University of Glasgow

A study of the interactions between *Alternaria linicola* and linseed

Neal Evans BSc. (Hons.) (Liverpool Polytechnic/CNAA)

Department of Plant Science
S.A.C., Auchincruive
Ayr UK.

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, at the Faculty of Science, University of Glasgow.

Dedication

This work is dedicated to my wife Lisa,
with all the love in the world.....

Abstract

The principal aim of the study was to further the knowledge of the interaction between *Alternaria linicola* and the host plant linseed (*Linum usitatissimum*). A novel detached cotyledon *in vitro* bioassay was developed to allow the quantification of disease resistance of *Linum* material to *A. linicola*. Differences were apparent between the disease response scores of four linseed varieties when tested with seven isolates of the pathogen which differed in aggressiveness. However, there was no significant difference between the disease response scores of the varieties and no change in the ranking of varieties over three experiments. This indicated that the varieties behaved in a predictable manner to each isolate during each test. Accordingly, in a subsequent study, 102 *Linum* accessions were challenged with an aggressive and a non-aggressive isolate. About 75 % of the accessions gave a moderate response, although there was a continuous distribution of resistance from high susceptibility to resistant. Accessions at both extremes of the disease response consisted of breeding material, currently grown varieties and near relatives of the host species. For example, one of the more resistant accessions tested was *Linum angustifolium*.

A sub-set of nine *Linum* accessions was chosen (a range of susceptible, moderately-resistant and resistant material) and the resistance response of the material to an aggressive and a non-aggressive *A. linicola* isolate was investigated using a whole seedling inoculation technique. A comparison of the response of the material during the seedling test with that of the detached *in vitro* assay indicated that the latter test systematically, but marginally, overestimated the disease response. The *in vitro* bioassay scores and the seedling test scores were positively correlated following inoculation with the more aggressive of the two isolates. It was suggested that the resistance response of material could be accurately predicted by the *in vitro* bioassay but that a certain level of isolate aggressiveness was necessary to differentiate between responses of the accessions. Since large isolate-line interactions with respect to resistance scores were not observed, the results implied that resistance was polygenically determined. These results indicate that the bioassay for disease resistance produces an accurate measure of resistance and provides plant breeders with a useful tool which can be utilised during breeding programs.

A microscopic analysis was carried out to examine differences in pathogen behaviour and host response in interactions between *Alternaria linicola* and three genotypes of *Linum usitatissimum* (previously identified as susceptible, moderately resistant and resistant to the

pathogen). Cotyledons of whole seedlings were inoculated with a conidial suspension of the pathogen and seedlings were incubated in a controlled environment cabinet. Observations of the infection process were carried out at 18, 24 and 40 h post-inoculation by UV fluorescence microscopy. Data of 15 variables of the interaction was analysed using multivariate analysis of variance (MANOVA) and canonical variates analysis (CVA). Significant differences in pathogen development were found among the *Linum* accessions at 18, 24, and 40 hours after inoculation. At 18 hours after inoculation, attempted penetration by the pathogen was relatively rare on all three accessions. Canonical variates analysis revealed that overall differences among accessions resulted from the successful penetration of the most susceptible accession. Beyond 18 h post-inoculation attempted penetration was more common in the moderate and resistant accessions also. Overall differences among accessions were found to result from differences in the rate of colonisation of the host tissue. Pathogen development was observed to occur at a quicker rate on susceptible material.

Subsequent studies demonstrated that *A. linicola* growing *in vitro* produced a wide range of secondary metabolites. Extracts of culture filtrate were taken by reverse phase chromatography. Crude extracts produced disease-like symptoms on linseed cultivars and a range of non-host species indicating the presence of phytotoxic components in the extract. Characterised *via* thin layer chromatography, these included the non-host specific phytotoxins tenuazonic acid, alternariol monomethyl ether, tentoxin and two destruxin-type compounds (which closely resembled destruxin A and destruxin B). The identity of four of the compounds was confirmed by two dimensional thin layer chromatography and proton nuclear magnetic resonance spectroscopy.

Linum leaf material infected with conidia of *A. linicola* and blastospores of *Melampsora lini* were extracted using a facilitated diffusion extraction technique. The resultant extracts contained a number of compounds which were fungitoxic to *Cladosporium cladosporioides* and, to a lesser extent, *A. brassicicola*. One such compound corresponded to the phytoalexin coniferyl alcohol which had previously reported to have been produced by flax seedlings inoculated with *M. lini*. Quantitative differences in the amount of the fungitoxic compounds produced between the inoculated and uninoculated resistant and susceptible host genotype combinations suggested that the production of fungitoxic compounds was greater in response to attempted colonisation and that as such, phytoalexin production was a component of the resistance reaction.

The main conclusions from the study were that due to the absence of qualitative differences in resistance response, resistance was polygenically determined. This observation, and the lack of a specific inhibitory response by a single host factor during the infection process indicated that the mechanism of resistance was multicomponent in nature. It is suggested that one of the major components in the interaction would appear to be the ability of the pathogen to produce non-host specific phytotoxins (some of which may be host-selective). At the cellular level, changes are induced in the host which result in the modification of the cell wall. Changes in the physical and/or chemical construction of the cell wall slow down the rate of pathogen ingress. Concurrent production of phytoalexin compounds by the host cells also has the effect of slowing or preventing pathogen ingress. Susceptible accessions apparently are induced too late or produce less of a resistance response in comparison to resistant accessions. The possibilities of an improvement in the resistance of linseed to *A. linicola* are discussed and it is suggested that such improvement would be both possible and beneficial.

Table of Contents

List of Figures


It should be noted that many figures were originally drawn in colour.

List of Tables

List of Plates

Publications

Aspects of the following study have been previously published in the following papers:

Evans, N., McRoberts, N., Hitchcock, D. & Marshall, G. (1995). Assessing linseed (*Linum usitatissimum*) resistance to *Alternaria linicola* using a detached cotyledon assay. *Annals of Applied Biology* **127**, 263-271.

Evans, N., McRoberts, N. & Marshall, G. (1995). Aspects of the physiological and biochemical interactions in the *Alternaria linicola* - linseed pathosystem. *Aspects of Applied Biology* **42**: *Physiological responses of plants to pathogens*, 341 - 344.

Acknowledgements

The following project was generously funded by the European Commission (DG VI)(contract number: 9001-CT90-0006). Due to the collaborative and multidisciplined nature of the study, there are many colleagues and friends to whom thanks are due:

My supervisors Drs Neil McRoberts and George Marshall, for their advice, encouragement and support during the study. I would especially like to thank Neil McRoberts for the many helpful discussions on various aspects of *Alternaria* pathogen/host interactions.

Dr Bob Hill of the Chemistry Department, Glasgow University for carrying out the ^{1}H-nmr analysis and for being so patient with my lack of knowledge of organic chemistry. Dr M. Païs (Institute de Chimie des Substances Naturelles, Gif-sur-Yvette, France) for the generous gifts of destruxin A and B. Mr John Findlay (Science Faculty SEM unit, Edinburgh University) for help with the SEM work and for his amusing stories.

Prof. W. Friedt (Giessen), Mons. G. Fouilloux (INRA, Versailles), Dr K. Rashid (Agriculture Canada), Dr E. Tybirk (Prodana), Dr L. Holly (Hungary) and Mr R. Whitehouse (ISP) for samples of seed. Drs I. Vloutoglou (formerly of Rothamsted, UK), R. Beale and S. Ball (both formerly of NIAB, UK) for *A. linicola* isolates.

Alistair Sword and David Hitchcock (BIOSS) for help and advice on the statistical analysis of the data and to Dave Arnott, Alan McEwan and Helen Love for help with computing matters. Also, members of the library staff for help with tracking down references and keeping me up to date with the Auchincruive gossip !

Colleagues of the Plant Science Department especially Janice Hampson and Jim Thomson for technical support. Also, good friends over the years, Dave, Lis, Martin, Mike, Aud L, Neil H., Jackie, Arlene, Tracy, Mike, Richard, Audrey M, Aileen and Caroline for keeping me smiling.

I would like to thank my parents, Keith and Val, my brother Mark, and also Beryl and Geoff White, for all of their love and support over the years. Finally, my biggest thank you goes to my wife Lisa for putting up with my grumbles and for keeping me semi-sane during "the bad bits". Thank you for believing in me and for being more supportive than anyone could ever have wished for.

Chapter 1

1.0 Introduction

1.1 The linseed host

1.1.1 The history and importance of linseed (*Linum usitatissimum* L.).

The Linaceae

The *Linaceae* is a family consisting of 12 genera which contain about 200 identified species of shrubby, often tree-like, herbaceous plants (Hutchinson, 1967; Durrant, 1976; Gill, 1987). Although examples of woody shrubs of this family are common (for example *Linum arboreum*, a woody much-branched shrub native to Crete), many of the species found within the *Linaceae* are annual herbs with a wiry stem. The only species of the *Linaceae* of agricultural importance is *Linum usitatissimum* L., an annual herb which shows polymorphism into two main cultivar groups. Linseed cultivars are generally short and highly branched to produce large quantities of seed, from which the valuable commodity linseed oil can be extracted. The taller flax cultivars are usually single stemmed and show only minor branching, the length and quality of fibres in the stem being the important character of the flax crop. The species is largely self-pollinating and the two groups have tended to remain fairly distinct (Turner, 1987).

Ancient history of linseed and flax cultivation

Linseed and flax varieties of *Linum* spp. have historically been grown in Europe since ancient times. Fragments of flaxen garments and nets have been found amongst the remains of neolithic Swiss lake dwellers (dated to 8,000 BC), although it is thought that these materials were derived from the strongly tillering and branching, dehiscent perennial species *Linum angustifolium* which was common in southern Europe and western Asia (Durrant, 1976; Turner, 1987). The great diversity of forms of *Linum* found in the Indian sub-continent suggested that early forms of the domesticated crop originated there and were bought into western Europe *via* the trade routes of the middle East (Tammes, 1928; Durrant, 1976; Turner, 1987). Forms of *L. usitatissimum* were probably not introduced into the U.K until the arrival of the Romans (Durrant, 1976).

Recent history of linseed and flax cultivation

Large scale production of flax was prevalent in Britain during the late 19th century-early 20th century and more recently flax and linseed production was dictated by the economic and trading constraints associated with the two world wars (Appel, 1991). After the resurgent war-time period, the area of both linseed and flax slowly declined during the 1950's, mainly due to the introduction and mass production of synthetic lubricants and fibres. Essential linseed oil was imported as required, predominantly from Canada or Argentina and linen became less fashionable as a textile (Appel, 1991).

Following the formation of the European Community and in the wake of the 1973 U.S. soya embargo, linseed was one of the industrial oilseed crops specified under the Common Agricultural Policy (CAP) of 1987. The aim of the resulting legislation was that the community would become self-sufficient in linseed (a requirement estimated at ~400,000 t per annum) removing the need for economically unstable imports (Appel, 1991). At that time only France was producing any significant amount of linseed and in order to entice farmers to grow the crop, large area aid subsidies were offered to make linseed economically viable.

The recent dramatic increase in the area of linseed cultivated in Britain from less than 2000 ha in 1984 to the peak of 156,000 ha during the 1993 season (Fig. 1.1) was produced as a direct result of the CAP policy. It should also be noted that the exponential increase in the U.K. linseed crop area contrasted with other EC member states which, with the exception of Germany (55,000 ha in 1993), did not experience the expected increase in the area of linseed grown. This phenomenon was probably a direct effect of the historical reliance of these countries on flax production, an eventuality predicted in 1987 by Turner. It is also a direct consequence of a cut in the area aid subsidy which has lead to a decline in the popularity of the crop in recent years.

Aspects of linseed production

Linseed holds a unique role in the British agricultural system being a non-food oilseed breakcrop which provides an alternative to oilseed rape or a leguminous field crop which can be used in rotation with traditional cereals. Linseed requires a low-input of fertilisers and agrochemicals allowing the grower to maximise the profit margin per hectare in comparison with other breakcrops. The linseed crop reportedly produced the greatest yield increase when

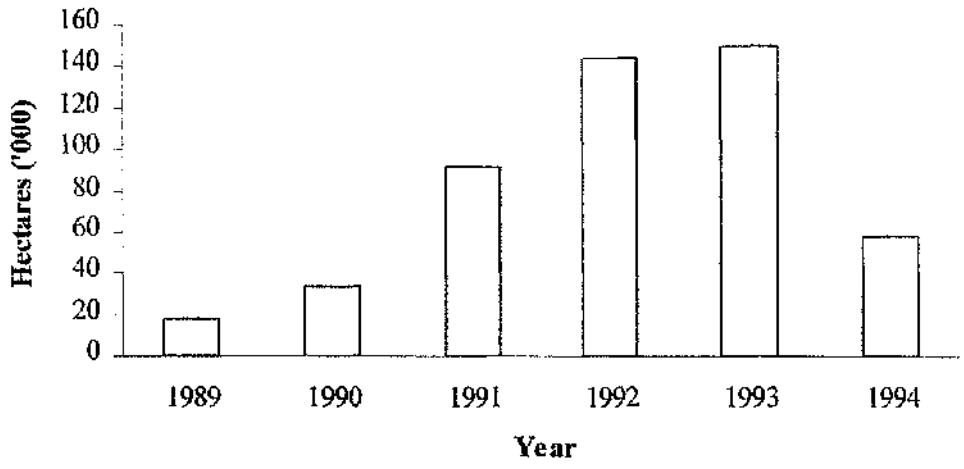


Fig. 1.1 The exponential growth of linseed as a non-food oilseed break crop in the U.K. (Source: Annual Agricultural and Horticultural Census, MAFF, 1995).

the crop was sown into a minimally cultivated seed bed using a conventional seed drill (Freer, 1995). Autumn sown linseed varieties are currently available and improved winter cultivars are undergoing trials. These provide further choices for winter cultivation and an alternative winter cover crop to prevent nitrogen leaching. Another advantage of linseed is that the crop is readily combinable using a conventional combine harvester provided the weather is dry at harvest time although dessication may be necessary for late maturing varieties.

1.1.2 The cytogenetics of the *Linum* genus.

Evidence from the literature suggests that the cytogenetics of the *Linum* genus are poorly understood. Basic chromosome number within the genus appears to be exceptionally variable (n = 8, 9, 10, 12, 14, 15, 16) but there are evident karyotypes at 2n = 18 and 2n = 30 with linseed and flax cultivars belonging to the second of these two groups. Tammes (1928) cites a number of authors who found *L. usitatissimum* to contain 30 chromosomes (diploid) and haploid lines containing 15 but also cites Martzenitzina (1927) and Emme & Schepeljeva (1927) who found *L. usitatissimum* 2n = 32.

It is reported that hybrids are readily obtainable between several species of the 2n = 18 group and between several of the 2n = 30 group, many showing one or more translocations (Gill & Yermanos, 1967). The *L. perenne* group (2n = 18) found in Eurasia is thought to be close to

the *L. pratense* group (2n = 18) of North America which was probably introduced *via* Siberia and Alaska. The higher numbered groups (2n = 30, 32 & 36, including *L. usitatissimum*) were introduced to Northern America at a much later date by European settlers (Durrant, 1976).

Although the cytotaxonomy of the *Linum* genus appears extremely confused, the present consensus is that modern forms of *L. usitatissimum* are derived from the closely related wild species of North Africa and Eurasia with 2n = 30 chromosomes: *L. africanum, L. angustifolium, L. corymbiferum, L. decumbens, L. nervosum* and *L. pallescens* (Durrant, 1976). Of these, *L. usitatissimum* appears to be nearest to the highly variable *L. angustifolium* (2n = 30, 32), a strongly branched and tillered perennial or biennial with dehiscent capsules. *L. usitatissimum* is easily crossed with this wild relative and differs from it cytologically by one translocation (Durrant, 1976). Tammes (1928) reported that the chromosome number of *L. angustifolium* is 2n = 30 and remarks on the ease with which this species will cross with *L. usitatissumum*, pointing out the fact that the progeny of such a cross are normal, fertile hybrids. The probable relationships and evolutionary pathways of linseed/flax are shown in Fig. 1.2.

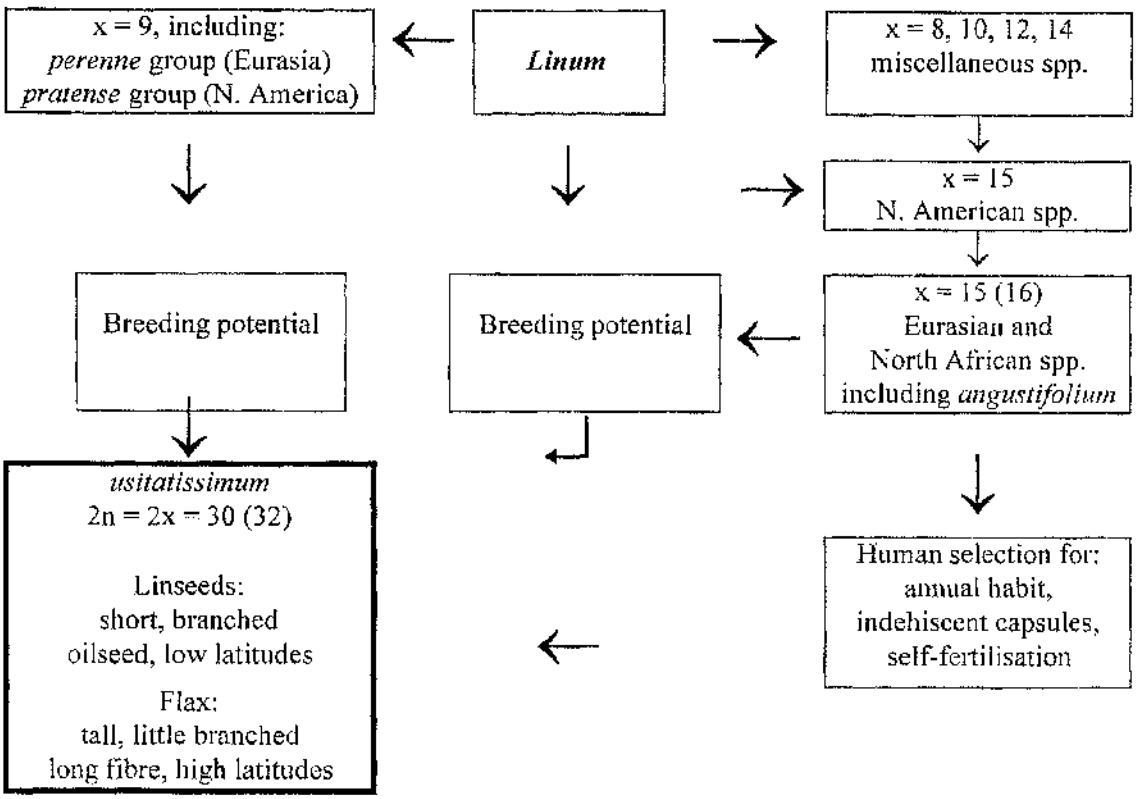


Fig. 1.2 Relationships and evolution of linseed and flax cultivars of L. usitatissimum (after Durrant, 1976).

1.2 The pathogen

1.2.1 The genus *Alternaria* and the pathogen *Alternaria linicola* Groves and Skolko.

The systematics of the genus Alternaria

Within the Deuteromycotina (Fungi Imperfecti) the dematiaceous Hyphomycetes are characterised by the production of pigmented conidia from simple conidiophores produced from pigmented or hyaline hyphae. Within this group, the genus *Alternaria* Nees (ex Fries) contains a large number of economically important plant pathogens with a wide range of host species. Conidia produced by *Alternaria* species are frequently catenate (Ellis, 1971), usually elliptical or obclavate euseptate porospores produced as an outgrowth of protoplasm through a pore in the apical wall of a conidiophore (Simmons, 1967). In catenate species, secondary and subsequent conidia are produced through a simple pore in the tip of a primary conidium or through the lateral outgrowth of any conidial or beak cell. This cell converts morphologically and functionally to act as a conidiophore (Simmons, 1967).

Differences in conidiophore and conidium morphology provide good characters for the taxonomic study of the *Alternaria*. However, variability in both cultural and morphological characteristics of axenic cultures have historically impeded an understanding of the *Alternaria* (Simmons, 1986; 1992). The systematics of the conidial fungi and of the dematiaceous Hyphomycetes in particular has been a matter of contention for many years (Kendrick, 1981a,b) and Simmons (1992) highlights the main problems with respect to the *Alternaria* in particular. The problem is compounded in that teleomorphs of most of the *Alternaria* have not as yet been obtained in axenic culture (Simmons, 1986). Present evidence suggests the *Alternaria* are the asexual phase of the genus *Lewia* Barr & Simmons within the Ascomycotina (Simmons, 1986; McRoberts, 1992).

Alternaria *species pathogenic on linseed/flax*

Three species, *Alternaria linicola* Groves & Skolko. *Alternaria alternata* (Fries) Keissler. and *Alternaria infectoria* Simmonds (anamorph of *Lewia infectoria* Barr & Simmons) are commonly found to be pathogenic on British linseed (Fitt & Ferguson, 1990). *Alternaria lini* Dey has been reported to infect linseed in India (Dey. 1933) although it is unclear from the descriptions given whether the causative agent observed is a separate species. or a pathogenic

form of *A. alternata* as suggested by Arya & Prasada (1953) and Saharan (1988). A further species, *Alternaria cheiranthi* (Lib.) Bolle has been reported on flax in the Ukraine (Grebenyuk, 1983) where in a study between 1976 - 1978, 72.3% of 820 stem samples were infected with this particular species, although this is the only report of this species attacking flax.

Morphologically, *A. linicola* is far removed from *A. alternata* and *A. infectoria* within the *Alternaria* genus. *A. linicola* produces hyphae which are septate, branched, hyaline to pale smokey olive, 4 - 7µm in diameter, with similarly coloured septate conidiophores of variable length, 5 - 8µm in diameter. Conidia of the species are singular, obclavate, gradually attenuated above into a long slender, sometimes branched beak, smooth, muriform, with 7 - 11 transverse septa, 150 - 300µm x 17 - 24µm (Groves & Skolko, 1944). In comparison, conidia of *A. alternata* and *A. infectoria* are found in long, often branched chains, each individual conidium being much smaller (20 - 63µm x 9 - 18µm and 20 - 70µm x 9 - 18µm respectively) with a much shorter, less distinct beak than those of *A. linicola* (Ellis, 1971).

A. linicola is regarded as the most serious of the seed-borne diseases affecting British linseed (Mercer *et al.*, 1991a). *A. alternata* and *A. infectoria* are not generally considered to be as pathogenic as *A. linicola* although these species have been found to produce symptoms on cotyledons and lower leaves. In contrast to *A. linicola*, it appears unlikely that *A. alternata* or *A. infectoria* cause sufficient damage to decrease yields (Fitt *et al.*, 1991a).

Characteristics and epidemiology of A. linicola

A. linicola is not strongly pathogenic on adult plants where an infection is manifest as dark brown lesions on the leaves, stems and capsules (Turner, 1987; Mercer *et al.*, 1991a). However, the pathogen causes serious economic damage early in the season and later during saprophytic growth following flowering when the developing seed can become infected. Seedlings may be seriously weakened or killed leading to as much as a 50 % reduction in the emergence of the crop, a 35 % reduction in seed yield and a reduction in oil yield and quality (Muskett & Colhoun, 1947; Mercer *et al.*, 1989). Levels of contamination caused by *A. linicola* are the commonest cause of the failure of UK linseed to meet the seed certification standard of less than 5 % infected (Mercer *et al.*, 1991a).

Like many *Alternaria* spp. which are phytopathogenic in temperate regions. *A. linicola* only causes a disease problem during moist seasons such as 1987 (Fitt *et al.*, 1991b) and 1993 (B. Freer, Pers. comm.). The primary source of inoculum for disease development in the growing crop has been shown to be the seed (Mercer, 1994; Vloutoglou *et al.*, 1995). As a result, the disease can be especially problematic and economically damaging if damp weather precedes germination and the harvest period. However, for much of the growing season, *A. linicola* does not appear to be particularly detrimental to the developing linseed crop. The pathogen is often only found to be present at low levels on the lower leaves and stems of the plant (Mercer *et al.*, 1991a) and if plants survive the seedling stage it is common for the actively growing upper stem and leaves to be relatively free of the pathogen until after flowering when infection again becomes apparent. Little is known of the mechanisms which underlie this phenomenon, or how the pathogen is able to re-infect the seed at the end of the growing season from what must surely be a depleted inoculum source.

Mercer *et al.* (1991a) suggested that sufficient inoculum exists on the lower leaves for it to multiply quickly at this time of the growing season if the climatic conditions favour the growth of the pathogen. This suggestion was supported by an observed increase in *A. linicola* spore numbers above and within the crop canopy immediately prior to capsule formation (Mercer *et al.*, 1991a). Recent work by Vloutoglou *et al.* (1995) has shown that the pathogen can survive over winter as thick-walled chlamydospores in hyphal or conidial cells in naturally infected crop debris and also on infected linseed volunteers and the alternate hosts *Veronica persica.* Vloutoglou *et al.* (1995) suggested that under suitable environmental conditions, these sources of inoculum, in conjunction with planted infected seed, formed the primary inoculum source at the beginning of the season and continued to provide a source of inoculum through to the middle of the season.

Inoculum dispersal by rain-splash is thought to play only a minor role in the movement of *A. linicola* from the lower to the upper parts of the linseed plant (Vloutoglou *et al.*, 1995). This observation contrasted with the similar movement of *Alternaria brassica* and *Alternaria brassicicola* conidia on Brassica crops where rain-splash has been described as important (Prasanna, 1984). Similarly, Fontem *et* al., (1991) observed that during a period of intense wet weather, rain-splash dispersal appeared to be the predominant cause of the spread of *A. brassicicola* spores during dark leaf spot epidemics on cabbage. Rain-splash is probably less important in the *A. linicola*/linseed interaction due to the narrow shape and small size of the leaves of the host plant.

Wind blown conidia appear to be the primary source of inoculum for the spread of *A. linicola* between plants and between different fields of linseed during the growing season (Fitt & McCartney, 1986), although details of the epidemiology of the pathogen are still not fully understood. Conidia of *A. linicola* are dispersed singly, in comparison with the conidia of *A. alternata* and *A. infectoria,* 50% of which were found to be dispersed in chains of up to seven spores. It is this, in conjunction with the differences in spore size and morphology, which produce the characteristic differences in the fall speed of the conidia of the three species (Fitt *et al*., 1991a). *A. linicola* conidia released singly have a fall speed of 1.0 cm s^{-1}, whereas for *A. alternata* and *A. infectoria* the fall speed range was 0.6 cm s^{-1} (single conidia) to 1.1 cm s^{-1} (chain of up to 3 conidia) which indicates that *A. linicola* conidia will settle out of still air at a faster rate than singular spores of the *A. alternata* and *A. infectoria.* In turbulent air the greater velocity of *A. linicola* spores and the larger spore size ensures a greater impaction velocity (Gregory, 1961) onto the smaller leaves of the linseed host in comparison to the two non-pathogenic species.

Comparison with the *Alternaria* pathogens of oilseed rape would suggest that inoculum of *A. linicola* from a heavily infected crop may be dispersed over a large distance in the air, although reports in the literature are somewhat contradictory. Humpherson-Jones & Maude (1982) trapped large numbers of viable conidia of *A. brassicicola* near to, and down wind from *Brassica oleracea* seed crops during harvesting. Although conidia were trapped at distances of up to 1800m from the crop, the density of inoculum was found to decrease rapidly away from the inoculum source. Recently however, Fontem *et al*., (1991) suggested that steep disease gradients and relatively slow isopathic rates observed in dark leaf spot (*A. brassicicola*) epidemics on cabbage were due to very localised spore dispersal, predominantly through rain-splash dispersal which, as mentioned above, is probably not an important route of disease spread in the *A. linicola*/linseed pathosystem.

Seed infection

Alternaria linicola is thought to infect the developing seed either during capsule development or later on in the season during capsule ripening (Mercer, 1994). Current evidence suggests that alternate wet and dry conditions during capsule maturation increases the levels of infection of the seed. K. Rashid (Pers. comm., 1992) suggested that the pathogen is able to ingress through the continually opening and closing margins of each segment of the developing

capsule. Mercer (1994) suggested that the pathogen grows either through the capsule wall or up the central stalk although the actual process is still not understood.

In infected seed, *Alternaria linicola* is located almost exclusively the outer cells of the testa and in the mucilaginous coat where the pathogen over winters in the form of resting hyphae (Mercer & Hardwick, 1991; Mercer *et al.*, 1991a). The levels of *A. linicola* on stored seed decrease with time but only comparatively slowly in comparison to other seed-borne pathogens (Mercer *et al.*, 1991a). Viable resting hyphae have recently been shown to survive successfully for at least five years (Mercer, 1994).

Climatic factors affecting disease development in the field

In axenic culture, *A. linicola* has different optimal growth temperatures depending on the growth media used. Typical temperature conditions in the field however, do not appear to limit the ability of seed-borne isolates of the pathogen to colonise seedlings or indeed to germinate from wind blown conidia and infect the growing crop later in the season. Although little work on the importance of climatic conditions for successful infection and epidemic development has been reported in the literature field observations suggest that cool, wet conditions after planting favour pathogen development in the growing crop (Mercer *et al.*, 1991a; Mercer, 1994).

Humpherson-Jones & Hocart (1983) observed that the optimal temperatures for *Alternaria* pathogens of brassica seed crops was species dependant. In controlled environment infection studies with cabbage, *A. brassicicola* and *A. brassicae* produced the greatest levels of infection at temperatures of 25°C and 15°C respectively. Both species required a minimum period of 16 hours at these temperatures for the initiation of infection and optimal disease development occurred after 48 - 72 hours. Importantly, Humpherson-Jones & Hocart (1983) found that at 10°C, *A. brassicicola* failed to produce significant infection after 96 hours whereas *A. brassicae*, the dominant species in the field, was found to produce numerous lesions at that temperature.

Vloutoglou *et al.* (1995) observed that *A. linicola* was effectively transmitted from the seed to the seedling but transmission levels were dependant on soil temperature and the proportion of infected seeds sown. The pathogen was transmitted most effectively at a temperature range of between 15-25°C and was significantly reduced at 10°C (Vloutoglou *et al.*, 1995) although

transmission from infected seed to linseed seedling roots was found to be greater at 8-10°C than at 20-24°C (Mercer, 1994).

After arriving on the phylloplane, moisture appears to be a limiting factor to conidial germination as is the case many other *Alternaria* species. Lacey (1986) suggested that *A. alternata* requires a minimal range of 90 - 92 % relative humidity (r.h.) for germination to occur. In an experiment with the same species on *Phaseolus* leaves, Dickinson & O' Donnell (1977) found that the highest levels of conidial germination and germ tube growth were achieved by keeping plants under continuous high r.h. conditions of 97%. In contrast, this study also found that changes in the physiological condition of the leaf (i.e. by either washing the leaf surface prior to inoculation or by adding sucrose with the inoculum) had very little effect on conidial germination and germ tube growth.

Webster & Dix (1960) reported that at 20°C, *A. alternata* required a relative humidity in excess of 89% for germination to occur. Chandrashekar & Ball, (1980) showed that at 25°C, an isolate of *A. alternata* from grey mangrove (*Avicennia marina* var. *australasica*), in Australia required an r.h. of only >75% to germinate, but maximum germination was only reached at 100% r.h. However, in a more detailed study, Dickinson & Bottomley, (1980), found large responses in the germination of conidia to r.h. at different temperatures with germination only occurring at 97-98% r.h when the temperature was between 5-10°C.

1.2.2 Control of *Alternaria linicola*

Chemical control

Historically, the most effective control of *A. linicola* was achieved through the application of the fungicide iprodione to infected seed. However, extensive use of this chemical lead to the selection of insensitive isolates. Data from the UK Seed Testing Station (DANI, Belfast) showed that the proportion of insensitive isolates within the *A. linicola* pathogen population increased dramatically from 2% of all seed samples tested in 1986 to 85% by 1988 (Mercer *et al.*, 1991a).

As a result of the problems with iprodione seed treatment, prochloraz has been the recommended for disease control in the UK for a number of years as the compound has been shown to give good control of *A. linicola* (Mercer *et al.*, 1988). The compound has been

successfully used as a seed treatment (Mercer, 1994). However, field trials with this fungicide as a spray treatment showed that there was no significant decrease in *A. linicola* levels on either the capsules or seed and that there was no increase in yield unless the chemical was used at an uneconomic rate (Mercer *et al.*, 1989; 1991b; 1992). It should be noted that disease levels during the trials were low due to dry, warm weather conditions which were not conducive to pathogen development.

Biological control

The phenomenon of microbial competition on the phylloplane is well known with antagonism between saprophytes and plant pathogens being utilised commercially for novel biological control methods (Baker & Cook, 1974; Reinecke, 1981). At the leaf surface *A. linicola* appears to be strongly dominant with a high degree of influence on other seed-borne pathogens such as *Fusarium* species. Mercer & Hardwick (1991) found that there was significantly less growth of *Fusarium avenaceum* in the presence of *A. linicola*. Similar results were found for *Botrytis cinerea* (Mercer & Jeffs, 1988).

Competition between *A. linicola* and other species of the *Alternaria* has also been observed in that *A. linicola* appears to be dominant over *A. alternata* and *A. infectoria* when atmospheric conditions are suitable for the pathogenic species' growth. Mercer *et al.* (1993) observed that although *A. alternata* had a significant effect on the growth of *A. linicola* in paired-culture tests suggesting a degree of potential antagonism, *A. alternata* had little effect on levels of *A. linicola* when both species were co-inoculated in seed tests.

A number of workers have realised the potential of biological control of linseed diseases considering the low-input status of the crop. The possibility of controlling *A. linicola* with bio-control measures would also prevent the selection of fungicide insensitive isolates from the pathogen population. Mercer *et al* (1991b) found that in field trials, spore suspensions of *Epicoccum nigrum* competed successfully with *A. linicola*. The level of control achieved was equivalent to that achieved with sprays of prochloraz but not as effective as sprays of iprodione (Mercer *et al.*, 1991b). However, low disease levels during the duration of the trials did not provide a clear indication of the usefulness of the organism as a biological control method. In more recent work, Mercer *et al* (1993) found that isolates of *Epicoccum nigrum* and *Trichoderma* spp. gave good control of *A. linicola* both in the laboratory, glasshouse and field

trials. The level of control achieved by a number of *Trichoderma* spp. (in particular isolates of *T. harzianum*) rivalled the level of control achieved by the fungicide iprodione.

Cultivar resistance

Although differences in the levels of *A. linicola* on specific cultivars have been observed in the field, it is uncertain whether observed differences are due to genetically inherited resistance mechanism or are caused by differences in relative maturity (Mercer & Jeffs, 1988; Turner, 1987). Recent evidence suggested that incidence of *A. linicola* was not correlated to maturity (Mercer & Ruddock, 1994), but as yet, cultivars are not recommended by the National Institute of Agricultural Botany with respect to disease resistance. The current status of what is understood of cultivar resistance and the possibilities of the use of the method for the control of *A. linicola* on linseed will be discussed more fully during the following chapter.

1.3 Aims of the project

Considering the problems associated with disease control as outlined above, disease pressure from the pathogen has been expected to increase over recent years due to the increased interest in the crop and the accompanying increase in the area of linseed grown in the UK. Although linseed is an ancient crop to Britain, very little is known about the biology of the pathogens which infect the crop. The main aim of the study was to further the knowledge of the understanding of the interactions between linseed and *A. linicola*.

Objectives:

1. To develop a bioassay capable of differentiating levels of resistance to *A. linicola* in linseed.
2. To use the bioassay to assess the levels of resistance to *A. linicola* in a ranges of linseed, flax and *Linum* accessions.
3. To describe the nature of the host/pathogen interaction at the cellular level.
4. To characterise the role of phytotoxin production by the pathogen in the infection process.
5. To investigate phytoalexin production by the host during the resistance response.

Chapter 2

2.0 An investigation of levels of resistance to *Alternaria linicola* in *Linum* material

2.1 Breeding for resistance to plant disease

Current status of disease resistance to Alternaria linicola *in linseed.*

It has only been in recent years, since the large increase in the area of linseed grown in the UK (Fig. 1.1), that disease resistance to fungal pathogens and to *A. linicola* in particular, has become of relative importance to the crop breeder. The main cause for concern has been that the large area under linseed has increased the selection pressure on the pathogen population. This, in combination with the problems associated with the control of *A. linicola* by chemical means, as outlined previously, has ensured that the possibilities of the use of cultivar resistance to control the pathogen have taken on a new significance. There was, therefore, an increased interest in the current levels of resistance exhibited by cultivars and a growing importance to assess sources of resistance for the breeding of improved cultivars. However, it would appear that one of the main reasons why breeding for disease resistance to *A. linicola* has not ranked highly amongst breeders selection criteria previously is that no discreet assay which allows the quantification of resistance levels to the pathogen has been developed. The principal aim of the following study was, therefore, to design such a test in order to investigate levels of resistance to the pathogen. It was also envisaged that the testing of a sufficiently large number of linseed accessions would allow inferences to be made as to the genetical system which controlled the resistance mechanism.

At the beginning of the 1990's, very little was known of levels of resistance to *A. linicola* in linseed and even to date, after extensive field trials for the recommended list, the National Institute of Agricultural Botany (NIAB) does not make recommendations to the grower on disease resistance of linseed cultivars (Anon. 1995). Mercer & Jeffs (1988) and Turner (1987) reported differences in the levels of *A. linicola* infection on different cultivars in field trials but suggested that the effect was due to differences in the maturity of the cultivars. More recent evidence from late in the growing season suggests that different cultivars have an effect on the incidence of *A. linicola* on capsules and seed following harvest, but that there is no correlation with cultivar maturity, from which it can be concluded that the observed effect is of genetical significance (Mercer & Ruddock, 1994). However, it was also observed that the ranking of the cultivar effect changed from year to year and that correlation between years was poor (Mercer & Ruddock, 1994). Mercer & Ruddock (1993) had previously reported that no correlation was observed between the incidence of *A. linicola* on capsules or seeds following harvest and the incidence of the pathogen on seedlings of the crop earlier in the season. From these observations Mercer & Ruddock (1994) suggested that seedling resistance and the resistance which determined differences in pathogen incidence between cultivars later in the season were governed by two separate mechanisms.

Possible mechanisms of resistance - The concept of monogenic control

During the history of the crop, it is unlikely that linseed/flax cultivars have been bred specifically for disease resistance to any of the important diseases; yield of seed or fibre and other agronomic characters being economically more important to the breeder and grower. In recent history, the exception to this has been the breeding for resistance against the rust pathogen, *Melampsora lini*. The work of Flor (1941; 1942 a, b; 1946; 1947; 1955) and subsequent studies (Lawrence *et al.*, 1981 a, b) has resulted in the genetics of the flax-*M. lini* interaction being one of the best characterised of all agricultural crops and their pathogens. The resistance reaction was observed to be controlled by major genes in both the pathogen and host which interacted in a specific manner to confer resistance or susceptibility. Flor concluded that the genetic interaction between the pathogen and the host was much closer than had been previously assumed and the 'gene-for-gene' hypothesis (Flor 1942b; 1955) was proposed.

Flor hypothesised that for each gene that conditions the resistance reaction in the host there is a corresponding gene in the parasite that conditions pathogenicity (Flor, 1942b, 1946, 1947). In the case of the flax-rust interaction, resistance was invariably dominant to susceptibility with genes which controlled high degrees of resistance being epistatic to genes which confer less resistance. Resistance alleles conditioned a characteristic resistance reaction type which was manifested by the extent of colonisation by the pathogen. Analysis of the complementary genes in the fungus indicated that avirulence was dominant. Linkage between the resistance genes was observed, as was multiple allelism, and subsequent work showed five closely linked genes. To the present time, 29 specific resistance alleles have been recognised which have been grouped into 5 linkage groups designated L, M, N, P and K, containing 13, 7, 3, 5 and 1 alleles respectively (Crute, 1985). Linkage between loci conferring specific virulence was also observed, but less frequently, and no evidence of multiple allelism was found in the pathogen (Lawrence *et al.*, 1981a; 1981b).

Each host gene for resistance possessed alternative alleles, *R* resulting in resistance, and *r* which did not confer resistance, and this locus interacted with a specific corresponding gene in the pathogen which also possessed alternative alleles; *Av* resulting in avirulence and *v* resulting in virulence. Hypothetically, the resultant pattern of conformative response, known as a quadratic check, showed that one of the four possible combinations of the corresponding alleles in the host and pathogen gave resistance (or avirulence) when *R* in the host coincided with *Av* in the pathogen and susceptibility for the three remaining combinations of *R/v*, *r/Av* and *r/v*.

The gene-for-gene interaction dictated that a different resistance gene with two allelic forms interacted with a different avirulence/virulence gene in the pathogen. The complementary interaction that this confers could not be illustrated for a single pair of corresponding genes in a quadratic check, as the specificity of the interaction was not depicted. Table 2.1 illustrates that the minimum number of complementary gene pairs required to represent the gene-for-gene interaction is two, which, as Day (1974) pointed out, was the model upon which Flor based the original hypothesis.

Subsequent development of the gene-for-gene hypothesis has shown that the complementary gene system was not, as first thought, a peculiarity of the flax/rust pathosystem, but was applicable to many plant-pathogen systems (Crute, 1985) including, potato-*Phytophthora infestans* (Black, 1952; Toxopeus, 1956) and apple-*Venturia inaequalis* (Bagga & Boone, 1960, 1968). Van der Plank (1978) listed 28 examples of pathosystems which exhibit gene-for-gene systems as the basis of the interaction and also suggested that this was likely to be only a small proportion of the total number of plant-pathogen interactions which would eventually be found to be controlled by the mechanism.

Pathogen alleles	Host alleles			
	R1R2	*R1r2*	*r1R2*	*r1r2*
Av1Av2	I[1]	I	I	C[2]
Av1v2	I	I	C[3]	C
v1Av2	I	C	I	C
v1v2	C	C	C	C

[1] I : incompatible interaction (resistant/avirulent).

[2] C : Compatible interaction (susceptible/virulent).

[3] _ : underlined characters indicate that *R2* does not confer incompatibility with *Av1* and likewise *R1* does not confer incompatibility with *Av2* demonstrating the specificity of the gene-for-gene interaction.

Table 2.1. An example of an hypothesised gene-for-gene interaction between two host loci (each with two alleles, R1 *or* r1 *and* R2 *or* r2*) and two corresponding loci in a pathogen (also with two allelic forms,* Av1 *or* v1 *and* Av2 *or* v2*). (after Johnson, 1992).*

Evidence for other gene-for-gene interactions in linseed/flax pathosystems have been published in the literature. Resistance to wilt, caused by *Fusarium oxysporum* f.sp. *lini*, was reportedly observed to be controlled by a single dominant gene and resistant varieties to the pathogen are reported from the United States (Burnham, 1932; Stevenson & Jones, 1933) and India (Singh *et al.*, 1956; Jeswani & Upadyaya, 1970; Kamthan *et al.*, 1981; Goray *et al.*, 1987). Work on powdery mildew (*Oidium lini*) also suggested that resistance was conferred monogenically by a dominant gene, designated *Ol*, and that this was present in all resistant cultivars of linseed (Goray *et al.*, 1989; Singh & Saharan, 1979).

Very little information has been published on the resistance mechanisms involved in linseed/flax diseases caused by *Alternaria* spp.. Kalia *et al.* (1965) identified resistance against the form of *Alternaria* blight endemic in India, *Alternaria lini*, to be under monogenic control. From the descriptions given by some of the Indian authors, it would appear that *A. lini* was a linseed infecting pathotype of the complex of pathogens which belong to the *A. alternata* anamorph group. Such a close relationship with the host plant would not be uncommon for a pathotype of *A. alternata* as many forms have an extremely narrow host range. Often causing major disease problems in sub-tropical and tropical regions, pathotypes of *A. alternata* have been associated with the production of host-specific toxins under monogenic control (Kohmoto *et al.*, 1987, also, see Chapter 4, Introduction).

There is no evidence from the literature at present to suggest that major genes are involved in the *A. linicola*/linseed pathosystem. The observations of Mercer & Jeffs (1988) and Turner (1987) suggest a continuous range of resistance response between resistant and susceptible cultivars as opposed to the discontinuous, large interactive differences which would be expected should the interaction follow the classic gene-for-gene pattern. Some evidence of toxin production by the pathogen has been published (Leduc, 1958). The description suggests the production of host non-specific toxins similar to those produced by the large spored species, *A solani* rather than the monogenically controlled host-specific toxins characteristic of pathotypes of the *A. alternata* anamorph.

Possible mechanisms of resistance - The concept of polygenic control

Flor (1941; 1946; 1965) observed some exceptions to the general trends upon which his gene-for-gene theory was postulated. Subsequent studies with other host-parasite pathosystems have shown other variances from Flor's model. Examples include, resistance being inherited as a recessive or

partially dominant trait, duplicate avirulence genes corresponding to a single resistance gene, and additive effects of resistance genes. Since the proposition of the gene-for-gene hypothesis, a large number of pathosystems have been shown not to fit into the gene-for-gene pattern and many examples are cited by Crute (1985). The evidence to date suggests that the genetics of resistance/susceptibility are more complex in nature than can be explained by a generalised model.

In an attempt to explain the different forms of genetic control observed, Van der Plank (1963) proposed that there were two distinct classes of resistance, vertical, which was effective only against certain races (e.g. was race-specific) and therefore conformed to a strict gene-for-gene system, and horizontal which was equally effective against all races (e.g. was race-non-specific) and was therefore not controlled by a gene-for-gene interaction. Van der Plank (1963) pointed out that vertical resistance was the key tool that breeders had been choosing during the breeding of new crop cultivars for many years, the successful inclusion of a resistance gene conferring a state of immunity, or no disease. Historically this lead to the so-called "boom" in the growth of large areas of resistant crop cultivars utilising vertical resistance genes. However, the inherent problem with reliance on vertical resistance genes was, and still remains, that resistance/susceptibility in the host is conferred by usually one or very few genes which in turn interact with one or very few virulence/avirulence genes in the pathogen. Thus, a small shift in the frequency of virulence alleles in the pathogen population (for example by deletion or mutation), coupled with strong selection pressure from the resistance genes of the 'resistant' host resulted in resistance being quickly lost (Johnson, 1978; 1992).

The loss of effectiveness of the resistance gene and subsequent loss of resistance was often referred to as the cultivar being "bust". Thus, the whole sequence of events, from an increase in the popularity and subsequent failure of many crop cultivars, became termed the "boom and bust" cycle. It was as a consequence of the repeated extensive incorporation of single genes into resistant cultivars and the subsequent failure of resistance that breeding tactics changed to involve the incorporation of series of genes rather than individual genes (Ellingboe, 1981). Table 2.1 shows that because of the specificity of the gene-for-gene interaction, resistance genes can be incorporated into the host in combinations and the pathogen must then evade the effect of each of the genes at a corresponding, specific locus. Thus the pathogen must evolve and accumulate the required virulence alleles in the correct combination. However, the eventual result is that change in the pathogen population will occur, but the key question for plant breeders and farmers is how long the change will take. Johnson (1992) suggested that in practice the 'life-span' of vertical resistance genes would be variable and depended on many factors, such as the extent of deployment of the

specific genes, the epidemiology and population size of the pathogen and the potential of the pathogen population to vary.

The much cited examples of the breakdown of resistance in many pathosystems have fuelled the great debate amongst plant pathologists and plant breeders. Many questioned the occurrence of a gene-for-gene relationship in all host-pathogen interactions and also whether reliance on dominant resistance genes was a necessary pre-requisite for successful plant breeding. Views in the literature appeared to differ depending on personal experience in the laboratory or field and the particular pathosystem being studied. Person (1959) suggested that evolution to resistance in the host would be followed by evolution to virulence in the pathogen in a step-by-step progression. Parlevliet (1981) and Ellingboe (1975) suggested that if this was the case, resistance would always follow a gene-for-gene relationship. In fact, Ellingboe (1975) argued that not only was all resistance controlled by a gene-for-gene interaction, but failure to demonstrate such an interaction was due to poor experimental technique ! Differences in opinion over the validity of Van der Plank's concept of disease resistance continue, and the consideration of recent experimental evidence has neither proved nor disproved the validity of HR or VR as real, genetically controlled phenomena.

However, following disease control failure, many breeders appeared to abandon the introduction of dominant major resistance genes into new cultivars, even in groups. It was realised that horizontal resistance was not just resistance "that had not yet been shown to be vertical" as rather curtly described by Ellingboe (1981). The possibility of producing cultivars showing "less disease" rather than immunity was a strange concept for many traditional breeders to grasp, although many realised that horizontal resistance could offer an alternative to the "boom and bust" treadmill. A recent review of the use of horizontal resistance in cultivated crops has indicated that not only does horizontal resistance work in the field, but no specific case of failure has so far been reported (Simmonds, 1991).

One of the earliest and most well known examples of successful breeding utilising horizontal resistance was described by Niederhauser *et al.* (1954) working on disease resistance of potato to *Phytophthora infestans*. Vertical resistance genes (*R*-genes) from, *Solanum demissum* had been introduced into numerous cultivars in Europe and North America in the 1930's. Failure of the resistance conferred by the R-genes was predictably swift and total. However, Niederhauser observed that the potato clones showed a highly repeatable variability of response to the pathogen and could be ranged from susceptible to fairly resistant (but not immune). Furthermore, the ranking of the material remained essentially stable over many years. Niederhauser suggested that

what was being observed was 'field resistance' and that being repeatable, resistance of this nature would be responsive to selection (Simmonds, 1991).

Unfortunately, very little of Niederhauser's early work on what is know recognised as being horizontal resistance was published. The important points are noted in Niederhauser *et al.* (1954). However, the early studies lead to the realisation that horizontal resistance was present in most, if not all, crop species at various levels and that an alternative strategy to the continual cycle of 'boom and bust' for disease resistance could be exploited.

The critical importance of horizontal resistance was that it was not discontinuous (i.e. there was no "all or nothing" response as observed with vertical resistance genes). Horizontal resistance, controlled by polygenes, produced a continuous distribution of resistance responses between host cultivars and pathogen isolates and as such, the selection pressure on the pathogen population was not as strong or specific. Indeed, Van der Plank (1963) defined horizontal resistance as being non-pathotype specific. He stated that an isolate x cultivar interaction constituted the effect of major genes and that if this situation was observed, resistance would be vertical due to the adaptation of pathotypes to a specific host genotype. If such an interaction was not observed, resistance would be horizontal (Van der Plank, 1963). However, there is some evidence that this is not necessarily true. As Simmonds (1991) states "it would be very surprising, on *a priori* grounds, if they [host x pathogen interactions] did not exist; in biological systems, interactions are nearly always found if sought". The example given by Simmonds (1991) to demonstrate this point concerns two pathogens of maize, *Colletotrichum graminicola* and *Bipolaris maydis* for which there was no evidence of vertical resistance in the host (Jenns *et al.*, 1982). Small interactions were found in single trials,which were variable and unrepeatable over separate trials, specificity was not observed, and resistance was unequivocally horizontal in nature. Although not the case in this example, large consistent interactions in respect to horizontal resistance would suggest the evolution of adapted pathotypes to a given genotype and the beginning of the failure of the horizontal resistance. For crops which have been breed using quantitative traits, large consistent interactions have not been reported in the literature and, indeed, this provides the basis of the argument that horizontal resistance is stable or 'durable'. Accordingly, the importance and advantages of the development of durable, usually race non-specific, resistance systems have been reported in recent years (Johnson, 1979; Simmonds, 1991). Evidence of durable resistance for facultative pathogens which have a similar biology to the *Alternaria* has been described recently and include *Septoria* spp., *Bipolaris* (formerly *Helminthosporium*) spp. *Cercospora* spp. and *Colletotrichum* spp. (Simmonds, 1991).

Current evidence from the literature concerning phytopathogens of the *Alternaria* which occur in temperate climates suggests that race specific resistance is not generally observed and that resistance mechanisms are polygenically controlled. Of the reports of *Alternaria* spp. which are pathogenic on *Brassica* spp., polygenic (quantitative) resistance was observed for *Alternaria brassicae* (Humpherson-Jones & Hocart, 1983; Prasanna, 1984; Bansal *et al.*, 1990) and *Alternaria brassicicola* (Prasanna, 1984).

Recent evidence of the interaction between linseed and *A. linicola* indicated that the mechanism of resistance was polygenic in nature. Klose *et al* (1993) observed that qualitative interactions were not observed between 16 linseed cultivars and two *A. linicola* isolates at a number of growth stages during glasshouse tests. Levels of resistance were continuous from susceptible to fairly resistant although some change in the ranking of resistance between the different cultivars tested was observed to occur at different growth stages. As large cultivar/isolate interactions were not observed, Klose *et al* (1993) concluded that resistance was under polygenic control and suggested that a good response to selection for improved resistance should be possible.

