	73	0.2
17-6-98	13.3	5.2	58	0.0
18-6-98	15.9	3.8	62	0.0
19-6-98	14.6	8.6	35	0.0
20-6-98	13.3	9.6	82	1.8
21-6-98	12.5	8.7	90	15.0
22-6-98	11.5	5.2	70	4.2
23-6-98	12.5	5.6	83	2.0
24-6-98	13.2	8.2	92	7.4
25-6-98	13.0	8.1	79	3.8
26-6-98	13.8	7.9	67	1.8
27-6-98	15.4	8.9	53	0.0
28-6-98	13.8	8.5	92	2.0
29-6-98	11.5	8.3	90	2.2
30-6-98	12.0	4.6	85	0.2

Appendix 15: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during July 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-7-98	11.8	6.0	69	1.2
2-7-98	12.2	6.1	90	0.2
3-7-98	13.6	5.8	59	0.0
4-7-98	15.0	10.3	49	0.0
5-7-99	16.3	12.3	49	0.0
6-7-98	14.7	8.9	90	8.8
7-7-98	15.1	8.3	77	0.0
8-7-98	12.1	8.8	75	0.2
9-7-98	12.6	7.9	77	4.4
10-7-98	10.9	4.5	87	0.0
11-7-98	10.3	5.8	76	0.0
12-7-98	10.1	4.9	61	0.0
13-7-98	14.0	6.2	59	0.0
14-7-98	16.3	6.0	53	0.0
15-7-98	14.0	8.6	58	0.0
16-7-98	14.4	5.3	51	0.0
17-7-98	15.5	10.7	45	0.0
18-7-98	15.5	8.1	73	0.0
19-7-98	15.6	9.6	62	0.0
20-7-98	13.8	10.0	80	0.0
21-7-98	12.6	8.8	92	0.4
22-7-98	13.4	5.3	82	0.0
23-7-98	15.5	6.7	88	0.0
24-7-98	13.3	7.6	64	0.0
25-7-98	13.9	5.2	69	0.0
26-7-98	14.0	7.3	87	0.0
27-7-98	11.9	8.8	84	0.2
28-7-98	8.9	3.3	91	32.6
29-7-98	10.7	5.1	57	8.4
30-7-98	11.4	5.2	88	3.4
31-7-98	13.2	4.6	88	3.2

Appendix 16: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during August 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-8-98	13.8	7.9	63	0.0
2-8-98	11.2	8.1	87	3.0
3-8-98	13.9	9.1	100	12.0
4-8-98	15.3	10.2	74	0.0
5-8-98	15.8	8.3	82	0.0
6-8-98	17.1	10.1	65	4.4
7-8-98	16.4	8.8	80	4.0
8-8-98	15.5	7.4	88	0.0
9-8-98	15.2	11.3	95	0.0
10-8-98	18.2	9.4	83	0.0
11-8-98	15.5	10.6	57	0.0
12-8-98	15.6	11.6	72	0.0
13-8-98	15.8	11.4	95	4.2
14-8-98	13.9	10.5	73	5.2
15-8-98	14.0	8.0	58	0.0
16-8-98	14.4	5.8	69	0.0
17-8-98	14.7	7.2	56	0.0
18-8-98	17.1	9.3	73	0.0
19-8-98	19.5	11.7	51	0.0
20-8-98	17.8	14.0	66	1.2
21-8-98	16.1	10.6	77	13.4
22-8-98	13.3	8.2	66	2.0
23-8-98	13.4	7.5	79	7.2
24-8-98	17.0	6.8	53	2.0
25-8-98	14.2	8.7	84	4.4
26-8-98	16.6	6.7	68	5.2
27-8-98	19.1	9.3	41	0.0
28-8-98	20.2	8.1	57	0.0
29-8-98	19.8	9.0	56	0.0
30-8-98	20.3	10.1	39	0.0
31-8-98	22.1	11.8	35	0.0