In a general review on the merits of the use of breeding for resistance utilising horizontal resistance, Simmonds (1991) not only gave examples where resistance had been both effective and reliable but suggested that the level of response of quantitative traits to selection was high. Of the *Alternaria*, Nash & Gardner (1988) demonstrated that resistance of tomato to *Alternaria solani* was quantitative in nature, heritable and showed good response to selection. Simmonds (1991) also provides a number of examples of quantitative breeding programmes targeted against pathogens which have a similar biology to *Alternaria* spp. where response to selection for quantitative traits has been successful. Jenkins *et al.* (1954) observed good response to selection of quantitative resistance of maize to the pathogen *Helminthosporium turcicum*, another anamorphic form of the *Pleosporaceae*.

It is likely that durable resistance to *A. linicola* will involve several physiological and biochemical components. Evidence from other phytopathogenic systems involving the *Alternaria* would suggest that following recognition, the resistance response would be multicomponent in nature and involve components including structural changes to the host cells and the production of defence related compounds by the host in response to physical and chemical stimuli from the pathogen. Such a structure of resistance response was suggested by Tewari (1991) as the mechanism of resistance of cruciferous species to *A. brassicae* and was also suggested as a general model of resistance by Heath (1991; 1995) for many plant pathogen interactions.

The development of *A. linicola* resistant varieties carrying durable resistance genes is of prime importance if linseed is to remain a viable proposition as a crop. At present the mechanism of resistance to *A. linicola* has not been fully characterised. Johnson (1978) suggested that the most obvious source of durable resistance for a breeding programme is a parent which has shown a good level of resistance for many seasons. In an extension of this, the co-evolution of natural plant populations with pathogen populations over many generations has effectively dictated the incorporation and maintenance of many useful resistance genes. As such, it would seem likely that a good source of resistance to *A. linicola* would come from a wild or near relative of *L. usitatissimum*, or from cultivars which have shown durability for a number of seasons in the field. Levels of resistance in such lines to *A. linicola* have not been described to date

Criteria for the development of a bioassay for resistance to Alternaria linicola

The aim of the following study was to develop a reproducible bioassay which could be used to assess the resistance of currently-grown linseed cultivars and a range of *Linum* material against field isolates of *A. linicola*. The second aim was to demonstrate that the response observed from the bioassay was indicative of the response during normal linseed plant growth under glasshouse conditions. Third, to use the data produced from the experiments to assess the underlying mechanism of the resistance response as a guide to possible breeding strategies for future studies.

A series of experiments were developed based on a number of studies reported in the literature. Because of the requirement for the resistance bioassay to be quick to carry out and to be reproducible, it was decided that a detached tissue assay under controlled environmental conditions would allow the systematic testing of large amounts of material. For this reason, an assay was developed in which cotyledons of linseed were removed from young seedlings, infected with isolates of the pathogen and were incubated for five days at a constant temperature/relative humidity.

The choice of the cotyledons as the test tissue was made for two reasons. First, the true leaves of linseed are long and narrow and were not conducive to the rapid application of inoculum droplets. Second, if an infected seed of linseed germinated successfully in the field and survived to reach the soil surface, the cotyledons would be the organs which were readily attacked in the field by *A. linicola*. As explained in Chapter 1, survival and strong growth of the seedling allowing the plant to outgrow the rate of infection would arise as a direct result of the resistance response of the cotyledons. The survival of the cotyledons at the vulnerable seedling stage of the growth cycle therefore determines whether the plant survives to develop further.

Due to inherent problems with the induction of *in vitro* sporulation of many of the *Alternaria*, and in this case, the *A. linicola* isolates, the use of a mycelial inoculum was developed for the purposes of inoculating the material during the assay. A regular supply of mycelium could then be cultured so that inoculum was available to coincide with the production of seedlings of the test material of the required growth stage. Also, considering the biology of the pathogen in the field, infection pressure from *A. linicola* at the seedling stage would be derived from mycelia growing over the surface of the seedling. The mycelia which are observed to attack the stem and cotyledons are produced from the infection hyphae which over winter in the mucilagenous layer surrounding the testa of the seed. It would appear that conidiogenesis does not occur in *A. linicola* until much later in the season during the movement of the pathogen from the lower leaves and stem upwards to the flowers and developing seed pods (Vloutoglou, 1994). Thus, infection of the cotyledons with a mycelial inoculum provides an exaggerated, but realistic, scenario of seedling attack as it would occur in the field.

Objectives of the bioassay study:

1. Develop a method to allow the quantification of the resistance response of *Linum* accessions to *A. linicola.*
2. Use the bioassay to assess variability in the aggressiveness of the pathogen population.
3. Assess the resistance response of a large number of genetically diverse *Linum* accessions (<100 accessions).
4. Validate the data from the *in vitro* bioassay by comparing with *in vivo* data.

2.2 Materials and methods

2.2.1 Growth of plant material

Seeds of the plant material were sown in Fison's Levington M3 compost in 9 cm pots and grown in a glasshouse under natural daylight supplemented for 16 h daily by 400 W mercury vapour lamps. The maximum temperature was 24° C during the day and fell to a minimum temperature of 9° C at night. Plants were removed from the glasshouse for testing when cotyledons had fully expanded and the first true leaves were beginning to unfold (G.S. 09-10; Freer, 1991) unless otherwise specified.

2.2.2 Culture of *Alternaria linicola* isolates and preparation of inoculum

Three 10 mm diameter plugs of each *A. linicola* isolate (Appendix 1.1) from five day-old V-8 agar cultures were used to inoculate 500 ml culture bottles (Duran) containing 250 ml of defined medium (Appendix 1.2). Liquid cultures of the pathogen were grown at 20° C in darkness for 30 days, the cultures being agitated every second day. The mycelial mat was filtered off under vacuum through a single layer of sterile muslin, mycelial mats from different bottles of each isolate being pooled, and washed with approximately 50 ml of sterile distilled water (SDW).

The mycelial mat was transferred to a pre-weighed receptacle and the fresh weight measured. The mycelium was homogenised in 20 ml SDW for one minute using a Waring blender with a pulverisor attachment. Inoculum solution was prepared by diluting the homogenate with SDW to a concentration of 0.02 g ml^{-1} fresh weight mycelium. One droplet of Tween 80 was added to each batch of inoculum in order to minimise clumping of the mycelial matter within the suspension and to maximise the spread of the inoculum droplet over the surface of the cotyledon. A water control solution was prepared using 60 ml of SDW with a droplet of Tween 80.

2.2.3 Preparation and inoculation of excised cotyledons

Cotyledons of the material to be tested were excised at the base of the petiole. Replicate cotyledons of each set of test material were embedded by the petiole in a Petri dish (10 cm x 10 cm, Sterilin) containing 80 mg l^{-1} benzimidazole water agar. Each cotyledon was inoculated

with a 25 µl droplet of mycelial suspension from one isolate. Each dish was sealed with Para-film to minimalise the possibility of contamination and to prevent the evaporation of the inoculation droplet. Dishes were incubated at 18° C (16 h photoperiod) for 5 days in a controlled environment cabinet (Conviron CMP3244).

2.3 Experimental design

2.3.1 Preliminary assay of four linseed cultivars with 7 isolates of *Alternaria linicola*

Seven isolates of *A. linicola* (Appendix 1.1, Al1-7) were tested against four widely grown cultivars of linseed; Antares, Barbara, Linda and McGregor, in order that a measure of levels of resistance in commercially available cultivars could be assessed. In each trial, a split plot design was used. The six replicate cotyledons of each cultivar (sub-plot) were tested with mycelial inoculum of one of the seven isolates or with control solution (main plot). Three trials were carried out on a randomised block design to test the repeatability of the technique and to assess whether the response of the *A. linicola* isolates and the linseed cultivars remained predictable over time.

2.3.2 Inoculation of linseed cultivars and *Linum* germplasm material with the non-aggressive isolate Al1 and the aggressive isolate Al6

Linseed, flax, *Linum angustifolium* (Hudson) and *L. usitatissimum* sub-species material were collected from EC project partners and from other sources as detailed in Appendix 1.4. Following a season in the field in order to bulk up seed stocks, material was tested with isolates Al1 and Al6 using the detached cotyledon bioassay described above.

Ten replicate cotyledons of test material were used per 10 cm x 10 cm dish together with five replicate cotyledons of each of the cultivars, Linda and Antares. The two "standard" cultivars were included in every test to act as reference material of a known resistance response to the two *A. linicola* isolates during the tests. Material was tested on three separate occasions in batches of 8 - 40 accessions chosen at random following adequate germination.

2.3.3 Inoculation of cotyledons of intact *Linum* seedlings in a glasshouse study

A sub-set of eight accessions tested using the detached cotyledon assay (2.3.2) were chosen for inclusion in a whole plant study in order to verify that the response observed during the *in vitro* test was indicative of the resistance response expressed during normal whole plant growth. Antares was also included as a reference cultivar as in 2.3.2. The criterion used for the selection of material to be included in 2.3.3 was that the test accessions should include a range of material reflecting the whole range of responses observed during the main detached bioassay (2.3.2), from susceptible to resistant accessions.

Seedlings were grown in the glasshouse as described above (section 2.2.1). At G.S. 09-10 (Freer, 1991), cotyledons were inoculated with a 25 µl droplet of mycelial suspension of either isolate A11, A16 or water control solution, prepared as described in section 2.2.2. Ten replicate seedlings of each accession were inoculated per plastic pot and two replicate pots of each treatment were used during the experiment. Pots were placed on large metal trays containing water and were covered in clear polyethylene sheeting to ensure a high relative humidity.

2.3.4 Scoring of cotyledons and the statistical analysis of the data

During the course of experiments, visual assessment of disease symptoms was made on a scale of 0 - 4 (Appendix 1.3) five days after inoculation. Analysis of variance (ANOVA) was carried out on the data for the preliminary study (2.3.1) and the whole plant study (2.3.3) using GENSTAT release 5.2.2. (NAG, Oxford) using the VAX mini-computer. Owing to an unequal number of accessions being tested in each batch and material being tested non-sequentially, the design of the main study (2.3.2) was sufficiently unbalanced that the use of ANOVA was considered inappropriate. Data for study 2.3.2 were analysed by restricted maximum likelihood (REML) analysis using GENSTAT release 5.3. on a Sun Unix computer of Biomathematics and Statistics, Scotland (BIOSS).

For the data of the main study (2.3.2), the ten scores (or five in the case of Antares and Linda) of each accession-*A. lini*cola combination on a plate were summed to give a total of 886 experimental units. The fixed sources of variation were due to cultivar, *A. lini*cola isolate and the cultivar-isolate interaction. The random sources of variation were due to batch (1-12) and plate within batch. Residual variation would be expected from the fact that the ten replicates of a factor combination did not produce the same score.

REML uses the structure of the experiment, particularly the control cultivar scores in this case to estimate the effect of each of the random sources of variation. The technique then estimates a hypothetical mean for each of the fixed effects for the score that would be achieved if the design were balanced for all other sources of variation. The fixed effects can then be compared on an "equal footing" and statistical significance for the fixed effects is indicated by a χ^2 statistic.

Data for the preliminary bioassay (2.3.1) and the main study (2.3.2) were further analysed using a number of multi-variate statistical techniques. For the preliminary study data (experiment 1), principal components analysis (PCP) was carried out on the sums of squares data produced from the ANOVA output. PCP operates on a units by variables data matrix and analyses the relationship between the variables so that the units can be represented in a smaller number of dimensions whilst retaining as much of the original variation as possible. PCP analysis carries out this task by finding linear combinations of a set of variates that maximise the variation within them. If the resulting component axes display most of the original variability, the underlying trends in the data can be said to be represented in a smaller number of dimensions.

Hence, for the preliminary bioassay (2.3.1), PCP was carried out in order to summarise the original variance observed, provide more information about the response of the four cultivars with the *A. linicola* isolates and to characterise underlying trends. The data matrix produced was plotted as a biplot in order that the response could be visualised. Gabriel first described the technique in 1981 and it has subsequently been used effectively during the study of a number of host-pathogen interactions (Phillips & McNichol, 1986; Anderson *et al.*, 1990).

The estimated mean data produced from the REML analysis of the main study data (2.3.2) was re-analysed using a number of statistical and graphical methods. Firstly, a similarity matrix was produced using a City Block co-efficient, a similarity measure which was designed for use with quantitative data:

City block distance measure $$d_{ij} = \frac{1}{p}\sum \left| x_{ik} - x_{jk} \right| / r_k$$ (Cain & Harrison, 1958)

where the distance (d_{ij}) between i and j, based on p variables x_k (k=1,...,p) and r_k is the range of the kth variable. The similarity matrix was then represented graphically as a dendrogram.

2.4 Results

2.4.1 Preliminary assay of four linseed cultivars with 7 isolates of *Alternaria linicola*

Differences between the seven *A. linicola* isolates accounted for the majority of the variance. There was a significant effect of *A. linicola* isolate even when the effect of the control solution was restricted out of the analysis ($P < 0.001$). There was no significant difference between cultivars ($P = 0.064$) although Fig. 2.1 illustrated that there were consistent responses to the seven isolates. There was no significant interaction between isolates and cultivars ($P = 0.072$) and Fig. 2.1 illustrated a consistent trend, in that DI's in response to Al2 and Al6 were higher than with other isolates. The ranking of the isolates with respect to virulence remained fairly constant with three distinct groupings, aggressive (Al2 and Al6), non-aggressive (Al1 and Al7) and intermediate (Al3, Al4 and Al5)(Fig. 2.1).

Cultivars behaved in a predictable manner with low scores against non-aggressive isolates and higher scores (denoting susceptibility) against the two aggressive isolates (Al2 and Al6)(Fig. 2.1). Over the three tests, Linda achieved the lowest mean DI in comparison to Barbara and Antares, whilst McGregor achieved the highest score (1.16 < 1.25 < 1.354 < 1.361 respectively).

PCP analysis indicated that the total amount of variance could be accounted for in the first three principal components (PC) (64.81 %, 29.24 % and 5.94 % respectively). The biplot produced from the PCP analysis (Fig. 2.2) illustrates the response of the four cultivars against the seven *A. linicola* isolates. Small angles between vectors indicate co-linearity and vectors with opposite directions are negatively correlated (Anderson *et al.*, 1990). An increase in distance away from the origin of the points for the cultivars indicates a large response in the interaction.

Isolates Al2, Al4 and Al6 were closely associated in the interaction achieving a negative score on the first PC axis. Conversely, isolates Al1, Al3, Al 5 and Al7 achieved a positive score on the first PC axis. Also, the vectors assigned to isolates Al2, Al3 and Al5 extend a considerable distance from the origin indicating that these isolates accounted for a large proportion of the variance in the interaction.

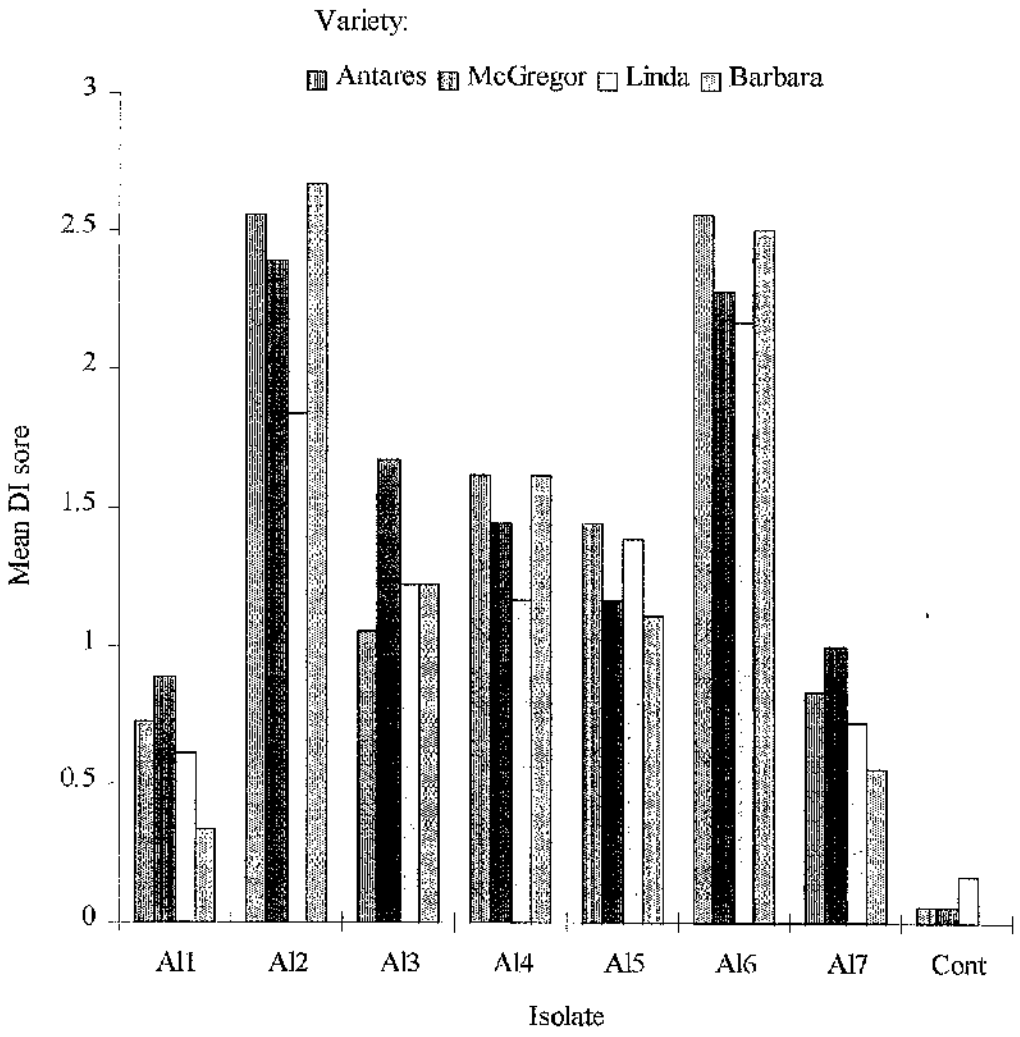


Fig. 2.1 Mean response of four linseed cultivars to seven isolates of Alternaria linicola *showing the characteristic level of resistance against aggressive, non-aggressive and intermediate isolates. SEDs = 0.34, df = 14 (for comparison between isolates) and 0.24, df = 48 (for comparison within isolates).*

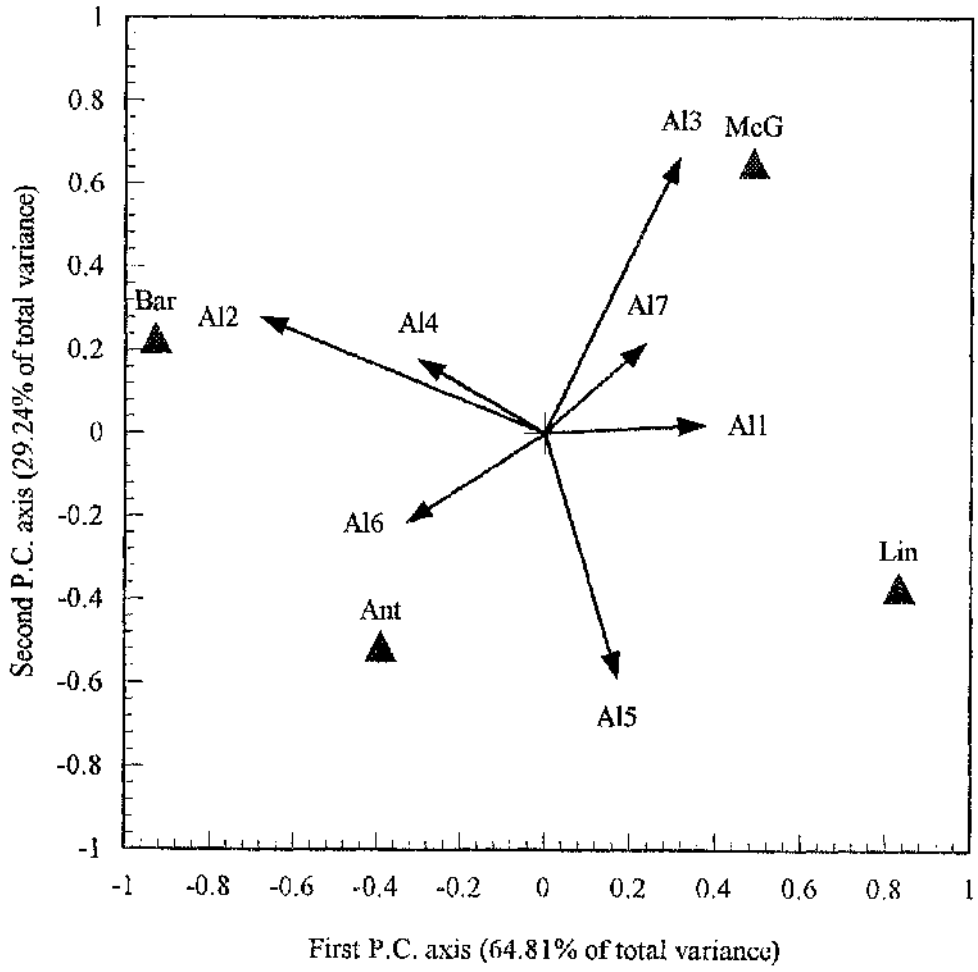


Fig. 2.2 Biplot of PCP analysis data from the preliminary bioassay (2.3.1) showing the interaction between seven A. linicola *isolates (Al1-7) and four cultivars of linseed, Antares (Ant), Barbara (Bar), Linda (Lin) & McGregor (McG).*

Fig. 2.2 also indicated that the PCP analysis associated the isolates with the cultivar upon which the highest score was achieved by that particular cultivar. The non-aggressive isolates Al3 and Al7 and to a lesser extent Al1, showed strong association with cv. McGregor the cultivar which had the highest mean DI score. Conversely, Al2. Al4 showed association with cv. Barbara which achieved the second lowest DI score and Al6 achieved the highest mean DI score on Antares with which it is clearly associated. None of the isolates were strongly associated with cv. Linda, the cultivar which achieved the lowest mean DI score.

2.4.2 Inoculation of linseed cultivars and *Linum* germplasm material with the non-aggressive isolate Al1 and the aggressive isolate Al6

Due to the underlying nature of REML, the estimated variance components (VC) decomposed the variance of a single observation and were used to compare the random effects over the whole data set. Of these, the effect of batch was fairly large (VC = 0.11) accounting for approximately 41 % of the residual effect (VC = 0.27). The effect of plate within batch, however, was almost negligible (VC = 0.01).

Of the fixed sources of variation, there was a significant effect of accession ($\chi^2 = 273.3$, df = 101, $P < 0.001$) and, as observed in the results described above (2.4.1), a large significant effect of *A. linicola* isolate ($\chi^2 = 1640$, df = 1, $P < 0.001$). The interaction between accession and *A. linicola* isolate was not significant ($\chi^2 = 101.1$, df = 101, $P = 0.51$) and for each accession the estimated mean for Al6 was greater than that for isolate Al1. Fig. 2.3 illustrates the relationship between the estimated summed mean scores of the accession material and indicates that, although much of the material gave an intermediate response, there was a continuous distribution of response from susceptible to resistant.

Table 2.2 indicates the ranking of accessions from resistant to susceptible using the estimated summed mean DI score of both isolates over three test occasions. The data presented in Table 2.2 illustrated the spread of response observed during the testing of the accessions and gives a quantitative measure of the resistance response.

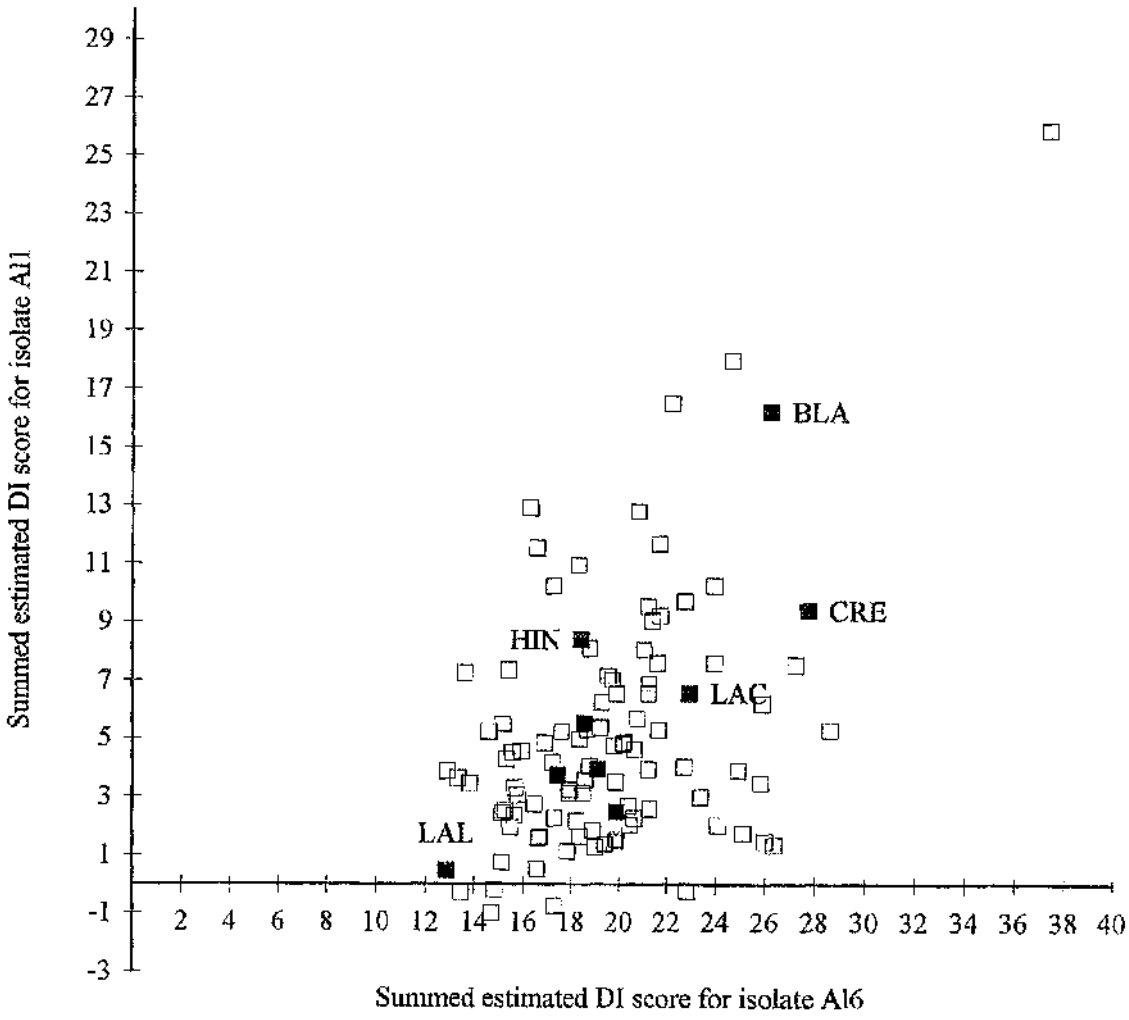


Fig. 2.3 Estimated summed mean data of DI scores for isolates A11 and A16 achieved against 102 accessions of Linum *material. Solid squares denote accessions which were chosen for inclusion in the whole seedling bioassay (2.3.3).*

Test accession	Mean	Test accession	Mean	Test accession	Mean	Test accession	Mean
L.u.elongatum elongatum	6.58	Vimy	10.2	Nusis	11.94	Norfolk Earl	14.08
L.u.u. albocoeruleum	6.66	Szegedi 62	10.23	Andro	12.0	Maros	14.41
L. angustifolium	6.86	*L.u.u. albidum*	10.34	Buenos Aires 106	12.04	*L.u.med. macranthum*	14.54
Ceres	7.32	Vimy	10.37	*L.u.med. mediterraneum*	12.26	Liral Monach	14.59
L.u.u. angustipetuleum	7.94	Rumänien II	10.4	*L.u.elongatum regale*	12.28	Japonais	14.6
Mikael	8.27	Aoyagi	10.43	Transylvanie	12.48	Indian 1212	14.61
L.u.u. album	8.38	Szegedi 62 VI-4-942	10.52	*L.u.elongatum roseum*	12.54	*L.u.u. pratense*	14.66
Maroc S.M.	8.49	*L.u.u. indicum*	10.59	Olajözön VI-4-41	12.58	*L.u.u. alumicum*	14.73
L.u.u. lutescens	8.55	Kiszombori 41	10.6	Laura	12.62	Bison	15.21
L.u.elongatum roseolum	8.63	Tadorna[F]	10.64	Hella	12.75	Culbert	15.38
Linda	8.71	DSV-7	10.69	Regina	13.07	Hester	15.46
Océan	8.8	Sandra	10.71	Royale	13.19	Fleischmann	15.78
Manchurian	8.9	*L.u. pekinense*	10.8	*L.u.u. choresmicum*	13.21	*L.u.u. caesium*	16.04
Szegedi 30 VI-4-940	9.01	Flanders	10.86	Hosszúháti 7131	13.22	Emeraude	16.22
Tadorna[G]	9.1	DSV-6	11.08	Farroupilha	13.34	Tape Parana	16.68
Szegedi 43 VI-4-861	9.16	Pinnacle	11.17	GI 704-74	13.36	Pro 9515	16.79
DSV - LU 1	9.41	DSV 1270/73	11.24	Viking	13.37	Hohenheimer.blau	16.96
L.u.elongatum crispum	9.47	*L.u.u. purpurascens*	11.3	Hinu	13.39	Madona	17.09
Somme	9.48	Clay	11.38	Dufferin[F]	13.43	*L.u.luteum*	17.39
Csanádi olajlen	9.6	Punjab	11.39	Opaline	13.46	Cresus	18.54
DSV-5	9.79	Ottawa 770 B	11.4	Victory	13.48	JWS	19.36
Linora FP862	9.8	Szegedi 43 VI-4-941	11.45	Cass	13.73	Blauwe-ster	21.21
Ariane	9.91	Antares	11.51	Rumänien I	13.74	Hindoukouch	21.3
Norman	10.0	Rio	11.53	Martta	13.86	*L.u.u. candidum*	31.64
Metcha	10.03	Szegedi olajlen	11.66	Dufferin[C]	13.88		
Szegedi 43	10.13	Vir	11.69	Bowman	14.04	SED = 3.161 (df = 101)	

Table 2.2. Mean estimated summed DIs of *Linum usitatissimum* accessions ranked from resistant to susceptible. (For sources, refer to Appendix 1.4).

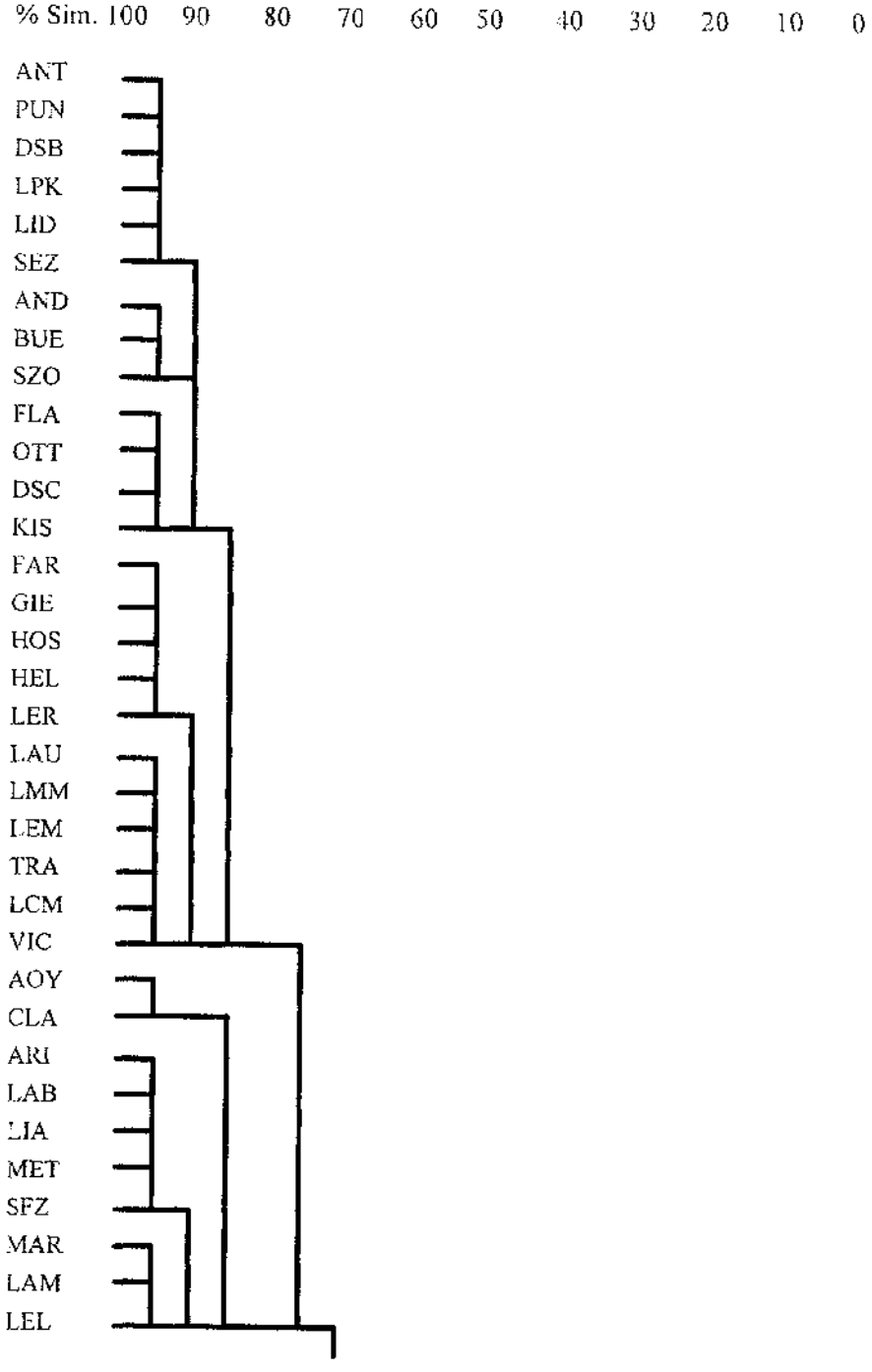
The dendrogram shown in Fig. 2.4 a, b and c illustrates the association of cultivars which responded similarly during testing with A11 and A16. The length of the horizontal lines to the connecting bars are proportional to the distance between material in relation to the resistance response and are given as percentage similarity. Four distinct clusters were observed representing four groups showing similarity of resistance response.

The first, and most obvious, sub-division was that *L. u. u.* candidum (LCD) showed only a 5% similarity with the 101 other accessions tested and was thus grouped individually (Fig. 2.4 c). The second of two major branches sub-divided the remaining accessions into a large group of lower scoring (moderately resistant and resistant) material (Figs. 2.4a and b.), which showed > 40 % similarity to the higher scoring (susceptible material) group (Fig 2.4c). The third major branch of the dendrogram occurred within the lower scoring material indicating that the resistant material (Fig 2.4b) showed 70 % similarity to the moderately resistant material observed on Fig. 2.4a.

Discounting the group formed by LCD, the three major groups showed varying degrees of sub-branching. The most complex was the susceptible group (Fig. 2.4 c) which showed a high level of branching at all similarity levels above 55 %. Of the sub-branches found within the group, only one small group which showed 95 % similarity contained more than two accessions, the group of 4 accounting for only a tenth of the total number of accessions in the susceptible group.

The material which achieved a moderate response (Fig. 2.4 a) was further sub-divided into three main groups at > 75, > 85 and > 90 % similarity. Above this level of similarity, the 34 accessions of the moderate group were sub-divided into only eight groups at the > 95 % level, seven of which contained more than two accessions.

Figure 2.4b indicated that the resistant accession group was observed to show a low amount of sub-branching at the higher levels of similarity. The group was sub-divided by three main sub-branches at > 80 %, > 85 % and > 90 % similarity and all sub-groups exhibited exceptionally low levels of sub-branching at a similarity of > 90%. The 25 accessions of the group were represented in only 5 groups at a similarity of > 90 %.



70 % similarity to LAL and other groups of accessions

Fig. 2.4a Accessions achieving a moderate response to two Alternaria linicola *isolates during an in vitro detached cotyledon bioassay. (For a guide to the three letter code of each accession, refer to Appendix 1.4).*

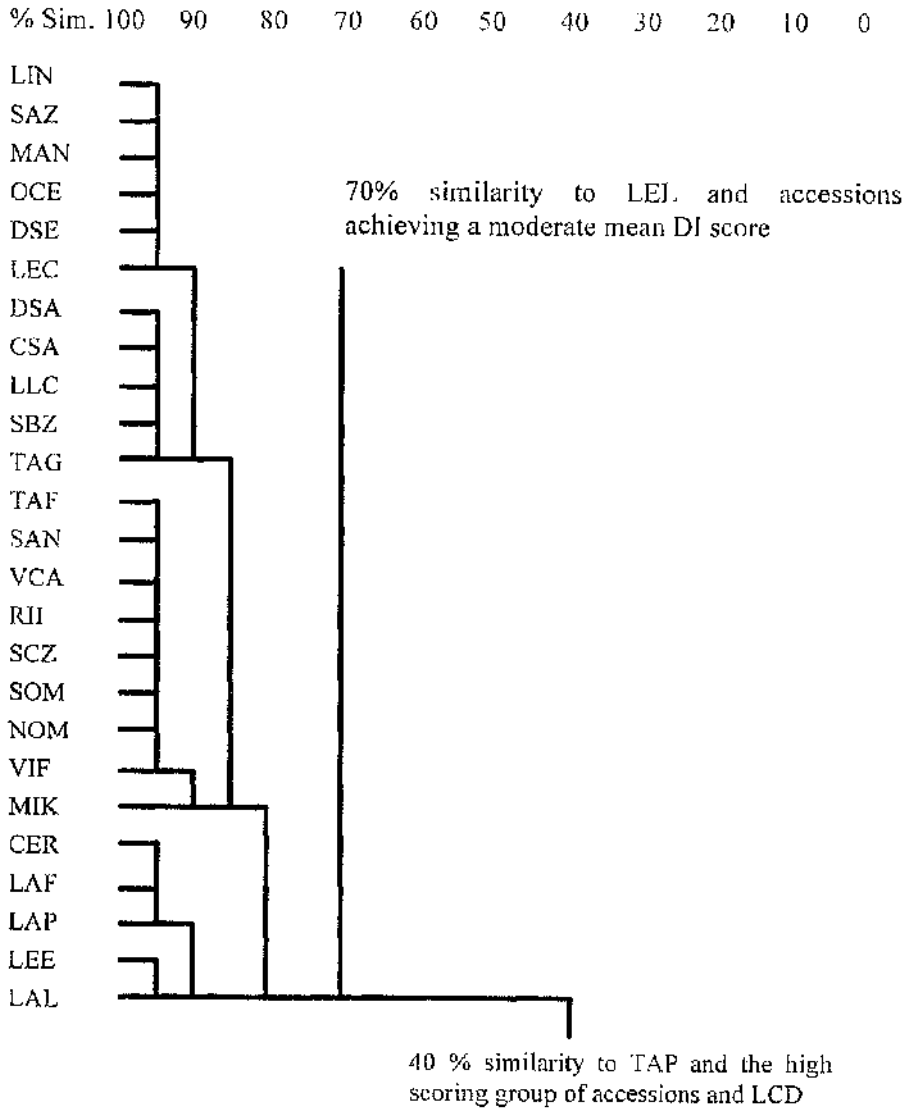


Fig. 2.4b Accessions achieving a low score (resistant) to two isolates of Alternaria linicola *during an in vitro detached cotyledon bioassay.. (For a guide to the three letter code for each accession, refer to Appendix 1.4).*

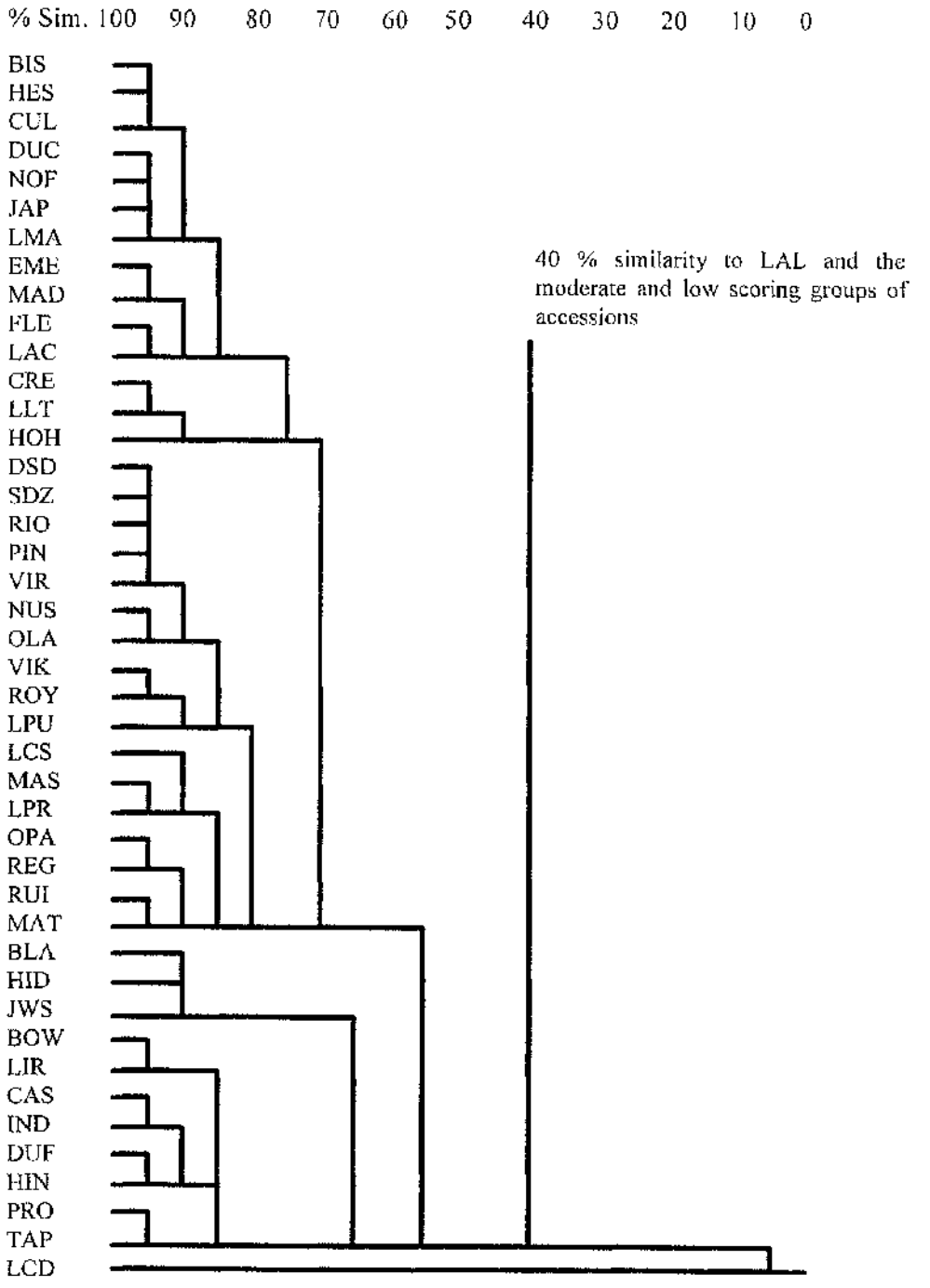


Fig. 2.4c Accessions achieving a high mean DI score to two Alternaria linicola *isolates during an in vivo detached cotyledon bioassay. (For a guide to the three letter code of each accession, refer to Appendix 1.4).*

2.4.3 Inoculation of cotyledons of intact *Linum* seedlings in a glasshouse study

The analysis of variance indicated that there was a significant effect of isolate ($P < 0.001$, Al1 mean = 0.874, Al6 mean = 1.357, SED. = 0.06) and accession ($P < 0.001$)(Fig. 2.5). There was no significant interaction between accession and cultivar ($P = 0.235$).

The DI scores for the *in* vitro assay (2.3.2) and the whole plant assay (2.3.3) were positively correlated, but the significance of the correlation was dependant on isolate. In the case of the non-aggressive isolate, Al1, the correlation between the DI scores was positive, but not significantly so ($r = 0.476$, $P > 0.1$), while the correlation with the aggressive isolate, Al6, was positive and significant ($r = 0.744$, $P > 0.05$). Overall, the DI scores recorded during the *in vitro* assay (2.3.2) were slightly higher than those recorded in the whole plant assay (2.3.3) indicating that the *in vitro* test slightly, but systematically, underestimated the resistance of the tested material (Fig. 2.6).

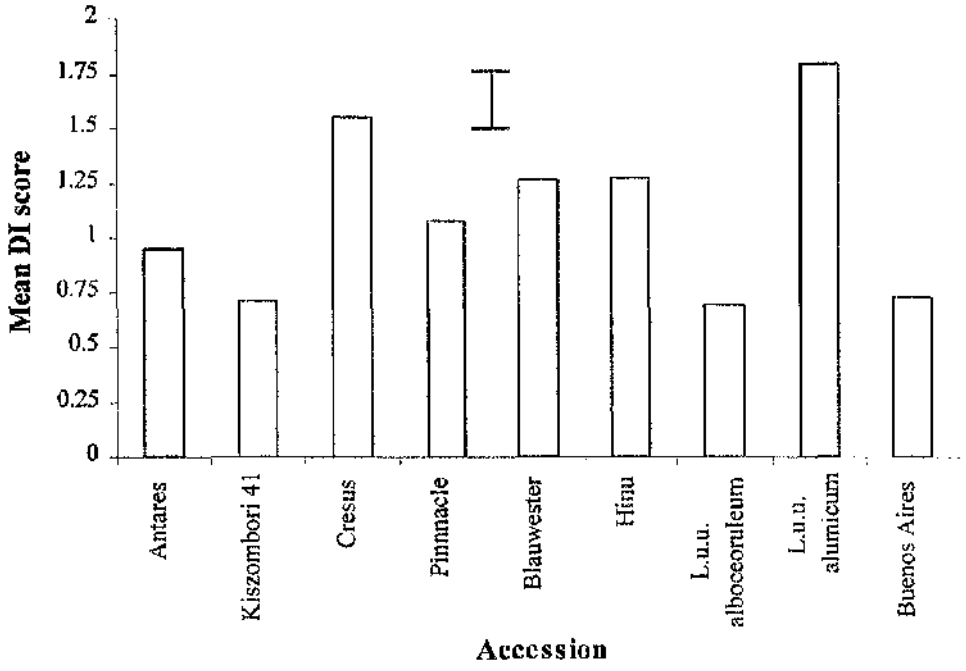


Fig. 2.5 Mean DI score achieved by nine Linum *accessions tested with two isolates of* Alternaria linicola *during a whole seedling study (2.3.3). Error bar = SED*

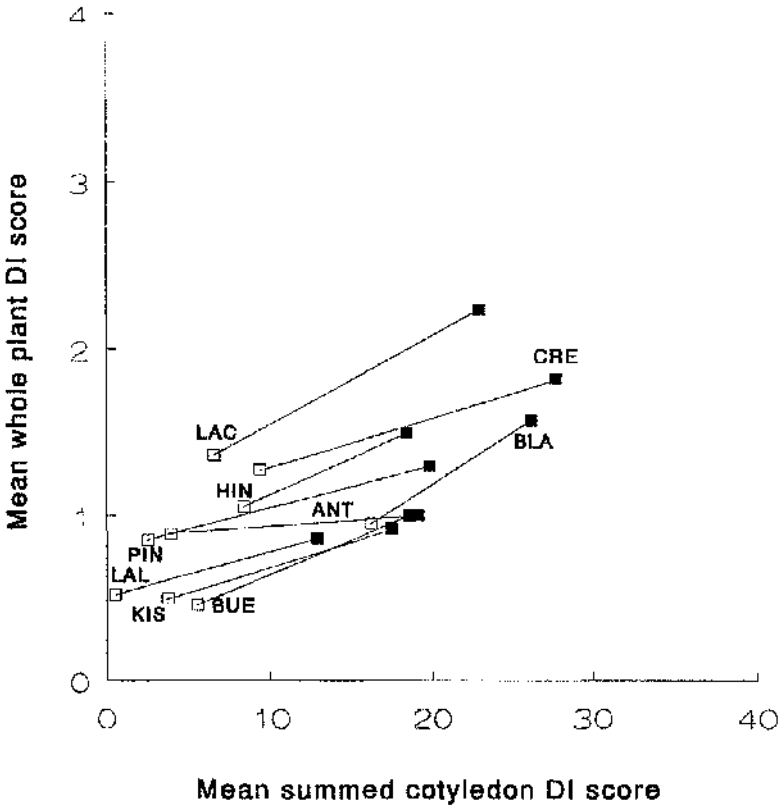


Fig. 2.6 Plot of mean DI scores from the whole seedling bioassay (2.3.3) against the estimated summed detached scores from the in vitro bioassay (2.3.2) illustrating the systematic response to the non-aggressive isolate , Al1 (□) and the aggressive isolate, Al6 (■). (For a guide to the three letter accession codes, refer to appendix 1.4, ANT = cv. Antares).

2.5 Discussion

General observations on the response of the linseed cultivars during the preliminary bioassay

Analysis of the preliminary study results (2.4.1) indicated that the majority of variance was accounted for by differences between isolates. In this experiment, and indeed, the main study (2.4.2) and the whole plant test (2.4.3), changes in the level of pathogenicity observed between batches of tests for each particular isolate proved to be slightly problematic. However, the results of the preliminary study indicated that no significant differences occurred between the four cultivars tested and also that the ranking of the cultivars remained similar throughout the study. Comparisons between different batches of the main experiment were therefore likely to be valid as the relative differences in the aggressiveness of the isolates were stable with time. The incorporation of the "standard" cultivars Antares and Linda into the experimental design of the main study also allowed the possibility of variation within the pathogen to be accounted for during the testing of the accessions (2.3.2).

The basic response of the cultivars during the preliminary assay (2.3.1) remained stable with time (2.4.1). Although the level of pathogenicity showed slight variation between the three tests, the response of each cultivar to a specific isolate, for example Al6, the more virulent of the two aggressive isolates, was always a characteristic high DI score. Conversely, inoculation with isolate Al1 always resulted in a low DI score. As there was no significant interaction between *A. linicola* isolates and cultivars the pattern of resistance observed remained the same, but the absolute score showed variation.

Effect of environmental conditions on infection during bioassays

An explanation for this could be that the experimental conditions fluctuated between tests. The fact that test dishes were sealed and placed in the same controlled environment cabinets for each test would appear to exclude this, however. Nonetheless, it could be reasoned that no matter how carefully an experiment is designed and undertaken, there may be errors which would affect the observations to some degree. From the work of Dickinson & O' Donnell (1977), Chandrashekar & Ball (1980) and Lacey (1986) it would appear that relative humidity is the most limiting environmental factors affecting the success of *Alternaria* spp. on the phylloplane and that as a genus, relatively high levels are required for successful germination (reported as 90 - 92 % and 97 % relative humidity, respectively by the previously cited

authors). Achieving and maintaining a very high relative humidity (for example, 95% as used in 2.3.1 and 2.3.2) can be extremely difficult and as relative humidity appears to be especially important for infection to occur with *Alternaria* pathogens, a slight drop in the level of relative humidity may have some influence on the outcome of an experiment.

Temperature would also appear to be an important factor which affects the optimal level of growth and development of pathogens such as *Alternaria* spp. (Humpherson-Jones & Hocart, 1983). From this, it would be quite conceivable that the relative humidity level and temperature of the test plates within the controlled environment cabinet could fluctuate to a degree which would have caused a slight shift in the level of the mean DI scores. Fluctuations in environmental conditions would explain the observation that the changes in DI were observed for all test plate/cultivar combinations of a specific test and were systematic.

However, although some inherent variability was encountered, the *in vitro* nature of the test ensured a level of control of the environmental conditions and greatly increased the ease with which uniformity of infection was achieved in comparison with whole-plant inoculation methods. Uniformity was important since the aim of the study was to develop a reproducible screening method for *Linum* breeding material.

Variation within the pathogen isolates during 2.3.1

Another reasonable explanation for differences in the mean DI scores would be that the length of time isolates were stored under axenic culture conditions affected the aggressiveness of isolates. Many authors have reported a loss in pathogenicity of fungal isolates following extended periods of axenic culture, particularly for species such as the *Alternaria*, and *A. linicola* in particular (Mercer & Mukherjee, pers. comm., 1992, Vloutoglou, pers. comm., 1993). It should be noted that problems of this nature were not encountered to such a degree during the main bioassay (2.3.2) and the whole seedling assay (2.3.3) following the re-vitalisation of stock cultures of *A. linicola* A1 isolates on a sand : peat : earth mixture (Schneider, 1958).

The effect of genotypic differences between the cultivars used during 2.3.1

Although the four cultivars used in the preliminary study were bred by four separate companies in geographically diverse regions (Antares - Co-operative de Semences de Lin,

France; Barbara - Cereal Research Institute, Hungary; Linda - Northrup King Semences, France; McGregor - Morden Agricultural Research Station, Canada) one would expect the breeding base from which they were produced to be fairly narrow as is the case with many cultivated crops. Also, *A. linicola* is not considered to be a major disease of any of these warmer, drier continental countries. Thus, breeding for resistance against the disease would not have figured highly as a criterion for selection. However, no significant difference was observed between the response of the four cultivars during the preliminary study (2.3.1). Even though there was probably not specific selection of *A. linicola* resistance during the breeding of the cultivars, the moderate DI scores suggest a background level which was effective against the isolates used during the test.

No field data exist on the resistance of linseed cultivars, particularly under UK conditions. However, linseed has been grown for many years and major problems with diseases such as *A. linicola* have not been encountered, except in the wettest of years, and this provides further evidence of a good level of background resistance in currently grown cultivars. Differences in the levels of *A. linicola* infection on growing crops of different cultivars were observed in field trials in Northern Ireland. Correlations between the response of cultivars over different years were not strongly positive and the ranking of the levels of resistance was observed to change from year to year. It was thus not concluded whether observed differences were genotypic in nature or otherwise (Mercer & Ruddock, 1993).

The use of Principal Components Analysis to investigate the interaction

The biplot of the interaction between the 4 linseed cultivars and the isolates of *A. linicola* (Fig 2.2) indicated that the majority of the variance was accounted for by the first two PC axis and the remaining variance was accounted for by the third PC axis. As only four cultivars were tested, this was to be expected, as the mathematical distance between four points requires only three dimensions to be fully represented graphically. However, as the majority of the variance within the data was due to differences between the Al isolates, as identified during the ANOVA, the first axis provides a measure of the aggressiveness of the isolates. Weak, non-aggressive isolates typically have a positive score on the first PC axis (Al1, Al3, Al5 and Al7) and strong, aggressive isolates have a negative score on the first PC axis (Al2, Al4 and Al6). A similar pattern can also be observed on the second PC axis which could be considered to detail the response of the cultivars to the isolates, isolate vectors being associated with the cultivar upon which the highest mean DI score was achieved.

One of the important points which can be made from the data collected for the preliminary bioassay (2.3.1) is that, even when testing a limited amount of linseed material, there was a diverse response produced against the different isolates (as illustrated graphically in Fig. 2.1). The level of the response of each cultivar was not equal to each specific isolate, which suggests the interaction to be polygenically controlled and the resulting resistance to be horizontal in nature.

General observations on the response of the accessions tested during the main bioassay

During the main bioassay (2.3.2), there was a large significant effect of isolate within the interaction. The effect of plate within batch was negligible. However, an effect of batch was observed which suggested some variation between batches during the study. The majority of this would be accounted for by differences between the two isolates as was observed for the preliminary study, although the size of the main detached bioassay (2.3.2) would ensure that inherent variation was amplified further. As the accessions were selected for the batches at random, some of the differences between batches may have arisen by chance if several resistant, or conversely, several very susceptible, accessions had been tested together in one batch but not in others. However, the inclusion of the "control" cultivars Antares and Linda in every dish of all batches during the study allowed REML to take account of much of this variance and weight the estimated scores for the accessions accordingly. As there was a significant effect of accession during the main study (2.3.2), some of the variation between batches was due to differences between disease responses of the different accessions.

As observed during the preliminary bioassay (2.3.1), there was no interaction of accession with *A. linicola* isolate during the main bioassay (2.3.2). The summed estimated DI for each accession was always higher for the aggressive isolate A16 than for the non-aggressive isolate A11, and the relationship can be clearly observed from Fig. 2.3. Much of the material gave a moderate response, similar in mean score to that observed for the commonly grown varieties tested during the preliminary study (2.3.1). A continuous distribution of response was observed, from material which was highly susceptible (for example; *L.u.u. candidum*, Blauwester, Hindoukouch and JWS) to resistant material which achieved a low score, even against the aggressive isolate A16 (for example; *L.u.u. albocoeruleum*, Ceres, *L.u.u. elongatum elongatum* and *L. angustifolium*). Note that five of the data points representing the resistant material (Ceres, *L.u.u. elongatum elongatum*, *L. angustifolium*, *L.u.u. angustipetuleum* and Mikael)

shown on Fig. 2.3 were estimated as negative values by REML analysis which denoted an extremely low score response for these accessions against A11 in comparison with the other 97 accessions.

Analysis of the data presented in Table 2.2, the estimated summed DI scores for the accessions tested averaged over the three test occasions, illustrated the trend observed from Fig. 2.3. As well as the general pattern observed for the accessions as a whole, it was interesting to note that the different types of accession tested, for example the primitive forms of *L. usitatissimum*, the breeding lines from Hungary or Germany or the established cultivars achieved the full range of resistance response from resistant to susceptible.

The range of resistance responses to the two Alternaria linicola *isolates*

The scores of the two extremes of the table illustrate the possibilities for resistance breeding and the possible eventualities of growing cultivars which have little resistance. The susceptible material, such as Blauwe-ster and Hindokouch, achieved a mean DI score per cotyledon of > 2.0, and the primitive form *L.u.u. candidum* was observed to produce an extremely susceptible response of a mean of 3.164 which, for a scale of 0-4, shows the extent to which the accession was attacked by the pathogen. The first five accessions of the resistant material, all of which achieved a mean score per cotyledon of < 0.8, contained three *L. usitatissimum* sub-species or primitive forms, the near relative *L. angustifolium* and the cv. Ceres. If, as suggested by Durrant (1976), *L. angustifolium* was the nearest "wild relative" to the cultivated *L. usitatissimum*, the potential for breeding and response to selection would, theoretically, be higher than from more distant species of the genus (Simmonds, 1991).

Another point of interest from Table 2.2 was the position of the "control cultivars", Linda and Antares. During the preliminary bioassay (2.3.1) it was observed that the resistance response of Linda was superior to that of the other three cultivars tested, Barbara, McGregor and Antares. Table 2.1 indicated that Linda achieved an estimated mean DI of 8.71 and gave a response which was characteristic of the top 10 % of the accessions tested. Antares achieved an estimated mean DI of 11.51 which, although not being that much higher than the score of Linda, grouped Antares with many other accessions which achieve a moderate resistance response.

The response of the primitive form *L.u.u. candidum* was extremely susceptible in comparison to the other accessions and this observation was highlighted by the analysis of similarity which was graphically represented in Fig. 2.4. *L.u.u. candidum* was observed to form an outlying group which was separated on the dendrogram at the first available branching point. When grown in the field at Auchincruive during the season of 1992 (during the bulking up of seed stocks prior to the testing of material, unpublished data), *L.u.u. candidum* was observed to be phenotypically very stunted, highly branched and white flowered and completely different from all of the other accessions. Plots of the accession were severely diseased even though levels of *A. linicola* on other material was low. The nearest field of commercially grown linseed which could have acted as a possible inoculum source, was estimated to be more than 45 miles away, in Lothian. There was also the possibility of disease carry over from flax trash from a previous crop or from alternate hosts (i.e. *Veronica* spp., Vloutoglou *et al.*, 1995) however levels of infection by *A. linicola* on other material was low. Inoculum levels on the seed were no higher than on other seed lots of the primitive forms received from Prof. Friedt, University of Giessen, Germany. From this it was assumed that *L.u.u. candidum* would be the most susceptible accession of the linseed material available for testing and this was found to be the case.

The dendrogram which represented the moderate resistance response accessions (Fig. 2.4 a) contained the accessions of the middle two columns of Table 2.2 and clearly represents the close association of much of the material with a moderate resistance response. Many of the sub-groups within Fig. 2.4 a, contain three to five accessions which show similarity at the 95 % level and this illustrates the likeness of the response of much of the moderately resistant material. The group contained a mixture of accessions including many linseed and flax cultivars and a number of the primitive forms or sub-species of *L. usitatissimum*.

Comparison of in vitro *and* in vivo *bioassay techniques*

The whole seedling study (2.3.3) verified that the resistance responses observed during the preliminary (2.3.1) and main bioassays (2.3.2) occurred *in planta* and that inoculation of cotyledons *in* vitro resulted in a representative score which could be used during a breeding programme. The *in vitro* test typically gave a slightly higher DI score than the whole plant assay. Although inoculated whole plant cotyledons appear, from this study, to phenotypically respond with "less disease" the differences in levels of disease caused by each *A. linicola* isolate for each accession differed systematically between the whole plant and *in vitro* screens.

Vloutoglou (1994) suggested that the softer construction of the cotyledon leaf of linseed in comparison with the true leaves probably accounted for the ease with which *A. linicola* can be successfully transferred from the seed to seedling.

As only inoculation with the aggressive isolate Al6 resulted in a significantly positive correlation between the DI scores of experiments 2 and 3, it is suggested that differences in disease resistance between the accessions would only be measurable when the disease symptoms were observed to reach a specific level. Although the resistance response of the accessions can be observed to show the same ranking (Fig. 2.6) when inoculated with the non-aggressive isolate Al1, the differences between the accessions in either the *in vitro* assay or the whole plant assay are less obvious. From this observation, the use of an aggressive isolate would be recommended for future testing of material in order for the observed response to be truly representative of the resistance level of the accession.

The resistance mechanism and the use of the bioassay by breeders

From the three studies reported here it is concluded that resistance was governed by a quantitative mechanism and that due to the range of the response of the material, there are probably many genes involved in the interaction between linseed and *A. linicola*. This would agree with similar studies carried out with other *Alternaria* species (Prasanna, 1984) in which monogenic resistance factors are also thought to be absent. Klose *et* al. (1993) also found that resistance of 16 linseed cultivars to *A. linicola,* tested at a number of stages during the plant growth period, was under polygenic control and suggested that good response to selection for improved resistance should be achieved. In comparison with the results of the experiments described in this chapter, Klose *et al.* (1993) similarly observed that of the two *A. linicola* strains used during their study (ALT I and ALT II), there was a consistent difference in disease level between the two strains over all of the cultivars tested. From this it would appear that the resistance response was also isolate specific.

The work reported here confirms that an effective screening test for resistance of linseed and *Linum* material to the pathogen *A. linicola* has been developed. The test concentrates on the seedling stage of plant growth, which is the stage at which *A. linicola* has its greatest economic effect on the crop in the field (Mercer *et al.*, 1991a). An added benefit of the test in a breeding situation is that the cotyledons can be detached from juvenile *Linum* seedlings without causing detrimental damage to the plant and subsequent development can occur through to seed

production. This means that valuable breeding lines or rare primitive forms of *Linum*, can be screened even if large seed stocks do not exist.

The test described compares well with other reports from the literature of bioassays for disease resistance which utilise plant explants. For example Cohen (1993) reported that the use of a leaf disk assay for the detection of resistance in melon to the pathogen *Sphaerotheca fuliginea* race 1 allowed the preliminary selection for resistance at the cotyledonary stage and also that the results correlated well with results obtained in whole plant experiments. A similar leaf disk assay allowed the detection of resistance to early blight (*Alternaria solani*) in juvenile potato plants which allowed the manipulation of environmental factors which had previously prevented tissue characterisation in the field (Bussey & Stevenson, 1991). Both tests utilise an advantage that the *in vitro* assay possesses which cannot be easily controlled in the field. In the case of the *in vitro* bioassays reported above (2.3.1 and 2.3.2), the use of excised cotyledons utilises the largest available surface area of the linseed plant upon which the inoculum droplet can be placed without the requirement for sporulation by the pathogen and the environmental parameters can be carefully controlled.

Aspects of the resistance response of Linum/Alternaria linicola *through the growing season*

However, some authors have suggested that the resistance response at the seedling stage is different in genetical control and mechanism to that observed later in the season. Evidence from the linseed-*A. linicola* pathosystem would currently support this theory. Mercer & Ruddock (1993) found no correlation between the incidence of *A. linicola* on the seed capsules and seed and that on inoculated seedling grown in the glasshouse. Similarly, Klose *et al.* (1993) observed changes in the rankings of the resistance response of 16 cultivars when infected with two strains of the pathogen at three different growth stages. From this, they suggested that the incidence of the disease on seedlings was governed by genetically-determined phytoalexin-type responses, whereas incidence on capsules and seeds was dependant on a complex of physiological, genetic and environmental effects, however, the authors provided no scientific basis or evidence for their suggestions. The current study suggests that both mechanisms, if indeed there are two, would be multicomponent in nature, especially if the biology of *Alternaria* pathogens generally is considered (Tewari, 1991), in which case, it is likely that a number of resistance mechanisms would be involved in such an interaction.

Rotem (1994) states that "all *Alternaria* diseases are characterised by a short period of susceptibility in seedlings, a long period of resistance in young to intermediate-aged plants, and increasing susceptibility in ageing plants" although he noted a number of exceptions (including *Alternaria solani* on potato, the juvenile stage of which is resistant, with susceptibility increasing with age). The generalisations made by Rotem would appear to fit the observations of researchers involved in work on the linseed-*A. linicola* pathosystem in that linseed was often observed to be heavily infected with the pathogen at the seedling stage and the stages following the onset of flowering, but the crop remains relatively healthy during the majority of the growing season.

Little evidence exists of the changes which take place to explain the observations of Mercer & Ruddock (1993) and Klose *et al.* (1993) as described above. Rotem (1994) observed that with the exception of potato, very little information exists about the association between juvenile and adult plant resistance to *Alternaria* spp. However, as the seedling stage of the host growth cycle forms one of the important periods in which *A. linicola* can cause economical losses in the linseed crop, the possibilities of increasing levels of resistance to the pathogen even for a small section of the growth cycle would be beneficial. Adding further resistance which was effective during the early stages of the growth season would reduce levels of inoculum for later in the season and slow the possible development of a disease epidemic.

Breeding using horizontal resistance

Despite the common supposition that polygenic resistance is cumbersome to handle in traditional breeding programmes, theoretically there is no reason to expect that increasing the level of resistance against pathogens such as the *Alternaria* should be possible (Simmonds, 1991). As a consequence of the polygenic nature of the resistance interaction and the likelihood of the physiological and biochemical resistance mechanism being multi-component in nature, it is likely that resistance to *A. linicola* would be durable. The large number of accessions which achieved a moderate response to the test isolates confirms that there appears to be a good level of background resistance genes either already incorporated into cultivars or that the potential exists for the introduction of useful levels from near relatives.

Rotem (1994) suggests that wild species offer a major source of resistance in many *Alternaria*-host pathosystems, but continues to point out that in the majority of cases the wild origin of a given cultivar has been long forgotten and breeders use some of the resistant offspring of the

original wild relative to further breeding efforts. Many examples of resistance which has been successfully transferred from wild relatives to crop cultivars which previously were not resistant are given by Knott & Dvorak (1976). There are very few reports of resistance from wild relatives against diseases caused by the *Alternaria* which have been transferred into crop cultivars but the best documented case involves the *Alternaria solani*-tomato systems were stocks of germ plasm have been characterised and maintained by a network of institutes in north America (Rotem, 1994). Accessions such as *Lycopersicon pimpinellifolium*, *L. hirsutum* and *L. peruvianum* were crossed with *L. esculentum* and the complex of resistance genes form the basis of resistance to *A. solani* in commercial tomato cultivars.

Similar work which examined the exploitation of resistance from wild relatives and sub-species of crop cultivars of the brassicas was described by Tewari (1991). All commercial cultivars of rape were found to be susceptible to *A. brassicae*, but those of the *Brassica napus* subsp. *oleifera* were less susceptible than those of *B. campestris* subsp. *oleifera*. Weed species such as *Camelina sativa*, *Capsella bursa-pastoris* and *Eruca sativa* showed a high degree of resistance and an accession of *B. campestris* subsp. *rapifera* showed an intermediate level of resistance. Tewari concluded that the resistance mechanism was multicomponent in nature and could be transferred to commercial rape cultivars by conventional breeding techniques and the use of interspecific biotechnological techniques.

Improvement of resistance to Alternaria linicola *in linseed*

Given the current economics of linseed production in the UK, in which *A. linicola* does not appear to cause disease problems except in the wettest of years, and even one fungicidal spray treatment is barely justifiable, it could be argued that any improvement in durable disease resistance would be a worthwhile aim. This is particularly true if linseed is to hold the position that the crop currently holds as a low-input break crop within the British agricultural system.

Some degree of understanding of the mechanism of the multicomponent resistance response would be beneficial, particularly from a plant breeders point of view. A knowledge of a resistance component and the timing and importance of the action of that component would aid the selection of the resistant character in subsequent generations. There are candidate components which can be expected to play a role in the resistance response and the level of resistance observed for a specific accession probably results from a complex of numerous interactions. In an attempt to elucidate some of the components of the interaction between

linseed and *A. linicola* and to understand the specialisation of *A. linicola* to the linseed host, the following study concentrates on three areas of host-pathogen interactions. The three areas studied have commonly been found to be involved in *Alternaria* diseases in many commercially important crop species (Rotem, 1994).

The first study area was an examination of evidence of physiological differences in the resistance response at the microscopic level. During the study it was the intention of the author to examine the structure of the host cell response of resistant/susceptible accessions and to analyse the behaviour of aggressive and non-aggressive isolates of the pathogen which take place during the attempted ingress of the pathogen. The second area of study was to investigate the production of secondary metabolites by the pathogen. Many of the *Alternaria* produce phytotoxic compounds, and of these, host-specific toxins form the component which allows specificity and pathogenicity to a particular host or host cultivar (Kohmoto *et al.*, 1987). The third area of study was to investigate the production of fungitoxic secondary metabolites such as phytoalexin compounds. Compounds such as these have commonly been isolated from many host species following challenge with an *Alternaria* pathogen (Rotem, 1994).

Chapter 3

3.0 A microscopic examination of the infection of three *Linum* accessions by *Alternaria linicola*

Chapter 3

3.1 Introduction

Pathogen development and the penetration process

The pre-penetration, penetration and infection processes for many plant-pathogen interactions have been well documented. The environmental and physical factors which limit the germination of spores and the penetration of host tissue are well understood and have been described for many different plant-pathogenic species (Wood, 1967). Ellingboe (1972) reviewed the processes associated with infection by the powdery mildews describing the processes in great detail and defining specific morphological stages in pathogen development. Wynn (1976) described the early stages of penetration in response to surface contact stimuli for bean rust fungus. Similarly, Dickinson (1949), gave a detailed account of the tropic responses of rust hyphae on contact with different surfaces and membranes. Similar reviews by Wynn (1981) and Aist (1981) cover the entire infection process of many phytopathogenic species.

Reviews of the known behaviour of a number of pathogens on the phylloplane are given in a book edited by Blakeman (1981), one chapter of which specifically compares infection by *Alternaria* spp. and *Cladosporium* spp. (Dickinson, 1981). Dickinson (1981) suggests that *Alternaria* spp. show a significant potential for pathogenicity over a range of plant species tested in comparison with the *Cladosporium* spp.

Although the amount of study of the processes involved in *Alternaria* spp./plant interactions (and *A. linicola*/linseed interactions in particular) is minimal in comparison to many biotrophic phytopathogens, the extent of what is currently known is given in Table 3.1. From this the *Alternaria* appear to be generally more opportunistic in nature in comparison with the more specialised biotrophic pathogens. However, a degree of caution is necessary as specialisation by a number of *Alternaria* spp. has been reported, although many of the differences observed between reported penetration events from different species could be due to the relatively small amount of quantitative histological study which has been carried out on the infection of plant material by the *Alternaria*.

Species	Penetration method observed on host	Reference
A. alternata	*Brassica*: Direct with appressoria	McKenzie *et al* (1988)
	Triticum: Stomatal	Dickinson (1981)
	Phaseolus: Stomatal or direct with/without appressoria	Saad & Hagedorn (1969)
	Brassica: Direct or occasionally stomatal with or without appressoria	McRoberts & Lennard (1996)
A. brassicae	*Brassica*: Direct with appressoria	Changsri & Weber (1963)
	Brassica: Stomatal or direct with or without appressoria	Tsuneda & Skoropad (1978)
		Tewari (1986)
		Mckenzie *et al* (1988)
	Brassica: Stomatal or direct with or without appressoria	McRoberts & Lennard (1996)
A. brassicicola	*Brassica*: Stomatal or direct with appressoria	Changsri & Weber (1963)
	Brassica: Direct with or without appressoria	McKenzie *et al* (1988)
	Brassica: Direct or occasionally stomatal with or without appressoria	McRoberts & Lennard (1996)
A. cucumerina	*Cucumeris*: Direct with appressoria	Jackson (1959)
A. helianthi	*Helianthus*: Direct with appressoria or stomatal without appressoria	Allen *et al* (1983)
A. longipes	*Nicotiana*: Direct with or without appressoria	Von Ramm (1962)
A. linicola	*Linum*: Direct or stomatal (by chance) with or without appressoria	Vloutoglou (1994)
A. porri	*Allium*: Stomatal	Angell (1929)
	Allium: Stomatal or through wounds	Walker (1952)
	Allium: Direct or stomatal	Fahim & El-Shehedi (1966)
A. raphani	*Brassica*: Stomatal or direct with appressoria	Changsri & Weber (1963)
	Brassica: Direct or stomatal (rarely) with or without appressoria	McRoberts & Lennard (1996)
A. solani	*Solanum*: Stomatal or direct with or without appressoria	Harrison *et al* (1965)
		McRoberts & Lennard (1996)
A. tagetica	*Tagetes*: Direct	Cotty & Misaghi (1984)

(Adapted from McRoberts, 1992)

Table 3.1. Penetration phenomena observed in Alternaria *species*

Development of Alternaria *pathogens on the phylloplane*

The exact penetration process of *A. linicola* on the linseed host has received attention from only one study to date. The work of Vloutoglou (1994) on *A. linicola* suggested that infection by the pathogen showed many similarities with those observed for phytopathogenic *Alternaria* species as detailed in Table 3.1. The results of Vloutoglou (1994) compared directly to species which infect the *Brassicae* in particular, in that following conidial germination and hyphal growth, appressoria form above cell junctions. Following penetration, *A. linicola* was observed to show a pattern of intercellular growth as observed by McRoberts & Lennard (1996) for *A. brassicae* and *A. brassicicola*.

Variation in the development pattern of *Alternaria* of the same species during different tests has been described by some authors and indicates large levels of variability between isolates of specific species of the genus. For example, McKenzie *et al.* (1988) observed that conidia of *A. brassicae* and *A. brassicicola* germinated to produce one or more germ tubes on Brassica leaf disks, whilst Tsuneda & Skoropad (1978) reported that *A. brassicae* germinated as described by McKenzie *et al.* (1988) or occasionally was observed to produce secondary conidia. Curiously, McKenzie *et al.* (1988) observed the phenomenon of secondary conidium production by *A. brassicicola*, but not by *A. brassicae*.

The *Alternaria* do not appear to produce profuse hyphal growth on the surface of the leaf prior to attempting penetration although the lack of quantitative study of the infection process of the *Alternaria* and variability between isolates of particular species ensure that the phylloplane activity of many phytopathogenic *Alternaria* species remains unclear. Current evidence of tropism by germ tubes of *A. linicola* was not observed and encounters (and subsequent penetration attempts) at stomata were described as being chance events by Vloutoglou (1994).

Considerable variation in penetration site selection has been demonstrated for the *Alternaria* (Table 3.2) and intraspecific variance also prevents the formation of a generalised model of *Alternaria* development. As an example, the results of McKenzie *et al.* (1988) agreed with those previously published by Tewari (1986) who found that the Brassica pathogens *A. brassicae* and *A. brassicicola* were able to penetrate the leaf surface directly with or without prior appressorium formation. Additionally, Tsuneda & Skoropad (1978) concluded that penetration by *A. brassicae* could take place with or without prior appressorial formation either

directly through the leaf surface or indirectly through the stomata. Previously, Changsri & Weber (1963) had reported that both Brassica pathogens only attempted penetration following appressorial formation and that infection by *A. brassicae* only took place directly into the host cells, whilst *A. brassicicola* was able to penetrate directly or indirectly *via* the stomata. Such discrepancies in descriptions of the penetration method of the *Alternaria* abound within the literature. During studies on the development of onion blight in Egypt, *A. porri* was observed to penetrate directly into the epidermis or indirectly into the stomata (Fahim & El-Shehedi, 1966). Studies on the infection of the same host by *A. porri* in the United States found that penetration occurred through the stomata (Angell, 1929) or through the stomata or wounds (Walker, 1952).

Penetration site selection

Studies of the stimuli for penetration site selection for many biotrophic phytopathogens have concentrated on the physical topography of the leaf surface. For example, appressorial formation by *Erysiphe graminis* f.sp. *hordei* was reportedly stimulated by the physical structure of the epicuticular wax of the barley host (Ellingboe, 1972) although normal appressorial formation was observed on barley leaves which had been treated to remove the epicuticular wax (Carver & Thomas, 1990). Similarly, the biotrophic rust pathogen *Uromyces phaseoli* showed a positive tropic response to the shape of the guard cells of the bean host before attempting indirect penetration through the adjoining stomatal pore (Wynn, 1976).

Studies of the effect of epicuticular wax on the site of attempted penetration of a number of *Alternaria* spp. have been reported in the literature although the stimuli for penetration site selection do not appear to be specific in contrast with many biotrophic pathogens (Rotem, 1994). Akai *et al.* (1969) reported that appressorium formation by *A. porri* was stimulated by epicuticular wax although the authors noted that the effect was probably rare amongst the *Alternaria*. Tewari & Skoropad (1976) and Skoropad & Tewari (1977) reported the higher level of resistance of a cultivar of *B. napus* to *A. brassicae* in comparison with a cultivar of *B. campestris* was due to a difference in wax thickness. The higher level of resistance was reportedly not due to the physical barrier presented by the thicker layer but was a result of the increased hydrophobicity of the leaf which in turn directly reduced the retention of water-borne inoculum and affected the development potential of the pathogen. McRoberts (1992) assessed the effect of wax thickness on the development of a number of *Alternaria* pathogens using three breeding lines of *B. oleracea* var. *gemmifera* which differed in wax thickness. No

effect was observed on conidial germination although germ tube production by *A. brassicae* was reduced on the leaf type with the thickest wax layer. As previously suggested by the similar work of Conn & Tewari (1989) and Berry (1992), McRoberts (1992) concluded that the thicker wax layer probably impeded the flow of exudates from the epidermal cells which formed the primary stimulus for conidial germination and pathogen development. To date, the importance of the role of biotic compounds or salt ions exudates from the underlying epidermal cells as a stimulus for penetration site selection has not been fully explained for phytopathogenic *Alternaria* spp.

In comparison, Vloutoglou (1994) observed that the pattern of growth by *A. linicola* was typical of many of the *Alternaria* as described by Rotem (1994). Penetration was most often direct through the epidermal cells and indirect penetration *via* stomata was infrequently observed. No tropism towards particular physiological features of the leaf was reported and the study did not investigate the importance of the phylloplane topography.

Host responses to penetration

Following successful penetration, the speed and effectiveness of the host plant defence mechanisms appear to be paramount to the resistance response of many biotrophic and facultative plant pathogens. Aist (1976) gave examples of numerous biotrophic pathogen/cereal crop interactions and highlighted the importance of the role of papillae in the prevention of successful cellular penetration and further pathogen development which accounted for differences in cultivar resistance. Aist (1981) had further suggested that the prevention of further pathogen development from a sub-cuticular phase was more important in a larger number of pathosystems than was previously recognised. Following further work, Aist & Gold (1987) suggested that papillae development was responsible for cultivar resistance derived from the *ml-o* resistance gene of barley and that the resistance reaction and papillae development was regulated by calcium ions.

A central role for papillae formation by the host as a resistance response to fungal attack has now been demonstrated for numerous non-biotrophic pathogens, for example *Plasmodiophora brassicae*/cabbage (Aist & Williams, 1971), *Phytophthora infestans*/potato (Ehrlich & Ehrlich, 1966) and *Colletotrichum graminicola*/oat (Politis, 1976). The components of the papillae which form in resistance to both biotrophs and facultative phytopathogens have been shown to include numerous defence-related secondary metabolites (e.g. polyphenols, peroxidase, pectin,

suberin, and glycoproteins such as β1, 3-glucans) many of which have, or share, biosynthetic pathways with related defence compounds such as the phytoalexins which are found in the cytoplasm.

Considering host responses to *Alternaria* pathogens, McRoberts & Lennard (1996) reported that papillae formation, through the localised deposition of callose (a β1, 3-glucan), played an important role in the response of a number of Brassicas to infection with three *Alternaria* species. Following penetration to a sub-cuticular position, McRoberts (1992) observed that papilla deposition at the site of attempted cell penetration prevented further development of *A. brassicae* and *A. brassicicola* and suggested that the speed of deposition was important in the resistance response of the different cultivars under field conditions. Considering *A. linicola*, the area of the interaction following penetration has not been fully investigated and development of the pathogen from a intercellular position and the response of the host was not described further by Vloutoglou (1994).

The phenomenon of the hypersensitive response has been described as the major component of the resistance reaction of many biotrophic and facultative pathogens (Bailey & O' Connell, 1989, Heath, 1981). Evidence of an important role for the hypersensitive response in the facultative pathogens appears to be less well defined in comparison to those involving biotrophs. Presently, hypersensitivity has not been described in response to attack by the *Alternaria* generally or *A. linicola* in particular. It should be noted that although hypersensitivity does not appear to occur with pathosystems involving the *Alternaria*, many components of the interaction between host and pathogen associated with the hypersensitive response in non-*Alternaria* pathosystems have been observed to be important in the interaction between hosts and *Alternaria* pathogens. For example, phytotoxin production (see Chapter 4) by the pathogen which has been suggested to be the trigger for many hypersensitive response reactions, and also phytoalexin elicitation (see Chapter 5), have been reported for some *Alternaria*/host interactions (Rotem, 1994).

Evidence from the literature suggests that the chemical and physical properties of the host plant phylloplane of many *Alternaria* spp. appear to play little part in the resistance response and the primary level of defence against pathogens of the genus results from the reaction of the host cells following cuticular penetration. In response, host plants of the *Alternaria* spp. have been shown to produce a number of physical and biochemical defence mechanisms which prevent or slow the rate of pathogen ingress (Rotem, 1994). From this, it seems likely that

possible differences in the speed and effectiveness of the host cell reaction would account for differences in the resistance response of different cultivars of hosts to respective *Alternaria* pathogens.

The following study had two principal aims: firstly, to investigate whether the differences in resistance response of *Linum* accessions previously observed during the *in vitro* bioassay (Chapter 2) could be discerned by a study at the microscopic level, and, secondly to investigate which pathogen-mediated, or host-mediated, components of the infection process were responsible for the differences in the resistance response. Three accessions of *Linum* which were representative of the range of resistance responses reported in 2.3.2 (i.e. resistant, moderately resistant and susceptible) were inoculated with conidia of *A. lini*cola isolate A16 and assessed microscopically for pathogen development and host response over a time course. Data was collected for 15 quantitative components of the infection interaction and were analysed using a combined exploratory and inferential approach as described by Krzanowski (1990) using a number of multivariate techniques.

3.2 Materials and methods

3.2.1 A microscopic investigation of the infection of three *Linum* accessions with *Alternaria linicola*

Production of conidia in vitro

Conidia of *A. linicola* isolate Al6 was produced using a modified version of the method described by Shahin and Shepard (1979). The isolate was stored at 5° C on a sterile sand : loam : peat mixture (1 : 1 : 2) as described by Schneider (1958) and was subcultured onto V8 agar containing 3 g l^{-1} $CaCO_3$ six days prior to the inducement of sporulation. Aerial mycelium was scraped from the plates and the agar was divided into ~5 mm^3 cubes with a sterile scalpel. Agar cubes were placed on Petri dishes containing sporulation (S) medium (Shahin & Shepard, 1979) and were saturated with 3 ml SDW.

Un-sealed S medium plates were incubated in darkness at 20° C and exposed to near UV radiation (12 h photoperiod) for 1 - 2 days after which time conidia had formed on the surface of the V8 agar blocks. Conidia were harvested by gentle rubbing of the V8 agar blocks with a sterile glass rod and were washed off with a minimal volume of SDW (containing a droplet of Tween 80). The solution was filtered through a single layer of muslin to remove extraneous fungal hyphae and adjusted to a concentration of 9000 conidia ml^{-1} using a haemocytometer.

Inoculation of plant material

Plant material (cvs Antares, Blauwe-ster and the closely related sub-species *L.u.u. albocoeruleum*) was grown as described previously (2.2.1). Material was removed from the glasshouse at GS 09 - 10 and each cotyledon was inoculated with a 25µl droplet of conidial suspension of Al6 (or control solution, SDW). True leaf material was not used during the experiment for two reasons; firstly, the narrow leaves were found to be difficult to inoculate with conidial suspension, and secondly, the material tested was chosen on the resistance response scores which were derived from the cotyledon bioassay described during the previous section (2.2.2). Following inoculation, plants were placed in a controlled environment cabinet at 18° C. 95% relative humidity, with a 16 hr photoperiod. Fifteen cotyledons of each accession/treatment combination were sampled at random 18, 24 and 40 h post-inoculation and were immediately fixed and cleared in ethanol for at least six hours prior to staining.

Staining, visualisation and scoring of inoculated cotyledons

Cleared inoculated cotyledons were stained for 24 h with 0.05 % (w/v) water soluble aniline blue (Gurr) in a pH 11 phosphate buffer. Following two rinses in SDW, cotyledons were stained with 0.005% (w/v) aqueous calcoflour white (Sigma) for 30 s, rinsed twice in SDW and mounted in aniline blue solution. Inoculated cotyledons were viewed under visible bright field illumination and uv fluorescence (50 W mercury vapour light source, 340 - 380 nm uv excitation filter, 430, 460 and 490 nm uv suppression filters) using a Leica Leitz DMRB microscope. An additional 5 cotyledons of each accession/treatment were harvested at each time period. These were cleared separately and observed unstained for the presence of lignin which was known to auto-fluoresce under uv radiation. No auto-fluorescent material was observed during the experiment. Stained conidia were observed for the occurrence of germination and measurements of fungal development and plant physiological response of were taken as detailed in Table 3.2. Germ-tubes were regarded as being fluorescing hyphal structures with a length of greater than 2 µm originating from non-fluorescing conidial cells. Photomicrographs were taken on either Kodakolor Gold II 35 mm print film (100 ASA) or Kodak Ektachrome 35 mm slide film (100 ASA) using a Leica Wild MPS52 camera unit controlled by a Leica Wild MPS48 exposure control system.

Data variate[1]	Abbreviation
Number of germ tubes	N.gt.
Germ-tube length	Gtl.
Number of branched germ-tubes	N.brgt.
Number of terminal appressoria	Ter.App.
Number of intercalary appressoria	Int.App.
Number of penetrations *via* cell wall junction	Cwj.pen.
Number of penetrations *via* cell periclinal wall surfaces	Cpw.pen.
Number of penetrations *via* stomata	Sto.pen.
Number of successful penetrations	Succ.pen.
Number of penetrations resulting in sub-cuticular growth	Sub.gth.
Number of penetrations resulting in intercellular growth	Inter.gth.
Number of penetrations resulting in intracellular growth	Intra.gth.
Number of penetrations with only localised host cell reaction	Loc.
Number of penetrations with non-localised host cell reaction	Non.Loc
Number of penetrations with no apparent host cell response	No.Res.

[1] All variates except Gtl. refer to counts per conidium and were assessed on germinated conidia only. Data for Gtl. are the mean values of the longest germ tubes of up to 30 germinated conidia on each replicate cotyledon.

Table 3.2 Physiological responses quantified by u.v. microscopy following the inoculation of three accessions of Linum *accessions with conidia of* A. linicola *isolate Al6.*

Statistical analysis of the components of the interaction.

The data were analysed using a combined exploratory and inferential approach as suggested by Krzanowski (1990). This approach was adopted for two main reasons. Firstly, owing to practical difficulties in obtaining consistent inoculation due to a problem with the controlled environment cabinet, the original experimental design became unbalanced. As a result, the data set contained a large number of missing values which prevented the use of standard ANOVA as a statistical testing method. Missing and present data was tested using a χ^2 test. The null hypothesis being tested in each case was that there was no difference among accessions with respect to the proportion of missing data. Secondly, it was of interest not only to test for differences between the response of the three accessions, but to identify those variables of the interaction which were responsible for differences between accessions.

The null hypothesis (H_o) being examined was that there was no difference between accessions with respect to fungal development or host plant response to attempted infection. In order to test for this, the data collected each sampling time were analysed using multivariate analysis of variance (MANOVA), a multivariate extension of univariate ANOVA (Krzanowski, 1990). Univariate ANOVA can be thought of as a method for assessing differences in variance between sample means testing for coincidence of variate points along a single line (e.g. in a single dimension). MANOVA extends the basic principles of the test but tests the hypothesis in several dimensions. Thus, if p variates are measured, rather than being located in a single dimension (e.g. along a single line), treatment means lie in a p-dimensional space and acceptance of H_o would require the points to occupy a coincident point within that space.

Both ANOVA and MANOVA make the assumption that the data which form the treatment means are normally distributed with a common variance σ^2, or in the multivariate case, the treatment means follow a multivariate normal distribution within the common dispersion matrix Σ. This in turn allows the construction of tests of the H_o which would imply that all treatment means are coincidental on the single line (univariate ANOVA) or are coincidental within the multivariate space (MANOVA). In both cases, rejection of the H_o occurs when at least one treatment mean is found to be too distant from the coincidental point for the H_o to be considered to be true at a given probability level. However, in the case of multivariate analysis, there may be a number of possible reasons for the rejection of H_o depending on the nature of the data and the way that the data is processed. For example, treatment means may differ greatly with respect to one or more variates or there may be more subtle differences amongst a larger number of the variates. Consequently, the different statistical tests which can be used during MANOVA are designed to give greater

weighting to some forms of variance in comparison with others depending on the nature of the differences within the data. For this reason it is usual to consider a number of test statistics when making inferences about treatments using MANOVA (Krzanowski, 1990)

Any differences between treatment means identified with respect to the H_0 formulated *a priori* were investigated further by investigating the sources of variation between the treatments. Identification of the dominant variates in the between-accession variance was then obtained using canonical variates analysis (CVA) on the data from each sampling time. CVA is a statistical technique which is related to PCP as used previously in section 2.3.4. The technique leads to the construction of a new set of variables (the canonical variates) from an initial multivariate set of data. In common with PCP, the canonical variates use linear combinations of the original data but during CVA the linear combinations are constrained so that they maximise the between-group (accessions in this context) to within-group variance. CVA thus identifies those variates which are responsible for differences between the groups.

Biplots of the points representing the accession groups and the group means can be plotted along with the latent vectors representing the data variates in a manner analogous to that described for PCP previously (Gabriel, 1981; Krzanowski, 1990). With g treatments there are, at most, g-1 canonical variates. Thus during the current study involving the testing of three accessions, CVA would be expected to represent all of the variance in the 3 x 15 data matrix for each sampling time in the two dimensions of the biplot. In addition, under the assumption that the original data conforms to approximate multivariate normality, confidence regions for the groups and group means can be drawn on the biplot. It can be shown that the confidence limits follow a χ^2 distribution such that, for a given confidence level (100-α) the confidence limits for a 2 dimensional biplot for group means and groups are respectively $\left(\chi^2_{\alpha,2} / n\right)^{1/2}$ and $\left(\chi^2_{\alpha,2}\right)^{1/2}$ where n is the number of individuals in a group. Differences between groups are indicated by non-overlapping confidence regions. An approximate χ^2 test was used to determine whether the CVA axes represent real effects within the interaction or whether they may have resulted from random effects.

The geometric analysis provided by CVA should agree with the test statistics generated in the MANOVA. However, as Krzanowski (1990) points out, the MANOVA hypothesis tests must be conducted on an *a priori* hypothesis, not on any suggestions which may arise from the interpretation of CVA biplots. A full account of the methods used are given in Krzanowski (1990), while CVA was also discussed by Digby *et al.*, (1987).

Analyses were conducted in Genstat 5.3 running under Open VMS on a DEC Alpha minicomputer. Probability values for the MANOVA test statistics were obtained by repeating the MANOVAs using Minitab (version 9.1) running under Windows (TM) (version 3.1) on a PC. The MANOVA results were identical between statistical packages but while the CVA information is available from Genstat, the package does not output the probabilities for the MANOVA test statistics, which are printed automatically by Minitab.

3.2.2 Analysis of the interaction of *A. linicola* isolate Al6 with three linseed accessions using Low Temperature Scanning Electron Microscopy (LTSEM).

Growth and inoculation of plant material

Conidia of isolate Al6 were prepared as previously described (3.2.1). Cotyledon material of cvs Antares, Blauwe-ster and *L.u.u. albocoeruleum* were prepared for *in vitro* culture and inoculated with conidial suspension using the method described previously (3.2.1). Plates containing inoculated cotyledons were placed in a controlled environment cabinet (18° C, 95% relative humidity., 16 hr photoperiod) for 24 and 40 h before being removed for analysis. Cotyledons were observed under a binocular microscope immediately prior to preparation in order to assess infection level and highlight areas which would be conducive to freeze-fracture.

Preparation of material for LTSEM

Following rough trimming with a sharp blade, 2 - 3 cotyledons were mounted vertically onto copper stubs using Tissue-Tek® carboxymethylcellulose adhesive. Material was frozen rapidly in nitrogen slush (-140° C) under argon using a cryo-preparation unit (Cambridge Instruments). Samples were transferred to the cryo-stage of the scanning electron microscope (Cambridge Instruments S250) for preliminary observation. If required, samples were etched at -70° C in order to remove ice crystals, following which, samples were removed from the cryo-stage and returned to the cryo-preparation unit. A number of samples were freeze-fractured using a cooled scalpel blade and fractured and unfractured samples were sputter-coated with gold at -140° C. Coated samples were returned to the cryo-stage of the microscope and viewed for signs of pathogen activity/host response. Photomicrographs were taken on 120 mm Kodak Print film.

3.3 Results

3.3.1 A microscopic investigation of the infection of three linseed cultivars with *Alternaria linicola*

MANOVA and analysis of the components in the interaction by CVA

Upon examining inoculated material, many of the inoculation droplets were observed to have dried out due to excessive air movement of the controlled environment cabinet. Following clearing and staining, it was observed that the number of conidia on much of the material was very low. It was also observed that all of the conidia which remained on the cotyledon surfaces had germinated. From this, it was concluded that many ungerminated conidia, or those which had not adhered sufficiently to the leaf surface, had been washed away during the clearing and staining procedure. In cases where low numbers of conidia were found on cotyledons, percentage data were calculated from all of the conidia present. A χ^2 test indicated that there were significant differences between the accessions with respect to the proportion of missing data at 18 and 24 h.a.i.. However, there was no consistency in the pattern of missing data between sampling times which indicated that although one or more of the accessions was particularly prone to missing data at any one sampling time, differences were due to a random effect. The χ^2 test results for 40 hours indicate that there were no significant differences among accessions.

Analysis of the data by MANOVA indicated that there were significant differences between the three linseed accessions at 18, 24 and 40 h (Table 3.3). However, canonical variates analysis indicated that three data variates describing the pre-penetration development of *A. linicola*, number of germ-tubes per conidium, number of branched germ-tubes per conidium and, germ-tube length, showed little variation among accessions at any of the sampling times and did not contribute significantly to overall differences among the accessions (Table 3.4).

At 18 h.a.i. none of the accessions were associated with successful infection although rare sub-cuticular growth was observed on the most susceptible accession (Blauwe-ster), and penetration events associated with localised responses or no apparent response were observed for the most resistant accession (*L.u.u. albocoeruleum*) (Table 3.4, Fig. 3.1, Fig. 3.6). Antares was found to differ from the other accessions in that there was no evidence of attempted infection or host plant response at 18 h.a.i. (Fig. 3.1a).

	18 hours		24 hours		40 hours	
Statistical Test	Statistic	*P* value	Statistic	*P* value	Statistic	*P* value
Wilk's lambda	0.010	0.001	0.0007	0.001	0.0596	0.001
Lawley-Hotelling trace	45.0	< 0.001	344.44	0.001	6.8283	0.001
Pillai's trace	1.51	0.081	1.7412	0.013	1.4737	0.001
approximate F	4.72[a]	0.001	14.24[b]	< 0.001	3.10[c]	0.001

[a] (df = 16), [b] (df = 12), [c] (df = 30)

Table 3.3 Results of MANOVAs of differences between three linseed cultivars at 18, 24 and 40 hours after inoculation with A. linicola *isolate Al6, with respect to 15 data variates.*

At 24 h.a.i. the susceptible accession Blauwe-ster was distinguished from the other two accessions by the occurrence of successful penetration events and intracellular growth (Fig. 3.1b, Fig. 3.5). There was little variation between Antares and *L.u.u. albocoeruleum* with respect to fungal development or plant response at 24 h.a.i. (Fig. 3.1b).

An approximate χ^2 test of the CVA data from 40 hours after inoculation indicated that the first canonical variate (D-squared(0)) was significant ($P < 0.0005$) and therefore represented a true treatment effect. The second canonical variate (D-squared(1)), however was not significant ($P = 0.07$). Thus any separation produced by the second canonical variate may have been due to random effects.

At 40 h.a.i. the variance in the interaction was more evenly distributed among the three accessions (Fig. 3.2) and a larger number of variates were observed to account for differences among the accessions (Table 3.4, Fig. 3.2) than at 18 and 24 h.a.i.. Starting in the top left quadrant of Fig. 3.2, the spread of the variate vectors clockwise around the origin approximately follows the sequence of events during successful infection by the pathogen. The most resistant accession (*L.u.u. albocoeruleum*) was found to be more closely associated with variates describing early events in the infection process than the less resistant accessions. For example, the incidence of appressoria was higher on *L.u.u. albocoeruleum* than on Antares or Blauwe-ster (Table 3.5), particularly appressoria located over cell wall junctions which occurred significantly more often on *L.u.u. albocoeruleum* (Fig. 3.3). Antares was associated with variates of pathogen development following penetration such as successful penetration, sub-cuticular growth (Fig. 3.4) and intra-cellular colonisation (Fig. 3.5). The susceptible

accession Blauwe-ster was associated with a high incidence of inter-cellular ramification by the pathogen (Fig. 3.5). Features of the interaction are illustrated in the photomicrographs in Plates 3.1 - 3.5.

	18 hours		24 hours		40 hours	
	axis 1	axis 2	axis 1	axis 2	axis 1	axis 2
% l.r.*	97	3	99	1	74	26
N.gt.	2.531	1.762	4.622	-4.846	-4.715	0.638
Gtl.	-1.659	-5.713	5.787	-4.503	0.364	2.770
N.brgt.	6.743	3.226	2.197	-1.136	-5.269	0.550
Ter.App.	-5.817	**39.743**	-2.567	**12.457**	4.287	2.970
Int.App.	-1.392	16.164	1.728	6.301	1.136	2.743
Cwj.pen.	1.812	**-33.646**	-2.630	**-17.660**	**-13.860**	**13.917**
Cpw.pen.	0.499	**-22.123**	-0.174	-3.375	-6.351	5.280
Sto.pen	3.376	-7.510	-3.322	-1.789	-1.429	4.428
Succ.pen.	**-21.197**	0.687	**-39.777**	-5.619	-0.951	**7.947**
Sub.gth	**14.096**	-5.252	**6.007**	6.751	5.370	4.975
Inter.gth	5.737	-6.095	0.129	-2.081	-6.260	**-14.008**
Intra.grth	1.638	-3.222	**-37.977**	-4.922	1.577	-1.656
Loc	**11.070**	3.620	1.243	**7.562**	**20.613**	**-22.162**
Non.Loc	-6.028	7.235	-0.558	0.554	3.942	-6.938
No.Res.	9.509	2.260	1.905	3.849	**9.253**	-9.649

* Per cent latent root

Table 3.4 Adjusted latent roots and latent vectors for canonical variates analyses of the interactions between Alternaria linicola *and three* Linum *accessions at 18, 24 and 40 hours after inoculation. The absolute values for the latent vectors indicate the relative importance of the original data variate to the ratio of within-accession:between-accession variance explained in the corresponding canonical variate axis; the three most important variates for each axis at each time are indicated by bold type. The abbreviations for the data variates are given in Table 3.2.*

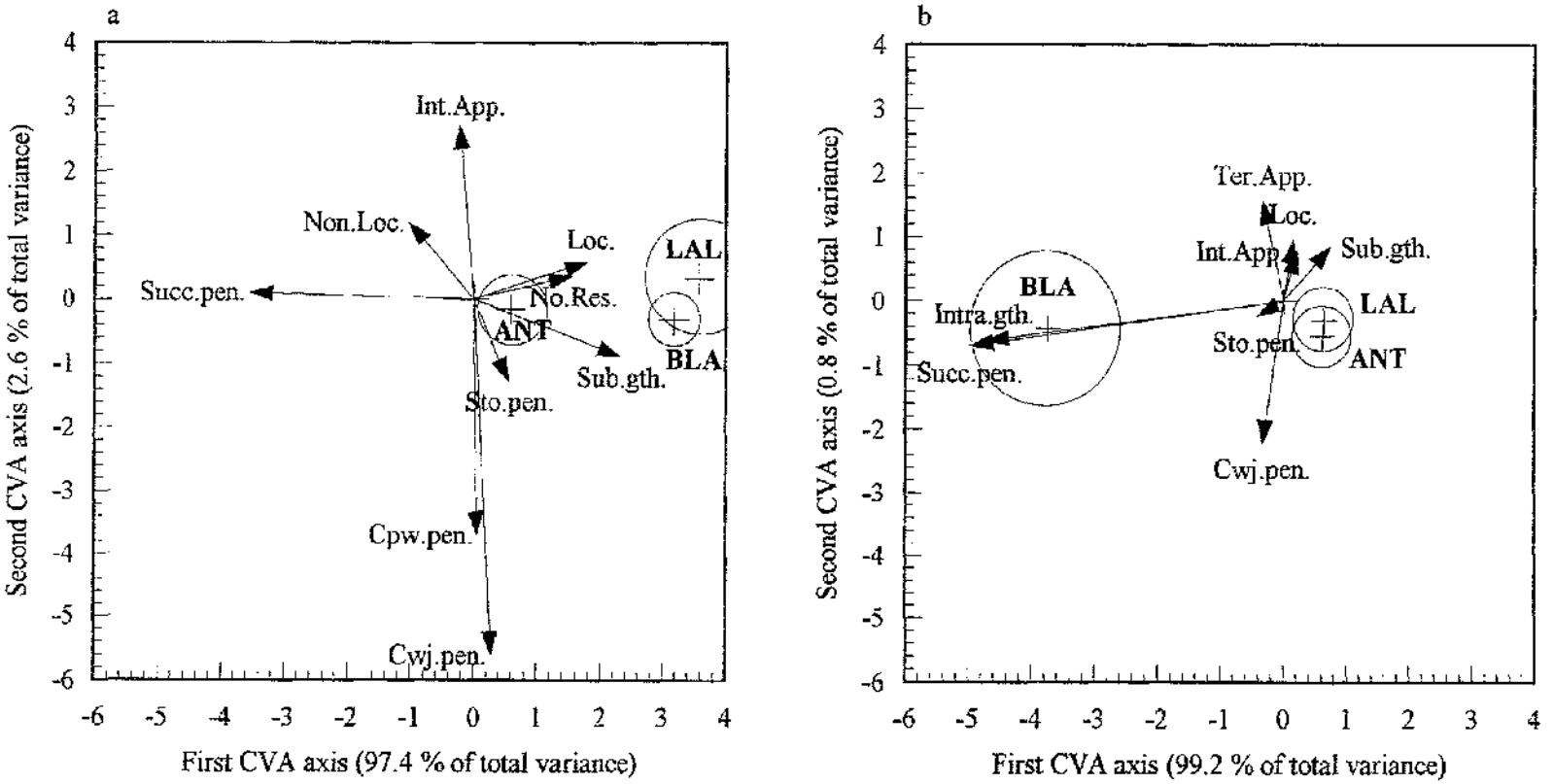


Fig. 3.1 Biplots of the association among three Linum *accessions inoculated with* A. linicola *(a) 18 and (b) 24 hours after inoculation following canonical variates analysis of 15 fungal development and host response variates. Confidence regions for accession means are shown by the circles, means are indicated by crosses. Vectors data points were scaled by a factor of 0.2 (a) and 0.125 (b).*

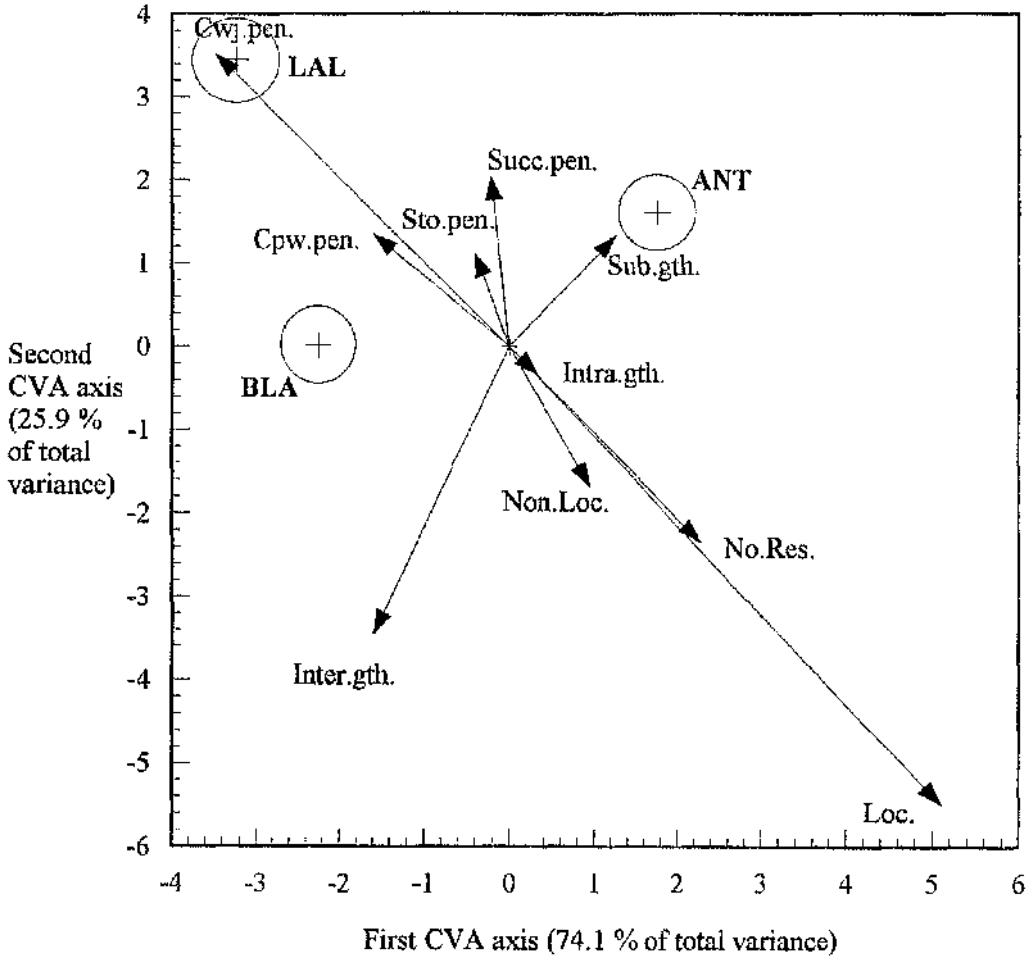


Fig. 3.2 Biplot of the association among three Linum *accessions inoculated with* A. linicola *40 hours after inoculation following canonical variates analysis of 15 fungal development and host response variates. Confidence regions for accession means are shown by the circles, means are indicated by crosses. Vector data points were scaled by a factor of 0.25.*

Accession	Hours post-inoculation	Terminal appressoria	Significant differences between accessions	Intercalary appressoria	Significant differences between accessions	Total number of appressoria	Significant differences between accessions
Antares		0.763		0.154		0.918	
Blauwe-ster	18	0.735	n.s.d.	0.208	n.s.d.	0.944	n.s.d.
L.u.u. albocoeruleum		0.622		0.2		0.822	
Antares		1.849		0.599		2.449	
Blauwe-ster	24	1.527	n.s.d.	0.5	n.s.d.	2.027	n.s.d.
L.u.u. albocoeruleum		1.905		0.725		2.631	
Antares		2.058	a-l[*]	0.783	a-l[‡]	2.841	a-l[#]
Blauwe-ster	40	2.515	b-l[†]	1.138	n.s.d.	3.653	b-l[§]
L.u.u. albocoeruleum		3.277	l-a[*], l-b[†]	1.641	l-a[‡]	4.919	l-a[#], l-b[§]

n.s.d. = no significant difference
[*] Antares-*L.u.u. albocoeruleum* 18 h, df = 19, $P < 0.001$.
[†] Blauwe-ster-*L.u.u. albocoeruleum* 18 h, df = 20, $P < 0.041$.
[‡] Antares-*L.u.u. albocoeruleum* 24 h, df = 17, $P < 0.001$.
[#] Antares-*L.u.u. albocoeruleum* 40 h, df = 18, $P < 0.001$.
[§] Blauwe-ster-*L.u.u. albocoeruleum* 40 h, df = 20, $P < 0.024$.