Appendix 17: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during September 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-9-98	19.5	13.9	46	0.0
2-9-98	26.5	9.6	50	0.0
3-9-98	22.7	14.6	68	2.8
4-9-98	17.7	10.2	84	0.0
5-9-98	14.2	7.6	59	0.0
6-9-98	15.3	9.0	72	2.8
7-9-98	14.9	8.5	54	0.2
8-9-98	17.1	8.6	46	0.0
9-9-98	18.7	10.0	50	0.0
10-9-98	20.5	13.1	65	0.0
11-9-98	21.9	13.0	63	0.0
12-9-98	15.2	11.10	85	4.8
13-9-98	14.6	9.9	60	1.6
14-9-98	15.4	7.2	75	0.0
15-9-98	14.4	9.0	70	1.4
16-9-98	15.7	9.0	88	5.6
17-9-98	21.3	9.9	63	0.0
18-9-98	22.6	15.8	35	0.0
19-9-98	16.2	10.6	68	1.0
20-9-98	18.3	6.9	59	0.0
21-9-98	22.3	10.2	35	0.2
22-9-98	22.3	18.1	69	0.0
23-9-98	14.5	10.4	74	33.4
24-9-98	12.9	6.1	63	2.0
25-9-98	17.2	8.7	42	0.0
26-9-98	21.2	13.7	43	0.0
27-9-98	25.2	9.3	48	0.0
28-9-98	27.6	13.6	76	4.0
29-9-98	21.4	12.9	74	0.0
30-9-98	27.4	10.1	60	0.0

Appendix 18: Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during October 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-10-98	27.1	19.6	29	0.0
2-10-98	21.2	15.4	90	1.2
3-10-98	16.5	10.5	71	6.4
4-10-98	18.1	10.1	55	0.0
5-10-98	20.7	11.9	37	0.0
6-10-98	14.3	9.6	57	21.8
7-10-98	15.2	9.0	89	2.6
8-10-98	15.9	7.5	63	0.0
9-10-98	14.8	9.3	65	1.6
10-10-98	16.1	6.6	68	1.4
11-10-98	18.2	9.7	62	0.0
12-10-98	19.6	14.6	90	0.0
13-10-98	15.0	10.1	49	0.2
14-10-98	25.2	9.0	41	0.0
15-10-98	21.2	9.2	57	1.6
16-10-98	28.8	10.1	56	0.0
17-10-98	34.1	22.5	32	0.0
18-10-98	16.9	13.4	63	0.0
19-10-98	14.7	9.6	90	2.2
20-10-98	13.5	7.7	65	3.8
21-10-98	17.2	10.4	75	0.4
22-10-98	18.3	6.7	66	0.0
23-10-98	25.5	11.3	43	0.0
24-10-98	28.8	14.1	33	0.0
25-10-98	18.8	12.0	69	0.0
26-10-98	15.6	11.2	61	0.0
27-10-98	15.0	7.0	60	6.6
28-10-98	18.5	5.9	45	0.0
29-10-98	20.9	13.0	32	0.0
30-10-98	20.4	10.1	56	0.0
31-10-98	18.2	11.3	52	0.0

Appendix 19. Weather conditions: temperature, relative humidity (RH) and rainfall recorded at Waite Campus during November 1998.

Date	Max.Temp.°C	Min.Temp.°C	RH (%)	Rainfall (mm)
1-11-98	22.3	7.9	44	0.0
2-11-98	27.6	11.2	30	0.0
0.0	30	19.2	21	0.0
0.0	24.9	17.5	28	0.0
5-11-98	20.8	12.4	79	0.6
6-11-98	31	12.6	48	0.0
7-11-98	24.7	15.5	87	6.2
8-11-98	19.8	8.1	55	0.0
9-11-98	25.5	11.8	48	0.0
10-11-98	23.3	16.1	29	0.0
11-11-98	15.6	13.8	89	17.6
12-11-98	19	11.9	65	0.2
13-11-98	20.6	10.7	76	0.0
14-11-98	28.2	11.2	49	0.0
15-11-98	18.3	12.8	72	0.8
16-11-98	20.5	9.6	61	0.0
17-11-98	20.6	9.4	59	0.0
18-11-98	19.7	7.9	51	0.0
19-11-98	23	12.4	52	0.0
20-11-98	28.5	12.2	56	0.0
21-11-98	24	14.1	42	0.0
22-11-98	21	9.9	56	0.0
23-11-98	25.6	11.7	54	0.0
24-11-98	27.8	15.4	31	0.0
25-11-98	30.3	15.7	38	0.0
26-11-98	32.7	16.8	29	0.0
27-11-98	26.7	13.1	82	3.4
28-11-98	19.2	11	42	0.2
29-11-98	21.2	9.2	58	0.0
30-11-98	25.3	12.4	53	0.0

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