Table 3.5 Mean number of terminal-, intercalary- and the total mean number of appressoria formed by germ tubes of A. linicola, *isolate Al6, on three accessions of* Linum.

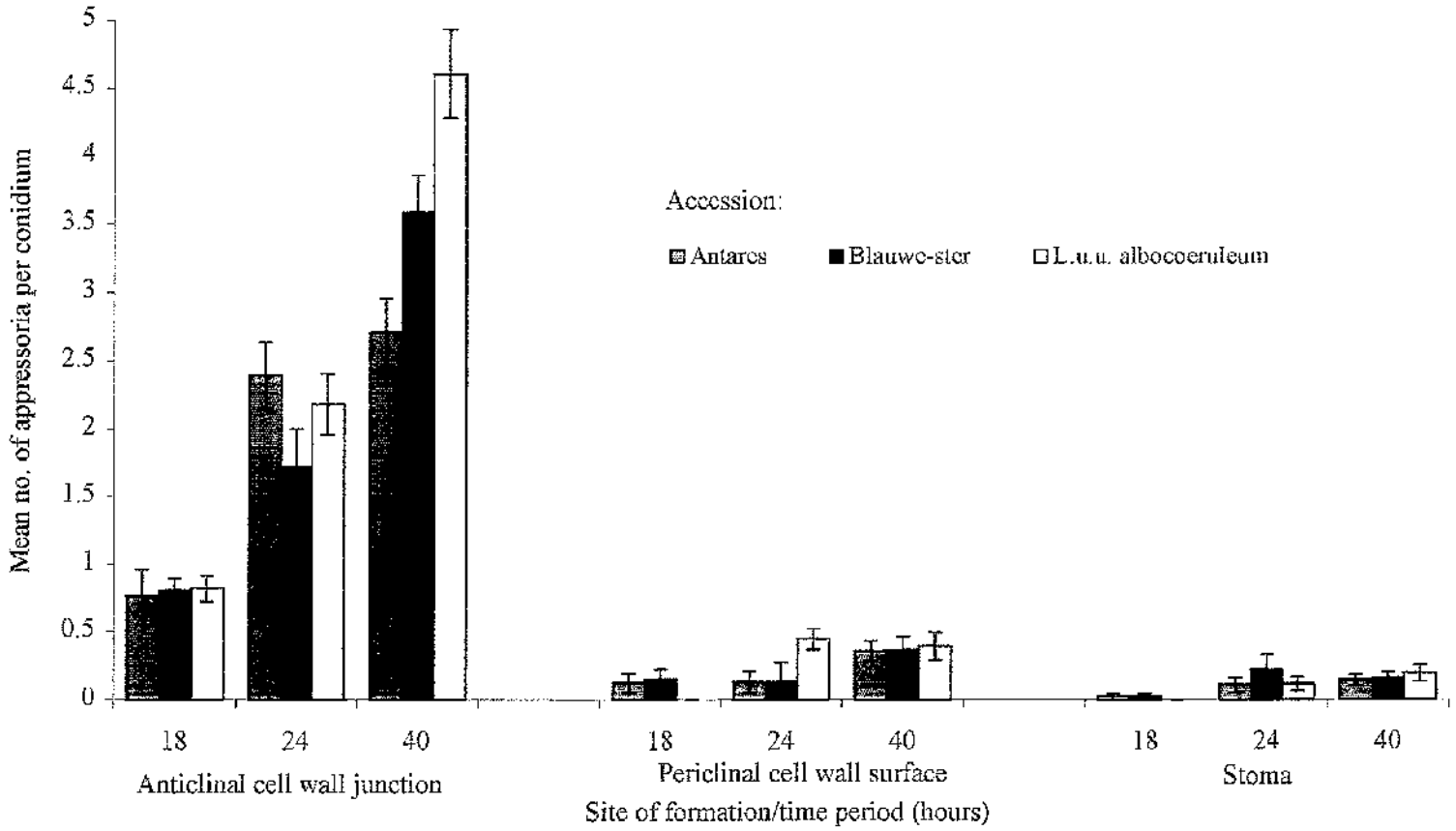


Fig. 3.3 Preference of sites of appressoria formation for A. linicola *isolate on three accessions of linseed. Error bars = SEMs*

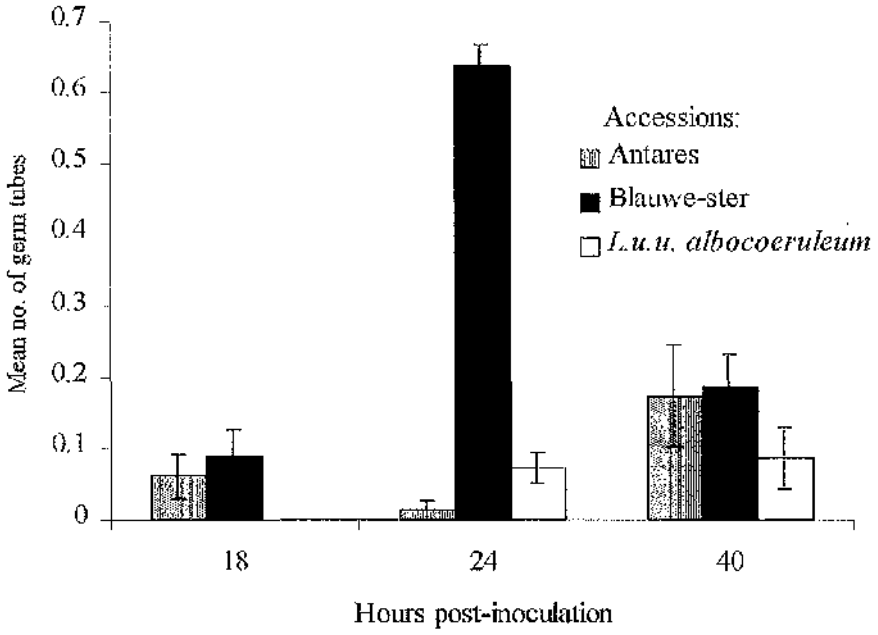


Fig. 3.4 Mean number of germ tubes per conidium observed to grow sub-cuticularly at three time points on three accessions of linseed. Error bars = SEM.

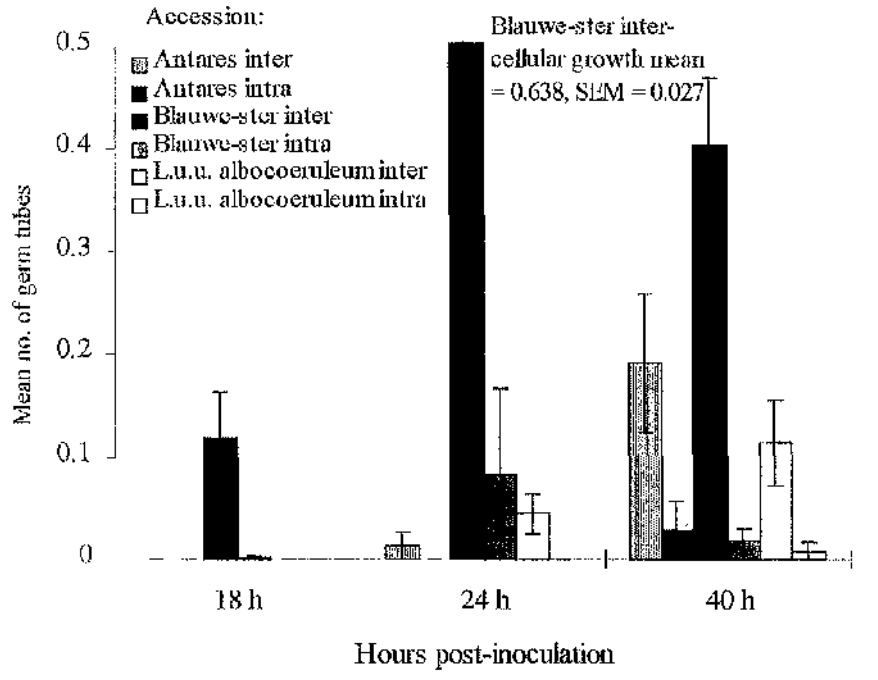


Fig. 3.5 Mean number of germ tubes per conidium observed to grow inter- or intra-cellularly at three time points after the inoculation of three accessions of linseed with A. linicola *isolate Al6. Error bars = SEMs.*

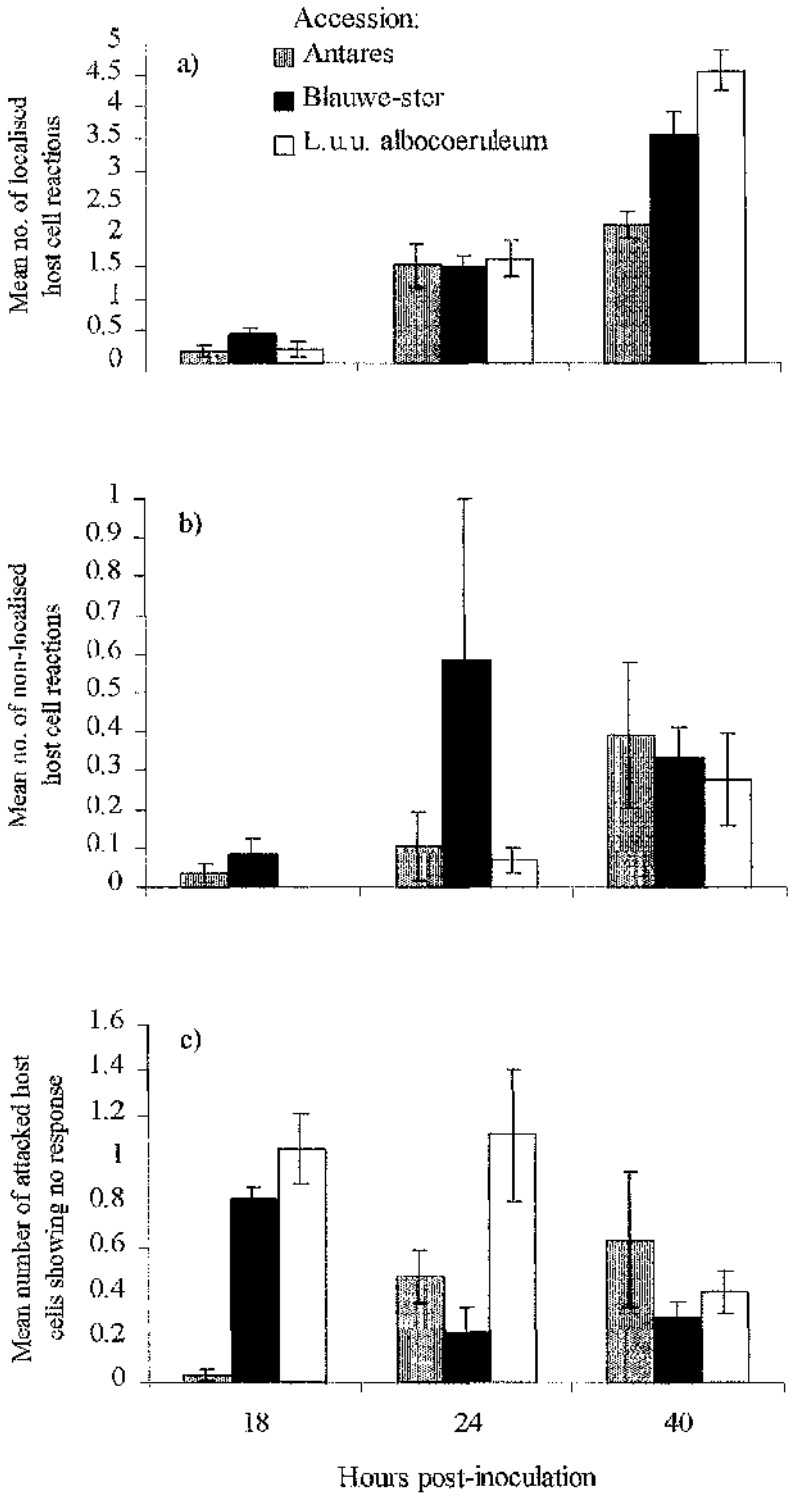
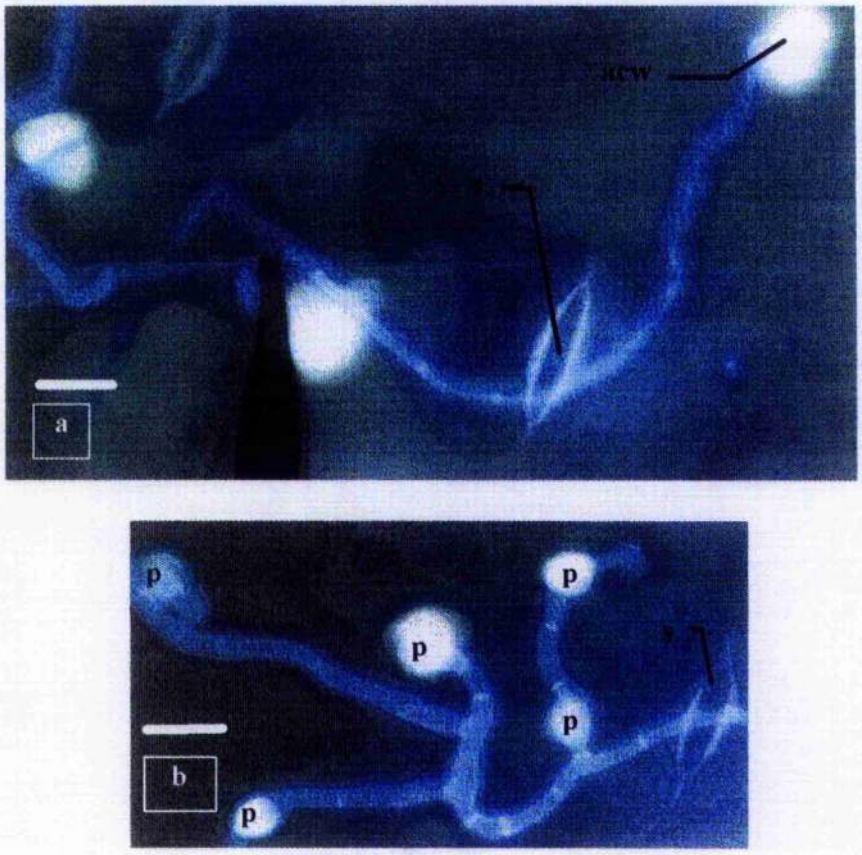


Fig. 3.6 Mean number of a) localised host cell responses, b) non-localised host cell responses and c) host cells showing no response, per conidium for three linseed accessions inoculated with conidia of A. linicola *isolate Al6. Error bars = SEMs, also note different Y axis scaling.*

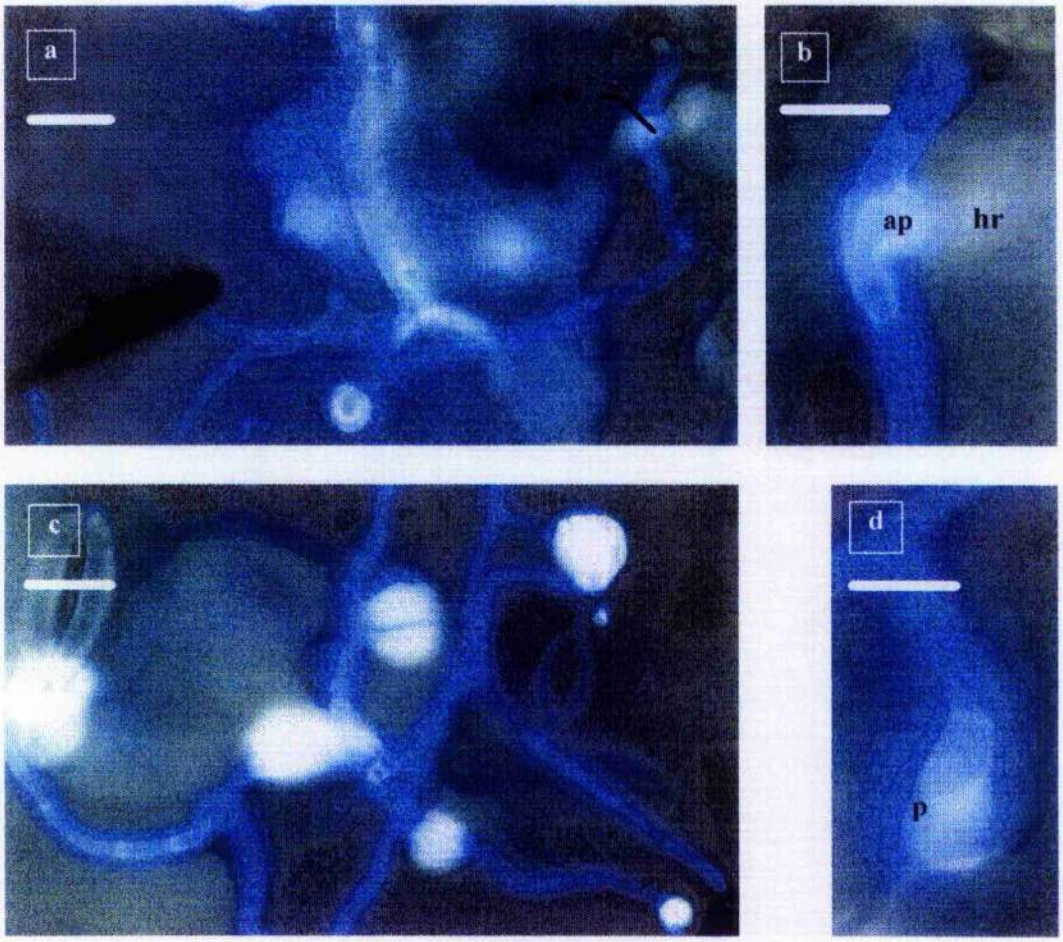
Plate 3.1 Photomicrographs showing the growth of germ tubes of A. linicola *isolate Al6 indicating the preference of epidermal cell wall sites for attempted penetration.*



a. A germ tube which has grown directly over an open stoma (s) and then attempted direct penetration *via* an anticlinal cell wall junction site (acw) on the accession *L.u.u. albocoeruleum* 40 h after inoculation. (Bar = 10 μm).

b. A germ tube which has grown directly over an open stoma (s) before it attempted five separate direct penetrations (p) at epidermal cell wall sites on *L.u.u. albocoeruleum*. (Bar = 5μm).

Plate 3.2 Attempted direct penetration at epidermal cell wall sites by A. linicola *isolate Al6 on accessions of Linum 40 hours after inoculation.*



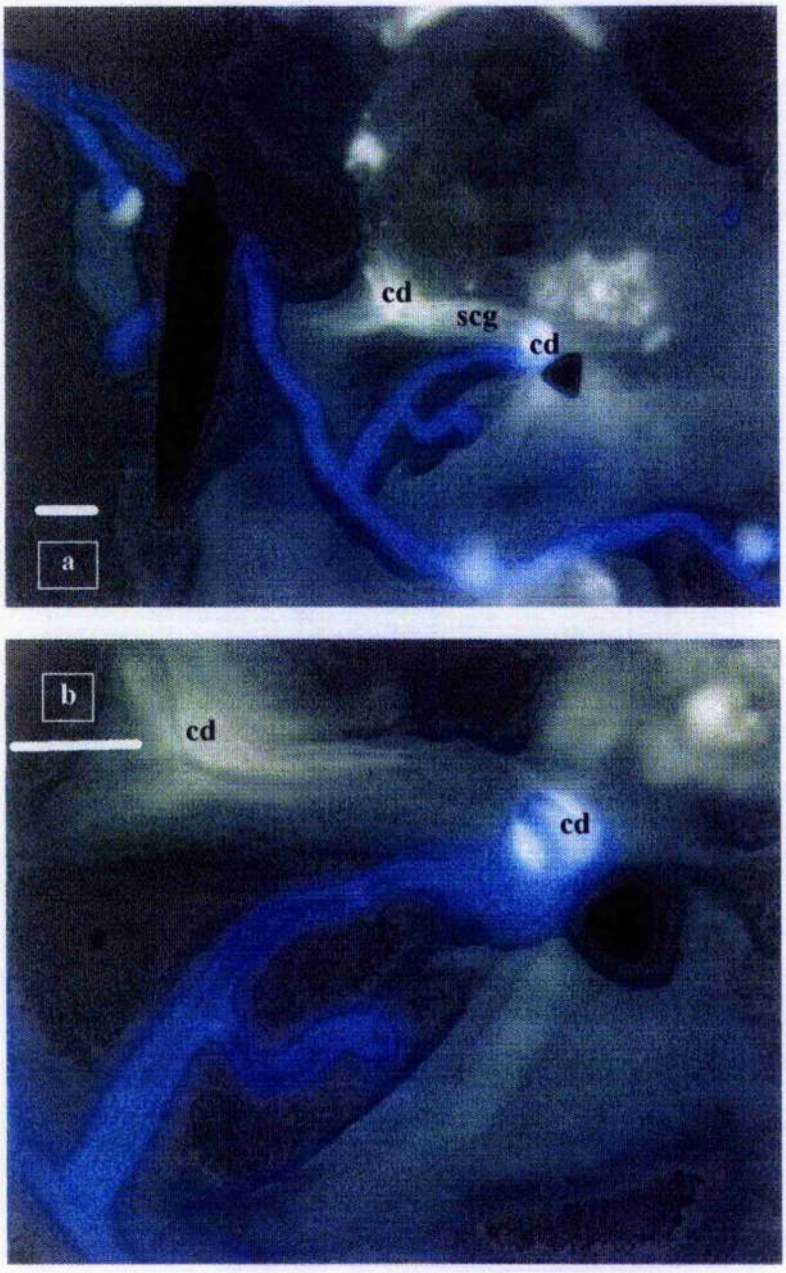
a. Attempted penetration at an anticlinal cell wall junction (acw) which has induced a localised host cell reaction preventing pathogen ingress. Fungal growth has continued to another cell junction site with no apparent response. (Bar = 10 μm).

b. Higher magnification of the penetration site in a. illustrating the formation of an appressorium (ap) by the pathogen and a localised response (hr) by the host cell. (Bar = 5 μm).

c. Well defined localised host cell responses induced by attempted direct penetration at epidermal cell wall sites on *L.u.u. albocoeruleum*. (Bar = 10 μm).

d. An attempted direct penetration (with a small infection peg [p]) at an anticlinal cell wall junction on *L.u.u. albocoeruleum* producing a localised host cell response. (Bar = 5μm).

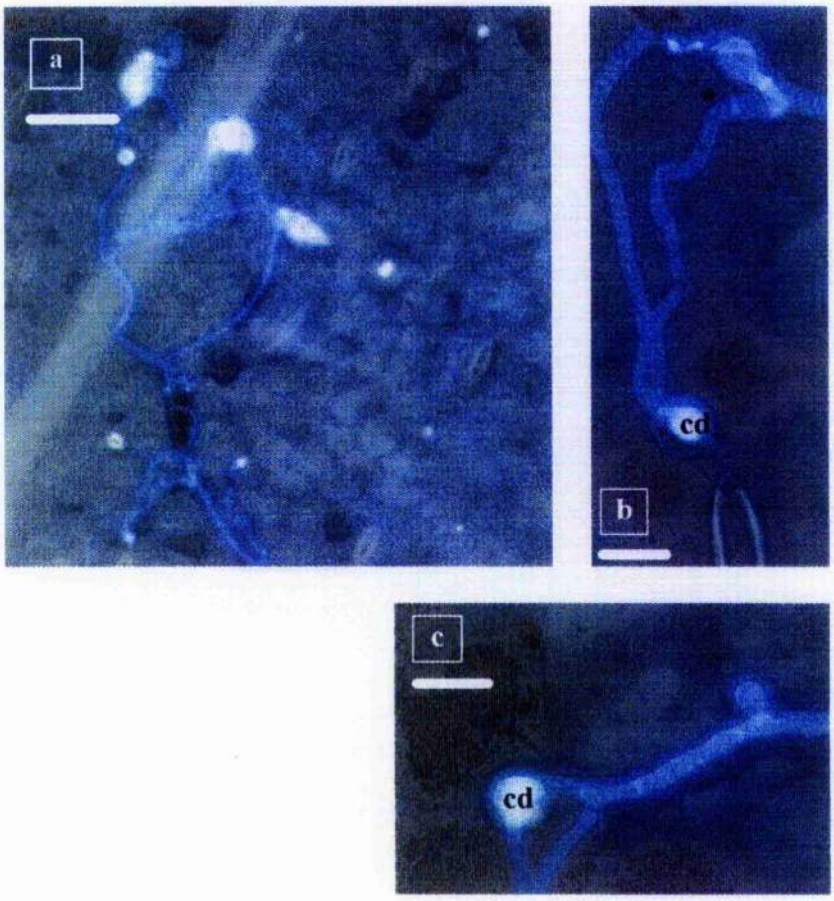
Plate 3.3 Attempted indirect penetration via an open stomatal pore by A. linicola *isolate Al6 on cv. Blauwe-ster.*



a. Unsuccessful indirect stomatal penetration attempt on Blauwe-ster (40 h.a.i). Growth of the germ tube continued to a sub-cuticular position at the apex of the stomata. Sub-cuticular growth (scg) continues along the cell wall junction inducing localised callose deposition (cd). (Bar = 5 μm).

b. High magnification of a. illustrating the localised callose deposition of the host response (cd). (Bar = 5 μm).

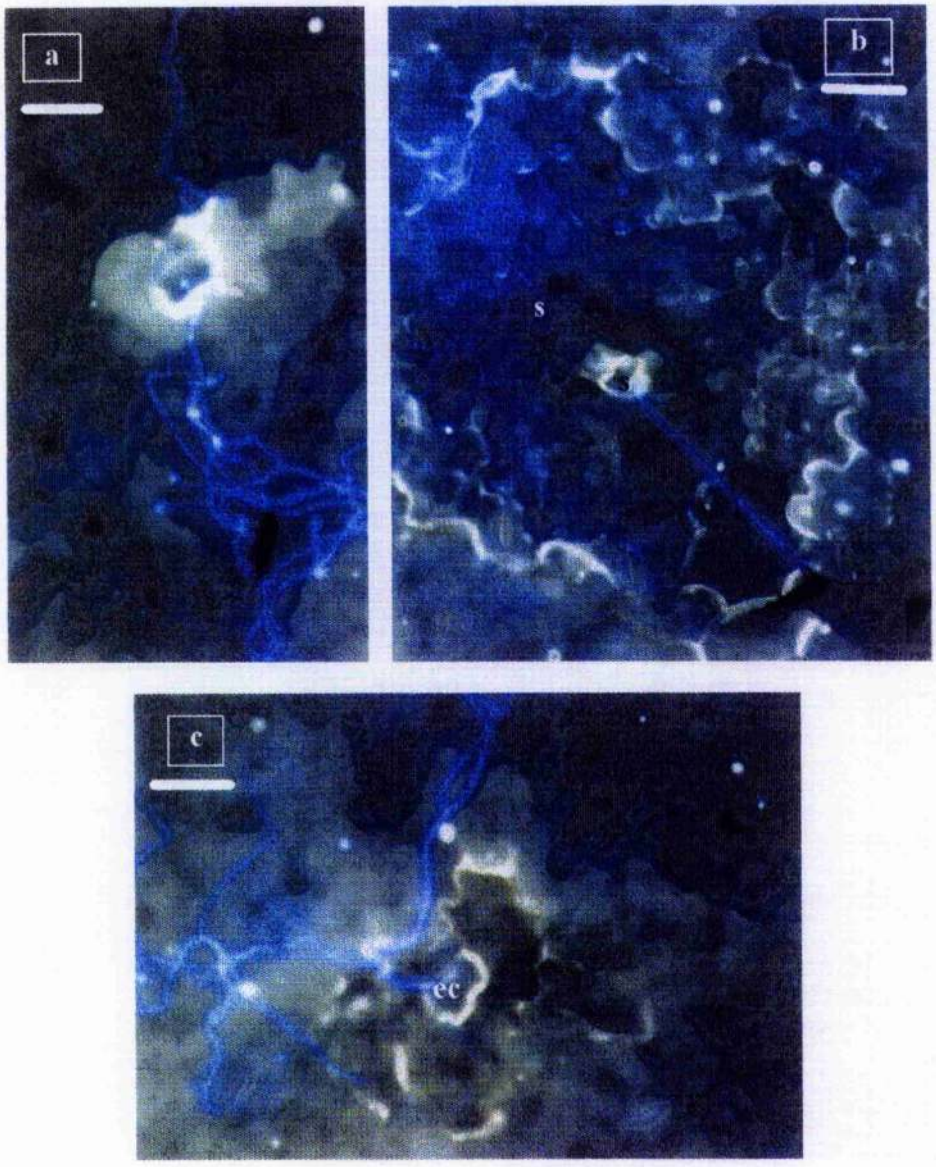
Plate 3.4 Localised host cell response observed on accessions of Linum *during attempted infection by* A. linicola *isolate Al6 40 h after inoculation.*



a. Low magnification photomicrograph of localised host cell responses at anticlinal cell wall junctions on cv. Blauwe-ster. (Bar = 50μm).

b. and c. Localised callose deposition (cd) in the walls of two epidermal cells adjacent to attempted penetration following the formation of appressorium at an anticlinal cell wall site. (Bar = 10μm).

Plate 3.5 Photomicrographs illustrating areas of non-localised host cell response observed during the infection of Linum *accessions during infection with* A. linicola *isolate Al6.*



a. Low magnification photomicrograph illustrating non-localised callose deposition at a stomatal site of *L.u.u. albocoeruleum* in response to attempted penetration. (Bar = 50 μm).

b. A large area of non-localised host cell reaction observed on the cultivar Blauwe-ster. The response arises from two successful indirect penetrations *via* stoma (s) from the germ-tube growing from bottom right to centre frame and the less obvious germ-tube growing from top left to centre of frame. (Bar = 50 μm).

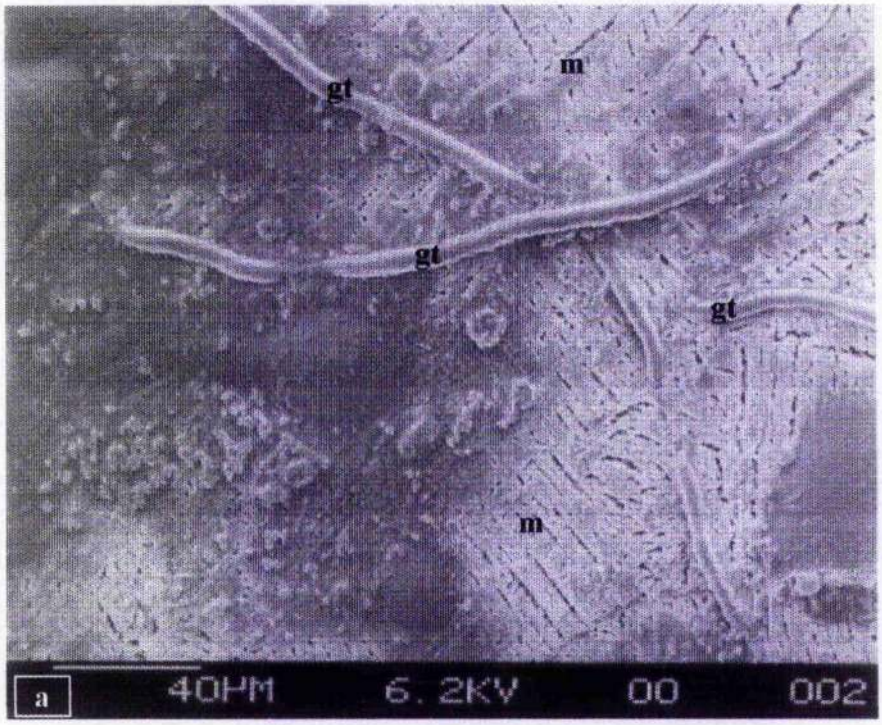
c. Low magnification photomicrograph illustrating non-localised response from epidermal cells of cv. Blauwe-ster following attempted direct penetration by Al6 at a guard cell/epidermal cell wall junction. Epidermal cell is fluorescing (ec) (Bar = 10 μm).

3.3.2 Analysis of the interaction of isolate Al6 with the three linseed cultivars using Low Temperature Scanning Electron Microscopy (LTSEM).

Preliminary studies indicated the presence of an electron dense substance which surrounded and often engulfed conidia of *A. lini*cola isolate Al6. This extraneous substance made observation of the interaction of the pathogen and the linseed host impossible (Plate 3.6). The material was not removable by etching and it was therefore assumed that the material was possibly the remnants of the trace of Tween 80 included in the conidial suspension to prevent clumping of the conidia. The experiment was repeated, the conidia being washed off the S medium plates and resuspended with SDW containing no Tween 80. However, the electron dense matrix was still observed following the use of the adapted method. Analysis was only made of conidia which could be observed at the edge of the matrix.

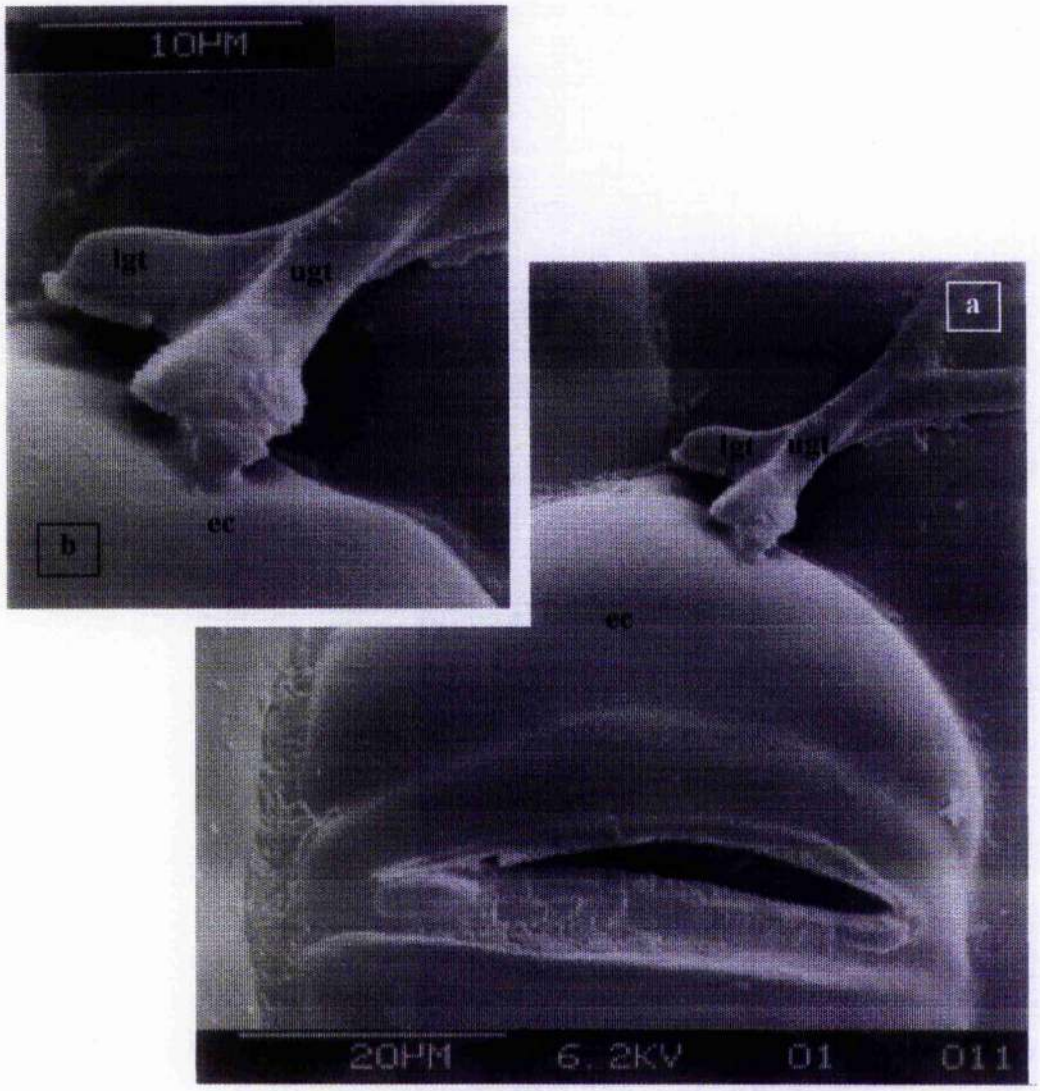
Analysis of germinated conidia indicated substantial growth across the phylloplane although no observable differences were noted with regards to selection of sites for attempted penetration between the three accessions. As observed under uv fluorescence microscopy, penetration was observed to be direct *via* epidermal cell wall sites (Plate 3.7) and indirect penetration *via* the stomata was not observed during the study. Intercellular growth of the infection hyphae was observed following direct penetration through the epidermal layer (Plate 3.8). Intercellular growth was often accompanied by a large amount of damage to adjacent cells although the infection hyphae did not appear to penetrate directly into palisade or mesophyll cells within the leaf (Plate 3.8).

Plate 3.6 Scanning electron micrograph illustrating the presence of large amounts of extraneous material surrounding germ tubes of the pathogen A. linicola *isolate Al6 on cotyledons of linseed cv. Antares 24 h after inoculation.*



a. Sheets of extramatrical material (m) appeared to thin towards the edge of the inoculation droplet. Developing germ tubes (gt) could be observed along the margins of the droplet. (Bar = 40 μm).

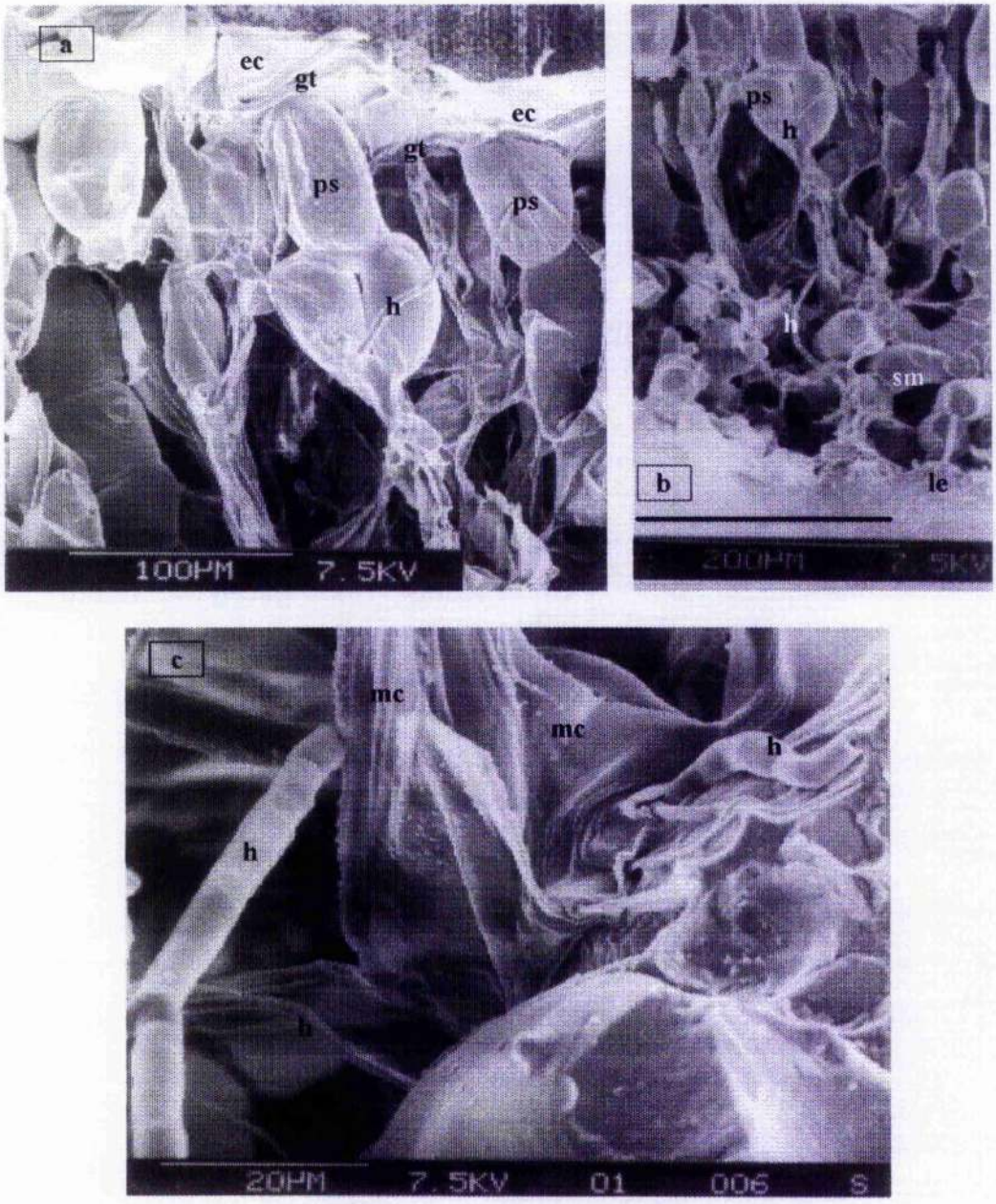
Plate 3.7 Scanning electron micrographs of A. linicola *isolate Al6 attempting direct penetration through epidermal cell wall sites of the* Linum *accession* L.u.u. albocoeruleum *24 hours after inoculation.*



a. Germ-tubes which have grow along the cleft between two epidermal cells. The lower germ-tube (lgt) continued along the junction between the two epidermal cells whilst the uppermost germ-tube (ugt) attempted direct penetration of the periclinal surface of the epidermal cell (ec) adjacent to the guard cell. (Bar = 20μm).

b. Higher magnification of the penetration event described in a.. The lower germ-tube (lgt) can be observed to be growing along the cleft between the anticlinal cell wall junction. The upper germ-tube (ugt) has produced a small appressorium and an infection peg which can be observed to be causing a depression in the surface of the host epidermal cell wall (ec). (Bar = 10μm).

Plate 3.8 Scanning electron micrographs illustrating the intercellular growth of A. linicola *isolate Al6 on the linseed cultivar Antares (40 h post-inoculation).*



a. Germ tubes (gt) of isolate Al6 observed to penetrate the upper epidermal layer (ec) and grow intercellulary (h) between the palisade cells (ps), many of which appear to have collapsed. (Bar = 100µm).

b. Intercellular development (h) through the spongy mesophyll layer (sm) vertically upwards towards the palisade cells (ps) following the penetration of the lower epidermis (le). (Bar = 200µm).

c. High magnification of a number of intercellular hyphae (h) growing amongst collapsed mesophyll cells (mc). (Bar = 20 µm).

3.4 Discussion

3.4.1 Initial pathogen development

General observations

Although very little work has been carried out on the infection process of the *Alternaria* in comparison with the biotrophic pathogens of the cereals, it would appear likely that the more generalised pattern of development observed for the facultative pathogens forms a basic model to our understanding of plant pathogen interactions. Of the *Alternaria*, very little work has been published on the development of *A. linicola* and the current study indicates that there are many similarities with a number of the other pathogens of the genus. *Alternaria linicola* follows a similar pattern to the *Brassicaceae* infecting species *A. brassicae* and *A. brassicicola* and the *Solanaceae* infecting species *A. solani*. Unlike many of the other *Alternaria* pathogens listed in Table 3.1, all four species show opportunistic potential as they are able to penetrate either directly or indirectly *via* stomata, either with or without the formation of appressoria. However, although the four species show some plasticity in their ability to colonise the host, each does show a preference for a particular method or point of ingress and, indeed, it may be specialisation to a weakness in the host defences which determines this phenomenon.

Requirements for adhesion and initial development

In general, the nature and condition of the host material, primarily the phylloplane environment and structure, dictate the success of a pathogen during the infection process (Rotem, 1994). As already discussed (section 1.2.1) the availability of free water appears to be a pre-requisite for the successful germination, adhesion and development of spores of many of the *Alternaria*. The importance of free moisture for the success of *A. linicola* was probably demonstrated during the present study in that the numbers of conidia of A16 present on the cotyledons of Antares, Blauwe-ster and *L.u.u. albocoeruleum* on which the inoculum droplet had dried out during the incubation period were very low. This could indicate the importance of water availability for the successful adhesion of the conidium to the leaf surface and for the germination and development of the pathogen.

The author suggests that the presence of an electron dense extra-matrical substance which was observed to surround the germinating conidia and developing germ tubes during the SEM

studies was produced for the purpose of adhesion. The substance had the characteristics of a polysaccharide compound (J. Findlay, Personal communication, 1994) which, as the compound was only observed around fungal structures, was apparently produced by the pathogen for this purpose. This analysis would agree with the observation and analysis of similar compounds which have been characterised as polysaccharides, proteins and glyco-proteins (Nicholson & Epstein, 1991). Many of these compounds have recently been implicated in pathogenesis as they exhibit enzymatic activity. Theoretically, compounds such as these could alter the leaf surface directly (Pascholati *et al*, 1993). However, no evidence of change to the cuticle or epidermal cell layer was observed directly beneath the mucilage during the present study.

Denny (1995) suggested that the role of bacterial extracellular polysaccharides may be two-fold, not only in terms of pathogenicity, but also for the protection and survival of the pathogen. During pathogen growth, it was suggested that extracellular polysaccharides protected the pathogen against desiccation at an extremely vulnerable stage in the life cycle, concentrated minerals and nutrients, reduced the contact of the pathogen from hydrophobic and charged macromolecules, as well as adhering the pathogen to the host surface. Denny (1995) also suggested that during pathogenesis, extracellular polysaccharides may prevent excessive contact with fungitoxic substances, promote water-soaking of the host tissues and aid the successful colonisation of the host by minimising the interaction of the pathogen with the host cells, so reducing the speed and extent of host response.

Development on the phylloplane

Following successful adhesion, the main characteristic affecting many pathogens ability to infect a plant is the morphology of the plant and more specifically, aspects of the topography of the leaf surface (i.e. cuticle thickness and structure, stomatal density and shape of the epidermal cells). Differences in the topography of the leaf surface have successfully been shown to account for the passive non-host resistance of many unrelated species (Royle 1976). Much of the work reviewed by Royle (1976) also explained examples of "age-related" and "tissue-related" resistance and some evidence for "environment-mediated" resistance.

As Antares, Blauwe-ster and *L.u.u. albocoeruleum* are closely related phyllogenetically, the different accessions could be expected to have a fairly similar fine topography. From this it would be expected that there would be little variation in the behaviour of *A. linicola* isolate

Al6 on the three accessions tested with respect to the topography and micro-environmental conditions of the cotyledons. However, differences in the response of the isolate on the three accessions were observed (Table 3.2, Fig. 3.1 and Fig. 3.2). Analysis of Fig 3.1 indicated that during the early stages of the interaction (18 h and 24 h post-inoculation) variates of importance were a number of growth parameters of the pathogen. These were further advanced and thus associated with, the less resistant accessions Blauwe-ster and, to a lesser extent, Antares. Differences in the speed of the development of the pathogen between the accessions were responsible for the significant differences between the accessions at these time points. As one would expect, host responses were observed to become important later in the interaction than fungal development (40 h post-inoculation) and whilst the resistant accession *L.u.u. albocoeruleum* was associated with variates of pathogen development, the less resistant accessions, particularly Blauwe-ster, were associated with variates describing cellular interactions.

No observations were made during the present study on the effect of differences in the waxy cuticle or age-related condition of the cotyledons between Antares, Blauwe-ster or *L.u.u. albocoeruleum* on infection by *A. linicola*. Considering the close phyllogenetical association between the three accessions, marked differences in the composition or structure of the cuticle would seem unlikely and no obvious differences were observed during the present study. Vloutoglou (1994) noted that the wax layer on cotyledons of linseed was thinner in comparison to that on the true leaves of the plant and suggested that this may be a factor in explaining the increased severity of infection by isolates of *A. linicola* which was observed on the cotyledons of seedlings.

Comparison with other phytopathogenic Alternaria *spp.*

The pattern of behaviour observed for *A. linicola* was similar to that observed for other large-spored *Alternaria* species on their host plants (Tsuneda & Skoropad, 1978; Allen *et al.*, 1983; Tewari, 1986; McRoberts & Lennard, 1996). The pre-penetration growth of *A. linicola* was thus apparently insensitive to minor variations in leaf surface morphology. As in interactions between other *Alternaria* species and their host plants, (Tewari, 1986; McRoberts & Lennard, 1996) resistance and susceptibility were found to be determined principally by events associated with attempted penetration and subsequent colonisation of the host tissue and the speed of the resistance response of the host plant cells. Thus, the data from the current study

suggest that differences in resistance among the accessions resulted from a reduction in the rate of the infection process.

Progress of disease development

Differences in the timing rather than the nature of resistance responses have previously been shown to be correlated with variations in host resistance in a number of plant/pathogen interactions (Hachler & Hohl, 1984; Ride, 1985; Aist and Gold, 1987). Further evidence that such a relationship could account for the variation in resistance among the *Linum* accessions used in the current study can be observed. For example, penetration events were found more frequently on the most susceptible accession, Blauwe-ster, than on the other two accessions at 18 h.a.i.. Later on in the interaction at 40 h.a.i., successful penetrations were observed on the most resistant accession, *L.u.u. albocoeruleum*. However, subsequent events in the infection process (sub-cuticular growth, and inter-cellular growth) were rare on the resistant accession. These events were common on the moderately-resistant accession, Antares, and the susceptible accession, Blauwe-ster.

The difference in the rate of infection by *A. linicola* on the three *Linum* accessions can be further illustrated by *a posteriori* comparisons of particular features of the interaction involving variates which occur during the latter stages of the interaction. The variates which were of importance in differentiating the accessions in the MANOVA (Table 3.2) were described by the results of the CVA analysis (Table 3.3). The occurrence of inter-cellular growth (Fig. 3.5) and non-localised host cell reactions (Fig. 3.6) were delayed on *L.u.u. albocoeruleum* in comparison with the more susceptible accessions suggesting that while *A. linicola* was able to infect the cotyledons of all three accessions, the infection process was delayed on the more resistant accession. At 40 h.a.i. the incidence of attempted penetrations with localised cell responses was higher on *L.u.u. albocoeruleum* than on the other two accessions (Fig. 3.6). These observations are consistent with the hypothesis that attempted penetration by the pathogen decreases in frequency once successful penetration has occurred, and thus the rate of successful penetration was delayed on *L.u.u. albocoeruleum* in comparison with the other accessions tested, leading to a higher frequency of *attempted* penetrations at 40 h.a.i..

Differences in appressorial formation between accessions

The general lack of variation in the growth pattern of *A. linicola* isolate Al6 on Antares, Blauwe-ster and *L.u.u. albocoeruleum* throughout the study was also observed in the frequency of appresssorial formation at either an intercalary or terminal position (Table 3.4). Significant differences in the overall number of appressoria formed and the position of those appressoria along the germ tube were not observed until late in the interaction (40 h). The total number of appressoria formed on *L.u.u. albocoeruleum* at this time was significantly higher than the number formed on either Antares or Blauwe-ster. Of these, significantly more of the appressoria on *L.u.u. albocoeruleum* were formed in a terminal position in comparison to Antares and Blauwe-ster and likewise, the number of intercalary appressoria formed was also significantly higher on *L.u.u. albocoeruleum* than on Antares (Table 3.4).

The data suggested that *A. linicola* isolate Al6 formed appressoria more readily on the resistant accession *L.u.u. albocoeruleum*. Analysis of the data for all accessions by CVA indicated that, although this was the case, the variables which were important in the interaction at the 40 h time point were penetration and host cell response variables (penetration, intercellular growth, sub-cuticular growth and localised host cell response). This again indicates that successful penetration of the other two accessions had already occurred by this time. Consequently, the majority of pathogen development on Antares and Blauwe-ster was occurring sub-cuticularly and/or intercellularly.

A similar phenomenon has been reported in the literature. Blazquez & Owen (1963) noted that resistant clones of *Hevea brasiliensis* (hevea rubber) induced the formation of more appressoria in comparison with susceptible clones during infection studies with the pathogen *Dothidella ulei*. The probable reason for this occurrence was the presence of larger quantities of leaf exudates which Blazquez & Owen suggested were inhibitory to germ tube growth and thus induced appressorial formation due to the initiation of a survival response from the pathogen. Whether differences in the amount of leaf exudate produced by *L.u.u. albocoeruleum* in comparison with Antares and Blauwe-ster was responsible for differences in appressorial formation frequency was not established during the present study.

In general, the majority of the appressoria formed by *A. linicola* isolate Al6 on Antares, Blauwe-ster and *L.u.u. albocoeruleum* were formed over anticlinal cell wall junctions (Fig. 3.3). Behaviour such as this has been observed for many phytopathogenic fungi and it has

been suggested that the selection of cell wall junction sites may be a positive response to a topographical or chemical stimuli (Preece *et al.*, 1967; Emmett & Parberry, 1975; Daniels *et al.*, 1991). Such a suggestion was made for the behaviour of *A. brassicae* which was observed to form appressoria at cell wall junction sites on oil seed rape (Tsuneda & Skoropad, 1978), although statistical evidence was not produced to substantiate the observation.

Comparative results on the preference of *A. linicola* for cell wall sites for appressoria formation on linseed material were published recently (Vloutoglou, 1994) along with similar results taken from Brassica host/non-hosts and a range of *Alternaria* spp. (McRoberts, 1992). No explanation was provided for the observed results by the former author. McRoberts (1992) attempted to explain site selection by studying many aspects of the interaction including leaf waxiness, however, no clear relationship was observed between different forms of waxiness and appressorial site selection.

Preference of site for appressorial formation was observed for *Alternaria longipes* on tobacco (Von Ramm, 1962) although seasonal variation was observed which was associated with the maturation and condition of the host tissue. Early in the season, Von Ramm observed that germ tubes grew longer before appressoria developed and penetration was only observed at certain sites. Later in the season, appressoria developed on short germ tubes close to the germinated conidium and direct penetration occurred, either with or without appressorial formation, regardless of the position of the pathogen on the plant surface. No explanation of the phenomena was given by the original author beyond the realisation that there was a correlation with plant age. However, Emmett & Parberry (1975) later suggested that the change in site selectivity observed by Von Ramm may have been associated with increased osmosis of exudates which accompanied the ageing of the host cells.

Sub-cuticular development of the pathogen

In general, following penetration of the cuticle, *A. linicola* was observed to grow sub-cuticularly for a short period, although growth of this nature was slightly delayed on the accession *L.u.u. albocoeruleum* due to a lack of successful penetration on the resistant accession. Similar growth was observed by Tewari (1986) who reported a short period of sub-cuticular growth by *A. brassicae* during the infection of *B. napus*. A possible suggestion for the period of sub-cuticular growth could be the ramification of the pathogen over the plant surface as observed before the penetration of the cuticle, but being sub-cuticular, the pathogen

is afforded shelter from excessive changes in the environmental conditions to which it is subjected. Another advantage to a phase of sub-cuticular growth is that metabolites produced by the pathogen would interact directly with the tissues of the host plant. Jones & Ayres (1972), for example, observed that subcuticular mycelium of *Rhyncosporium secalis* caused permeability changes in the underlying barley epidermal cells which promoted nutrient leaching. This in turn provided more nutrients for the developing pathogen.

Sub-cuticular growth of *A. linicola* isolate Al6 during the present study was intercellular along the cleavage lines of the anticlinal cell wall junctions of the epidermal cells. The probable reason for this directed growth was a similar thigmotropic response to the physical presence of the indentation of the cell wall junctions or a chemotropic response to the presence of chemical exudates or nutrients leaching from the host cells into the cell wall junction region as was observed during appressorial site choice.

Sub-cuticular growth was always observed to result in the penetration of the epidermal layer, an event which also took place directly (without sub-cuticular growth) and also occasionally indirectly through ingress to the sub-stomatal space *via* the stomata. The observation that periods of sub-stomatal growth lead to penetration to a sub-epidermal level is important in the interaction and suggests the conditioning of the host tissue by enzyme or toxin production by the pathogen. It may be the case that similar successful penetration without sub-cuticular growth, either direct or indirect, may be possible providing the pathogen can evade host responses for a long enough period to allow the conditioning of the host tissue ensuring it to be conducive to penetration.

A growth pattern of sub-cuticular growth, followed by the conditioning of the host tissue by fungal metabolites was suggested by Tewari (1986) during the infection of *B. napus* by *A. brassicae*, however, experimental evidence of conditioning was not given by the author. Similar evidence of pre-conditioning of epidermal cells by members of the *Alternaria* was observed during the infection of sicklepod (*Cassia obtusifolia* L.). Van Dyke & Trigiano (1987) suggested that the observed necrosis of the epidermal cells of sicklepod was due to the secretion of toxins by the weakly virulent *Alternaria cassiae*.

Following either a period of subcuticular growth, or direct/indirect penetration of the cuticle, *A. linicola* isolate Al6 grew intercellularly to a sub-epidermal position. Very little intracellular growth was observed and in this respect *A. linicola* follows the behaviour which appears to be

almost universally characteristic for *Alternaria* phytopathogens (Jackson, 1959; Von Ramm, 1962; Tewari, 1986). Isolate A16 was then observed to spread through the cell levels of the leaf, in this case through the palisade cells into the spongy mesophyll cells, ramification being associated with cell necrosis and collapse. As the pathogen was not often observed to physically puncture and invade adjacent host cells from the intercellular position, this would suggest the production and action of (a) compound(s) which were toxic to the surrounding host cells. Further discussion of the importance of toxin production in the *A. linicola*/linseed interaction will take place during the following chapter of this work.

3.4.2 The host response and the effect on pathogen development

The importance of the host response

During the present study, it would appear that much of the difference in the response of *A. linicola* isolate A16 to the linseed accessions Antares, Blauwe-ster and *L.u.u. albocoeruleum* was determined by active host mechanisms. This is particularly important in the latter stages of the interaction from 24 h.a.i. onwards. From the behaviour of the pathogen described and discussed to this point, it would appear that development progresses from adherence and germination through to penetration, ingress and development, in a genetically predetermined manner. Such a hypothesis has been suggested for many fungal pathogens including many phytopathogenic *Alternaria* spp. (section 3.1).

Localised callose deposition and papillae formation

The exclusion of the pathogen from penetration on *L.u.u. albocoeruleum* was characterised by the high number of localised host cell reactions which were observed on this accession. Many of the localised host cell responses produced by this accession were extremely small localised callose deposits which fluoresced with a bright yellow colour when stained with aniline blue (Eschrich & Currier, 1964). Although the callose structures were not observed to completely engulf the advancing fungal body, the callose structures resembled papillae (Aist, 1976) on many occasions. These consisted of characteristic semi-circular deposits in both of the anticlinal cell walls between which the pathogen was attempting to penetrate. At sites such as these, the pathogen was effectively prevented from developing further due to the physical presence of the callose structures.

Apart from the callose (usually a 1, 3 β-glucan) component, the chemical nature of the papillae-like formations could not be explained further from the observations taken. Unstained material was not observed to auto-fluoresce which would have indicated the presence of lignin or lignin pre-cursors, compounds which are often associated with papillae (Aist, 1976). Many studies have suggested further functions for papillae in the resistance response such as the restriction of the passage of solutes from the host cell (Wheeler, 1974), a permeability barrier protecting the host cells from exogenous toxic compounds (Hanchey & Wheeler, 1971; Mercer *et al.*, 1974; Skipp *et al.*, 1974; Wheeler, 1974). No evidence of another role for the papillae-like structures apart from the physical exclusion of the pathogen from the attacked cell was observed during the present study. However, if papillae formation was incited by the presence of a fungal toxin, the function of reducing the host cell permeability to exogenous toxin compounds should not be dismissed. Indeed, it seems unlikely that the host cell walls within which the papillae-like callose structures were formed were penetrated prior to callose deposition, as mechanical evidence of the action of infection pegs was not observed. This in itself suggests the response of the host cell was chemically induced, possibly by the production of a toxin(s) by the pathogen. This hypothesis would agree with evidence which indicates chemical stimuli, and the presence of toxic metabolites in particular, to be sufficient in the incitement of papillae formation (Hanchey *et al*, 1968; Luke *et al*, 1966).

Non-localised callose deposition

Failure of a localised host cell response to inhibit pathogen development appears to have led to a spread in the amount of callose formation within the host cell being attacked. This response was observed as a characteristic of failed localised responses in the accession *L.u.u. albocoeruleum* and, more characteristically, in Antares and Blauwe-ster. It should also be noted at this point that localised cell reactions were not common in the susceptible accession Blauwe-ster. Quite often, a generalised whole cell reaction was accompanied with a localised or whole cell reaction in the adjacent host cell, especially if the pathogen was observed to be developing sub-cuticularly between two epidermal cells. This response was also probably incited by the production of toxic metabolites by the pathogen as intercellular hyphae were not always observed to be within the near vacinity of the host reaction.

Evidence of the production of defence-related compounds

In the majority of cases of localised and non-localised response, the pathogen was observed to cease to develop further. This observation was particularly true for fungal infection bodies in areas which had sustained a high concentration of attack and where, consequently, many of the host cells were necrotic. A possible explanation would be the production of antifungal metabolites, such as phytoalexins, by the host plant cells in response to damage caused by *A. linicola*. The role of phytoalexins in the defence response of many plants against phytopathogenic fungi has been demonstrated over many years. Phytoalexin compounds were readily produced by cultivars of flax (*L. usitatissimum*) in response to infection with *M. lini* (Keen, 1978) which clearly indicates the ability of the host species to synthesise fungitoxic compounds. Similarly, a range of host plants, including cruciferous host and non-hosts, produce phytoalexins following challenge by species of the *Alternaria* (Conn *et al.*, 1988) which suggests phytoalexin elicitation by *A. linicola* would not be unexpected.

Many studies have linked disease resistance to the accumulation of uv-fluorescent material such as callose and lignin in host cells (Kidger & Carver, 1981; Mansfield *et al.*, 1974, Mayama & Shishiyama, 1976; 1978; Mayama & Tani, 1982; Mayama *et al.*, 1982). A commonly reported occurrence from studies with biotrophic pathogens such as *E. graminis* was the formation of fluorescent material immediately prior to host cell collapse during the hypersensitive response. In many cases, a good correlation has also been established between callose formation, or an increased activity of components of the callose (1, 3-β-glucan) biosynthetic pathway, and disease resistance in cultivars of crop species (Carver *et al.*, 1991; Carver *et al.*, 1994a; Carver *et al.*, 1994b; Ralton *et al.*, 1988; Schmele and Kauss, 1990; Tiburzy & Reisener, 1990). However, some studies on non-host resistance have indicated that callose deposition does not play a role in restricting pathogen growth (Perumalla & Heath, 1989).

As observed above, callose production, in the form of localised and non-localised host cell reactions, was greater in the resistant *L.u.u. alboccerulcum* and the moderately resistant Antares. From this, it would appear that changes in the composition and structure of the host cell walls are strongly implicated in the disease resistance response of *Linum* spp. to *A. linicola* although no evaluation of metabolic changes in the host cells was made during the present study. It could be suggested that the lack of ability of accessions such as Blauwe-ster to undergo physical defence responses is probably one of the factors which results in the breakdown of resistance and leads to susceptibility to *A. linicola*.

There is also strong evidence in the literature of a link between the timing of the occurrence of UV-fluorescent compounds in host cells and the production of phytoalexin compounds. Such an association in the *Linum/A. linicola* interaction studied would account for the observation that pathogen development was halted in regions which contained areas of epidermal cells which were either necrotic or showing a non-localised host cell reaction. If such compounds were produced during the interaction, extreme localised production at, or around, localised papillae-like reactions would account for the cessation of growth without the total engulfment of the fungal hyphae as was observed.

Important features of the interaction between A. linicola *and linseed*

Analysis of observations made during the present study suggest that the following description best explains the factors which are involved in the *A. linicola*/linseed interaction and those which are of importance in the determination of the resistance response:

1. Differences in the topographical nature of the three accessions appeared to have little effect on the development of the pathogen but the pathogen showed a preference for anticlinal cell wall junctions for sites of appressorium formation.
2. The majority of factors which are important in the interaction are the more specific active responses of the host to the development and penetrative attempts of the pathogen.
3. It is suggested that chemical components produced by the pathogen elicit a response from the host and it is differences in the speed and quantity of host cell reactions which appear to be the most important factors preventing pathogen ingress and development and thus determining resistance/susceptibility.
4. Changes in the construction of the host cell walls forms the primary response. However, continued pathogen development and ingress results in cell damage which elicits a secondary response through the production of phytoalexin compounds.

The remaining two chapters of this study investigate two components of plant pathogen interactions which have been suggested from the microscopic observations described above. The first of these will investigate phytotoxin production in *A. linicola* and considers the possibility that toxin accounts for the specialisation of this species on linseed. The second study will investigate whether linseed cultivars produce fungitoxic compounds as a defence response to attack by *A. linicola*.

Chapter 4

4.0 Secondary metabolite production by *Alternaria linicola*

4.1 Introduction

Phytotoxin production in host-pathogen interactions

Plant pathologists have known for many years that some fungal and bacterial plant pathogens produce compounds which are toxic to plants, indeed Gäumann (1954) stated that "microorganisms are pathogenic only if they are toxigenic". Although Gäumann's statement was considered extreme by most pathologists, many species of fungal pathogens are currently known to produce toxins. Some compounds (termed host specific toxin [HST]) are highly specific to the host plant species or even cultivars of one particular host species. Host-specific compounds cause disease-like symptoms on tissues of the host plant or cultivar whilst no detrimental effect is observed on the tissues of non-host species. The ability of some pathogen isolates to produce phytotoxins has caused many of the worlds major plant disease epidemics. For example, the southern leaf blight epidemic of maize in the United States (1970-1971) had a major impact on the US economy and was a repetition of an earlier epidemic of oats in North America. Both of these epidemics were caused by phytopathogens of *Bipolaris* spp. (known formerly as *Helminthosporium* spp.) which were able to colonise newly, and widely, introduced cultivars of maize and oat respectively through the production of a previously undetected host-specific phytotoxin (Scheffer & Briggs, 1981). Also, in human terms, the most terrible famine yet recorded occurred in the Indian sub-continent in 1943 when rice crops failed due to disease caused by *Bipolaris oryzae* which produced the toxin ophiobolin (Scheffer & Briggs, 1981).

The range of phytotoxins produced by pathogens of the Alternaria

Although phytotoxic compounds have been isolated from biotrophic pathogens (e.g. *Puccinia recondita* f.sp. *tritici* [Jones & Deverall, 1978]) the majority of the work on phytotoxins (particularly the HSTs) has been carried out on the so-called 'saprophytic species', mainly on *Alternaria* spp., *Helminthosporium* spp. and also on a few less well known species (Nishimura & Kohmoto, 1983a; b). A list of the phytotoxins which have been extracted from phytopathogenic *Alternaria* species, including host-specific and non-host specific toxins and the producing organisms are given in Table 4.1. Compounds which have been extracted from *Alternaria* species include a wide range of secondary metabolites. The compounds tend to fall

Phytotoxin	Chemical Group	Host specific	Site/mode of action	Producing spp.	Reference
ACR-toxins	α-pyrones	Yes	Mitochondria/disruption of membranes	*A. alternata* (citrus [rough lemon] infecting anamorph)	Kohmoto *et al.*, 1979
ACT-toxins	not determined	Yes	Plasma membrane	*A. alternata* (citrus [tangerine] infecting anamorph)	Pegg, 1966
AF-toxins	trienoic acids	Yes	Plasma membrane/electrolyte leakage	*A. alternata* (strawbwerry infecting anamorph)	Nishimura *et al.*, 1978
AK-toxins	trienoic acids	Yes	Plasma membrane/electrolyte leakage	*A. alternata* (Japanese pear infecting anamorph)	Otani *et al.*, 1985
AL-toxins	Not known	Yes	Mitochondria and rough ER/not known	*A. alternata* f.sp. *lycopersici*	Gilchrist & Grogan, 1976
AM-toxins	cyclic depsipeptides	Yes	Plasma membrane and chloroplast/disruption of membrane, electrolyte leakage	*A. alternata* (apple infecting anamorph)	Ueno *et al.*, 1975
AT-toxins	peptide	Yes	Mitochondria	*A. alternata* (tobacco infecting anamorph)	Nishimura *et al.*, 1980
Alternariol	dibenzo-α-pyrone	No	Unknown/chlorosis	*A. alternata* *A. dauci*	Raistrick *et al.*, 1953 Freeman, 1965
Alternariol monomethyl ether	dibenzo-α-pyrone	No	Unknown/chlorosis	*A. alternata* *A. alternata* (*kikuchiana*) *A. cucumerina* *A. dauci* *A. solani*	Raistrick *et al.*, 1953 Kameda *et al.*, 1973 Starratt & White, 1968 Freeman, 1965 Pollock *et al.*, 1982a,b
Alternaric acid	dihydro 1, 4 pyrone	No	Unknown (possibly plasma membrane)/chlorosis (probable electrolyte leakage).	*A. porri* *A. solani*	Brian *et al.*, 1949 Brian *et al.*, 1949
Brefeldrin	polyketide	No	Unknown/chlorosis and wilting	*A. carthami*	Tietjen *et al.*, 1983
Dihydrobrefeldrin	polyketide	No	Unknown/not stated		

Table 4.1 Phytotoxins produced by species of the Alternaria.

Phytotoxin	Chemical Group	Host specific	Site/mode of action	Producing spp.	Reference
Curvularin	heterocyclic	No	Unknown/not stated	*A. cinerariae*	Robeson & Strobel, 1981
αβ-Dehydrocurvularin	heterocyclic	No	Unknown/not stated	*A. cucumerina*	Starratt & White, 1968
Radicinin	heterocyclic	No	Unknown/not stated	*A. chrysanthemi*	Robeson *et al*, 1982
				A. radicina	Grove, 1964
Deoxyradicin	heterocyclic	No	Unknown/not stated	*A. helianthi*	Robeson & Strobel, 1982
Radicinol	heterocyclic	No	Unknown/not stated	*A. chrysanthemi*	Robeson *et al*, 1982
Tenuazonic acid	tetramic acid	No	General cell disruption/ inhibition of protein and nucleic acid synthesis causing chlorosis and necrosis	*A. alternata*	Kinoshita, 1972
				A. brassicae	Kinoshita, 1972
				A. brassicicola	Kinoshita, 1972
				A. cheiranthi	Kinoshita, 1972
				A. crassa	Kinoshita, 1972
				A. raphani	Kinoshita, 1972
				A. solani	Kinoshita, 1972
				A. temuissima	Davis *et al*., 1977
Tentoxin	cyclic depsipeptide	No	Chloroplast membrane /electrolyte leakage ?	*A. alternata*	Templeton *et al*., 1967
Zinniol	tetraketide	No	Unknown/not stated	*A. carthami*	Cotty & Misaghi, 1984
				A. dauci	Barash *et al*., 1981
				A. macrospora	Cotty & Misaghi, 1984
				A. porri	Cotty & Misaghi, 1984
				A. solani	Cotty & Misaghi, 1984
				A. tagetica	Cotty *et al*., 1983
				A. zinniae	Starratt, 1968
Destruxin B and the destruxin group	cyclic depsipeptides	No	Plasma membrane	*A. brassicae*	Bains & Tewari, 1987 Ayer & Pena-Rodriguez, 1987a

Table 4.1 cont'd Phytotoxins produced by species of the Alternaria.

into two main structural groups; the dibenzo-α-pyrones (or polyketides), or the nitrogen containing group including the cyclic depsipeptides, the tetramic acid, tenuazonic acid (TeA), the amides (AK-toxins) and zwitter ions (AL-toxins)(Stinson, 1985).

The largest group of metabolites produced by members of the *Altenaria*, the dibenzo-α-pyrones, contains many HST and non-host specific compounds. The relatively simple structure of compounds of the group has ensured that many have received the attention of chemists and biochemists and as a result the biosynthetic pathway of many of the compounds has been reported (Stinson, 1985). Three of the group, alternariol (AOH), altenariol monomethyl ether (AME) and alternaric acid (AcA) were found to possess phytotoxic properties (King & Schade, 1984). Alternariol and AME appear to be fairly ubiquitous amongst the *Alternaria* and have been positively isolated and characterised from *A. alternata* (Raistrick *et al.*, 1953), *A. alternata* f.sp. *kikuchiana* (Kameda *et al.*, 1973), *A. cucumerina* (Starratt & White, 1968) and the large spored species of *A. dauci* (Freeman, 1965) and *A. solani* (Pollock *et al.*, 1982a,b).

The biosynthesis of common Alternaria *phytotoxins*

The polyketide compounds to which this group of phytotoxins belong are formed by head-to-tail condensations of malonyl CoA units with single acetyl CoA. The biosynthetic routes for AOH and AME were studied in *A. alternata* by Thomas (1961a, b) who suggested head-to-tail condensations of acetate units. Gatenbeck & Hermodsson (1965) also studied the biosynthetic pathways of the compounds in *A. alternata* and further determined that acetate underwent carboxylation to form malonate, the polycondensing agent to produce AOH. Stinson & Moreau (1986) demonstrated that the cytosolic enzyme alternariol-O-methyltransferase was responsible for the conversion of AOH to AME in *A. alternata via* S-adenosyl-L-methionine which acted as the methyl donor.

Stinson (1985) also describes the biosynthetic pathway of tenuazonic acid (TeA), a polyketide which is a tetramic acid derivative. Tenuazonic acid is generally regarded as the most important toxin produced by the *Alternaria* mainly due to its widespread occurrence and toxicity not only to plants but to animals and insects as well (King & Schade, 1984). The compound has been extracted from several pathogenic species of the genus and as such it has been suggested that TeA production appears to be ubiquitous within the *Alternaria* (Kinoshita *et al.*, 1972; Steele & Mirocha, 1971).

Tenuazonic acid appears to cause general cell disruption by the inhibition of protein and nucleic acid synthesis and has been shown to cause chlorosis and necrosis on a wide range of plants (Templeton, 1972, King & Schade, 1984). Gatenbeck & Sierankiewez (1973) observed that specific analogues of TeA were produced by *A. alternata* depending on the provision of either L-valine or L-isoleucine in culture medium. This response to culture nutrient conditions was partly explained by Stoessel (1981)who proposed that the biosynthesis of TeA involved a condensation reaction between acetoacetic acid and L-isoleucine.

Characteristics of the host-specific toxins produced by pathoforms of the Alternaria alternata *anamorph*

Much of the work on HSTs has been carried out in Japan on the apple, pear, strawberry and citrus infecting pathotypes of *A. alternata* for which the HSTs have been shown to be a necessary determinant of pathogenicity (Nishimura & Kohmoto, 1983b). The production of HST appears to be essential for a given pathotype of *A. alternata* to be pathogenic on its respective host and thus accounts for the very narrow host range which is characteristic of HST-producing pathotypes of this species. Morphologically the different pathotypes of *A. alternata* show many similarities and all conform to the measurements of the type species as described by Simmons (1967).

Perhaps the most dramatic example of the narrow host range of some pathotypes of *A. alternata* can be illustrated by reference to the black spot disease of Japanese pear caused by the pear pathotype of *A. alternata* (previously known as *A. kikuchiana* Tanaka). The pathogen produces a number of phytotoxic compounds, designated the AK-toxins, which have been demonstrated to be the sole determinant of pathogenicity on pear cultivars. Ten cultivars, including the most popularly grown cultivar cv. Nijisseiki, were extremely susceptible to the pathogen, whilst the remaining 26 cultivars tested were immune (Otani *et al.*, 1972; 1973; 1975).

The HSTs, although showing highly host specific action, have been observed to have similar sites of action through the modification of the plasma membrane or mitochondrial modification or chloroplast modification (Table 4.1)(Kohmoto *et al.*, 1987). HSTs have also recently been observed to produce similar effects on the specific organelle of the host cell on which damage is caused (Park, 1994). It was noted that in addition to the observed membrane specificity,

only partial modification of the target structure occurred and general disruption of the host cell contents was not observed (Park *et al.*, 1981a; Park *et al.*, 1981b; Kodama *et al.*, 1990; Nutsugah *et al.*, 1993). Park (1994) suggested that this contrasted to the general disruption caused by the degradation of all cellular membranes by non-specific metabolites such as tenuazonic acid, in both susceptible and resistant tissues early in the interaction.

The main effect of the modification of the plasma membrane by HSTs (and in a less specific manner, the non-HST compounds also) appeared to be a depolarisation of the membrane potential. This resulted in the rapid loss of electrolytes (mainly potassium ions) from toxin treated susceptible cells (Otani *et al.*, 1985) and in the case of AK-toxin induced depolarisation of the membrane potential of susceptible pear cells within 30 minutes (Otani *et al.*, 1989).

The primary site of action of the cyclic depsipeptide HSTs produced by the apple infecting pathotype of *A. alternata* (AM-toxin) was also reported as being the plasma membrane and resulted in the rapid loss of electrolytes (Kohmoto *et al.*, 1987). Dysfunction of the chloroplast resulting in the inhibition of CO_2 fixation during photosynthesis indicated a second site of action for the AM-toxins. However, work by Shimomura *et al.* (1991) indicated that specificity of the pathogen was only dependant on the dysfunction of the plasma membrane. Tewari (1983) reported that toxins produced by *A. brassicae* in brassicae leaf tissue had similar sites of action to those observed for AM-toxins. The AM-toxins are structurally similar to the other cyclic depsispeptide phytotoxins which are often produced by members of the *Alternaria*, the destruxin compounds and also the common fungal metabolite tentoxin (TT). Studies have indicated that similarities in the structure of the compounds produce both shared and unique properties in the molecules which in turn determine the toxicity of the compounds. The cyclical nature of the three compounds was suggested as being important to the toxicity of the molecules since phytotoxic activity was lost when linear analogues were produced (Nishimura & Kohmoto, 1983a; Ayer & Pena-Rodriguez, 1987a; Edwards *et al.*, 1987). The similarities in the structures and sites of action between the three cyclic peptide toxins suggests conservation of biosynthetic pathways within the *Alternaria* even though speciation has occurred and the host ranges of the producing pathogens (or pathotypes of *A. alternata*) are narrow but are diverse from one another.

Chapter 4

Phytotoxins of the medium and large spored species of Alternaria

Studies of phytotoxin production by larger-spored members of the *Alternaria* has taken place on a relatively small scale in comparison with the work on HSTs produced by pathotypes of *A. alternata*. In contrast to the small-spored members of the Alternaria and the many pathoforms of the *A. alternata* anamorph in particular, the large- spored pathogens do not appear to produce HST-type compounds. Much of the work that has been undertaken has been carried out on the production of metabolites by the brassica infecting *Alternaria* pathogens, primarily *A. brassicae*.

Ayer & Pena-Rodriguez (1987a) and Bains & Tewari (1987) isolated the depsipeptide destruxin B from *A. brassicae* which was described as being host-specific by the latter authors. The claim of host-specificity for the compound was later discredited and found to be an artefact of the two different bioassays which Bains & Tewari (1987) used to test phytotoxic activity on host and non-hosts. Leaf surfaces of host species were treated with aqueous solutions of the toxin preparation whilst non-host species were tested by a cut stem method which was later found to account for the lack of phytotoxicity on the non-hosts tested (Buchwaldt & Green, 1992). Buchwaldt & Jensen (1991) and Buchwaldt & Green (1992) demonstrated that destruxin B was released by germinating spores and developing germ tubes of *A. brassicae* and was readily extracted from brassica infected with the pathogen. Using a bioassay where leaves of host and non-host species were inoculated with aqueous solution, Buchwaldt & Green (1992) found significant differences among the taxonomic plant groups tested in the level of susceptibility to destruxin B. The results indicated that, as observed from infection studies with *A. brassicae*, *Brassica* species were the most sensitive to the toxin and sensitivity decreased as the relatedness of the plant groups tested became decreased. As phytotoxic activity was observed on plant families such as *Chenopodium*, *Hordeum*, *Nicotiana* and *Phaseolus*, Buchwaldt & Green (1992) concluded that destruxin B was not host-specific but was host-selective in nature.

The role of destruxin B in the pathogenicity of *A. brassicae* on the *Brassicaceae* has not been established to date. Tewari (1983; 1986) and Bains & Tewari (1985) suggested that the role of destruxin B may be to condition the host by disrupting the plasma membrane of the host cells in advance of the developing pathogen. Evidence from work on HSTs of the anamorphs of *A. alternata* has demonstrated that the toxins produced by the germinating conidia are the primary determinant of pathogenicity allowing the penetration of the susceptible host (Daly, 1987).

Kohmoto *et al.* (1987) suggested such a role for AK-toxin which was thought to predispose the susceptible pear tissue through the suppression of the host defences. Buchwaldt & Green (1992) suggested that destruxin B acted as a virulence factor which contributed to the aggressiveness of *A. brassicae* by conditioning the host tissue to pathogen ingress and that this determined the susceptibility of the host.

An interesting observation from the work on *A. brassicae* was that as crude extracts have been further purified, the host specificity of the components of the extract has increased (Degenhardt, 1978; Bains & Tewari, 1985; 1987; McKenzie *et al.*, 1988; Buchwaldt & Green, 1992). McRoberts (1992) suggested that in the light of the evidence that TeA was produced *in vitro* by *A. brassicae*, *A. brassicicola* and *A. raphani* (Bruce *et al.*, 1984), the presence of non-HSTs such as TeA in crude extracts of cultures of these species may account for the lack of specificity of the extracts. McRoberts (1992) continued to raise the question that, although non-HSTs had not been isolated from *in vivo* studies to date, the possibility that compounds with a wide host range were produced would degrade the relative importance of any HST compounds in the pathogenicity of these producing species of *Alternaria*. It could be suggested that there appear to be two groups amongst the *Alternaria* pathogens, opportunistic saprophytic species (pathotypes of *A. alternata*) which have evolved HSTs enabling pathogenicity on an extremely narrow host range, and pathogens which appear to be more generalised (such as *A. solani*) and are pathogenic on a wider range of hosts through host-selective and non-HST toxins. This hypothesis would suggest a degree of divergence towards speciation within the *Alternaria* and would fit the morphological differences between the two groups (e.g. small- and large-spored).

The use of phytotoxin sensitivity in breeding programmes

Following the discovery of the importance of phytotoxins in the pathogenicity of many pathogens, there was considerable interest in the use of toxins as a screening tool which would allow the testing of large volumes of breeding material in a short space of time. However, the use of bioassays utilising toxin tolerance as a technique for the selection of resistance to toxin-producing pathogen has had mixed success to date. Often, resistant material can be selected by the use of exposure to toxins, but in the majority of studies to date, regeneration from callus or protoplasts can prove to be difficult and resistance may not be inherited. For example, resistant tobacco cells could be selected using AT-toxin in an *in vitro* protoplast bioassay (Nishimura, 1987). However, although Kumashiro (1983) treated tobacco cells with TeA and

selected resistant cells, following regeneration, the plants were as susceptible as the explant donors since resistance was not inherited.

MacDonald & Ingram (1985) reported the selection of *A. brassicicola* resistant lines of *B. napus* from the regeneration of secondary embryoids which had been exposed to culture extracts *in vitro*. However, resistant lines could also be regenerated from non-exposed cultures which suggested that resistance was a response to the culture environment rather than the toxicity of the culture filtrate. Reviews by Daub (1986) and Vassil (1990) summarise the merits and problems associated with the use of toxins for *in vitro* selection. Both authors note that no crop variety produced by this method has yet been released for commercial cultivation.

The genetics of phytotoxin production in relation to host/pathogen interaction

In general, the genetics of toxin production and host interaction are not presently very well understood. For some pathogens for which the production of HST confers pathogenicity on a narrow selection of cultivars, cultivar-race specificity has been suggested. For many of the interactions which involve the *Alternaria*, analogous comparisons have been made to the gene-for-gene theory as either a single, or a very small number of alleles, appear to be involved in the interaction. For example (Clouse & Gilchrist, 1987) observed that tomato lines homozygous for the recessive allele ASC^- were highly susceptible to AL-toxin producing isolates of *A. alternata* f.sp. *lycopersici* whilst heterozygous, or non ASC^- allele, carrying tomato lines were resistant. However, evidence for *Alternaria* pathogens which are not thought to produce HSTs indicate that there is little evidence of race specificity and the lack of qualitative resistance response in general suggests that the interaction is polygenic in nature (Humpherson-Jones *et al.*, 1980; Prassana, 1984).

Heath (1985) suggested that HST producing pathogens were unusual fungi which were able to overcome the previously durable resistance of host plants by effectively killing off the host cell before the resistance response could be elicited. Following cell death, Heath (1985) suggested that colonisation and development on necrotic tissue would continue unabated as the HST producing pathogens were saprophytic in nature. However, Scheffer (1983) had previously pointed out that HST producing pathogens were not always observed to induce cell death before colonisation. The colonisation of live cells has since been demonstrated by Kohmoto *et al.* (1987) who observed that AK-toxin producing pathotypes of Japanese pear appeared to inactivate the active host defences but did not kill the host cells.

Evidence for phytotoxin production in Alternaria linicola

Although many *Alternaria* pathogens have been studied for phytotoxin production, there has been a single report in which phytotoxin production was suggested as a component of the *A. linicola*/flax pathosystem (Leduc, 1958). During microscopy studies, Leduc (1958) observed the production of a compound immediately in front of the advancing hyphae and suggested the production of compounds such as alternaric acid and/or alternarin which had been recently extracted from *A. solani* (Brian *et al.*, 1952).

The results of the microscopy work reported above (Chapter 3) suggested that a compound, such as a toxin may have been produced and that this may account for the difference in the aggression of the isolates of the pathogen. The suggestion that phytotoxins may negate some aspects of the host defence mechanism (Kohmoto *et al.*, 1987) would appear to fit into the model proposed during the previous chapter that the host cell responses in susceptible accessions of *Linum* (e.g. Blauwe-ster) did not occur with the frequency and speed of those observed in resistant material. Furthermore, differences in the aggressiveness of the *A. linicola* isolates were observed during Chapters 2 and 3 and infection by the more aggressive isolate resulted in a greater extent of tissue damage to the host. This suggests that, if produced in aggressive isolates, toxin producing isolates were able to infect material at a much quicker rate than non-aggressive isolates.

The following study was initiated to investigate the nature and phytotoxic activity of metabolites produced by isolates of *A. linicola* using two methods of separation.

The aims of the study were:

1. To characterise metabolite production by isolates of *A. linicola in vitro*.

2. To compare metabolites produced by *A. linicola* with known *Alternaria* metabolites.

3. To assess the phytotoxicity of crude and purified extracts from *A. linicola* on host and non-host species.

4. To characterise the chemical nature of phytotoxic metabolites *via* 2-dimensional t.l.c. and ^{1}H-NMR.

4.2 Materials and methods

4.2.1 Extraction and characterisation of metabolites from isolates of *Alternaria linicola*

Growth and preparation of cultures

Liquid cultures of *A. linicola* isolates A11 - A14 were grown in 250 ml of DCM as previously described (2.2.2). Four culture bottles of each isolate were incubated at 18° C for 30 days in the dark, bottles being agitated by hand for 30 s every second day. The four cultures of each isolate were filtered through a single layer of muslin and the resultant mycelial mat was homogenised with ~ 50 ml of the culture filtrate using a food blender for 30 s. The combined culture filtrate and homogenised mycelial mat of each isolate was then divided into two equal volumes (500 ml). One portion was extracted directly using the organic solvent chloroform. The second portion was prepared for reverse phase chromatography (RPC) by being further filtered through Whatman No. 1 paper (x 2) and a ceramic filter (filter size 6 µm) before being freeze-dried to a total volume of 100 ml.

Organic extraction of culture filtrate by partitioning with chloroform was carried out by placing 250 ml of the culture filtrate in 500 ml extraction funnels with 100 ml of chloroform. Extraction involved two mixing stages and the total standing time to allow separation of the aqueous and solvent layers was 10 minutes. The lower solvent layer was removed by filtration through Whatman 1PS (phase separation) paper containing anhydrous K_2SO_4 in order to remove aqueous contaminants, and collected in a round bottomed flask.; Solvent from replicate flasks was pooled and the extraction process was repeated twice with two 50 ml volumes of chloroform per extraction flask. Extract was rotary-evaporated to dryness at 35° C and re-suspended in a minimal amount of chloroform.

Freeze-dried samples of culture filtrate to be extracted by RPC were further filtered through 0.45 µm microdiscs (Sigma) to prevent clogging of the columns during sample application. RPC was carried out using the method described by Buchwaldt & Jensen (1991). Bond-Elut C_{18} columns (Varian, USA) were placed on a Vac-Elut manifold connected to a vacuum pump and were activated by the addition of 10 ml of methanol per column. Following a column wash with 10 ml of sterile distilled water, the sample was loaded onto the column sequentially in 10 ml aliquots followed by 10 ml sterile distilled water until the total volume of 100 ml of culture filtrate had been equally divided between two columns (2 x 50 ml).

The first fraction of highly polar material was eluted from the column with a 10 ml aliquot of 25 % aqueous acetonitrile. The second fraction of medium polarity contained a mixture of metabolites and was eluted with a 20 ml aliquot of 40 % aqueous acetonitrile. The first 3 ml of this fraction was collected and re-chromatographed through the column followed by the remaining 17 ml of 40 % aqueous acetonitrile. Unwanted low-polarity material was flushed from the column with 10 ml of 100 % aqueous acetonitrile and discarded. Following flushing, columns were used to extract metabolites from culture filtrate of the same isolate at a later date. To prevent the possibilities of cross-contamination a new set of tubes was used for the culture filtrate of each individual isolate. The two fractions produced for each isolate were evaporated to dryness at 35° C and re-suspended in a minimal amount of 25 % and 40 % aqueous acetonitrile respectively.

Visualisation by thin layer chromatography

Metabolite profiles of the crude extracts were observed following separation by thin layer chromatography (t.l.c.). 20 µl aliquots of each extracted fraction were spotted onto the pre-absorbent layer of LK6DF pre-channelled plates (Merck) 1 cm from the active interface (origin). Following adequate drying, plates were developed in a solvent system of 95:5 v/v chloroform : methanol until the solvent front had reached a pencil line previously scored through the silica bands 15 cm from the active interface. Plates were removed and dried overnight in a fume hood.

Developed t.l.c. plates were observed under a UV source at 254 and 366 nm. UV-quenching compounds were detected at 254 nm, whilst fluorescent compounds were detected at 366 nm. R_F values were calculated for all metabolites observed. Metabolites from crude extracts were compared with available standard *Alternaria* toxin compounds (tentoxin [TT], tenuazonic acid [TeA] and alternariol mono-methyl ether [AME]) (Sigma).

4.2.2 Comparison of metabolites produced *in vitro* by two *Alternaria linicola* isolates and three *Alternaria* species.

Growth of cultures, extraction and separation by t.l.c.

Two *A. linicola* isolates (A11 and A16), an isolate of each of *A. solani* (As), *A. brassicae* (Ab) and *A. brassicicola* (Ac) and an uninoculated control were grown in 4 x 250 ml DCM as described above (2.2.2). Following a reduction in volume from 1 litre to 200 ml by freeze-drying, semi-purified fractions of each isolate were produced by RPC as described above. Aliquots of 20 µl of each extract were developed on LK6DF plates using a 7:3 v/v dichloromethane : acetone solvent system which was found to separate the standard compounds to a greater extent than a 95:5 chloroform : methanol solvent system as used during the previous experiment. As well as TT, TA and AME, two other fungal toxin compounds, destruxin A and destruxin B (M. Païs, Institute de Chimie des Substances Naturelles) were also developed on the plates for comparison.

Concentrated bands of destruxin A and B could be characterised by a very faint blue fluorescence at 366 nm. Presence or absence was confirmed by spraying the t .l.c. plate with a 5 % w/v phosphomolybdic acid solution (in 5 % aqueous. H_2SO_4 and containing a trace of ceric sulphate). Treated plates were heated at 150° C for fifteen minutes and destruxin-type compounds (cyclodepsipeptides) were characterised by the presence of a blue band (Ayer & Pena-Rodriguez, 1987).

4.2.3 Bioassay of phytotoxic activity of extracts from *Alternaria linicola* isolates A11 and A16 on four linseed cultivars and on nine non-host plant species.

Growth of cultures and plant material

Crude extract and semi-preparative fraction 1 and 2 extracts of liquid cultures of *A. linicola* isolates A11 and A16 were produced by partitioning with chloroform and by RPC as described above (4.2.1). Fraction 2 RPC extracts were redissolved in 22 % aqueous acetonitrile following evaporation to dryness, as preliminary studies indicated that 40 % aqueous acetonitrile was phytotoxic to the test material. Aliquots of the extracts were used in two experiments, firstly to test for phytotoxic activity against cotyledons of the linseed cultivars Antares, McGregor, Barbara and Linda and secondly to test for phytotoxic activity against a

range of non-host species. The species tested during the second experiment were the crucifers *Brassica rapa*, *Brassica napus*, *Sinapis arvensis*, *Raphanus raphasnistrum* and *Raphanus sativus*, *Camelina sativa* and *Capsella bursa-pastoris*, and two non-cruciferous species, *Nicotiana tabacum* and *Phaseolus vulgaris*.

Bioassay design, scoring and analysis

Linseed and non-host plant material was grown in the glasshouse as previously described (2.2.1) and a detached cotyledon bioassay was conducted as described previously (2.2.3). Nine treatments were used during the two experiments as detailed in Table 4.2. As treatments 1, 4 and 8 contained chloroform and 7 contained methanol, 10 µl of the treatment was firstly applied to antibiotic filter paper discs which were allowed to dry in a fume hood. The treated antibiotic filter paper disc was then placed directly onto the leaf surface to be tested and moistened with 20 µl of sterile distilled water.

Treatment number	Extract or Treatment
1	A11 chloroform extract (crude)
2	A11 reverse phase chromatography, Fraction 1
3	A11 reverse phase chromatography, Fraction 2
4	A16 chloroform extract (crude)
5	A16 reverse phase chromatography, Fraction 1
6	A16 reverse phase chromatography, Fraction 2
7	Tenuazonic acid (1 mg ml^{-1} in methanol)
8	Chloroform control
9	22 % aqueous acetonitrile

Table 4.2 Description of the crude and semi-preparative extract treatments applied to linseed cotyledons and non-host species to test for phytotoxicity

Five replicate cotyledons of each cultivar or three replicate cotyledon/first leaves of non-host plant species were placed in a single dish for the linseed bioassay and non-host bioassay, respectively. Material was tested in both bioassays by placing either a 10 µl droplet of the extract or a moistened antibiotic paper for extracts 1, 4, 7 and 8, directly onto the upper surface of the leaf. Each treatment dish was tested with only one extract or treatment and Petri dishes were sealed with para-film to prevent possible cross contamination through vapour effects.

Petri dishes were incubated in a controlled environment cabinet as previously described and treated material was scored after 5 days using a 0 - 4 DI scoring system (Appendix 1.3). Analysis of the data was carried out by analysis of variance using GENSTAT on a VAX mini-computer.

4.2.4 Purification and phytotoxic activity of metabolite extract from *Alternaria linicola* isolate Al6

Purification method

The remaining stocks of the RPC fraction 2 extracted from Al6 during the previous experiment were separated on 6 preparative t.l.c. plates (LK6DF) using a 7:3 v/v dichloromethane : acetone solvent system. At 254 nm and 366 nm, the plates were divided into 12 regions at R_F values which appeared to differentiate the main bands observed (Table 4.3).

Region label	R_F value of region	Amount eluted from plate (mg)
1	0 - 0.16	4.4
2	0.16 - 0.19	3.5
3	0.19 - 0.24	2.6
4	0.24 - 0.28	2.6
5	0.28 - 0.40	2.5
6	0.40 - 0.45	2.0
7	0.45 - 0.54	2.5
8	0.54 - 0.65	2.0
9	0.65 - 0.85	3.7
10	0.85 - 0.89	2.8
11	0.89 - 0.94	3.0
12	0.94 - 1.0	3.5

Table 4.3 R_F values and weights of semi-purified metabolite bands produced by reverse phase chromatography Fraction 2 from culture filtrate of Al6 run on six preparative plates in a 7:3 dichloromethane : acetone solvent system.

Bands were scraped into 1.5 ml Eppendorf tubes to each of which was added 0.5 ml of 40 % aqueous acetonitrile. The tubes were vortexed for ~ 30 s and left to stand for 5 min. Following another 30 s vortex to mix the silica and acetonitrile, Eppendorfs were centrifuged

for 5 min at 13,000 r.p.m in a microfuge. The supernatant (40 % acetonitrile) was carefully removed from each Eppendorf using a 200 µl micro-pipette and placed in a pre-weighed glass vial. The silica washing and mixing process was repeated twice, firstly with 40 % acetonitrile and lastly with 100% acetonitrile. Samples were dried under nitrogen using a sample concentrator at 30° C. weighed and diluted to a concentration of 5 mg ml^{-1} with 20 % acetonitrile.

Bioassay design, scoring and analysis

The twelve redissolved metabolite bands were tested on cv. Antares cotyledons as described previously (4.2.3). Cotyledons were also inoculated with the standards destruxin A and destruxin B (both at a concentration of 5 mg ml^{-1}) and a 20 % acetonitrile control solution. Ten replicate cotyledons of Antares were inoculated with a 10 µl droplet of each treatment solution. Petri dishes were sealed with para-film and incubated in a controlled environment chamber as previously described (4.2.3). Cotyledons were scored after five day using the DI scoring system of 0 - 4 (Appendix 1.3) and data were analysed by ANOVA using MS Excel 5.0 on a PC.

4.2.5 Purification and characterisation of metabolites produced *in vitro* by *Alternaria linicola* isolate Al6 by two dimensional t.l.c. and proton NMR

Growth of cultures, extraction and purification of metabolite bands

Cultures of isolate Al6 were grown in 6 litres of DCM (24 x 250 ml) as previously described for 30 days (2.2.2). Fraction 2 extract was produced by RPC as described in section 4.2.1 but using Isolute C_{18} columns (International Sorbent Technology, UK). The fraction was dried *in vacuo* at 30° C and taken up in a minimal amount of 40 % acetonitrile. Preparative t.l.c. was carried out using two LK6DF plates in a 7:3 dichloromethane : acetone solvent system. One channel of each plate was loaded with the standards TA, AME and destruxin A and B samples.

Plates were observed under short and long wave UV radiation and bands corresponding to the R_F values of the standards were marked on the plates. Corresponding bands on the Al6 extract channels were scraped from the plate and eluted as previously described (4.2.4). The recovered fractions were dried under nitrogen at 30° C and taken up in a minimal amount of 40 % acetonitrile. Further purification was carried out by running the recovered material on a

further preparative plate from which the fractions were again separated and recovered into pre-weighed vials, as described above. After being weighed, fractions were taken up in a minimal volume of 40% acetonitrile.

Two dimensional t.l.c (2D-t.l.c)

Fractions were analysed by 2D-t.l.c. with the standard *Alternaria* toxin compounds to which the R_F of the fraction corresponded using foil backed silica analytical t.l.c. plates (Merck, Art. 5554) (Fig. 4.1). Twenty five µl of the fraction sample was carefully spotted onto the point at the convergence of the origins of each dimension. Fifteen µl of standard was spotted in the centre of the standard lane (Standard spot 1, Fig 4.1). After being dried in a fume-hood, plates were assessed for the presence of a band corresponding to the standard toxin sample in both dimensions. Following analysis at 254 nm and 366 nm, plates were sprayed with 5% phosphomolybdic acid solution to test for the presence of cyclodepsipeptide compounds (section 4.2.2)

Confirmation of the purified compounds structures by proton nuclear magnetic resonance spectroscopy (^{1}H-nmr)

Four metabolites from Al6 (Altlin 1-4) were analysed using proton nuclear magnetic resonance spectroscopy (^{1}H-nmr) in order to confirm observations made from 2D-t.l.c. Samples were analysed using a Brüker AM 200 SY NMR Spectrometer at 200 MHz. Samples were evaporated to dryness and re-dissolved in either deutero-chloroform (Altlin 1, 3 and 4) or deutero-acetone (Altlin 2) as the carrier solvent. The internal standard used was tetramethylsilane (TMS). Analysis of the spectra produced was carried out by reference to published spectra for the standard compounds or compound groups (Cole and Cox, 1981; Gupta *et al.*, 1989).

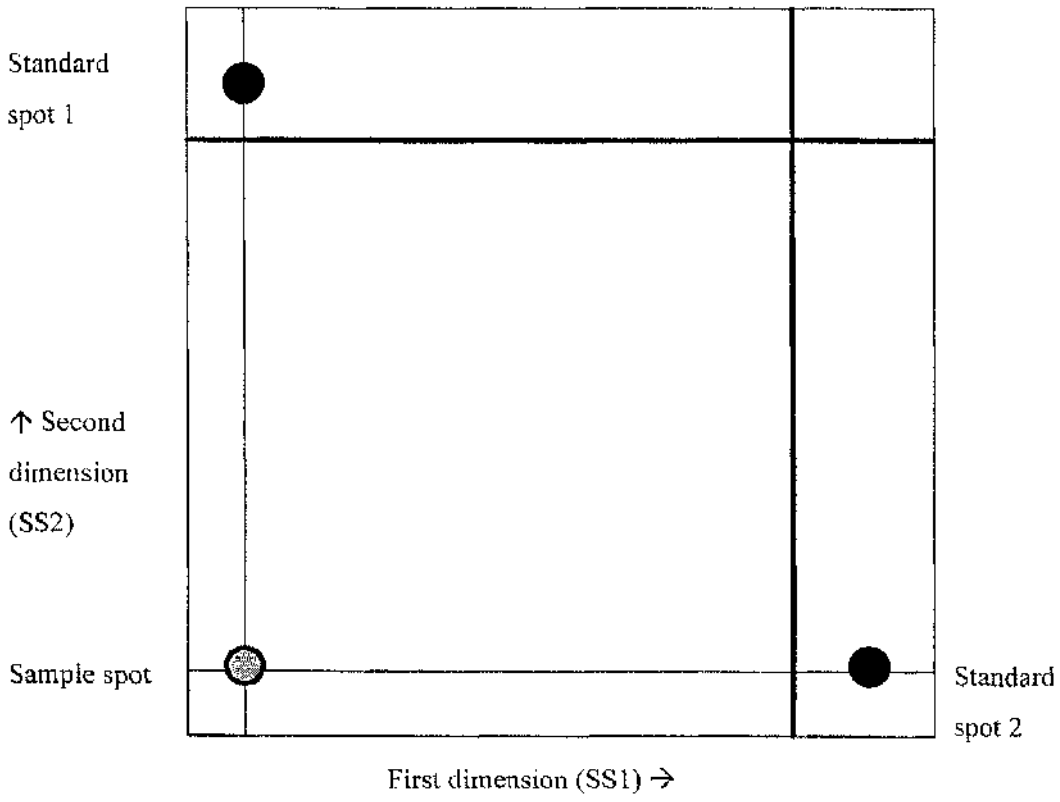


Fig. 4.1 The design of 2D-t.l.c. plates used to characterise the presence of the standard Alternaria *toxins, tenuazonic acid, alternariol mono-methyl ether and destruxins A & B in purified fractions produced from culture filtrate of isolate Al6 by RPC. Plates were developed in the first dimension (Fig. 4.1) in solvent system 1 (SS1; 7:3 v/v dichloromethane : acetone). After being dried in a fume-hood, the second standard sample was spotted into the centre of the other standard lane (Standard spot 2, Fig. 4.1). The plate was then developed in the second dimension in solvent system 2 (SS2; 95:5 v/v chloroform : methanol).*

4.3 Results

4.3.1 Extraction and characterisation of metabolites from isolates of *Alternaria linicola*

Metabolite bands observed under UV radiation

Similar metabolite profiles (Figs. 4.2 and 4.3) were obtained from the isolates of the pathogen by the two extraction methods, although some differences in intensity of bands was observed. Metabolites extracted by the semi-preparative RPC technique appeared to be more distinct and darker (Fig. 4.2) or brighter (Fig. 4.3) than those observed from crude chloroform extracts. Differences in metabolite production between the different isolates were observed with a number of isolates also producing compounds with R_F values corresponding with those of TT, TeA and AME. The control cultures were observed to produce a faint, single, quenching band which occurred at an R_F value of 0.04 - 0.08.

Under UV radiation at 254 nm (Fig. 4.2), metabolites which quenched fluorescence at R_F values and corresponded to TeA were observed for isolates Al2, Al3 and more faintly Al4. Isolate-specific bands were observed for Al1 (R_F 0.37-0.39) and Al4 (R_F 0.4-0.42) and isolates Al2 and Al3 were observed to produce a metabolite at R_F 0.22-0.23.

At 366 nm, many more minor metabolites were observed (Fig. 4.3). Prominent bands included isolate-specific metabolites as observed above with further isolate-specific metabolites being observed for isolates Al3 (R_F 0.33-0.36 and R_F 0.42-0.44) and Al4 (R_F 0.4-0.42 and R_F 0.51-0.54). An isolate specific band observed for Al1 at 254 nm (R_F 0.37-0.39) was also faintly observed for Al2 at 366 nm. Isolate Al1 was observed to produce a metabolite which fluoresced faintly at 366 nm at an R_F value of 0.68-0.72 corresponding to the standard AME. Two fluorescent bands were observed from the control cultures at R_F 0.04 - 0.11 and R_F 0.12 - 0.18, both of which were present for all cultures grown in DCM.

R_f value																	
0.68-0.72			***														
0.58-0.62	***																
0.4-0.42													**	**	**		
0.370.39				**	**	**											
0.26-0.31		***					**	**	**	**	**	**	*	*	*		
0.22-0.23							**	**	**	**	**	**					
0.19-0.22				**	**	**				**	**	**	**	**	**		
0.12-0.18				**	**	***	**	**	**	**	**	**	**	**	**		
0.09-0.11													**	**	**		
0.04-0.08				*			*						**	*	*	*	*
Isolate	TT	TeA	AME	Al1	Al1	Al1	Al2	Al2	Al2	Al3	Al3	Al3	Al4	Al4	Al4	Cont	Cont
Sample				O/E	F1	F2	O/E	F1	F2	O/E	F1	F2	O/E	F1	F2	O/E	F2

*Fig. 4.2 Schematic diagram of UV quenching bands observed at 254 nm for organic chloroform extracts (O/E) and reverse phase chromatography extracts (fraction 1 [F1] and fraction 2 [F2]) of isolates Al1 - Al4 cultured in vitro for 30 days. Intensity of quenching: High-low; *** - * (solvent system 95:5 v/v chloroform : methanol).*

R_f value																	
0.68-0.72			***		*	*											
0.58-0.62	**+			*+	*+	*+				*+	*+	*+	*+	*+	*+		
0.510.52													**	**	**		
0.42-0.44										**	**	**					
0.4-0.42													**	**	**		
0.370.39				**	**	**	*	*+	*+								
0.33-0.36										**	**	**					
0.26-0.31		**+		*	*+	*+	*	++	++	+	+	+					
0.22-0.23							**	**	**	**	**	**					
0.19-0.22				**	**	**	**	**	**	**	**	**	**	**	**		
0.12-0.18				*	**	**	**	***	***	**	***	***	**	***	***	**	**
0.04-0.11				**	**	**	**	**	**	**	**	*	**	*	*	**	**
Isolate	TT	TeA	AME	Al1	Al1	Al1	Al2	Al2	Al2	Al3	Al3	Al3	Al4	Al4	Al4	Cont	Cont
Sample				O/E	F1	F2	O/E	F1	F2	O/E	F1	F2	O/E	F1	F2	O/E	F2

*Fig. 4.3 Schematic diagram of fluorescent bands observed at 366 nm for organic chloroform extracts (O/E) and reverse phase chromatography extracts (fraction 1 [F1] and fraction 2 [F2]) of isolates Al1 - Al4 cultured in vitro for 30 days. Intensity of fluorescence: High-low; *** - *; colours: * = blue, + = yellow (solvent system 95:5 v/v chloroform : methanol).*

4.3.2 Comparison of metabolites produced *in vitro* by two *Alternaria linicola* isolates and three *Alternaria* species.

Metabolite bands observed under UV radiation

Differences were observed among the metabolites produced *in vitro* by the two *A. linicola* isolates (Al1 and Al6) and among the three other *Alternaria* spp., *A. solani*, *A. brassicae* and *A. brassicicola* (Figs. 4.4 and 4.5). However, a degree of homogeneity in banding pattern was also observed with many metabolites being shared among test isolates, particularly when visualised at 366 nm when a larger number of metabolite bands were observed (Fig. 4.5). One species-specific metabolite band was observed to quench fluorescence at 254 nm at an R_F of 0.06 (As) although this did not correspond to an R_F value for any of the standard compounds. A single quenching band was observed from the extract from the control culture at R_F 0.03 and this was observed for all cultures.

Both *A. linicola* isolates and the three *Alternaria* spp. were observed to produce compounds with R_F value comparable with the standard *Alternaria* toxins, TeA and AME (Figs 4.4). Quenching was also observed at an R_F position which was similar to that produced by the destruxin B sample (Al1, Al6, *A. solani* and *A. brassicae*). The degree of quenching observed for Al6 was fairly strong. However, for isolate Al1, *A. solani* and *A. brassicae* the level of quenching at the destruxin B R_F value was faint.

A similar pattern was observed at 366 nm although a greater number of metabolite bands were produced (Fig. 4.5). As observed on Fig. 4.3, the majority of bands were visualised as blue or blue/yellow in colour and many of the compounds fluoresced brightly. Two species-specific bands were observed at R_F 0.28 (*A. solani*) and R_F 0.57 (*A. brassicae*) although neither of these corresponded to standard compounds. As observed at 254 nm, compounds which showed similar R_F values to TeA and AME were observed for all test isolates to varying degrees whilst destruxin B occurred in all test isolates except *A. brassicicola*. Also, as observed at 254 nm, at 366 nm a single fluorescent band was observed from the control culture extract at R_F 0.03 and this was observed for all cultures.

R_F value											
0.78-0.82					***	**	**	**	**	*	
0.70-0.74		***				*	**	*	*		
0.64-0.69	***										
0.53-0.57						**	**	**	**		
0.34						**	**				
0.32						**		**			
0.19-0.23				***		**	**	**	**	**	
0.17-0.18						**	**	**	**	**	
0.16							**	**			
0.08								**		**	
0.06								**			
0.04-0.05						**				**	
0.03						*	*	*	**	*	**
Extract/standard	Dest A	Dest B	TT	TeA	AME	A11	A16	As	Ab	Ac	Con

*Fig. 4.4 Schematic diagram of UV quenching bands observed at 254 nm for reverse phase chromatography extracts (F2) of isolates A11 and A16 and isolates of A. solani (As), A. brassicae (Ab) and A. brassicicola (Ac) cultured in vitro for 30 days. Intensity of quenching: High-low; *** - * (solvent system 7:3 v/v dichloromethane : acetone).*

R_F value											
0.78-0.82					*#*	*	*+	**		*	
0.70-0.74		*				*	**	**	**	*	
0.64-0.69	**										
0.57									**		
0.55						*+	*+	*+			
0.52									**	**	
0.44-0.45						**	**		**	**	
0.34-0.36						**	**	*+	*+	*+	
0.28								**+			
0.24						*+	**+		*+	*+	
0.19-0.23				***		*+	*+	*+	*+	*+	
0.17-0.18						**	**	**	**	**	
0.16							**	**			
0.08						**		**		**	
0.06								**	**	**	
0.04-0.05						*+	*+	*+			
0.03						**§	**§	**§	**§	**§	**§
Extract/standard	Dest A	Dest B	TT	TeA	AME	Al1	Al6	As	Ab	Ac	Con

*Fig. 4.5 Schematic diagram of fluorescent bands observed at 366 nm for reverse phase chromatography extracts (F2) of isolates Al1 and Al6 and isolates of A. solani (As), A. brassicae (Ab) and A. brassicicola (Ac) cultured in vitro for 30 days. Intensity of fluorescence: High-low; *** - *; colours: * = blue, + = yellow, # = brown, § = red (solvent system 7:3 v/v dichloromethane : acetone).*

4.3.3 Bioassay of phytotoxic activity of extracts from *Alternaria linicola* isolates A11 and A16 on four linseed cultivars and on nine non-host plant species

Disease-like symptoms on four linseed cultivars

Extracts from both isolates of the pathogen (A11 and A16) caused disease-like symptoms on cotyledon material of Antares, Linda, Barbara and McGregor. There was no significant difference between the response of the four cultivars to the toxin treatments ($df = 144$, $P = 0.760$). Significant differences were observed between the toxin treatments ($df = 144$, $P < 0.001$, Fig. 4.6). There was no cultivar-toxin treatment interaction ($df. = 144$, $P = 0.614$). For both isolates, extracts produced by RPC caused greater damage to the leaf material than that from extract produced by the organic solvent extraction technique.

Of the two RPC fractions, the second fraction from each isolate produced greater disease-like symptoms, significantly more severe in the case of the A16 fractions(Fig. 4.6). Disease-like symptoms caused by the non-host specific standard TeA were minor in comparison to the levels of tissue damage produced by the RPC fractions, but were similar to the damage observed for the crude extracts produced by the organic solvent extraction method. Toxicity was not apparent from the chloroform control treatment, but a low level of toxicity was observed for the acetonitrile control treatment.

Disease-like symptoms on non-host species and the control cultivar Antares

The disease-like symptoms apparent on non-host species were similar to those observed on linseed. Significant differences were observed among the different species, for the different treatments and also, there was a species-treatment interaction ($df = 180$, all $P < 0.001$). Disease-like symptoms, comparable with those observed on the linseed cotyledons, were observed for many of the *Brassica* and cruciferous species (Fig 4.7). The RPC fractions produced the greatest amount of tissue damage (Fig. 4.8). All brassica species except turnip (*B. rapa*) and the two other cruciferous species were observed to be as severely damaged as Antares (Table 4.4). The non-cruciferous species, *N. tabacum* and *P. vulgaris* showed a pattern of less severe disease-like symptoms in comparison, although *P. vulgaris* developed severe disease-like symptoms (mean = 2.0) when inoculated with A16 RPC fraction 2.

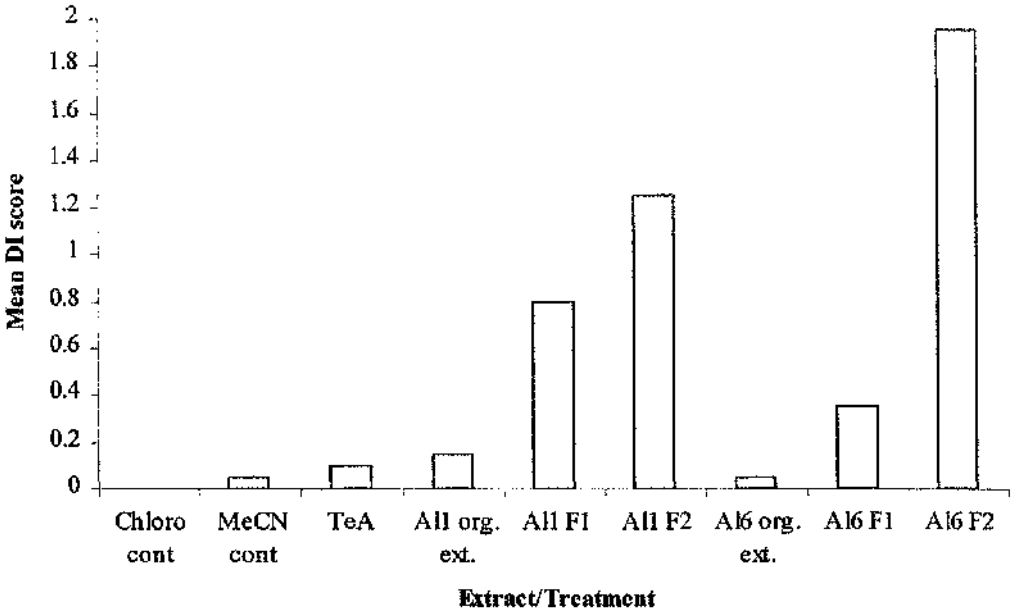


Fig. 4.6 Mean disease-like symptoms caused by extracts from culture filtrates of A. linicola *on four linseed cultivars. SED = 0.144 (df = 144).*

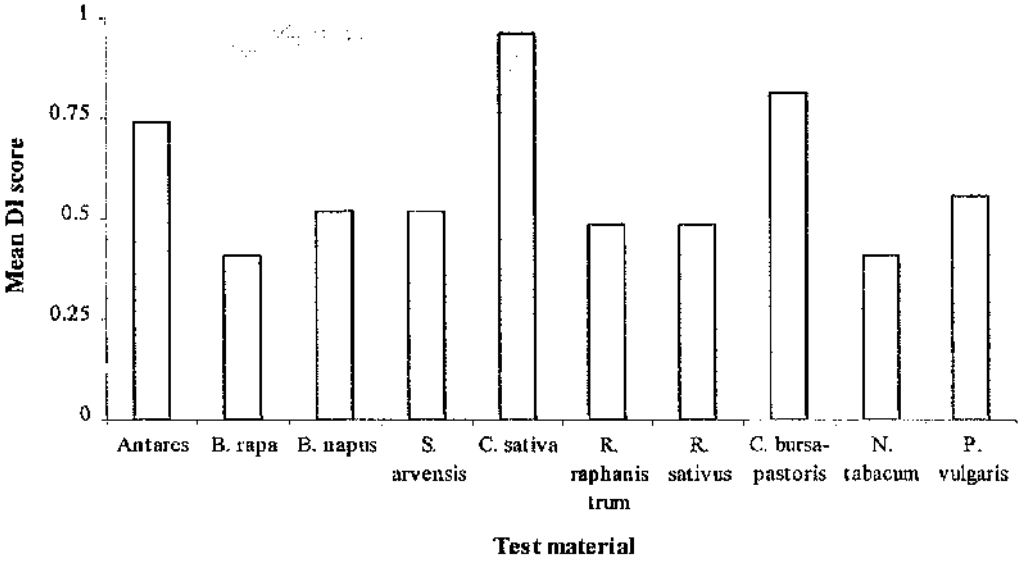


Fig. 4.7 Mean disease-like symptoms caused by nine extract or toxin treatments on nine non-host plant species of A. linicola *and the host cultivar Antares. SED = 0.1304 (df = 180).*

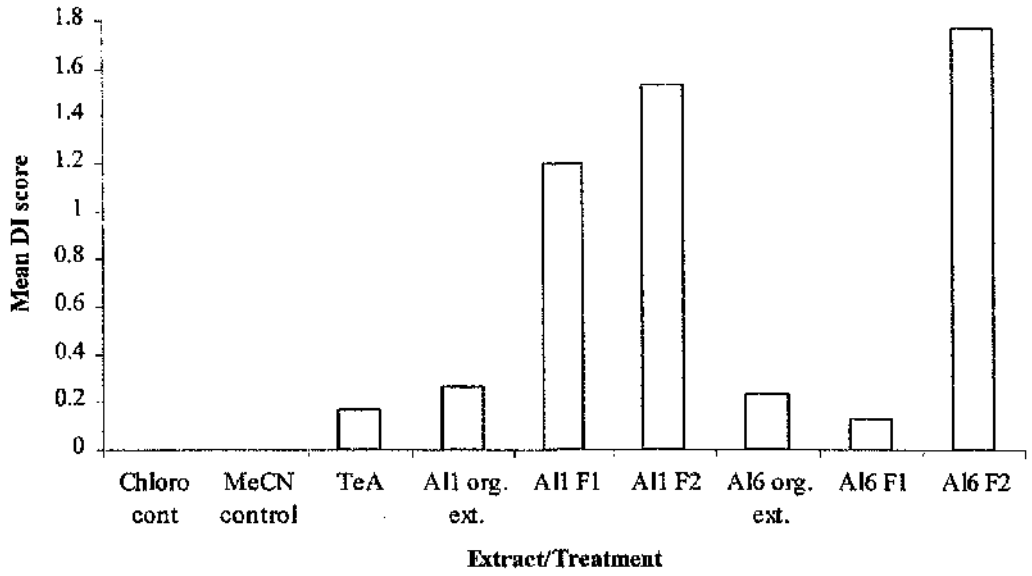


Fig. 4.8 Mean disease-like symptoms caused by extracts from culture filtrate of A. linicola *on nine non-host plant species and the linseed cultivar Antares. SED = 0.1304 (df = 180).*

	Treatment								
Species	MeCN cont	Chloro cont	TeA	Al1 org. ext.	Al1 F1	Al1 F2	Al6 org. ext.	Al6 F1	Al6 F2
Antares	0	0	0	0	1	2.33	0.33	0.33	2.66
B. rapa	0	0	0.33	0.33	0.33	1	0.66	0	1
B. napus	0	0	0.66	0.33	0.66	1.33	0	0	1.66
S. arvensis	0	0	0.33	0	1.33	0.66	0	0.33	2
C. sativa	0	0	0	1.33	2.33	3	0	0	2
R. raphanistrum	0	0	0.33	0	1	1	0	0	2
R. sativus	0	0	0	0	1.33	1.33	0.33	0	1.33
C. bursa-pastoris	0	0	0	0.66	2	2.33	0	0.33	2
N. tabacum	0	0	0	0	1	1.33	0	0.33	1
P. vulgaris	0	0	0	0	1	1	1	0	2
SED = 0.39 (df = 180)									

Table 4.4 Disease-like symptoms caused by fractions of crude extracts of A. linicola culture filtrate on nine non-host plant species and Antares.

4.3.4 Purification and phytotoxic activity of metobolite extract from *Alternaria linicola* isolate A16

Analysis of preparative plates

Analytical t.l.c. plates of the products of the purification process indicated that much of the material scraped from the original preparative plates had been lost during the extraction. Bands were observed at the correct R_F value for each scraped band but fluorescence was very faint (data not shown).

Severity of disease-like symptoms caused by purified metabolites

Disease-like symptoms observed on the treated cotyledons were slight and there was no significant difference between damage caused by the different metabolite bands alone ($df = 117$, $P = 0.451$). Only one cotyledon inoculated with metabolites from band 10 ($R_F = 0.85 - 0.89$) showed signs of toxicity, achieving a score of 1. Severe disease-like symptoms were observed for both the destruxin compounds A and B which achieved mean scores of 3.8 and 2.7 respectively, ensuring a large significant difference in comparison with the other treatments ($df = 135$, $P < 0.001$)

4.3.5 Purification and characterisation of metabolites produced *in vitro* by *Alternaria linicola* isolate A16 by two dimensional t.l.c. and proton nmr

2D-t.l.c. results

Details of the four metabolite bands which corresponded to the standards TeA, AME and the two destruxin compounds are given in Table 4.5. Two compounds, Altlin 1 and Altlin 2 produced the characteristic R_F values of the *Alternaria* phytotoxin compounds TeA and AME respectively. Altlin 3 and Altlin 4 produced R_F values which corresponded to the standards destruxin A and B respectively in the first dimension only (SS1; solvent system 1). Movement of the two compounds by SS2 (solvent system 2) was not exactly matched to that observed for the standard compounds.

Compound	Weight extracted (mg)	R_F value in the 1st dimension (SS1*)	R_F value in the 2nd dimension (SS2†)
Altlin 1	1.8	0.19-0.23	0.23-0.25
Altlin 2	2.4	0.79-0.81	0.68-0.74
Altlin 3	2.4	0.65-0.68	0.34-0.38
Altlin 4	2.1	0.70-0.74	0.38-0.42
TeA		0.2	0.25
AME		0.8	0.72
Destruxin A		0.68	0.1
Destruxin B		0.74	0.57

Table 4.5 Weight and R_F values of four metabolites purified from crude extracts of isolate Al6 with the comparative R_F values of four pathotoxins commonly produced by Alternaria *spp. (* SS1; 7:3 dichloromethane : acetone; † SS2; 95:5 chloroform : methanol.*

Confirmation of compound structures by proton nuclear magnetic resonance spectroscopy

Altlin 1 was confirmed as TeA. The ^{1}H-nmr spectrum for TeA in deutero-chloroform showed a complex multiplet (δ 0.9-1.5 ppm) for the alkyl side chain, a multiplet at δ 3.9 ppm for the proton next to nitrogen and a broad singlet at δ 2.1 ppm for the methyl group (Cole & Cox, 1981). [δ_H ($CDCl_3$) 0.9-1.5 (9H, m, H-6 - H-9), 3.95 (1H, m, H-5), and (3H, br s, CH_3) ppm] The nmr spectrum for Altlin 1 and the chemical structure of TeA are given in Appendix 2.1.

Altlin 2 was confirmed as AME by ^{1}H-nmr spectroscopy. The ^{1}H-nmr spectrum of AME in deutero-acetone showed a singlet at δ 2.77 ppm corresponding to the aromatic methyl group, a singlet at δ3.58 ppm for the methoxyl group and a complex multiplet at around δ 7.6 ppm attributable to the four aromatic protons (Cole & Cox, 1981). [δ_H (d_6-acetone) 2.77 (3H, s, Ar-CH_3), 3.58 (3H, s, OCH_3) and 7.6 (4H, m, Ar-H) ppm]. The nmr spectrum for Altlin 2 and the chemical structure of AME are given in Appendix 2.2.

The ^{1}H-nmr spectra for Altlin 3 and Altlin 4 indicated that the two compounds showed the typical features of destruxin-type compounds, for example the large signals at δ 5 ppm (Gupta *et al.*, 1989). Altlin 3 and Altlin 4 showed distinct similarities with the nmr spectra of destruxin A and B respectively. However, the poor quality of the purified fractions and the complexity of destruxin group of compounds prevented complete characterisation of the two samples. The nmr spectrum for Altlin 3 and Altlin 4 and the chemical structures of destruxin A and destruxin B are given in Appendices 2.3 and 2.4, respectively.

4.4 Discussion

Extraction procedure

The first step towards the successful isolation and characterisation of fungal metabolites such as phytotoxins is to achieve a reliable and effective extraction technique. During this study, the semi-preparative reverse phase chromatography method produced cleaner samples than were produced by the classical organic solvent extraction system. Chromatographic techniques have been the preferred method of sample preparation for phytotoxic compounds for a number of years because of the ability of the C_{18} substrate to bind high and mid-polarity compounds such as tenuazonic acid (TeA) and destruxin compounds, respectively (Samuels *et al*, 1988; Buchwaldt & Jensen, 1991 Buchwaldt & Green, 1992). The advantage of this particular method arises from the subsequent flushing of the bound sample with a high polarity solution which in effect allows the semi-purification of the target compound(s).

Range of metabolites produced by Alternaria linicola

As a genus, the *Alternaria* are known to produce a wide range of secondary metabolites. In this respect, *A. linicola* does not appear to be an exception. Although only four isolates were extracted and compared during the first of the phytotoxin studies reported here (4.3.1), the differences in banding patterns which were observed between isolates indicated variation in their ability to produce metabolites *in vitro*. Similar variation in the spectrum of toxic metabolites that an isolate is able to produce *in vivo* could account for the differences in virulence observed between isolates of the pathogen as observed in section 2.3.1. Quantitative differences between metabolites produced *in vitro* and those produced *in vivo* during natural infection may also account for this effect.

Among the many bands produced, the *A. linicola* isolates were observed to produce at least one, and usually two, of the standard host non-specific toxins; tentoxin (TT), TeA and/or alternariol monomethyl ether (AME). Thus isolates produced a complex of compounds, some of which were the non-host specific toxins, TT, TeA and AME and some uncharacterised metabolites. Cotty & Misaghi (1984) suggested that the ability of many *Alternaria* spp. to produce a number of host non-specific compounds provides the pathogens with an ecological advantage in that they would be able to infect a wider range of host species and would be less limited by habitat availability. If this argument was extended further to consider differences in

the ability of a pathogen to infect different cultivars of a species, a range of phytotoxic metabolites may provide the pathogen with a greater ability to overcome the hosts defences and undoubtedly account for some forms of cultivar resistance.

Alternaria spp. have been observed to produce a wide range of host specific and host non-specific phytotoxins (see section 4.1) and many of the former have been implicated as determinants of pathogenicity (Yoder, 1980). It is suggested that many of the compounds observed during the present study could collectively produce a synergistic effect in terms of relative toxicity to the host in that the action of one compound is enhanced by the action of a second toxic compound resulting in a greater toxicity effect than the summed effect of the two individual compounds. A similar example of such an interaction was observed by Degenhardt (1978) who isolated and partially purified two groups of phytotoxins from *A. brassicae* and *A. raphani*. These unidentified, ninhydrin-positive, high molecular weight compounds were not observed to show host specific activity independently, but produced increased toxicity on *Brassica* species through a synergistic interaction.

Unfortunately, as standards for host specific compounds usually associated with pathotypes of *A. alternata* (e.g. AK-, AF-, AT-, AM-, ACT-, ACR- or AAL-toxins) were not available for comparison during the present study, the presence of formally characterised host specific toxins was not determined. This problem was confounded as insufficient quantities of the uncharacterised compounds could be extracted to allow bioassays to be carried out on susceptible and resistant cultivars and lead to subsequent characterisation. Also the groups of unknown compounds within the semi-purified bands produced during the work in 4.3.4 were either not phytotoxic or, perhaps more likely, were not at concentrations which were high enough to produce phytotoxicity. However, the possibility that *A. linicola* produces host specific toxin should not be discounted at this point and the presence of such a compound(s) could possibly explain the apparent limited host range of the pathogen.

Variation in metabolite banding pattern between different Alternaria *species*

A comparison of extracts of two *A. linicola* isolates (A11 and A16) with extracts from isolates of *A. solani*, *A. brassicae* and *A. brassicicola* (4.3.2) indicated variation in the number and relative amounts of metabolites produced between the four species. Although the study considered only one isolate of each species and did not take into account the possibilities of intra-specific variation between isolates of the species, previous studies have indicated a high

degree of similarity between a range of isolates of both *A. brassicae* and *A. brassicicola* (McRoberts, 1992).

The pattern of compounds produced by *A. solani* showed the largest degree of homogeneity with the metabolite profiles observed from the *A. linicola* isolates with eight of the ten compounds visualised at 254 nm and ten of the twelve compounds visualised at 366 nm also being produced by either one or both *A. linicola* isolates. The similarity of metabolite spectra produced by the two isolates, and the close morphological similarity between *A. linicola* and *A. solani* may indicate that the two species are closely related within the *Alternaria*. Further evidence of a close phyllogenetical relationship between the two species is suggested from an analysis of the biology of the pathogens. Since *A. linicola* and *A. solani* can be regarded as infecting a narrow range of host species (solely linseed, and a limited number of the *Solanaceae*, respectively) this implies a certain amount of co-evolution between the pathogens and hosts. The two brassica-infecting species examined during the study showed a lower level of homogeneity with respect to the banding pattern of metabolite compounds produced by *A. linicola* isolates.

These results could indicate a closer evolutionary relationship between *A. linicola* and *A. solani* in comparison to *A. brassicae* and *A. brassicicola* through the conservation of a greater number of metabolic pathways between the two large spored, long beaked *Alternaria* spp. Recent evidence indicates that *A.brassicae* and *A. brassicicola* are closely related within the *Alternaria* as sections of nuclear ribosomal DNA from both species were amplified and characterised. Sequences for each species were highly conserved and analysis with *A. alternata* and *A. raphani* suggested that all four members of the genus were closely related (Jasalavich *et al.*, 1995). However, although *A. brassicae* is also classified as a large spored, long beaked *Alternaria* spp., loss of metabolic similarities to *A. linicola* and *A. solani* may have occurred during the co-evolution and specialisation of this particular pathogen with *Brassica* spp.. Further studies, possibly using the genetic techniques described by Jasalavich *et al.* (1995), would be needed before such a hypothesis could be clarified.

Differences between the metabolite profiles of *A. linicola*, *A. solani*, *A. brassicicola* and *A. brassicae* during the present study broadly agree with a previous report of differences in metabolite production between *Alternaria* spp. Cotty and Misaghi (1984) found that unlike *A. solani*, *A. brassicae* did not produce zinniol in culture and suggested differences in the metabolic pathways between the *Brassicae* infecting species and *A. solani*. Cotty and Misaghi

(1984) also noted that zinniol was not detected in three pathogenic species, *A. alternata*, *A. citri* and *A. raphani* all of which were small spored, short beaked *Alternaria* species. In contrast, zinniol was produced by six of the eight large spored, long beaked *Alternaria* spp. tested. Cotty and Misaghi (1984) suggested that the evolutionary conservation of zinniol production within the large spore-long beaked species of the *Alternaria* was indicative of the importance of the toxin in the pathogenesis of these species on their particular hosts.

Production of common Alternaria *phytotoxins by* Alternaria linicola

Comparison with the standards suggested that both *A. linicola* isolates and the isolates of *A. brassicae*, *A. brassicicola* and *A. solani* produced compounds that co-chromatographed with the host non-specific toxins TT, TeA and AME. TT, AME and TeA have commonly been reported to be produced by many *Alternaria* spp. including the type species *A. alternata* (King & Schade, 1984, also see section 4.1). The timely arrival of the gifts of destruxin A and destruxin B samples (M. Païs) shortly before the onset of the study also allowed extracts to be screened for the presence of destruxin compounds.

Tentoxin

The presence of TT in extracts from isolates of *A. linicola* was not unexpected as TT is a common cyclic depsipeptide of *A. alternata* (described as *A. tenuis* by Templeton, 1972) which is structurally related to the destruxins and also the host specific AM-toxins. Differences in structure, where these compounds show similarities and dissimilarities, are thought to account for subsequent differences in the specific activity of the three toxins on different hosts (Nishimura & Kohmoto, 1983a; Ayer & Pena-Rodriguez, 1987a: Edwards *et al.*, 1987). With this in mind, it could be the case that TT was responsible for the levels of phytotoxicity observed on some of the non-hosts tested during the present study and the higher levels of phytotoxicity observed on linseed and some of the brassica species was due to the host-selective nature of the destruxin-type depsipeptide component of the extracts.

During the present study, TT was only detected from culture filtrate of weakly aggressive *A. linicola* isolates A11, 3 and 4 (4.3.1) and was not observed to be produced by A12 and A16 (4.3.1 and 4.3.2), the more aggressive of the isolates tested during the initial cotyledon bioassay reported earlier (2.3.1). Tentoxin is often regarded as being a weak phytotoxin, causing chlorosis through the inhibition of chlorophyll formation, but not necrosis (Fulton *et*

al, 1965) and. indeed, the insensitivity of seedling growth of many plant families was demonstrated by Durbin & Uchytil (1977). *Alternaria alternata* is usually regarded as a saprophyte (with the exception of the virulent pathotypes which produce HSTs). Given that *A. alternata* commonly produces TT it could be suggested that TT is a compound produced by the weaker *Alternaria* and that the three weakly pathogenic *A. linicola* isolates which produced the compound during the present study have retained the TT biosynthetic pathway through their evolution. In contrast the more aggressive isolates evolved to produce much more potent compounds at the expense of the TT biosynthetic pathway.

Altenariol monomethyl ether

The polyketide AME, a reportedly common toxin produced by *A. alternata* isolates (King & Schade, 1984) was observed to be produced by both *A. linicola* isolates (A11 & A16) and by *A. solani*, *A. brassicae* and *A. brassicicola* during 4.3.2 and was subsequently isolated and characterised from A16 (4.3.5). During 4.3.1 however, detectable amounts could only be extracted from A11 using reverse phase chromatography and the compound was not detected in culture filtrate from A12 - 4. The absence of the compound from extracts of these three isolates may be an artefact of the single, 30 day sampling time used during the experiment; Wei & Swartz (1985) found that maximum production of AME by *A. alternata* in synthetic liquid culture occurred at 10-14 days. However, the same authors found that *A. brassicae* did not produce detectable levels of AME until after 20 days of culture. Wei & Swartz (1985) suggested a difference in behaviour of the two species in culture. Thus *A. linicola* may, like *A. brassicae*, produce the compound at a later stage of culture and by 30 days of culture, A11 had produced just enough of the compound for detection by t.l.c..

Tenuazonic acid

As mentioned previously (4.1) TeA is generally regarded as the most important toxin produced by the *Alternaria* mainly due to its widespread occurrence and toxicity to plants, animals and insects (King & Schade, 1984). As the compound appears to be a ubiquitous metabolite of the *Alternaria* (Kinoshita *et al.*, 1972; Steele & Mirocha, 1971), the results of the present study, where TeA was observed to be produced by all *Alternaria* isolates tested suggest an important role for the compound in the life cycle of the *Alternaria*. However, the widespread occurrence of TeA among the *Alternaria* and thus its lack of specificity would exclude the compound from the status of being a determinant of pathogenicity. This not withstanding, all of the

evidence in the case in *A. linicola* suggests that TeA may be involved in determining virulence levels on linseed cultivars, possibly through synergistic effects with other metabolites (e.g. AME, TT or the destruxin type-compounds).

Destruxins

Compounds which co-chromatographed with the destruxin B sample were observed for *A. linicola* isolates Al1 and Al6 and for the isolates of *A. solani* and *A. brassicae* (4.3.2). The further extraction of Altlin3 and 4 from isolate Al6, and confirmation of the identity of the two compounds as cyclodepsipeptide molecules (possibly destruxins A and B, respectively) by ^{1}H-nmr (4.3.5) means that *A. linicola* certainly produces destruxin-type compounds *in vitro*. Whether the destruxin compounds are produced *in vivo* in the case of the *A. linicola*/linseed interaction was not ascertained.

A number of destruxin compounds, including destruxin B, have been implicated in *Alternaria brassica*/oil seed rape interactions. Ayer & Pena-Rodriguez (1987a) and Bains & Tewari (1987) first extracted and identified destruxin B from cultures of *A. brassicae*. Bains & Tewari (1987) described destruxin B as a host-specific phytotoxin since their studies indicated that the toxin only caused symptoms on *Brassica* spp. Unable to reproduce the results of Bains and Tewari (1987) and following detailed studies, Buchwaldt & Jensen (1991) and Buchwaldt & Green (1992) suggested the compound was better described as being host-selective. This definition was considered more acceptable as toxicity was observed on a wider range of species including non-hosts such as *Nicotiana tabacum*, *Solanum tuberosum* and *Triticum aestivum*, but extreme phytotoxicity was only observed on the *Brassicae*.

The destruxin compounds are not unique to *Alternaria* spp. and should not be regarded solely as phytotoxins (Kodaira, 1961; Païs *et al.*, 1981). This not withstanding, this is the first report of a destruxin type compound being isolated from *A. linicola* which is only the second *Alternaria* spp. from which the compounds have been isolated and, in this case, partially characterised. Whether the compounds played an integral part on the determination of the pathogenicity of *A. linicola* on linseed was not elucidated during this study. One of the reasons for this was that a study of the *in vivo* production of toxin compounds by the pathogen was inconclusive. During the study, successful infection of whole seedlings was not achieved due to a loss of pathogenicity in the isolates used during the study (Evans, unpublished results). However the similarity of the results of the present study (4.3.3) with the results of

Buchwaldt & Green (1992) would suggest a possible role for destruxin-type phytotoxins in the pathogenicity of *A. linicola* on linseed and may account for the higher aggressiveness of *A. linicola* to linseed than is observed for the commonly isolated species *A. alternata* and *A. infectoria*.

Phytotoxicity of metabolites extracted from Alternaria linicola

During the study of phytotoxicity of crude extracts on non-host species and Antares (4.3.3), differences in the levels of the production of destruxin-type compounds such as destruxin B may have accounted for the differences which were observed in the phytotoxicity of the extracts from Al1 and Al6. The extracts (particularly the RPC mid-polarity second fraction) achieved a high phytotoxicity score on linseed which suggested a level of host-selectivity for the active component of the extract. Although the phytotoxicity scores of linseed were similar to those of the Brassica and crucifer species tested, there was a significant difference between the phytotoxicity scores of linseed and the non-hosts bean and tobacco, neither of which generally showed such high phytotoxicity scores.

Before the phytotoxic effect of the crude and semi-prepared extracts was tested (4.3.3), dilution of the samples was carried out to a concentration of 5 $\mu g\ ml^{-1}$ of extract in solvent. Considering the comparative differences in purity of the samples as outlined above, fraction 2 therefore contained less impurities and more metabolites per unit volume in comparison with the organic extract and fraction one and this may account for the higher phytotoxicity index. Similarly, the extremely high phytotoxicity index of the second fractions from the two isolates relative to the TeA standard was probably due to the synergistic effect of the various compounds in the extract in comparison with the individual mode of action of the purified standard. These observations highlight two important points. Firstly, as pointed out by Scheffer & Briggs (1981), in practise the culture fluids of almost any micro-organism can be toxic to plants and it is whether the constituents of such an extract are, or can be shown to be involved in disease causation which is the most important, and inevitably, hardest question to answer in toxin studies. The second problem concerns the problem of working with crude and semi-preparative extracts, where the exact concentration of the components of the extract, and also the respective molarities of the component compounds, are not known. Unfortunately these two statements seem interlinked, in that, during the present study, the individual testing of the constituents of the crude extracts was not possible as the precise constituents of the extract were not known

Phytotoxicity on cultivars of the host species

Comparing the phytotoxic effect of the crude and semi-preparative extracts on linseed cultivars (4.3.3) there was no significant difference between cotyledons of Antares, McGregor, Barbara and Linda. The lack of differences appears surprising considering the differences between the four cultivars during the cotyledon bioassay study (2.3.1) especially with respect to the differences in aggressiveness between A11 and A16. However, the constituents of the crude extract were not known and the chances of a toxic compound which was cultivar-specific acting alone (if, indeed such a compound were present) would be minimal. The biology of the *Alternaria* in general would suggest that many components of pathogen-host interactions have a role in the response of the host to the pathogen. As a result, the influence of one specific component, in this case phytotoxin activity, may not differentiate differences in resistance that may exist between a set of relatively genetically uniform cultivars A phytotoxin bioassay using a more diverse set of cultivars, from different ends of the resistance spectrum demonstrated in 2.3.2 for example, may have produced phytotoxic differences between the cultivars.

Buchwaldt & Jensen (1991) observed that the second fraction of the reverse phase chromatography extraction method contained the majority of destruxin compounds and Buchwaldt & Green (1992) confirmed that this fraction produced the highest phytotoxicity index scores on hosts. Differences between the phytotoxicity of the crude and semi-preparative extracts were observed during the present study. The second fraction produced by reverse phase chromatography produced significantly more toxigenic effects on the cotyledon material. This, along with the intensity and clarity of the metabolite bands on t.l.c. plates (4.3.1) indicated that metabolite bands of these fractions from A11 and A16 were brighter and sharper. The phytotoxicity bioassay provides more evidence that this method was more efficient at extracting the active components from the culture medium.

Phytotoxicity on non-host species

When tested on a range of non-host species (4.3.3), the crude and semi-preparative extracts caused levels of damage to the tissue which, in the majority of cases, were comparable with those observed on linseed. Significant differences were observed, however, particularly in the mean levels of phytotoxicity which were caused on *B. rapa* and *C. sativa* in particular which

were significantly less affected and more affected than linseed, respectively. The level of phytotoxicity observed on *C. sativa* was high in comparison with published work where *C. sativa* was regarded as being resistant to *A. brassicae* during cotyledon inoculation studies by Tewari (1991). However, the results of Buchwaldt & Green (1992) agreed with the results reported here, as they indicated that although *C. sativa* was relatively resistant to *A. brassicae* during infection studies, the destruxin B sample was extremely phytotoxic to *C. sativa*. This observation also provides further evidence that multiple determinants of pathogenicity occur in *Alternaria*/host pathosystems.

The role of phytotoxins in the A. linicola*/linseed interaction*

If the range of compounds produced *in vitro* during the present study were produced *in vivo* during pathogenesis, the fact that metabolites in the *A. linicola* extracts appear to show a level of host-selectivity may explain differences in disease resistance among linseed accessions, some being more susceptible to toxins than others, depending on the breeding history of a particular line. This would also provide further evidence that the interaction between pathogen and host in this pathosystem, as in other *Alternaria* pathosystems, is multi-component in nature and that the mechanism may even operate at the cultivar level. However, further work would be needed in this area of the *A. linicola*/linseed interaction before the true nature of the effect of phytotoxins produced by the pathogen could be assessed.

The phytotoxicity of the crude extracts on the non-host species showed differences in the response of the material to the extracts. The linseed cultivar Antares achieved the highest phytotoxicity score for the reverse phase chromatography second fraction from isolate Al6 and also scores highly for the second reverse phase chromatography fraction from the non-aggressive isolate Al1. Many of the *Brassica* spp. achieved a similar high score to the host species suggesting the presence of a non-host specific or host-selective compound(s) in the extracts. The infection of non-host species with the pathogen was not investigated during the present study. However, Buchwaldt & Green (1992) found that phytotoxicity scores for destruxin B on the host species and on non-host species followed the same pattern as inoculation studies with *A.brassicae* but generally returned a higher score. The high phytotoxicity scores in relation to actual infection scores were not explained by the authors although the most probable reason would be that the destruxin B sample being tested was at a higher concentration than occurred *in vivo*. A similar concentration difference may possibly explain the lack of phytotoxicity in 4.3.4 when no disease-like symptoms were observed on

cotyledons of Antares as much of the metabolite material was lost during the purification process. Further purification of the extracts using one of the many HPLC protocols which have been published for *Alternaria* metabolites would perhaps have produced much cleaner compounds that would then have been easier to bioassay and ultimately characterise (Buchwaldt & Jensen, 1991; Gupta *et al*, 1989; Païs *et al*., 1981).

As already stated, the results of the present study indicate the presence of a number of host non-specific metabolites and other uncharacterised metabolites which, it was suggested earlier, may act synergistically on the host plant. Cotty & Misaghi (1984) suggest that in the case of *Alternaria* species such as *A. solani*, the broader host range and relative persistence of the pathogen through time is conditioned by the pathogen's ability to produce a number of non-specific toxins as opposed to a single host-specific toxin. The result of the action of a number of host non-specific toxins as opposed to a single (or a few) host-specific toxins would be to reduce the selection pressure on the host genotype as the infected plant would be less diseased. Although the evolutionary interactions between pathogen and host are probably more complex than the suggestions of Cotty & Misaghi (1984) allow, this may slow the evolutionary process in the pathogen. In effect, this would prolong the number of seasons over which growers continue to plant a specific cultivar as disease levels from year to year are percieved as being acceptable. The pathogen would be able to infect the particular host cultivar without further genetic change within the pathogen population. In this case, the balance between the pathogen and host would effectively be closer to a status of equilibrium, whereas, the action of an HST usually renders the host extremely susceptible. If such a scenario exists in the interaction between *A. linicola* and linseed, which has only been cultivated on a moderate to large scale in the UK in recent years, selection pressure on isolates of the pathogen would be small. This may account for the fact that the cultivars which have been grown have remained fairly constant with no major introductions. Thus the pathogen has been equipped with the correct toxins to potentially inhabit the crop given conducive environmental conditions.

Chapter 5

5.0 Phytoalexin production by the linseed host

5.1 Introduction

The origins of phytoalexin research

The term phytoalexin was defined by Müller & Börger (cited by Deverall, 1982) in 1940 from the greek for "warding-off agents in plants". Subsequent studies have established that the elicitation of phytoalexins is one of the major causes of reduced pathogen development in many pathosystems. Paxton (1981) defined phytoalexins as being anti microbial compounds of small molecular weight that accumulate after infection. The Angiosperms universally appear to be able to synthesise phytoalexins to one degree or another and correlation between the cessation of fungal development and the accumulation of phytoalexin compounds to fungistatic levels provided initial proof of a fundamental role in non-specific resistance (Bailey & Mansfield, 1982; Heath, 1991).

However, a role for only a few phytoalexins has been unequivocally proven to date, the most well defined example being the case of pisatin produced by pea (*Pisum sativum*). Van Etten *et al.* (1989) observed that the pathogen *Nectria haematococca* was unable to successfully invade pea in the absence of a P-450 monooxygenase which allowed the pathogen to detoxify the pisatin elicited by the pea. Low virulence isolates on pea were invariably highly sensitive to pisatin *in vitro* and were found to be deficient in pisatin demethylase activity. High virulence segregated with high pisatin demethylase activity and insensitivity to pisatin *in vitro*. Van Etten *et al.* (1989) suggested that this indicated that pisatin was physiologically important as a resistance mechanism in pea against *Nectria haematococca* unless the pathogen had the ability to detoxify the defence compound.

Occurrence and diversity of phytoalexins

Although it is generally acknowledged that Angiosperms produce phytoalexins, there is a wide array of chemical structures among the compounds and these differ in concentration and form between different species and plant groups. Stoessl (1980) observed that the wide array of compounds described as phytoalexins were produced by a multitude of biosynthetic pathways and that although single plants usually produce only a small number of compounds, the type of compounds, and the biochemical pathways through which they are produced, are often common amongst related plant groups. However, exceptions to Stoessl's observation have been reported in that identical phytoalexins have been found in species from distantly related

taxa, for example, the *Fabaceae* and *Zingiberaceae* (Kumar *et al.*, 1984). Indeed, even phytoalexins produced by different species within a genus (e.g. medicarpin, a phytoalexin reported in species of the genus *Melilotus* [Ingham, 1977]) may be produced quite differently at the molecular level. An example of this is observed in two cultivars of French bean (*Phaseolus vulgaris*). Both accumulate isoflavonoid phytoalexins on exposure to an elicitor from the cell wall of the bean pathogen *Colletotrichum lindemuthianum*. However, distinct differences are observed in the number of genes expressed by, or the relative number of transcripts from, members of two gene families. These genes code for key enzymes produced during the biosynthetic pathway which lead to isoflavonoid phytoalexin production (Ellis *et al.*, 1989).

Phytoalexin elicitation by pathogenic Alternaria *species*

There are a number of reports of phytoalexins being produced by crop species in response to infection by members of the *Alternaria*. Dorozhkin *et al.* (cited by Rotem, 1994) reported that the production of rishitin and lyubimin by potato (*Solanum tuberosum*) conferred immunity to infection by *A. solani*. Kulshreshtha & Chauhan (1985; 1987) reported the detection of phytoalexins in raddish (*Raphanus sativus*) infected with *A. alternata* and sesame (*Sesamum indicum*) infected with *A. sesami*.

Work by Tewari's group on the interaction of the *Alternaria* with members of the *Cruciferae* has produced firm evidence of phytoalexin production in response to *A. brassicae* (Conn *et al.*, 1988). Following the infection of a range of cruciferous and brassica species with *A. brassicae*, Conn *et al.* (1988) reported the isolation of a range of phytoalexin compounds including brassinin and cyclobrassinin. The authors suggested that differences in the qualitative and quantitative levels of production of the phytoalexin compounds may have accounted for the differences observed in the susceptibility of the test species to *A. brassicae*.

Conn *et al.* (1988) reported that turnip (*Brassica campestris* ssp. *rapifera*) was less susceptible to infection by *A. brassicae* and was found to produce more phytoalexins than two rape cultivars (*Brassica campestris* ssp. *oleifera*). Also, the closely related non-crop species *Capsella bursa-pastoris* and *Camelina sativa* were resistant to *A. brassicae*, and these species were found to produce a wider range of phytoalexins, including two metabolites not produced by the turnip or rape cultivars. Conn *et al.* (1988) hypothesised that the production of one or more of the *C. sativa* and *C. bursa-pastoris* specific metabolites was associated with the higher

level of resistance of these two species to *A. brassicae* in comparison with the other crucifers tested. From further work, two novel indole phytoalexins, camalexin and methoxycamalexin were subsequently isolated from *C. sativa* (Browne *et al.*, 1991). Jejelowo *et al.* (1991) observed that large quantities of the two compounds were produced, even when only very few conidia were placed on the *C. sativa* leaf surface. Jejelowo *et al.* (1991) found that the concentration of phytoalexins produced by *C. sativa* in response to inoculation with *A. brassicae* increased linearly with conidial concentration and suggested that this was due to a similar increase in the concentration of the fungal elicitor molecules present within the inoculation droplet. The authors concluded that the phytoalexins slowed germination and inhibited germ-tube growth of *A. brassicae in vitro*.

Phytoalexin production by Linum *species*

Littlefield (1973) examined phytoalexin production in the flax/flax rust (*M. lini*) pathosystem and observed that the effect of the defence compound was restricted to the immediate area of infection. Littlefield (1973) suggested that a close relationship existed between the development of the pathogen on the surface of the leaf and the elicitation of phytoalexin production and also observed that phytoalexin accumulation was always much more rapid and occurred to a higher concentration during an incompatible non-host/host-pathogen interaction in comparison to a compatible interaction (Keen & Littlefield, 1978). Keen later used the flax/rust pathosystem to develop a facilitated diffusion technique which permitted the rapid extraction of phytoalexins from plants such as flax and soybean (Keen, 1978).

Initial elicitation and detoxification

There are many reports that the production of, or the action of, phytotoxins elicit the onset of phytoalexin biosynthesis (Anderson, 1991). Tewari (1991) suggested that phytoalexins were elicited in response to the production of destruxin B in the *A. brassicae*/crucifer interaction. However, there are studies which provide strong evidence that in some interactions, phytotoxin production by the pathogen suppresses host defences. Toxin production by the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* directly suppressed phytoalexin production in the bean host (Gnanamanickam & Patil, 1977). Good evidence from the *Alternaria* pathogens suggests a similar role for the host specific AK toxins of the pear infecting pathotype of *A. alternata* (Hayami *et al.*, 1982; Nishimura & Kohmoto, 1983b; Kohmoto *et al.*, 1987).

Recently, phytoalexin detoxifying genes have been isolated from certain pathogens (Schäfer *et al.*, 1989) which, for the first time the 50 years of phytoalexin research, provided indirect proof that phytoalexins play a central role in some non-specific plant/pathogen interactions. Schäfer *et al.* (1989) isolated the pisatin demethylase genes from the pea pathogen *Nectria haematococca* and transferred these into the maize pathogen *Cochliobolus heterostrophus*. Recombinant *C. heterostrophus* was found to be pathogenic on maize and pea which suggested resistance to phytoalexins (the ability to detoxify pisatin in this case) to be important in pathogenicity. It is also interesting to note that this work demonstrates evidence of a common mechanism observed during host, and in this case, non-host resistance.

Genetic control of phytoalexin biosynthesis

Recent advances in biotechnology have allowed the isolation and characterisation of genes involved in phytoalexin biosynthesis and have also provided the potential for the transfer of phytoalexin genes from producers to non-producers (Fischer & Hain, 1994). For example, genes from grapevine which code for stilbene synthase, an enzyme involved in the biosynthesis of the stilbene phytoalexin resveratrol, have been isolated and inserted into the tobacco genome. Following inoculation with a suitable pathogen, the transgenic tobacco plants were found to produce resveratrol although not at levels equivalent to those found in the original grapevine (Hain *et al.*, 1990). Subsequent analysis indicated an explanation for the lower expression levels of the phytoalexin; 6-8 stilbene synthase genes occurred in the grapevine, whereas only one of these had been transferred to the transformed tobacco (Hain *et al.*, 1993). Similar success has been achieved with the expression of a trichodiene synthase gene from *Fusarium sporotrichioides* in transgenic tobacco (Hohn & Ohlrogge, 1991). Although from a fungal source, trichodiene synthase, a sesquiterpene cyclase, has been shown to be involved in the biosynthesis of cyclic sesquiterpenoids which are defensive compounds which are found in plant, fungi and insects. The authors suggest that the system may provide a model for the study of a role for novel sesquiterpenoids in disease resistance (Hohn & Ohlrogge, 1991).

Utilising phytoalexin production during breeding for disease resistance

Tewari (1991) and Tewari & Conn (1993) suggested that it should be possible to transfer the phytoalexin mediated multiple component resistance observed in crucifers in response to *A.*

brassicae from resistant to susceptible genotypes through either conventional breeding or biotechnological techniques. Following work on phytoalexin production by *L. usitatissimum*, Littlefield (1973) similarly suggested that the phytoalexin interaction in response to *M. lini* was one of a multiplicity of factors which were involved in disease resistance, and that breeding using phytoalexin production as a marker for selection may be possible. Evidence from the literature would suggest that, as a component of a quantitative resistance mechanism, a phytoalexin mediated resistance response should be heritable and show a good response to selection (Simmonds, 1991).

The aim of the study was:

1. To investigate the *in vivo* production of phytoalexin compounds by *Linum* accessions inoculated with *A. linicola* and the rust pathogen *M. lini*.
2. To compare extracted compounds to the common phytoalexin coniferyl alcohol.
3. To assess the fungitoxic nature of the inoculated leaf extracts.

5.2 Materials and Methods

5.2.1 The *in vivo* extraction of secondary metabolites following the inoculation of *Linum* species with *Alternaria linicola* and *Melampsora lini*

Seedlings of *L.u.u. albocoeruleum* and *L. usitatissimum* cv. Bison (flax) were grown in the glasshouse to GS 13 as previously described (2.2.1) in deep pots (16 cm diameter). Pots of whole seedlings were sprayed with 20 ml of a conidial suspension of either *A. linicola* (Al6) prepared as previously described (3.3.1), with *Melampsora lini* (race 1, 18,000 conidia ml^{-1}) or sterile distilled water control. The pots were covered in large plastic bags supported on short stakes and were kept well watered in order to maintain a high relative humidity.

After 72 h, seedlings were cut at the base of the stem, weighed and placed in 250 ml buchner flasks. Whole seedlings were extracted using a modified version of the facilitated diffusion technique described by Keen (1978). For each cultivar/treatment, 15 ml g^{-1} 70 % v/v aqueous methanol was added to each flask which was stoppered, evacuated for 30 s using a vacuum pump (Speedivac 2, Edwards, UK) and sealed. Seedlings were vacuum infiltrated for 1 h. The extract was filtered through a single layer of sterile muslin and sterile Whatman No 1. paper before being evaporated to dryness *in vacuo* at 40° C.

Crude leaf extracts were resuspended in 70% (v/v) aqueous methanol to 5 mg ml^{-1}, of which 20 µl of each crude extract and the standard phytoalexin, coniferyl alcohol (CoA, 5 mg ml^{-1}) (Sigma, UK) were spotted onto LK6DF pre-channelled silica gel plates. Plates were developed in a chloroform : methanol (49:1 v/v) solvent system. Two-dimensional t.l.c. was also carried out with 10 µl of each extract using the plate design as described previously (Fig. 4.1). The plates were developed in the first dimension with chloroform : methanol (49:1 v/v) and in the second dimension with dichloromethane : acetone (7:3 v/v). Plates were observed at 254 nm and 366 nm for fluorescence quenching or fluorescent bands respectively.

Plates were sprayed with a thin layer of spore suspension of either *Cladosporium cladosporioides* (~20, 000 blastospores ml^{-1}) or *A. brassicicola* (~10,000 conidia ml^{-1}) in half strength Czapek-Dox liquid culture medium, placed in sealed trays with damp paper towel and incubated at room temperature. Fungal development was examined daily and areas of inhibition were noted and compared to the channel containing CoA and to banding patterns observed under UV radiation.

5.3 Results

5.3.1 The *in vivo* extraction of secondary metabolites following the inoculation of *Linum* species with *Alternaria linicola* and *Melampsora lini*

Extraction and visualisation by t.l.c.

Extract from material infected with Al6, and to a lesser degree, *M. lini*, was observed to be of a darker green/brown colour in comparison with the brilliant green extract produced from uninoculated material. Following separation by t.l.c., a number of quenching bands could be observed at 254 nm (Plate 5.1, Table 5.1). The darkest and most obvious of the quenching bands was that of coniferyl alcohol (CoA) observed at R_F 0.28-0.37 in channel 1, whilst very faint quenching bands were observed at R_F 0.8-0.83 for all of the inoculated material extracts except cv. Bison inoculated with *M. lini* (channel 7).

At 366 nm, many more bands were observed (Plate 5.1). The majority of compounds observed fluoresced with a pink/bright red colour and although there was some homogeneity between the control and inoculated extracts, shared bands were fainter on channels containing extracts from uninoculated material. Table 5.2 indicates that fluorescent bands were not observed for the standard compound CoA, although some quenching was observed at R_F 0.28-0.37 and R_F 0.57-0.62. Quenching was not observed at these R_F values for any of the cvs/treatments, although some red fluorescence was observed at a comparable R_F value (0.33-0.37) for the extract from *L.u.u. albocoeruleum* (LAL) inoculated with Al6.

Cultivar/treatment	R_F value of quenching band				
CoA	0.03	0.12	0.28-0.37*	0.62	
LAL/SDW					
Bison/SDW					
LAL/Al6	0.03-0.06				0.80-0.83
Bison/Al6	0.03-0.06				0.80-0.83
LAL/*M. lini*	0.03-0.05				0.80-0.83
Bison/*M. lini*	0.03-0.05				

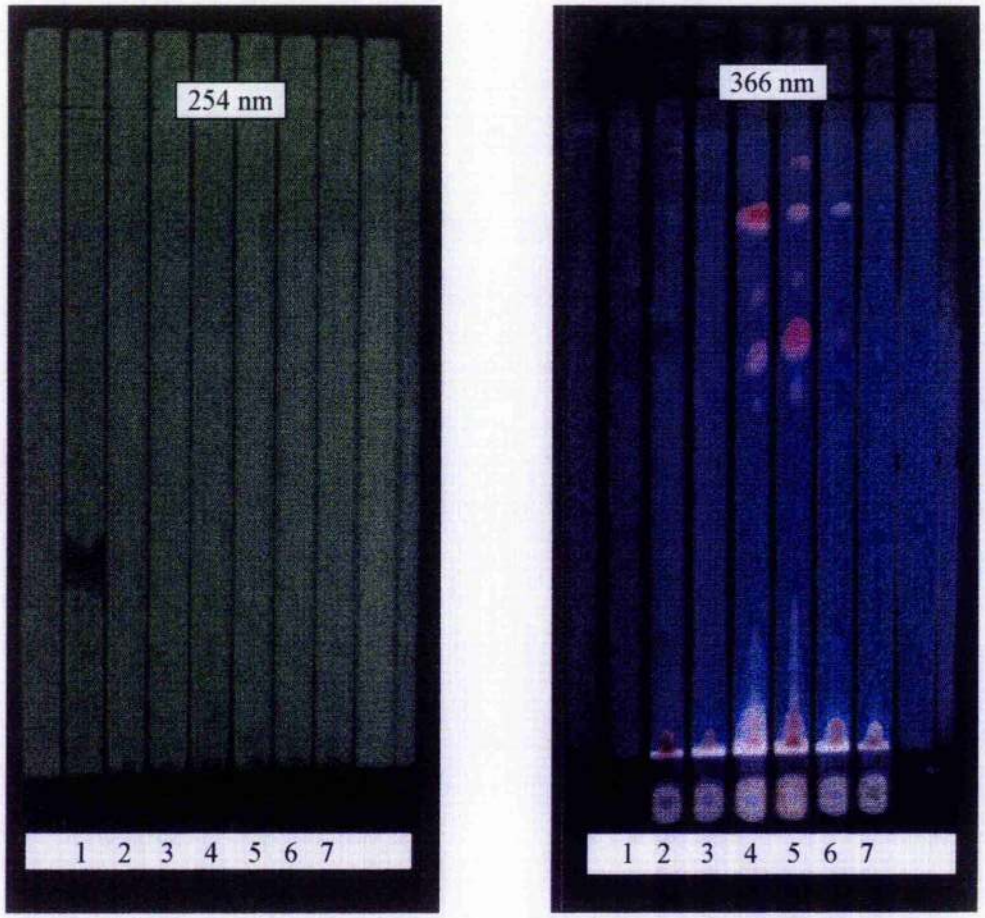
* Very dark quenching

Table 5.1 R_F values of bands quenching fluorescence at 254 nm for extracts of inoculated and uninoculated plants of L.u.u. albocoeruleum *and Bison.*

Cultivar/ treatment	R_F values of bands fluorescing at 366 nm											
CoA						0.28-0.37			0.57-0.62			
LAL/cont	0.01	0.02-0.03	0.04†		0.12†	0.15†		0.53	0.59-0.64		0.82-0.83	0.89-0.93
Bison/cont	0.01	0.02-0.03	0.04†	0.06†	0.12†	0.15†					0.82-0.83	0.89-0.93
LAL/Al6	0.01	0.02-0.03	0.04†	0.06†	0.12†	0.15†	0.33-0.37	0.51-0.55	0.58-0.64	0.68-0.73	0.78-0.84	
	Faint red band 0-0.2											
Bison/Al6	0.01	0.02-0.03	0.04†		0.12†	0.15†	0.44-0.47	0.51-0.55	0.58-0.64	0.7-0.74	0.78-0.84	0.87-0.91
	Faint red band 0-0.47											
LAL/*M. lini*	0.01	0.02-0.03	0.04†	0.06†	0.12†	0.15*		0.52-0.57	0.61-0.66		0.8-0.84	0.89-0.91
	Faint red band 0-0.23											
Bison/*M. lini*	0.01	0.02-0.03						0.55-0.57	0.58-0.66		0.8-0.84	
	Faint red band 0-0.2											

† indicates bright blue coloured band. Boxed figures indicate quenching bands. All other bands were pink/bright red. R_F values given correspond to distinct bands, non-distinct bands are designated by the shaded areas of the table.

Table 5.2 R_F values for bands fluorescing at 366 nm for extracts produced from inoculated and uninoculated linseed material.



Lanes:
1: Coniferyl alcohol
2: *L.u.u. albocoeruleum*/SDW
3: cv. Bison/SDW
4: *L.u.u. albocoeruleum*/Al6
5: cv. Bison/Al6
6: *L.u.u. albocoeruleum*/*M. lini*
7: cv. Bison/*M. lini*

Plate 5.1 Illustrating quenching of fluorescence at 254 nm with a band only being visualised for the standard CoA. At 366 nm, many more metabolites are visualised including many which do not correspond to CoA

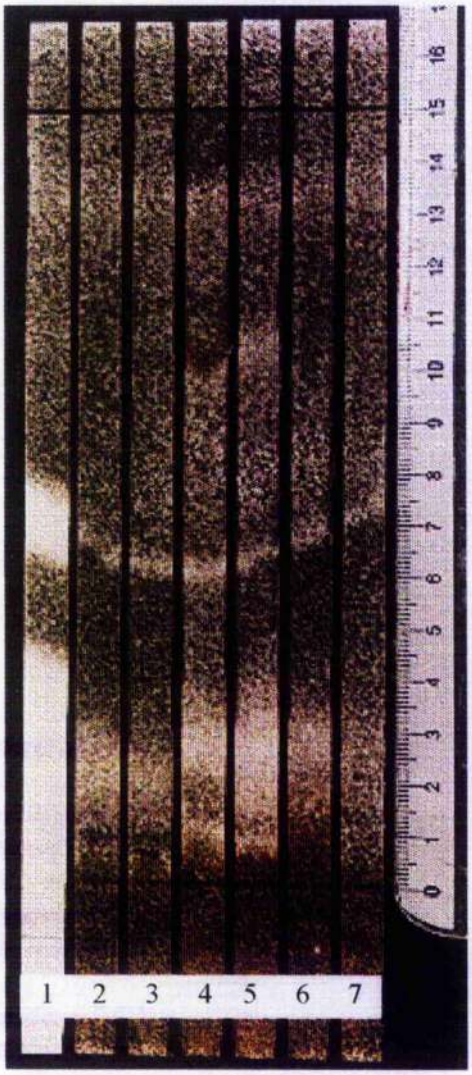
Bioassay for fungitoxic properties

Large areas of inhibition in the growth of *C. cladosporioides* were observed at R_F values corresponding to the fractions of the standard phytoalexin CoA (R_F 0-0.3 and R_F 0.42-0.53) for the extracts of linseed lines inoculated with Al6 and to a lesser extent *M. lini* (Plate 5.2, Table 5.3). Areas of inhibition were also observed on the channels containing extract from uninoculated material (channels 2 & 3, Plate 5.2) but inhibition was less distinct in comparison with those observed for inoculated treatments. Plate 5.2 indicates that other areas of inhibition occurred at R_F values which did not correspond to CoA R_F values. These bands were widespread among the treatments, but again for commonly shared bands, inhibition was always greater on those channels containing extract from material inoculated with Al6 (channels 4 & 5).

Cultivar/treatment	R_F value of zones of growth inhibition				
CoA	0-0.3		0.42-0.53		
LAL/SDW		0.13-0.2	0.42-0.43		
Bison/SDW		0.12-0.2	0.40-0.42		
LAL/Al6	0.06-0.09	0.14-0.24	0.38-0.43	0.52-0.56	0.63-0.67
Bison/Al6	0.06-0.1	0.14-0.24	0.39-0.43	0.52-.57	0.64-0.68
LAL/*M. lini*		0.14-0.24	0.42-0.46	0.52-0.57	
Bison/*M. lini*			0.46-0.5	0.54-0.57	

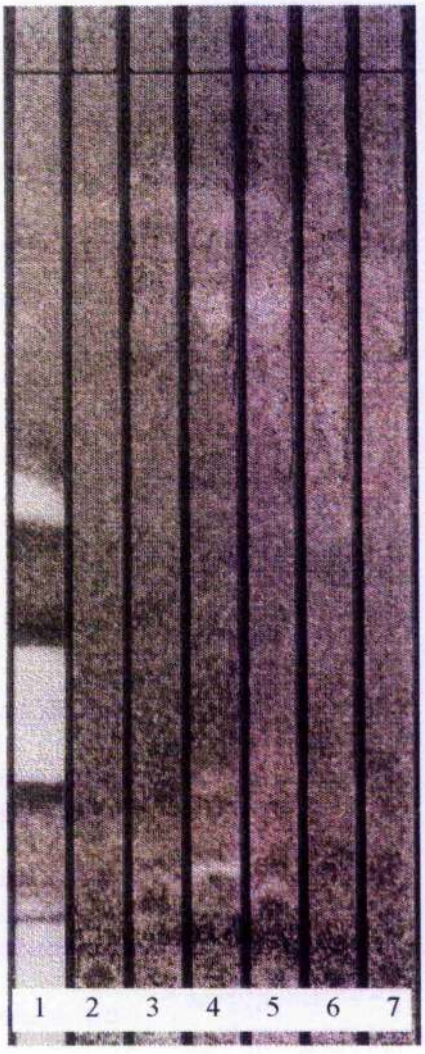
Table 5.3 R_F values of zones of growth inhibition of C. cladosporioides *grown on a t.l.c. plate containing the extracts of inoculated and uninoculated linseed material.*

In comparison with plates sprayed with *C. cladosporioides*, plates sprayed with conidia of *A. brassicicola* were observed to show fewer and less distinct zones of growth inhibition (Plate 5.3, Table 5.4). Zones of inhibition on the channel containing CoA were well defined with inhibition occurring at R_F 0.16-0.31 and RF 0.46-0.52. Corresponding bands were not observed for either of the extracts obtained from uninoculated material. Faint bands of inhibition were observed for both of the extracts obtained from LAL (both Al6 and *M. lini* inoculated, R_F 0.49-0.51 and R_F 0.44-0.51 respectively) and from cv. Bison inoculated with Al6 (R_F 0.47-0.51)(Table 5.4).



Lanes:
1: Coniferyl alcohol
2: *L.u.u. albocoeruleum*/SDW
3: cv. Bison/SDW
4: *L.u.u. albocoeruleum*/Al6
5: cv. Bison/Al6
6: *L.u.u. albocoeruleum*/*M. lini*
7: cv. Bison/*M. lini*

Plate 5.2. Inhibition of growth of Cladosporium cladosporioides *by components of crude leaf extracts from* Linum *material inoculated with conidia of Al6 and* M. lini.



Lanes:
1: Coniferyl alcohol
2: *L.u.u. albocoeruleum*/SDW
3: cv. Bison/SDW
4: *L.u.u. albocoeruleum*/Al6
5: cv. Bison/Al6
6: *L.u.u. albocoeruleum*/*M. lini*
7: cv. Bison/*M. lini*

Plate 5.3. Inhibition of the growth of A. brassicicola *by components of crude leaf extracts from* Linum *material inoculated with conidia of Al6 and* M. lini.

Cultivar/treatment	R_F value of zones of growth inhibition				
CoA	0.16-0.3	0.46-0.52			
LAL/SDW					
Bison/SDW	0.03-0.04			0.68-0.72	0.82-0.85
LAL/Al6	0.05-0.06		0.49-0.51	0.68-0.72	0.82-0.85
Bison/Al6	0.05-0.04		0.47-0.51	0.68-0.72	0.82-0.85
LAL/*M. lini*			0.44-0.51	0.68-0.72	0.82-0.85
Bison/*M. lini*					

Table 5.4 R_F values of zones of growth inhibition of A. brassicicola *grown on a t.l.c. plate containing the extracts of inoculated and uninoculated linseed material.*

Inhibition of C. cladosporioides *on 2D-t.l.c. plates*

The zones of inhibition observed on 2D-t.l.c. plates sprayed with *C. cladosporioides* corresponded to the zones of inhibition attributable to the CoA standard in each dimension (Plate 5.4). Small areas of inhibition were observed for extracts from uninoculated LAL plant material. The largest and most distinct zones of inhibition were observed on plates containing extracts from LAL and cv. Bison which had been inoculated with Al6. Corresponding areas were also observed with extracts from material inoculated with *M. lini*.

Areas of inhibition were not observed on 2D-t.l.c. plates at the higher R_F values which were previously observed under 366 nm after single dimension t.l.c (Plate 5.1, Table 5.2) and which had previously shown faint areas of inhibition. (Plate 5.2, Table 5.3). Zones of growth inhibition were not observed on any of the 2D-t.l.c. plates sprayed with *A. brassicicola* suspension (data not shown).

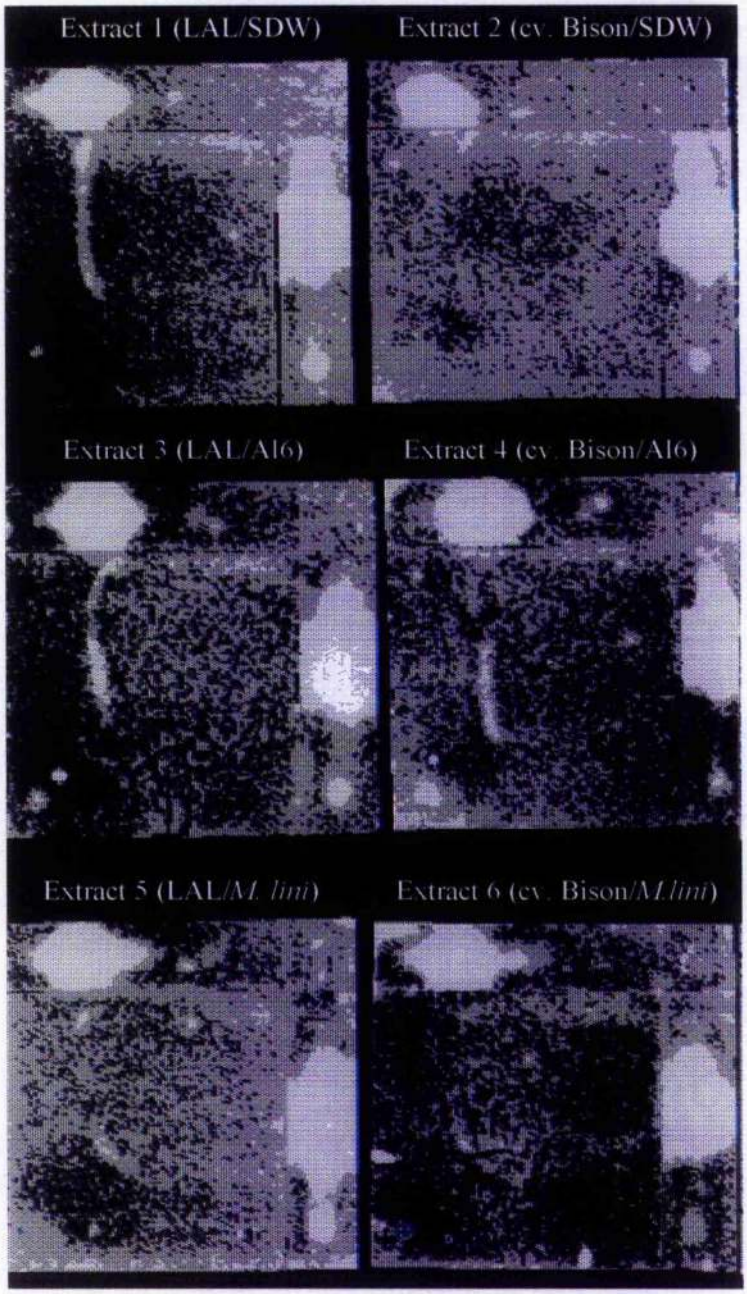


Plate 5.4 2D-t.l.c. plates of extracts from inoculated and uninoculated Linum *plants sprayed with* Cladosporium cladosporioides *illustrating the production of the phytoalexin CoA. White areas correspond to areas of growth inhibition. (Solvent system: First dimension [horizontal, left to right], chloroform : methanol, 49:1 v/v and second dimension [vertical, bottom to top], dichloromethane : acetone, 7:3 v/v. LAL =* L.u.u. albocoeruleum*).*

5.4 Discussion

General observations

The results of the microscopy study (Chapter 3) suggested that a component of the resistance mechanism of the *A. linicola/Linum* interaction involved a reduction in the rate of disease development. The development of the pathogen on the accession *L.u.u. albocoeruleum* was observed to be significantly slower in comparison to the more susceptible accession Blauwester. One of the possible explanations tentatively proposed for the observed response was the production of phytoalexin compounds by the host in direct response to attempted infection by *A. linicola* and that quantitative or qualitative differences in the production of phytoalexins between different accessions might explain the differences in disease resistance response. The aim of the current study was to investigate both of these questions with the long term aim of assessing the possibility of utilising the production of defence related compounds through breeding to improve disease resistance of linseed to the pathogen.

Visually, extracts from *Linum* material inoculated with either *A. linicola* or *M. lini* were noticeably darker and browner in shade in comparison with the control extracts and contained a greater number of bands which could be visualised following t.l.c. This suggested that extracts from infected material contained compounds which were not extracted from healthy uninoculated material and which were therefore present as a direct result of infection by *A. linicola/M. lini*. This could have been due to the effect of damage which was caused to the cells of the host plant during the interaction with the pathogen. If, as hypothesised during the previous chapter, phytotoxins interfered with the function of the plasma membrane of the host cells, compounds which leaked out could have caused the colour changes observed. Further indirect evidence of such an effect was suggested by differences in the colouration of the extracts from material inoculated with *A. linicola*, which were darker in comparison to those extracted from material inoculated with *M. lini*. As demonstrated during the previous chapter, *A. linicola* is capable of producing non-host specific toxins *in vitro*, however there is no evidence to support the production of phytotoxins by *M. lini*. Thus, the darker colouration may be produced as a direct result of the extra damage caused by the production of non-host specific toxins by the *Alternaria* pathogen.

The dark coloration of the inoculated extracts could be that the compounds associated with the breakdown of the cell metabolism during senescence were also extracted and these non-

pathogen induced compounds were acting as part of the defence mechanism of the host plant in a passive manner. For example, Anderson, (1991) found that the brown coloration of necrotic cells was due to the presence of oxidised polyphenols. Anderson (1991) suggested that these compounds had an adverse effect on the metabolism of the pathogen as polyphenols were known to bind to proteins and so inactivate enzyme activity.

Phytotoxins such as the AM-toxins are known to be site specific to the chloroplast and although the site of action of the structurally related destruxins is not known, damage to the chloroplasts by the action of a phytotoxin may cause phytochromic components to leak from the attacked cells producing the deep brown colour. During the extraction process, some chlorophyll would be expected to be extracted along with other cell contents which would account for the bright green colour of the control extracts. However this did not account for the faint red banding which was observed in the inoculated extracts during t.l.c. but not in the control extracts. Unfortunately, the red banding observed on the t.l.c. plates made the visualisation of other bands within those R_f values problematic.

Extraction technique

Keen (1978) suggested that problems could be incurred during the initial extraction and subsequent t.l.c. of the two major phytoalexins of the flax/*M. lini* interaction, coniferyl alcohol (CoA) and coniferyl aldehyde, due to the complexing or degradation of these two phenylpropanoid compounds with/by contaminants. Extracts produced during the present study may have contained less red pigmentation if partitioning of the extract with ethyl acetate had been carried out as suggested by Keen (1978). However, the results achieved by Conn *et al.*, (1988) using a revised method of that described by Keen (1978), which was used during the present study, did not suggest that contamination by extraneous compounds occurred either during t.l.c. or subsequent bioassay. A possible explanation for the large amounts of non-distinct red fluorescence observed on t.l.c. plates (Table 5.2, Plate 5.1) was that the relative amount of cell damage caused by *A. linicola* was much greater, particularly to the cell and cell organelles in comparison with that observed for *M. lini* by Keen (1978). Evidence from the present study would agree with this hypothesis as the non-distinct faint red banding observed from the inoculated extracts was much more concentrated in extract derived from linseed accessions inoculated with the isolate of *A. linicola* in comparison with those inoculated with *M. lini*.

Phytoalexins and the hypersensitive response

For many years, the association between phytoalexin production and the hypersensitive response has been well understood. There are many good examples of histological and biochemical data to support the co-incidental timing of localised cell death, the production and accumulation of phytoalexin compounds from the surrounding cells, and the subsequent cessation of pathogen development (described for many pathosystems by Mansfield, 1982). Hypersensitive response reactions (e.g. necrotic flecking) were not observed in linseed infected with *A. linicola*. Even resistant material such as *L.u.u. albocoeruleum* was observed to develop non-localised symptoms before the cessation of the growth of the pathogen. However, recent evidence concerning the role of phytoalexins and resistance genes in the response of host and non-host plants to infection has suggested that the process of cell death in the classical description of the hypersensitive response is not a necessary requirement for the elicitation of phytoalexin biosynthesis (Keen, 1992; 1993). Many abiotic and biotic elicitors have been examined which induce a resistance response from the host and often confer a level of acquired resistance to subsequent infection by the same, or a different pathogen (Kuc, 1995). Thus it has become clear that phytoalexins may play a larger part in the response of plants to pathogens in general, not only in interactions involving a hypersensitive response, but in interactions involving a more subtle expression of disease resistance. Following work on phytoalexin production by *L. usitatissimum*, Littlefield (1973) similarly suggested that a multiplicity of factors was involved in disease resistance, and thus the presence or absence of one particular component would not necessarily indicate resistance or susceptibility.

Increased elicitation due to pathogen attack

The data produced during the present study agreed with the hypothesis of Mansfield (1982) and the results of Keen & Littlefield (1978) as three levels of intensity of inhibition of the growth of *Cladosporium cladosporioides* were observed. Mansfield (1982) had suggested that a feature of the role of phytoalexins in disease resistance was that phytoalexin accumulation was always much more rapid and occurred to a higher concentration during an incompatible non-host/host-pathogen interaction in comparison with a compatible interaction. Keen & Littlefield (1978) observed results which agreed with Mansfield's hypothesis during work on the flax/*M. lini* pathosystem. Levels of fungistatic compounds produced from linseed material treated with sterile distilled water (control) during the present study were low in comparison with inoculated material. Of these, the treatment of the resistant accession *L.u.u.*

albocoeruleum with *A. linicola* isolate Al6 (an incompatible interaction) produced brighter fluorescing bands and larger areas of growth inhibition in comparison with Bison/Al6 (moderate/susceptible), LAL/*M. lini* (race 1) (susceptible) and Bison/*M. lini* (race 1)(susceptible). This suggests a definite role for phytoalexin-type compounds in the resistance response of the incompatible interaction between *L.u.u. albocoeruleum* and *A. linicola* as has been proven for the incompatible interaction of a resistant flax cultivar and *M. lini* (Keen, 1978; Keen & Littlefield, 1978; Littlefield, 1973).

Production of coniferyl alcohol

Distinct bands were observed for the standard CoA following t.l.c. which caused large areas of growth inhibition to *C. cladosporioides,* and to a lesser extent, *A. brassicicola.* A number of the extracts produced bands which co-chromatographed with the standard CoA bands and caused growth inhibition. As mentioned above, the brightest fluorescence and largest area of growth inhibition were observed for extracts from the incompatible interaction between *L.u.u. albocoeruleum* and *A. linicola* isolate Al6. The difference in relative concentration of phytoalexin produced during the incompatible interaction in comparison with that of the more compatible interactions can be clearly seen from the bioassay of 2D-t.l.c. plates shown on Plate 5.4. Unfortunately the compounds which caused the areas of inhibition were not characterised further and so a level of uncertainty remains as to whether the inhibitory compound(s) were actually CoA or a similar compound which was inhibitory to fungal growth.

Other phytoalexin compounds produced in response to members of the Alternaria

Comparison of the current study results with those produced by Tewari's group (Conn *et al.*, 1988), who studied phytoalexin production in response to elicitation by *A. brassicae*, indicated that a number of the bands observed on t.l.c. plates during the present study fluoresced with a bright blue colour and matched compounds produced by resistant members of the *Cruciferae*. One such unidentified compound produced by *Camelina sativa* and *Capsella bursa-pastoris*, corresponded to R_f values of bands produced by *L.u.u. albocoeruleum* and Bison in response to infection with *A. linicola* isolate Al6 (Conn *et al.*, 1988). Similarly, a second band (with a higher R_f value) produced by *L.u.u. albocoeruleum* and Bison infected with Al6 corresponded to that of the cyclobrassinin standard used by Conn *et al.* (1988) which was also produced in *C. bursa-pastoris*, four rapeseed cultivars (*Brassica campestris* ssp. *oleifera*) and turnip (*Brassica campestris* ssp. *rapifera*). Conn *et al.* (1988) hypothesised that the production of

one or more of the bands which were specific to *C. sativa* and *C. bursa-pastoris* was associated with the higher level of resistance of these two species to *A. brassicae* in comparison with the other crucifers tested. Indeed, two novel indole phytoalexins, camalexin and methoxycamalexin were subsequently isolated from *C. sativa* (Browne *et al.*, 1991).

The work of Tewari's group in conjunction with the results of the present study pose some questions as to the relationship between the *Alternaria* pathogens and their hosts. The differences in resistance between *C. sativa* and *C. bursa-pastoris* and the other crucifers tested appears to be directly linked to qualitative (and possibly quantitative) differences in phytoalexin production (Conn *et al.*, 1988). The similarities in the response of the linseed material tested here and the crucifers tested by Tewari's group suggests that, although not closely related to the *Brassicaceae*, *Linum* spp. may produce a number of compounds similar to those which are hypothesised to provide a higher level of resistance to infection by *A. brassicae* in the resistant brassicas. The results of Tewari's group and the observations made in this study suggest that the *Cruciferae* and *Linum* may show a similar response to the members of the *Alternaria*.

Stimulus for phytoalexin elicitation

Littlefield (1973) observed that the effect of phytoalexins in the flax/flax rust pathosystem was restricted to the immediate area of infection which suggested a close relationship between the development of the pathogen on the surface of the leaf and the elicitation of phytoalexin production. Jejelowo *et al.* (1991) found that the concentration of phytoalexins produced by *C. sativa* in response to inoculation with *A. brassicae* increased linearly with conidial concentration and suggested that this was due to a similar increase in the concentration of the fungal elicitor molecules present within the inoculation droplet. An investigation of the locality and timing of phytoalexin production was not carried out during the current study. However the work mentioned above, along with evidence from other pathosystems, suggests that there is usually a definite stage of the infection process at which phytoalexin production is elicited and pathogen development subsequently ceases (Anderson, 1991; Bailey, 1982; Mansfield, 1982).

Although the timing of phytoalexin production was not studied *in vivo* during the current, it could be suggested that phytoalexin elicitation may have begun at the point of, or immediately after, phytotoxin production. There are many reports that the production of, or the action of,

phytotoxins elicit the onset of phytoalexin biosynthesis (Anderson, 1991). Tewari (1991) suggested that such an interaction existed in the *A. brassicae*/crucifer interaction in response to the production of destruxin B. However, there are studies which provide strong evidence that in some interactions, phytotoxin production by the pathogen suppresses host defences. Gnanmanickam & Patil (1977), for example, observed that toxin production by the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* directly suppressed phytoalexin production in the bean host. Evidence from the *Alternaria* pathogens suggests a similar role for the host specific AK toxins of the pear infecting pathotype of *A. alternata* (Hayami *et al.*, 1982; Nishimura & Kohmoto, 1983b)

Metabolism and/or tolerance of phytoalexins

The ability of pathogens to metabolise phytoalexin and related defence compounds produced by their host has been well documented in the literature (Fischer & Hain, 1994; Van Etten *et al.*, 1982; 1989). During the present study, many of compounds which prevented the blastospores of *C. cladosporioides* from germinating and developing had only a slightly fungistatic effect on the conidia of *A. brassicicola* (Plates 5.2 and 5.3). This could indicate that in comparison to *Cladosporium* spp., some members of the *Alternaria* are more able either to tolerate and/or metabolise compounds such as CoA. The extent of the difference of response between *Alternaria* isolates/spp. and whether tolerance or the level of metabolism was sufficient to negate the role of the phytoalexin in the resistance response was not investigated during the current study. Due to the multicomponent nature which is characteristic of the quantitative resistance response observed, it would seem likely that the production of phytoalexins by the host would be only one of many defence responses produced and would not neccessarily determine resistance or susceptibility alone.

The role of phytoalexins in the A. linicola/Linum *interaction*

The importance of phytoalexin production in the resistance response of linseed to *A. linicola* cannot be fully ascertained from the studies in this work. Further analysis of compounds produced *in vivo* preferably at the histological level and characterisation of the phytoalexins would provide an explanation of the interaction of compounds derived from the host with those produced by the pathogen. The evidence of the results of this chapter along with those from the previous Chapter 4 (phytotoxin production) suggests a biochemical interaction between pathogen and host which is correlated with differences in the speed of pathogen

development and host response. The differences in the timing and speed of phytoalexin production may explain differences in resistance response. Thus, disease/resistance response is multicomponent in nature and layered in time and the linseed/*A. linicola* interaction is in keeping with the current model of quantitative disease resistance (Kuc, personal communication; Kuc, 1995).

As suggested by Tewari (1991) and Tewari & Conn (1993) for phytoalexin mediated resistance of crucifers in response to *A. brassicae*, the transfer of this type of resistance from resistant to susceptible genotypes, through either conventional breeding or biotechnological techniques, should be possible. If similar transfers were possible within *Linum* there would be an opportunity to enhance the disease resistance of crop varieties within the genus by the employment of disease resistance whose mechanism is, at least partly, understood.

Chapter 6

6.0 General discussion and suggestions for future studies

6.1 General discussion and conclusions

Specific aspects of the results of the current study and an evaluation of the significance of these findings to previously published work have already been discussed in the preceding chapters. The following general discussion will relate the major points of relevance from this study to what is currently known about the biology of the *Alternaria*, including *A. linicola*, and more specifically, the interaction of the pathogen with the linseed host.

General observations on the Alternaria *as plant pathogens*

Along with the other Hyphomycete species which are currently regarded to be anamorphic forms of the *Pleosporaceae* (e.g. *Stemphylium*, *Drechslera*, *Curvularia*, *Nimbya* and *Bipolaris*), the *Alternaria* show a general capacity for saprophytic growth on a wide range of plant species. Many of the *Alternaria* are more than mere saprophytes and show a close association at the species level with either a single or a few particular host species of a plant family. Dickinson (1981), for example, suggested that the unspecialised but opportunistic association of *A. alternata* with many cereals represented an intermediate niche, the pathogen being neither a necrotroph or a biotroph. Indeed, some *Alternaria* species show a higher level of specificity to their hosts as observed for some host specific toxin producing species. However, current evidence from the literature (Vloutoglou *et al.*, 1995) and the results from the present study suggest that *A. linicola* appears to be typical of the *Alternaria* in general, as opposed to the HST-producing pathoforms of the *A. alternata* anamorphs, but the pathogen shows a high level of pathogenicity on the linseed host.

Pathogen development and the host response

Studies of the infection process of *Alternaria* species on host and non-host plants have shown that the pathogens are not adversely affected by differences in the topography of the leaf surface in comparison with some biotrophic pathogens such as the rusts and mildews (McRoberts & Lennard, 1996; Tewari & Skoropad, 1986). Similarly, differences in the leaf topography of the resistant, moderately resistant and susceptible accessions of *Linum* (if present) inoculated with *A. linicola* isolate A16 did not affect the ability of the pathogen to germinate, develop and penetrate the leaf surface. It would appear that, as observed for some other *Alternaria*/host interactions, the resistance response is controlled by factors which limit pathogen development either during or following penetration. Limitation of the infection of individual host cells through the production of callose-containing papillae would appear to be an early defence response of linseed, although whether or not this is accompanied by the biosynthesis of phytoalexins and/or fungal inhibitory

enzymes such as chitinase was not ascertained; although phytoalexins were shown to be produced by two *Linum* genotypes when challenged with *A. linicola*.

Rather than being regarded as the determinant resistance mechanism, many studies have shown that phytoalexins form only a component of an overall more complex resistance mechanism (Keen, 1993; Kuc,1995). The current hypothesised role of phytoalexin elicitation and biosynthesis fits in well with recent evidence of roles for compounds such as pathogenesis-related proteins, β1, 3-glucans, chitins and chitosans, lignin and callose, lipoxygenases, and the more recently investigated, active oxygen species (Baker & Orlandi, 1995; Kuc, 1995). Many of these compounds could play an active role in the interaction of *A. linicola*/linseed. For example callose (a β1, 3-glucan) was observed to be produced during the present study and a role for this compound in disease resistance has been recognised for many years. The multicomponent interaction of the pathosystem could conceivably include many such factors.

Phytoalexin production by the *Linum* species (flax) has been demonstrated previously in response to infection with to the pathogen *M. lini* (Keen, 1978). The current study has shown that phytoalexin elicitation occurs in response to other pathogenic fungal species in *Linum* and indeed, although not fully characterised, a compound with similar properties to coniferyl alcohol (produced in response to *M. lini*) was produced in response to elicitation by *A. linicola*. Whether or not phytoalexin production was elicited by the action of the phytotoxic compounds produced by the pathogen or by another component of the interaction was not determined during the present study. Closer analysis of the inoculated leaf extract, by reverse phase chromatography for instance, may have provided an answer to the question of whether both sets of compounds were produced simultaneously (or whether phytoalexin production was slightly delayed and followed phytotoxin production). One of the major problems with such a study would be deciding which compounds were produced by the pathogen and which by the host.

A classical hypersensitive response was not observed during the infection of linseed material. The first explanation for this could be that such a response is not elicited by *A. linicola*, although further study would be required to prove that this was the case. A more likely reason would be that *Linum* may have evolved to prevent the ingress of *A. linicola* actively, as opposed to inactively through the death of host cells in front of the advancing infection hyphae. The results of the current study would indicate a dual action role of papillae and phytoalexin production, both of which form components of the horizontal resistance response. Although these components are also observed during a hypersensitive response, cell death was not observed in the case of linseed. These

components nullify the need for the physical encapsulation or the isolation of the fungus with dead cell material as characteristically observed during a classic hypersensitive response.

An alternative scenario could be that hypersensitivity may be the natural response of the host. However, a typical hypersensite response may not occur for two reasons; firstly, the pathogen may not elicit a response, or, secondly, the action of an inhibitory compound produced by the pathogen may block and prevent recognition of the pathogen. However, the production of phytoalexins suggests that the host has recognised the presence of the pathogen possibly through the detection of phytotoxin production. Thus, rather than recognition being blocked, it would follow that it is the ability of the host to respond quickly to the recognition stimuli which is the important factor in the resistance response. As phytotoxin production by *A. linicola* has been shown during the current study, this component of the interaction would seem the most likely candidate preventing response. As previously mentioned, a similar role has been suggested for the AK-toxins produced by Japanese pear infecting anamorphs of *A. alternata* (Nishimura, 1987).

The possible role of phytotoxins in the interaction

Toxin production has been proven to be a determinant of pathogenicity for many of the great epidemic causing diseases of recent times (Daly, 1987) although, admittedly, the level of disease caused by *A. linicola* is hardly comparable. However, the traditional role of phytotoxic compounds, that of the induction of cell membrane damage allowing necrotrophic feeding of the pathogen, has been reviewed and extended on the evidence of more recent studies to include an overall general effect on the overall host/pathogen interaction. The suggestion of Nishimura (1987) that host specific compounds may also suppress the resistance reaction of the host has been shown to include phytotoxins which are far less specific than host-specific toxins such as the AK-toxins. For example, Gnanamanickam & Patil (1977) observed that Phaseotoxin from *Pseudomanas syringae* pv. *phaseolicola* suppressed phytoalexin production in beans and more recently, Vidhyasekaran *et al.* (1992) found that toxin from *Helminthosporium oryzae* suppressed phenol metabolism in rice and so negated the biosynthesis of many of the host plants defence compounds. These studies suggest that the non-host-specific phytotoxins isolated from *A. linicola* play a suppressive role in the interaction, and that such a factor may be sufficient to account for the pathogenicity of this species on linseed in comparison to *A. infectoria* or *A. alternata* which have low pathogenicity on linseed and occur as saprophytes. This would also explain the close association of the pathogen with linseed.

A host-selective role was suggested for destruxin B on *Brassicae* host species of *A. brassicae* (Buchwaldt & Green, 1992). Many non-host specific toxins appear to be implicated in the aggressiveness of a particular isolate of a pathogen. As such, the production of host non-specific toxin, or groups of non-specific compounds (which possibly produce a synergistic effect), by *A. linicola* probably account for the close association of the pathogen with linseed. Compounds such as TeA, AME and the partially characterised destruxin-type compounds appear to be important in the aggressiveness of isolates of *A. linicola* as differences were observed in metabolite production between the isolates.

Components of the interaction

The mechanisms which determine the interaction of *A. linicola* with linseed appear to be a diverse and complex multicomponent system which is under quantitative genetic control. Based on the results of the present study and recent evidence from the literature (Keen, 1993), Fig. 6.1 shows a hypothesised model of the probable interactions which occur between *A. linicola* and linseed. Fig. 6.1 is presented only as a tentative model and does not accommodate all possible interactions between the pathogen and host, for example, the effect of toxins on Ca^{2+} flux or the possibility that phytoalexins are elicited by glyco-proteins or substances produced by the pathogen other than phytotoxins are not included.

The results of the study reported here greatly increase our knowledge of the pathogen/host interaction between *A. linicola* and linseed. In summary four main points arise from the study:

1. A range of *Linum* accessions tested using a novel *in vitro* bioassay were found to produce a continuous distribution of resistance responses, from susceptibility to resistance.
2. Pathogen ingress and development was delayed on resistant accessions which actively responded to the presence of the pathogen through the elicitation of defence mechanisms. Susceptible accessions appeared to produce little response to pathogen development.
3. *Alternaria linicola* was observed to produce phytotoxins during *in vitro* culture and it is hypothesised that these may suppress early host responses.
4. Resistant accessions of *Linum* produced phytoalexin compounds when challenged by an isolate of the pathogen. During the same experiment, less resistant material (i.e. Bison) produced the same compounds although less was elicited. Extracted compounds from both host accessions subsequently inhibited fungal growth.

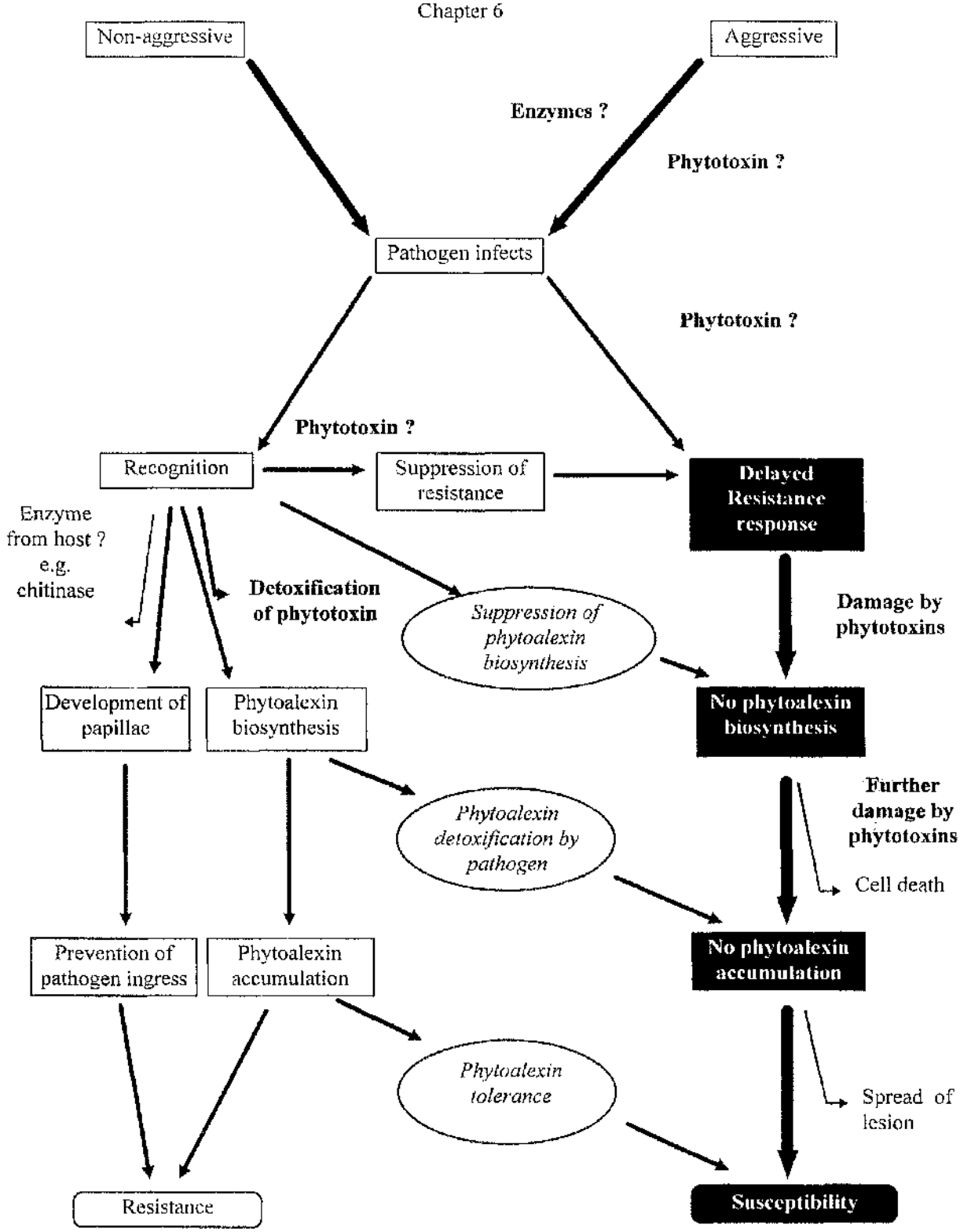


Fig. 6.1 Diagram of a hypothesised mechanism of the interaction between A. linicola *and linseed indicating a role for phytotoxins produced by the pathogen and cell wall changes and phytoalexin production in the host.*

Genetic control of resistance and prospects for the future improvement of linseed

The lack of qualitative gene effects between accessions tested with isolates of the pathogen during the present study (Evans *et al.*, 1995) was consistent with the quantitative response of many dicotyledonous crop species to non-biotrophic pathogens such as the *Alternaria* (Simmonds, 1991). Such interactions are generally considered not to be under major gene-for-gene control. Similar mechanisms of resistance suggest that the concepts of non-host resistance and quantitative host resistance share common aspects. Thus, the interaction between *A. linicola* and linseed may be genetically controlled by a number of components which produce the layered structure of the resistance response. Such a structure of resistance response was suggested by Tewari (1991) as the mechanism of resistance of cruciferous species to *A. brassicae* and was also suggested as a general model of resistance by Heath (1991; 1995).

Recent field trial results (Mercer & Ruddock, 1993) and the moderate-to-resistant response of the majority of accessions tested during the bioassay of the present study suggest that an adequate level of horizontal resistance exists within linseed to prevent excessive problems from *A. linicola* in all but the wettest of seasons. As suggested by Harlan (1976), such a relationship suggests that the crop and pathogen are near to a state of endemic balance. This ensures that, *A. linicola* can usually be isolated from the crop but levels of infection are tolerated and generally cause no appreciable disease problem. This state of "equilibrium" benefits pathogen and host in that the pathogen survives whilst the host is damaged to a degree (which continues to supply selection pressure for the maintenance of useful resistance genes within the host population) but continues to develop to maturity and in so doing, provides a substrate for the pathogen to grow on (Harlan, 1976).

The major plant disease epidemics of this century have been attributed to the intervention of man, usually through the introduction of large areas of monocrop carrying single "immunity" resistance genes. Pathogenic isolates are quickly selected and the resistance is destroyed. In this concern, linseed appears to have been fortunate in that, particularly in the UK, the crop has not been grown extensively except in recent years and, perhaps more importantly, the intensive breeding for resistance to which many cereal crops have been subjected has not occurred. The moderate to resistant response of the accessions tested during the current study indicated that

linseed appeared to have a good general combination of resistance genes which provide an adequate level of horizontal resistance to *A. linicola*.

During most seasons in the field, *A. linicola* appears to be constrained by environmental conditions such as moisture availability and temperature, both of which affect sporulation, disease development and infection (Vloutoglou, 1994). The *in vitro* aspect of the cotyledon test (where temperature was controlled at a near optimum level and there was ample moisture available) ensured that the disease response of the accessions was a true representation of resistance levels in the material. As such, there remains the possibility of increasing the level of horizontal resistance and, as suggested by Simmonds (1991), the successful selection of horizontal resistance against pathogens such as the *Alternaria* should be high.

In many ways the *Alternaria* typify the mid point between necrotrophy and complete biotrophy and as such tend to act as a model for the "average" plant pathogen. The conclusions of the present study highlight the need for further study of plant pathogen interactions amongst the pathogenic species of the *Alternaria*.

6.2 Suggestions for future studies

Generally, in comparison with the interactions between biotrophic pathogens such as the rusts, mildews and smuts, very little is understood about the interactions between the *Alternaria* and their hosts. The situation has, somewhat, improved in recent years through the work of the Japanese on host specific toxins and the Canadians (J.P. Tewari's group) on the general physiological and biochemical interactions between *A. brassicae* and crucifers. The work reported during the present study not only answers some of the previously unknown questions about the levels and underlying mechanism of the resistance of linseed to *A. linicola*, but also produced more general and intriguing questions. From the studies detailed above, disease resistance appears to be determined by the speed of pathogen development/host response following successful penetration. Further detailed study would be needed to ascertain the importance and timing of the various physical and biochemical changes which occur at the cellular level during this time although by the very nature of the multicomponent system which controls the interaction, it is unlikely that susceptibility would be mediated by a specific factor.

Although a number of host non-specific toxins were demonstrated to be produced by *A. linicola*, some of which may show host-selectivity (i.e. the destruxin compounds), there still

remains the question of the narrow host range of the pathogen. It may be the case that *A. linicola* does produce a HST but that the extraction and analysis methods used during the present study were not or sensitive enough to define the presence of such a compound. Alternatively, an HST may be produced only *in vivo*. Further toxin studies, possibly involving *in vivo* work, followed by analysis of extracts by HPLC which would be much more analytically sensitive, may answer some of these questions.

An interesting aspect of further work on phytotoxins would be to closely assess the effect of phytotoxins at the cellular level, both in whole plant material and by the use of *in vitro* culture. During the current study it was hoped that extracts could be sufficiently purified in large enough quantities to allow studies on electrolyte leakage and more specifically the role of calcium which has been suggested to mediate the action of phytotoxins during the process of cell wall damage. Unfortunately during the present studies, only enough purified phytotoxin could be isolated to allow the characterisation of the compounds by ^{1}H-nmr and electrolyte leakage could not be studied. The use of an HPLC system following reverse phase extraction would probably be more efficient at cleaning the samples which, as the ^{1}H-nmr spectra of Altlin 1-4 indicate (Appendix 2.1-2.4), were found to be contaminated with unidentified lipid compounds.

The results of the present study, although indicating a multicomponent mechanism of resistance, do not explain the differences in resistance response which are observed at the beginning of the season (seedling stage) and at the end of the season (onset of flowering/seed set). The results also do not account for the observation that mid-season growth of linseed appears to be relatively free of *A. linicola*. Such a pattern of disease response shows similarities with observations which have been made on the phenomena of acquired resistance. Both localised and systemic acquired resistance (SAR) have been described for many host plant species following pre-inoculation with a non-invasive -pathogen, or an attenuated version of the phytopathogen (Hammerschmidt & Kuc, 1995). Pre-inoculation is thought to trigger, or induce, the hosts natural defence system and depending on the host-pathogen combination exacts localised or systemic resistance to subsequent attempted infection. The exact mechanism by which acquired resistance is induced and functions is not completely understood but the mechanism of resistance is thought to be multicomponent in nature. Also, acquired resistance is often lost during and after flowering.

The author suggests that resistance of linseed to *A. linicola* may be acquired. to some extent. Such a pattern of response has been observed between linseed and *A. linicola* over many seasons: infection at the seedling stage may induce multicomponent resistance, if seedlings survive initial infection, acquired resistance prevents harmful levels of disease during the growing season. after flowering, acquired resistance fails and disease levels increase. It would be interesting to investigate aspects of this hypothesis. If such a system was in operation, phytoalexins may afford the host plant localised protection at the site of infection, or as is more likely, the resistance mechanism may prove to be more complex, with phytoalexin production being only one component of a systemic mechanism.

References

Aist, J.R. (1976). Papillae and related wound plugs of plant cells. *Annual Review of Phytopathology* **14**, 145-163.

Aist, J.R. (1981). Development of parasitic fungi in plants. In: *Biology of Conidial Fungi* (Cole, G.T. & Kendrick, B., eds.), Volume 2. Academic Press, New York. pp. 75-100.

Aist, J.R. & Gold, R.E. (1987). Prevention of fungal ingress: The role of papillae and calcium. In: *Molecular Determinants of Plant Diseases* (Nishimura, S., Vance, C.P. & Doke, N., eds). Japan Scientific Societies Press, Tokyo/Springer-Verlag, Berlin. pp. 47-58.

Aist, J.R. & Williams, P.H. (1971). The cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae*. *Canadian Journal of Botany* **49**, 2023-2034.

Akai, S., Fukitomi, M., Ishida, N. & Kunoh, H. (1969). An anatomical approach to the mechanisms of fungal infection in plants. In: *The Dynamic Role of Molecular Constituents in Plant-Parasite Interactions* (Mirocha, C.J. & Uritani, I., eds). American Phytopathological Society, St Paul. pp. 1-20.

Allen, S.J., Brown, J.F. & Kochman, J.K. (1983). The infection process, sporulation and survival of *Alternaria helianthi* on sunflower. *Annals of Applied Biology* **102**, 413-419.

Anderson, A.J. (1991). Phytoalexins and Plant Resistance. In: *Mycotoxins and Phytoalexins* (Sharma, R.P. & Salunkhe, D.K., eds). CRC Press, Boca Raton. pp. 569-594.

Anderson, W.F., Beute, M.K., Wynne, J.C. & Wongkaew, S. (1990). Statistical procedures for assessment of resistance in a multiple foliar disease complex of peanut. *Phytopathology* **80**, 1451-1459

Angell, H.R. (1929). Purple blotch of onion (*Macrosporium porri*, Ell.). *Journal of Agricultural Research* **38**, 467-487.

Anon (1995). NIAB Recommended Varieties of Oilseed Crops 1995. *National Institute of Agricultural Botany, Cambridge*. pp. 39-49.

Appel, R.S.W. (1991). The linseed market in the United Kingdom. *Aspects of Applied Biology* **28**. *Production and Protection of linseed*. pp. 1-6.

Arya, H.C. & Prasada, R. (1953). *Alternaria* blight of linseed. *Indian Phytopathology* **5** (1952). 33-39.

Ayer, W.A. & Pena-Rodriguez, L.M. (1987). Metabolites produced by *Alternaria brassicae*, the black spot pathogen of canola. Part 1. The phytotoxic components. *Journal of Natural Products*, **50** (3), 400-407.

Bagga, H.S. & Boone, D.M. (1960). Genes in *Venturia inaequalis* controlling pathogenicity to crabapples. *Phytopathology* **58**, 1176-1182.

Bagga, H.S. & Boone, D.M. (1968). Inheritance of resistance to *Venturia inaequalis* in crabapples. *Phytopathology* **58**, 1183-1187.

Bailey, J.A. (1982). Mechanisms of phytoalexin accumulation. In: *Phytoalexins* (Bailey, J.A. & Mansfield, J.W., eds.) Blackie, Glasgow. pp. 289-312.

Bailey, J.A. & Mansfield, J.W. (1982). *Phytoalexins*. Blackie, Glasgow, 334 p.

Bailey, J.A. & O'Connell, R.J. (1989). Plant cell death: A determinant of disease resistance and susceptibility. In:*Phytotoxins and Plant Pathogenesis* (Graniti, A., Durbin, R.D. & Ballio, A., eds). NATO ASI Series, Volume H27, Springer-Verlag, Berlin & Heidelberg. pp. 275-283.

Bains, P.S. & Tewari, J.P. (1985). Purification and properties of the phytotoxin(s) produced by *Alternaria brassicae*. *Phytopathology* **75**, 163.

Bains, P.S. & Tewari, J.P. (1987). Purification, chemical characterisation, and host specificity of the host specific toxin produced by *Alternaria brassicae*. *Physiological and Molecular Plant Pathology* **30**, 259-271.

Baker, K.F. & Cook, J. (1974). *Biological control of Plant Pathogens*. W.H. Freeman and Company, San Francisco. 433p.

Baker, C.J. & Orlandi, E.W. (1995). Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* **33**, 299-321.

Bansal, V.K., Seguin-Swartz, G., Rakow, G.F.W. & Petrie, G.A. (1990). Reaction of brassica species to infection by *Alternaria brassicae*. *Canadian Journal of Plant Science* **70**, 1159-1162.

Barash, I., Mor, H., Netzer, D. & Kashman, Y. (1981). Production of zinniol by *Alternaria dauci* and its phytotoxic effect on carrot. *Physiological Plant Pathology* **19**, 7-16.

Berry, L.A. (1992). Surface characteristics of brassica leaves and their influence on infection by fungal pathogens. PhD Thesis, Edinburgh University.

Black, W. (1952). A genetical basis for classification of strains of *Phytophthora infestans*. *Proceedings of the Royal Society of Edinburgh, B* **65**, 36-51.

Blakeman, J.P. (1981). *Microbial Ecology Of the Phylloplane.* Academic Press, London, New York, Toronto, Sydney, San Francisco. 502 p.

Blazquez, C.H. & Owen, J.H. (1963). Histological Studies of *Dothidella ulei* on Susceptible and Resistant *Hevea* Clones. *Phytopathology*, **53**, 58-65.

Brian, P.W., Elson, G.W., Hemming, H.G. and Wright, J.M. (1952). The phytotoxic properties of alternaric acid in relation to the etiology of plant diseases caused by *Alternaria solani* (Ell. & Mart.) Jones & Grout. *Annals of Applied Biology* **39**, 308-321.

Brian, P.W., Curtis, P.J., Hemming, H.G., Unwin, C.H. & Wright, J.M. (1949). Alternaric acid, a biologically active metabolic product of the fungus *Alternaria solani*. *Nature* **164**, 534.

Browne, L.M., Conn, K.L., Ayer, W.A. & Tewari, J.P. (1991). The camalexins; new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). *Tetrahedron* **47**, 24, 3909-3914.

Bruce, V.R., Stack, M.E. & Mislevic, P.B. (1984). Incidence of *Alternaria* species in small grains from the U.S.A. *Journal of Food Science* **49**, 1626-1627.

Buchwaldt, L. & Green, H. (1992). Phytotoxicity of destruxin B and its possible role in the pathogenesis of *Alternaria brassicae*. *Plant Pathology* **41**, 55-63.

Buchwaldt, L. & Jensen, J.S. (1991). HPLC purification of destruxins produced by *Alternaria brassicae* in culture and leaves of *Brassica napus*. *Phytochemistry* **30** (7), 2311 - 2316.

Burnham, C.R. (1932). The inheritance of *Fusarium* wilt resistance in Flax. *Journal of the American Society of Agronomy* **24**, 734-748.

Bussey, M.J. & Stevenson, W.R. (1991). A leaf disk assay for detecting resistance to early blight caused by *Alternaria solani* in juvenile potato plants. *Plant Disease* **75**, 385-390.

Cain, A.J. & Harrison, G.A. (1958). An analysis of the taxonomists' judgement of affinity. *Proceedings of the Zoological Society of London* **131**, 85-98.

Carver, T.L.W. & Thomas, B.J. (1990). Normal development by *Erysiphe graminis* on cereal leaves freed of epicuticular wax. *Plant Pathology* **39**, 367-375.

Carver, T.L.W., Robbins, M.P. & Zeyen, R.J. (1991). Effects of two PAL inhibitors on the susceptibility and localized autofluorescent host cell responses of oat leaves attacked by *Erysiphe graminis* D.C. *Physiological and Molecular Plant Pathology* **39**, 269-287.

Carver, T.L.W., Zeyen, R.J., Robbins, M.P., Vance, C.P. & Boyles, D.A. (1994a). Suppression of host cinnamyl alcohol dehydrogenase and phenylalanine ammonia lyase increases oat epidermal cell susceptibility to powdery mildew penetration. *Physiological and Molecular Plant Pathology* **44**, 243-259.

Carver, T.L.W., Zeyen, R.J., Bushnell, W.R. & R.J., Robbins, M.P. (1994b). Inhibition of phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase increases quantitative susceptibility of barley to powdery mildew (*Erysiphe graminis* D.C.). *Physiological and Molecular Plant Pathology* **44**, 261-272.

Chandrasheka, M. & Ball, M.C. (1980). Leaf blight of grey mangrove in Australia caused by *Alternaria alternata*. *Transactions of the British Mycological Society* **75**, 413-418.

Changsri, W. & Weber, G.F. (1963). Three *Alternaria* species pathogenic on certain cultivated crucifers. *Phytopathology* **53**, 643-664.

Clouse, S.D. & Gilchrist, D.G. (1987). Interaction of the ASC locus in F_8 paired lines of tomato with *Alternaria alternata* f.sp. *lycopersici*. *Phytopathology* **77**, 80-82.

Cohen, R. (1993). A leaf disk assay for the detection of resistance of melons to *Sphaerotheca fuliginea* race 1. *Plant Disease* **77**, 513-517.

Cole, R.J. & Cox, R.H. (1981). *Handbook of Toxic Fungal Metabolites*. Academic Press. 937 pp.

Conn, K.L. & Tewari, J.P. (1989). Interactions of *Alternaria brassicae* conidia with leaf epicuticular wax of canola. *Mycological Research* **92**, 240-242.

Conn, K.L., Tewari, J.P. & Dahiya, J.S. (1988). Resistance to *Alternaria brassicae* and phytoalexin-elicitation in rapeseed and other crucifers. *Plant Science* **56**, 21-25.

Cotty, P.J. & Misaghi, I.J. (1984). Zinniol production by *Alternaria* species. *Phytopathology* **74**, 785-788.

Cotty, P.J. & Misaghi, I.J., & Hine, R.B. (1983). Production of zinniol by *Alternaria tagetica* and its phytotoxic effects of *Tagetes erecta*. *Phytopathology* **73**, 1326-1328.

Crute, I.R. (1985). The genetic bases of relationships between microbial parasites and their hosts. In: *Mechanisms of Resistance to Plant Diseases*, (R.S.S. Fraser, Ed.). Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht-Boston-Lancaster. pp 80-142.

Daly, J.M. (1987). Toxins as determinants of plant disease. In: *Molecular Determinants of Plant Diseases* (Nishimura, S., Vance, C.P. &Doke, N., eds). Japan Scientific Society Press, Tokyo/Springer-Verlag, Berlin. pp. 119-126.

Daniels, A., Lucas, J.A. & Peberdy, J.F. (1991). Morphology and ultrastructure of W and R pathotypes of *Pseudocercosporella herpotrichoides* on wheat seedlings. *Mycological Research*, **95**, 385-397.

Daub, M.E. (1986). Tissue culture and the selection of resistance to pathogens. *Annual Review of Phytopathology* **24**, 159-186.

Davis, N.D., Diener, U.L. & Morgan-Jones, G. (1977). Tenuazonic acid production by *Alternaria alternata* and *Alternaria tenuissima* isolated from cotton. *Applied Environmental Microbiology* **34**, 155-157.

Day, P.R. (1974). *Genetics of Host-Parasite Interaction.* W.H. Freeman and Company, San Francisco. pp. 92-110.

Degenhardt, K.J. (1978). *Alternaria* blackspot of rapeseed and mustard: phytotoxins and other aspects of host-parasite interaction. Ph.D. thesis, University of Saskatchewan, Saskatoon.

Denny, T.P. (1995). Involvement of bacterial polysaccharides in plant pathogenesis. *Annual Review of Phytopathology*, **33**, 173-197.

Deverall, B.J. (1982). Introduction. In: *Phytoalexins* (Bailey, J.A. & Mansfield, J.W., eds). Blackie, Glasgow & London. pp. 1-20.

Dey, P.K. (1933). An *Alternaria* blight of the linseed plant. *Indian Journal of Agricultural Science* **5**, 881-896.

Dickinson, C.H., (1981). Biology of *Alternaria alternata, Cladosporium cladosporioides* and *C. herbarum* in respect of their activity on green plants. In: *Microbial Ecology of the Phylloplane* (J. Blakeman, ed.). Academic Press, London. pp. 169-184.

Dickinson, C.H. & Bottomley, D. (1980). Germination and growth of *Alternaria* and *Cladosporium* in relationship to their activity in the phylloplane. *Transactions of the British Mycological Society* **74**, 309-319.

Dickinson, C.H. & O' Donnell, J. (1977). Behaviour of phylloplane fungi on *Phaseolus* leaves. *Transactions of the British Mycological Society* **68**, 193-199.

Dickinson, D. (1949). Studies in the Physiology of Obligate Parasitism. II. The behaviour of the germ-tubes of certain rusts in contact with various membranes. *Annals of Botany* **13**, 219-236.

Digby, P.G.N., Galwey, N. & Lane, P. (1987). *Genstat 5: A Second Course.* Clarendon Press, Oxford. 233p.

Durbin, R.D. & Uchytil, T.F. (1977). A survey of plant insensitivity to tentoxin. *Phytopathology*, **67**, 602-603.

Durrant, A. (1976). Flax and Linseed. In: *Evolution of Crop Plants* (N.W. Simmonds ed.) Longman, London. pp. 190-193.

Edwards, J.V., Lax, A.R., Lillehoj, E.B. & Boudreaux, G.J. (1987). Structure-activity relationships of cyclic and non-cyclic analogues of the phytotoxic peptide tentoxin. *Journal of Agriculture and Food Chemistry* **35**, 451-456.

Ehrlich, M.A. & Ehrlich, H.G. (1966). Ultrastructure of the hyphae and haustoria of *Phytophthora infestans* and hyphae of *P. parasitica*. *Canadian Journal of Botany* **44**, 1495-1503.

Ellingboe, A.H. (1972). Genetics and Physiology of Primary Infection by *Erysiphe graminis*. *Phytopathology* **62**, 401-406.

Ellingboe, A.H. (1975). Horizontal Resistance: an artefact of experimental procedure ? *Australian Plant Pathology Society Newsletter* **4**, 44-46.

Ellingboe, A.H. (1981). Changing concepts in host-pathogen genetics. *Annual review of Phytopathology* **19**, 125-143.

Ellis, M.B. (1971). *Dematiaceous Hyphomycetes*. Commonwealth Mycological Institute, Kew, UK. pp. 7-21.

Ellis, J.S., Jennings, A.C., Edwards, L.A., Mavandad, M., Lamb, C.J. & Dixon, R.A. (1989). Defence gene expression in elicitor-treated cell suspension cultures of french bean cv. Imuna. *Plant Cell Reports* **8**, 504-507.

Emme, H. & Schepeljeva, H. (1927). Versuch einer karyologischen Artanalyse von *L. usitatissimum* L. *Bulletin of Applied Botany of Genetics and Plantbreeding* Leningrad **17** (3), 265-272.

Emmett, R.W. & Parberry, D.G. (1975). Appressoria. *Annual Review of Phytopathology*, **13**, 147-167.

Eschrich, W. & Currier, H.B. (1964). Identification of callose by its diachrome and fluorochrome reactions. *Stain Technology* **39**, 303-307.

Evans, N., McRoberts, N., Hitchcock, D. & Marshall, G. (1995). Assessing linseed (*Linum usitatissimum*) resistance to *Alternaria linicola* using a detached cotyledon assay. *Annals of Applied Biology* **127**, 263-271.

Fahim, M.M. & El-Shehedi, A. (1966). The mode of penetration of *Alternaria porri* into onion leaves. *Transactions of the British Mycological Society* **49**, 79-80.

Fischer, R. & Hain, R. (1994). Plant disease resistance resulting from the expression of foreign phytoalexins. *Current Opinion in Biotechnology* **5**, 125-130.

Fitt, B.D.L. & Ferguson, A.W. (1990). Responses to pathogen and pest control in linseed. *Proceedings of the Brighton Crop Protection Conference - Pests and diseases*, 733-738.

Fitt, B.D.L. & McCartney, H.A. (1986). Spore dispersal in splash droplets. In: *Water, fungi and plants* (Ayres, P.G. & Boddy, L., eds.). Cambridge University Press. pp. 87-104.

Fitt, B.D.L., Coskun, H. & Schmechal, D. (1991a). Biology of three *Alternaria* species on linseed: a comparison. *Aspects of Applied Biology* **28**. *Production and protection of linseed*, 101-106.

Fitt, B.D.L., Ferguson, A.W., Dhua, U. & Burhenne, S. (1991b). Epidemiology of *Alternaria* species on linseed. *Aspects of Applied Biology* **28**. *Production and protection of linseed*, 95-100.

Flor, H.H. (1941). Inheritance of rust reaction in a cross between the flax varieties Buda and J.W.S. *Journal of Agricultural Research* **63**, 369-388.

Flor, H.H. (1942a). Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* **32**, 653-669.

Flor, H.H. (1942b). Inheritence of pathogenicity in a cross between physiological races 22 and 24 of *Melampsora lini*. *Phytopathology* **32**, 5 (Abstract).

Flor, H.H. (1946). Genetics of pathogenicity in *Melampsora lini*. *Journal of Agricultural Research* **73**, 335-357.

Flor, H.H. (1947). Inheritance of reaction to rust in flax. *Journal of Agricultural Research* **74**, 241-262.

Flor, H.H. (1955). Host-parasite interaction in flax-rusts - its genetics and other implications. *Phytopathology* **45**, 680-685.

Flor, H.H. (1965). Tests for allelism of rust-resistance genes in flax. *Crop Science* **5**, 415-418.

Fontem, D.A., Berger, R.D., Weingartner, P. & Bartz, J.A. (1991). Progress and spread of dark leaf spot in cabbage. *Plant Disease* **75**, 269-274.

Freeman, G.G. (1965). Isolation of alternariol and alternariol monomethyl ether from *Alternaria dauci* (Kuhn) Groves and Skolko. *Phytochemistry* **5**, 719-725.

Freer, J.B.S. (1991). A development stage key for linseed (*Linum usitatissimum*). *Aspects of Applied Biology* **28**, Production and Protection of linseed, 33-40.

Freer, J.B.S. (1995). Leaner linseed costs. *Crops* **13** (3), 14-15.

Fulton, N.D., Bollenbacher, K. & Templeton, G.E. (1965). A metabolite from *Alternaria tenuis* that inhibits chlorophyll production. *Phytopathology*, **55**, 49-51.

Gabriel, K.R. (1981). Biplot display of multivariate matrices for inspection of data and diagnosis. In: *Interpreting Multivariate Data* (Barnett, V., ed.), Wiley & Sons, Chichester, UK, pp 147-173.

Gatenbeck, S. & Hermodsson, S. (1965). Enzymic synthesis of the aromatic product of alternariol. *Acta Chemica Scandinavica* **19**, 65-71.

Gatenbeck, S. & Sierankiewcz, J. (1973). On the biosynthesis of tenuazonic acid from *Alternaria tenuis*. *Acta Chemica Scandinavica* **27**, 1825-1827.

Gäumann, E. (1954). Toxins and plant disease. *Endeavour* **13**, 198-204.

Gilchrist, D.G. & Grogan, R.G. (1976). Production and nature of a host-specific toxin from *Alternaria alternata* f.sp. *lycopersici*. *Phytopathology* **66**, 165-171.

Gill, K.S. (1987). *Linseed.* Indian Council of Agricultural Research, New Delhi. 386 p.

Gill, K.S. & Yermanos, D.M. (1967). Cytogenetic studies on the genus *Linum*. II. Hybrids among taxa with 15 as the haploid chromosome number. *Crop Science* 7, 623-627.

Gnanamanickam, S. & Patil, S.S. (1977). Phaseotoxin suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. *Physiological Plant Pathology* **10**, 168-179.

Grebenyuk, N.V. (1983). Distribution of fungi on flax stems. *Mikologiya i Fitopatogogiya*, **17**, 185-189.

Goray, S.C., Khosla, H.K., Upadhayaya, Y.M., Naik, S.L. & Mandloi, S.C. (1987). Inheritance of wilt resistance in linseed. *Indian Journal of Agricultural Sciences*, **57**, 625-627.

Goray, S.C., Khosla, H.K., Upadhayaya, Y.M., Nigam, P.K. & Mandloi, S.C. (1989). Genes conditioning resistance of linseed to powdery mildew (*Oidium lini*). *Madras Agricultural Journal* **76**, 391-394.

Gregory, P.H. (1961). *The Microbiology of the Atmosphere*. Leonard Hill [Books] Ltd/Interscience Publishers Inc., London/New York. 251p.

Grove, J.F. (1964). Metabolic products of *Stemphylium radicinum*. Part I. Radicinin. *Journal of the Chemical Society* 1964, 3234-3239.

Groves, J.W. & Skolko, A.J. (1944). Notes on seed-bourne fungi: II. *Alternaria*. *Canadian Journal of Research*, **22**, 217-234.

Gupta, S., Roberts, D.W. & Renwick, J.A.A. (1989). Insecticidal cyclodepsipeptides from *Metarhizium anisopliae*. *Journal of the Chemical Society Perkin Transactions* **1**, 2347-2357.

Hachler, H. & Hohl, H.R. (1984). Temporal and spatial distribution of collar and papillae wall appositions in resistant and susceptible tuber tissue of *Solanum tuberosum* infected by *Phytophthora infestans*. *Physiological Plant Patholgy* **24**, 107-118.

Hain, R., Bieseler, B., Kindl, H., Schröder, G. & Stöcker, R.H. (1990). Expression of stilbene synthase gene in *Nicotiana tabacum* results in the synthesis of the phytoalexin resveratrol. *Plant Molecular Biology* **15**, 325-335.

Hain, R., Reif, H.J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P.H., Stöcker, R.H. & Stenzel, K. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361**, 153-156.

Hammerschmidt, R. & Kuc, J. (1995). *Induced Resistance to Disease in Plants.* Kluwer Academic Publishers, Dordrecht/Boston/London. p182.

Hanchey, P. & Wheeler, H. (1971). Pathological changes in the ultrastructure: tobacco roots infected with *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* **61**, 33-39.

Hanchey, P., Wheeler, H. & Luke, H.H. (1968). Pathological changes in ultrastructure: effects of victorin on oat roots. *American Journal of Botany* **55**, 53-61.

Harlan, J.R. (1976). Diseases as a factor in plant evolution. *Annual Review of Phytopathology* **14**, 31-51.

Harrison, M.D., Livingston, C.H. & Oshima, N. (1965). Epidemiology of potato early blight in Colorado, 1: Initial infection, disease development and the influence of environmental factors. *American Potato Journal* **42**, 279-291.

Hayami, C., Otani, H., Nishimura, S. & Kohmoto, K. (1982). Induced resistance in pear leaves by spore germination fluids of non-pathogens to *Alternaria alternata* Japanese pear pathotype and suppression of the induction by AK-toxin. *Journal of the Faculty of Agriculture of Tottori University* **17**, 9-18.

Heath, M.C. (1981). A generalised concept of host-parasite specificity. *Phytopathology* **71**, 1121-1123.

Heath, M.C. (1985). Implications on non-host resistance for understanding Host-Parasite interactions. In: *Genetic Basis of Biochemical Mechanisms of Plant Disease* (Groth, J.V. & Bushnell, W.R. eds). American Phytopathological Society Press, St Paul, Minnesota. pp. 25-42.

Heath, M.C. (1991). Evolution of resistance to fungal parasitism in natural ecosystems. *New Phytologist* **119**, 331-343.

Heath, M.C. (1995). Thoughts on the role and evolution of induced resistance in natural ecosystems, and its relationship to other types of plant defenses against disease. In: *Induced Resistance to Disease in Plants*. (Hammerschmidt, R. & Kuc, J., eds). Kluwer Academic Publishers. Dordrecht / Boston / London. pp. 141-151.

Hohn, T.M. & Ohlrogge, J.B. (1991). Expression of fungal sesquiterpene cyclase gene in transgenic tobacco. *Plant Physiology* **97**, 460-462.

Humpherson-Jones, F.M. & Hocart, M.J. (1983). Infection requirements of *A. brassicae* and *A. brassicicola*. National Vegetable Research Station Annual Report 1983. pp. 63-64.

Humpherson-Jones, F.M. & Maude, R.B. (1982). Studies on the epidemiology of *Alternaria brassicicola* in *Brassica oleracea* seed production crops. *Annals of Applied Biology* **100**, 61-71.

Humpherson-Jones, F.M., Maude, R.B. & Ainsworth, L.F. (1980). *Alternaria* disease of brassica crops. *National Vegetable Research Station Annual Report* 1980. pp. 69-70.

Hutchinson, J. (1967). *The genera of flowering plants (*Angiospermae*): Dicotyledons*, Volume 2. Oxford University Press, London. pp. 595-600.

Ingham, J.L. (1977). Medicarpin as a phytoalexin of the genus *Melilotus*. *Zeitschrift für Naturforschung, Teil C* **32**, 449-452.

Jackson, C.R. (1959). Symptoms and host-parasite relations of *Alternaria* leaf spot of cucurbits. *Phytopathology* **49**, 731-733.

Jasalavich, C.A., Morales, V.M., Pelcher, L.E. & Séguin-Swartz, G. (1995). Comparison of nuclear ribosomal DNA sequences from *Alternaria* species pathogenic to crucifers. *Mycological Research* **99** (5), 604-614.

Jejelowo, O.A., Conn, K.L. & Tewari, J.P. (1991). Relationship between conidial concentration, germling growth, and phytoalexin production by *Camelina sativa* leaves inoculated with *Alternaria brassicae*. *Mycological Research* **95**, 928-934.

Jenkins, M.T., Robert, A.L. & Findley, W.R. (1954). Recurrent selection as a method for concentrating genes for resistance to *Helminthosporium turcicum* leaf blight in corn. *Agronomy Journal* **46**. 89-94.

Jenns, A.E., Leonard, K.J. & Moll, R.H. (1982). Variation in the expression of specificity in two maize diseases. *Euphytica* **31**, 269-279.

Jeswani, L.M. & Upadhyaya, Y.M. (1970). Genetics of wilt resistance in linseed. *Indian Journal of Genetics and Plant Breeding* **30**, 104-108.

Johnson, R. (1978). Practical breeding for durable resistance to rust diseases in self pollinating cereals. *Euphytica* **27**, 529-540.

Johnson, R. (1979). The concept of durable resistance. *Phytopathology* **69**, 198-199.

Johnson, R. (1992). Past, present and future opportunities in breeding for disease resistance, with examples from wheat. *Euphytica* **63**, 3-22.

Jones, D.R. & Deverall, B.J. (1978). The use of leaf transplants to study the cause of hypersensitivity to leaf rust, *Puccinia recondita*, in wheat carrying the Lr20 gene. *Physiological Plant Pathololgy* **12**, 311-319.

Jones, P. & Ayres, P.G. (1972). The nutrition of the subcuticular mycelium of *Rhynchosporium secalis* (barley leaf blotch): permeability changes induced in the host. *Physiological Plant Pathology* **2**, 383-392.

Kalia, H.R., Chand, J.N. & Ghai, B.S. (1965). Inheritance of resistance to *Alternaria* blight of linseed. *Journal of Research. Ludhiana* **2**, (2), 104-105.

Kameda, K., Aoki, H., Tanaka, H. & Namiki, M. (1973). Studies on metabolites of *Alternaria kikuchiana* Tanaka, a phytopathogenic fungus of Japanese pear. *Agricultural and Biological Chemistry* **37**, 2137-2146.

Kamthan, K.N., Mishra, D.P. & Shukla, A.K. (1981). Independant genetic resistance to wilt in linseed. *Indian Journal of Agricultural Sciences* **51**, 556-558.

Keen, N. (1978). Phytoalexins: efficient extraction from leaves by a facilitated diffusion technique. *Phytopathology*, **68**, 1237-1239.

Keen, N.T. (1992). The molecular biology of disease resistance. *Plant Molecular Biology* **19**, 109-122.

Keen, N.T. (1993). An overview of active disease defense in plants. In: *Mechanisms of plant defense responses* (Fritig, B. & Legrand, M., eds). Kluwer Academic Publishers, Dordrecht. pp. 3-11.

Keen, N.T. & Littlefield, L.J. (1978). Association of phytoalexins with resistance in flax to *Melampsora lini*. *Proceedings of the American Phytopathological Society* **4**, 101-102.

Kendrick, W.B. (1981a). The History of Conidial Fingi. In: *Biology of Conidial Fungi*, (Cole, G.T. & Kendrick, B., eds) Volume 1. Academic Press, New York. pp. 3-18.

Kendrick, W.B. (1981b). The Systematics of Hyphomycetes. In: *Biology of Conidial Fungi*, (Cole, G.T. & Kendrick, B., eds) Volume 1. Academic Press, New York. pp. 21-42.

Kidger, A.L. & Carver, T.L.W. (1981). Autoflourescence in oats infected with powdery mildew. *Transactions of the British Mycological Society* **76**, 405-409.

King, A. D. & Schade J. E. (1984). *Alternaria* toxins and their importance in food. *Journal of Food Protection* **47**, 886-901.

Kinoshita, T. Renbutsu, Y., Khan, I., Kohmoto, D. & Nishimura, S. (1972). Distribution of tenuazonic acid production in the genus *Alternaria* and its pathological evaluation. *Annals of the Phytopathological Society of Japan* **38**, 397-404.

Klose, A., Bauers, F. & Paul, V.H. (1993). Pathogenicity of two isolates of *Alternaria linicola* on 16 cultivars of *Linum usitatissimum*. *Bulletin OILB/SROP* **16** (9), 100-108.

Knott, D.R. & Dvorak, J. (1976). Alien germ plasm as a source of resistance to disease. *Annual Review of Phytopathology* **14**, 211-235.

Kodaira, Y. (1961). Toxic substances to insects produced by *Aspergillus ochraceus and Oospora destructor*. *Agricultural and Biological Chemistry* **25**, 261-262.

Kodama, M., Park, P., Tsujimoto, T., Otani, H., Kohmoto, K. & Nishimura, S (1990). Ultrastructural studies on action site of host-selective AT-toxin produced by *Alternaria alternata* tobacco pathotype in tobacco leaf cells. *Annals of the Phytopathological Society of Japan* **56**, 637-644.

Kohmoto, K., Otani, H. & Nishimura, S. (1987). Primary action sites for host-specific toxins produced by *Alternaria* species. In: *Molecular Determinants of Plant Disease* (Nishimura, S., Vance, C.P. & Doke, N., eds). Japan Scientific Societies Press, Tokyo/Springer-Verlag, Berlin. pp. 127-143.

Kohmoto, K., Scheffer, R.P. & Whiteside, J.O. (1979). Host-selective toxins from *Alternaria citri*. *Phytopathology*, **69**, 667-671.

Krzanowski, W.J. (1990). *Multivariate Analysis: A user's perspective*. Clarendon Press, Oxford, UK. 563 p.

Kuc, J. (1995). Phytoalexins, stress metabolism, and disease resistance in plants. *Annual Review of Phytopatholgy* **33**, 275-297.

Kulshreshtha, B.M. & Chauhan, R.K.S. (1985). Production of phytoalexins in the roots of raddish (*Raphanus sativus*) after inoculation with *Alternaria alternata*. *Indian Phytopathology* **38**, 286-291.

Kulshreshtha, B.M. & Chauhan, R.K.S. (1987). Chromatogram bioassay: A quick method for the detection of phytoalexin in fruits of sesame (*Sesamum indicum* L.). *National Academy of Science Letters (India)* **10**, 49-50.

Kumar, S., Shukla, R.S., Singh, K.P., Paxton, J.D. & Husain, A. (1984). Glycecollin: a phytoalexin in leaf blight of *Costus speciosus*. *Phytopathology* **74**, 1349-1352.

Kumashiro, T. (1983). Selection for tenuazonic acid tolerant cells of tobacco and characteristics of the regenerates. *Japanese Journal of Breeding* **33** (suppl. 1), 194-195.

Lacey, J. (1986). Water availabilty and fungal reproduction: Patterns of spore production, liberation and dispersal. In: *Water, fungi and plants* (Ayres, P.G. & Boddy, L., eds.). Cambridge University Press, Cambridge. pp 65-87.

Lawrence, G.J., Mayo, G.M.E. & Shepard, K.W. (1981a). Interactions between genes controlling pathogenicity in the flax rust fungus. *Phytopathology* **71**, 12-19.

Lawrence, G.J., Shepard, K.W. & Mayo, G.M.E. (1981b). Fine structure of genes controlling pathogenicity in flax rust, *Melampsora lini*. *Heredity* **46**, 297-313.

Leduc, A. (1958). Sur les micromycetes altenaroides dc la flore fungique des graines des lins. *Revue Générale de Botanique* **65**, 541-580.

Littlefield, L.J. (1973). Histological evidence for diverse mechanisms of resistance to flax rust, *Melampsora lini* (Ehrenb.) Lev.. *Physiological Plant Pathology* **3**, 241-247.

Luke, H.H., Warmke, H.E. & Hanchey, P. (1966). Effects of the pathotoxin victorin on ultrastructure of root and leaf tissue of *Avena* species. *Phytopathology* **56**, 1178-1183.

MacDonald, M.V. & Ingram, D.S. (1985). *In vitro* selection for resistance to *Alternaria brassicicola* in *Brassica napus*ssp. *oleifera* (winter oilseed rape) using partially purified culture filtrates. *Cruciferae Newsletter* **10**, 97-100.

Mansfield, J.W. (1982). The role of Phytoalexins in disease resistance. In: *Phytoalexins* (Bailey, J.A. & Mansfield, J.W., eds). Blackie, Glasgow and London. pp. 253-288.

Mansfield, J.W., Hargreaves, J.A. & Boyle, F.C. (1974). Phytoalexin production by live cells in broad bean leaves infected with *Botrytis cinerea*. *Nature* **252**, 316-317.

Martzenitzina, K.K. (1927). The chromosomes of some species of the genus *Linum* L. *Bulletin of Applied Botany of Genetics and Plant Breeding, Leningrad*, **17** (3), 253-264.

Mayama, S. & Shishiyama, J. (1976). Detection of cellular collapse in albino barley leaves inoculated with *Erysiphe graminis hordei* by UV-fluorescence microscopy. *Annals of the Phytopathological Society of Japan* **42**, 618-620.

Mayama, S. & Shishiyama, J. (1978). Localized accumilation of fluorescent and U.V.-absorbing compounds at the penetration sites in barley leaves infected with *Erysiphe graminis hordei*. *Physiological Plant Patholgy* **13**, 347-354.

Mayama, S. &Tani, T. (1982). Microspectrophotometric analysis of the location of avenalumin accumilation in oat leaves in response to fungal infection. *Physiological Plant Pathology* **21**, 141-149.

Mayama, S., Hayashi, S., Yamamoto, R., Tani, T., Ueno, T. & Fukami,H. (1982). Effects of elevated temperature and α-aminooxy acetate on the accumilation of avenalumins in oat leaves infected with *Puccinia coronata* f.sp. *avenae*. *Physiological Plant Pathology* **20**, 305-312.

McKenzie, K.J., Robb, J. & Lennard, J.H. (1988). Toxin production by *Alternaria* pathogens of oilseed rape (*Brassica napus*). *Crop Research* **28**, 67-81

McRoberts, N., (1992). Aspects of the Biology of Brassica/Alternaria Host/Pathogen systems. Ph.D. thesis, University of Edinburgh, Edinburgh, UK.

McRoberts, N. & Lennard, J.H. (1996). Pathogen behaviour and plant cell reactions in interactions between *Alternaria* species and leaves of host and non-host plants. *Plant Pathology*, (In Press).

Mercer, P.C. (1994). Seed-borne pathogens of linseed in the UK. *1994 BCPC Monograph No. 57: Seed Treatment: Progress and Prospects*. pp. 179-187.

Mercer, P.C. & Ruddock, A. (1994). Effect of cultivar on a range of linseed diseases in Northern Ireland in 1993. *Tests of Agrochemicals and cultivars* **15** *(Annals of Applied Biology* **124**, Supplement), 100-101.

Mercer, P.C. & Ruddock, A. (1993). Effects of cultivars and iprodione on the incidence of *Alternaria linicola* in capsules and seeds of linseed in Northern Ireland. *Tests of Agrochemicals and cultivars* **14** *(Annals of Applied Biology* **122**, Supplement), 146-147.

Mercer, P.C., Ruddock, A., Mee, E. & Papadoplous, S. (1993). Biological control of Alternaria diseases of linseed and oilseed rape. *Bulletin OILB/SROP* **16** (9), 89-99.

Mercer, P.C., Ruddock, A. & McGimpsey, H.C. (1992). Effect of a single fungicide spray on a range of linseed cultivars. Tests of Agrochemicals and cultivars **13** (*Annals of Applied Biology* **120**, *Supplement*), 74-75.

Mercer, P.C. & Hardwick, N.V. (1991). Control of seed-borne diseases of linseed. *Aspects of Applied Biology*, **28**, *Production and protection of linseed*, 71-78.

Mercer, P.C., Hardwick, N.V., Fitt, B.D.L. & Sweet, J.B. (1991a). *Status of diseases of linseed in the U.K.* Home-Grown cereals Authority Review. (OS3). 76 p.

Mercer, P.C., McGimpsey, H.C. & Ruddock, A. (1991b). Evaluation of chemical and biological agents against seed-borne diseases of linseed in N. Ireland. *Tests of Agrochemicals and cultivars* **12** *(Annals of Applied Biology* **118**, Supplement), 44-45.

Mercer, P.C., McGimpsey, H.C. & Ruddock, A. (1989). Effect of seed treatment and sprays on the field performance of linseed. Tests of Agrochemicals and cultivars **10** (*Annals of Applied Biology* **114**, *Supplement*), 50-51.

Mercer, P.C. & Jeffs, M. (1988). The presence of seed-borne pathogens on three cultivars of linseed. *Tests of Agrochemicals and Cultivars* **9** *(Annals of Applied Biology* **112**, *Supplement*), 82-83.

Mercer, P.C., McGimpsey, H.C. & Ruddock, A. (1988). The control of seed-borne pathogens of linseed by seed treatment. Tests of Agrochemicals and cultivars **9** (*Annals of Applied Biology* **112**, *Supplement*), 30-31.

Mercer, P.C., McGimpsey, H.C., Black, R. & Norrie, S. (1985). The chemical control of *Alternaria linicola* on the seed of linseed. *Tests of Agrochemicals and Cultivars (Annals of Applied Biology* **110**, Supplement) **6**, 56-57.

Mercer, P.C., Wood, R.K.S. & Greenwood, A.D. (1974). Resistance to anthracnose of French bean. *Physiological Plant Pathology* **4**, 291-306.

Muskett, A.E. & Colhoun, J. (1947). *The diseases of the Flax Plant.* W. & G. Baird, Belfast. 112 p.

Nash, A.F. & Gardner, R.G. (1988). Heritability of tomato early blight resistance derived from *Lycopersicon hirsutum*. *Journal of the American Society for Horticultural Science* **113**, 264-268.

Nicholson, R.L. & Epstein, L. (1991). Adhesion of Fungi to the Plant Surface: Prerequisite for Pathogenesis. In: *The fungal spore and disease initiation in plants and animals* (Cole, G.T. & Hoch, H.C., eds). Plenum Press, New York. pp. 3-23.

Niederhauser, J.S., Cervantes, J. & Servin, L. (1954). Late blight in Mexico. *American Potato Journal* **31**, 233-237.

Nishimura, S. (1987). Recent development of host-specific toxin research in Japan and its agricultural use. In: *Molecular Determinants of Plant Diseases* (Nishimura, S., Vance, C.P. & Doke, N., eds). Japan Scientific Society Press, Tokyo/Springer Verlag, Berlin. pp 11-25.

Nishimura, S. & Kohmoto, K. (1983a). Host-specific toxins and chemical structures from *Alternaria* species. *Annual Review of Phytopathology* **21**, 87-116.

Nishimura, S. & Kohmoto, K. (1983b). Roles of Toxins in Pathogenesis. In: *Toxins and Plant Pathogenesis*. (Daly, J.M. & Deverall, B.J., eds). Academic Press, Sydney. pp. 137-155.

Nishimura, S., Tsutsumi, M. & Kohmoto, K. (1980). Detection of toxin mediating the pathogenicity of the *Alternaria alternata* causing brown spot of tobacco. *Annals of the Phytopathological Society of Japan* **46**, 387 (Abstr.).

Nishimura, S., Kohmoto, K., Kuwata, M. & Watanabe, M. (1978). Production of a host-specific toxin by the pathogen causing black spot of strawberry. *Annals of the Phytopathological Society of Japan* **44**, 359 (Abstr.).

Nutsugah, S.K., Park, P., Otani, H., Kodama, M. & Kohmoto, K. (1993). Ultrastructural changes in pigeon pea cells caused by a host-specific toxin from *Alternaria tenuissima*. *Annals of the Phytopathological Society of Japan* **59**, 407-415.

Otani, H., Tomiyama, K., Okamoto, H., Nishimura, S. & Kohmoto, K. (1989). Effect of AK-toxin produced by *Alternaria alternata* Japanese pear pathotype on membrane potential of pear cell. *Annals of the Phytopathological Society of Japan* **55**, 466-468.

Otani, H., Kohmoto, K., Nishimura, S., Nakashima, T., Ueno, T. & Fukami, H. (1985). Biological activities of AK-toxins I and II, host-specific toxins from *Alternaria alternata* Japanese pear pathotype. *Annals of the Phytopathological Society of Japan* **51**, 285-293.

Otani, H., Nishimura, S., Kohmoto, K., Yano, K. & Seno, T. (1975). Nature of specific susceptibility to *Alternaria kikuchiana*in Nijisseiki cultivar among Japanese pears. V. Role of host-specific toxin in early step of infection. *Annals of the Phytopathological Society of Japan* **41**, 467-76.

Otani, H., Nishimura, S. & Kohmoto, K. (1973). Nature of specific susceptibility to *Alternaria kikuchiana*in Nijisseiki cultivar among Japanese pears. II. Effect of host-specific toxin on the permeability of pear leaves (supplementary report). *Journal of the Faculty of Agriculture of Tottori University* **8**, 14-20.

Otani, H., Nishimura, S. & Kohmoto, K. (1972). Nature of the specific susceptibility to *Alternaria kikuchiana* in Nijisseiki cultivar among Japenese pears. I *Journal of the Faculty of Agriculture of Tottori University* 7, 5-12.

Païs, M., Das, B.C. & Ferron, P. (1981). Depsipeptides from *Metarhizium anisopliae*. *Phytochemistry*, **20**, (4), 715-723.

Park, P. (1994). Ultrastructural studies on host responses to *Alternaria* host-specific toxins. In: *Host Specific Toxin: Biosynthesis, Receptor and Molecular Biology* (Kohmoto K. & Yoder, O.C., eds). Faculty of Agriculture, Tottori University, Japan. pp. 97-108.

Park, P., Nishimura, S., Kohmoto, K. & Otani, H. (1981a). Comparative effects of host-specific toxins from four pathotypes of *Alternaria alternata* on the ultrastructure of host cells. *Annals of the Phytopathological Society of Japan* **47**, 488-500.

Park, P., Nishimura, S., Kohmoto, K. & Otani, H. (1981b). Two action sites of AM-toxin I produced by the apple pathotype of *Alternaria alternata* in host cells; ultrastructural study. *Canadian Journal of Botany* **59**, 301-310

Parlevliet, J.E. (1981). Race-non-specific disease resistance. In: *Strategies for the Control of Cereal Disease*, (Jenkyn, J.F. & Plumb, R.T., eds.). Blackwell Scientific Publications, Oxford. pp. 47-54.

Pascholati, S.F., Deising, H., Leite, B., Anderson, D. & Nicholson, R.L. (1993). Cutinase and non-specific esterase activities in the conidial mucilage of *Colletotrichum graminicola*. *Physiological and Molecular Plant Pathology* **42**, 37-51.

Paxton, J.D. (1981). Phytoalexins - a working definition. *Phytopathologische Zeitschrift* **101**, 106-109.

Pegg, K.G. (1966). Studies of a strain of *Alternaria citri* Pierce, the causal organism of brown spot of Emperor mandarin. *Queensland Journal of Agricultural Science* **23**, 15-28.

Perumalla, C.J. & Heath, M.C. (1989). Effect of callose inhibition on haustorium formation by the cowpea rust fungus in the non-host, bean plant. *Physiological and Molecular Plant Pathology* **35**, 375-382.

Person, C.O. (1959). Gene for gene relationships in host:parasite systems. *Canadian Journal of Botany* **37**, 1101-1130.

Phillips, M.S. & McNichol, J.W. (1986). The use of biplots as an aid to interpreting interactions between potatoe clones and populations of potato cyst nematodes. *Plant Pathology* **35**, 185-195.

Politis, D.J. (1976). Ultrastructure of penetration by *Colletotrichum graminicola* on highly resistant oat leaves. *Physiological Plant Pathology* **8**, 117-122.

Pollock, G.A., DiSaotino, C.E., Heimsch, R.C. & Coulombe, R.A. (1982a). The distribution, elimination and metabolism of ^{14}C-alternariol monomethyl ether. *Journal of Environmental Health Science* **B17**, 109-124.

Pollock, G.A., DiSaotino, C.E., Heimsch, R.C. & Hillbelink, D.R. (1982b). The subchronic toxicity and teratogenicity of alternariol monomethyl ether produced by *A. solani*. *Food and Chemical Toxicology* **20**, 899-902.

Prasanna, K.P.R. (1984). Studies of *Alternaria brassicae* and *Alternaria brassicicola* infection of cruciferous crop plants. Ph.D. thesis, University of Edinburgh, UK.

Preece, T.F., Barnes, G. & Bailey, J.M. (1967). Junctions between cells as sites of appressorium formation by plant pathogenic fungi. *Plant Pathology* **16**, 117-118.

Raistrick, H., Stickings, C.E. & Thomas, R. (1953). Studies in the biochemistry of microorganisms, 90: Alternariol and alternariol monomethyl ether, metabolic products of *Alternaria tenuis*. *Biochemistry Journal* **55**, 421-433.

Ralton, J.E., Howlett, B.J., Clarke, A.E., Irwin, J.A.G. & Imrie, B. (1988). Interaction of cowpea with *Phytophthora vignae*: inheritence of resistance and production of phenylalanine ammonia-lyase as a resistance response. *Physiological and Molecular Plant Pathology* **32**, 89-103.

Reinecke, P. (1981). Antagonism and Biological Control on aerial surfaces of the Graminae. In: *Microbial Ecology of the Phylloplane* (Blakeman, J.P., ed.). Academic Press, London. pp. 383-395.

Ride. J.P. (1985). Non-host resistance to fungi. In: *Mechanisms of resistance to plant diseases* (Fraser, R.S.S., ed.). Martinus Nijhoff/Dr W. Junk Publishers, Dordrecht/Boston/ Lancaster. pp. 29-61.

Robeson, D.J. & Strobel, G.A. (1981). αβ-Dehydrocurvularin and cuvularin from *Alternaria cinerariae*. *Zeitschrift für Naturforschung* **36c**, 1081-1083.

Robeson, D.J. & Strobel, G.A. (1982). Deoxyradicin, a novel phytotoxin from *Alternaria helianthi*. *Phytochemistry* **21**, 1821-1823.

Robeson, D.J., Gray, G.R. & Strobel, G.A. (1982). Production of the phytotoxins radicinin and radicinol by *Alternaria chrysanthemi*. *Phytochemistry* **21**, 2359-2362.

Rotem, J. (1994). *The Genus* Alternaria. The American Phytopathological Society, St Paul. 326 p.

Royle, D.J. (1976). Structural features of resistance to plant diseases. In: *Biochemical Aspects of Plant-Parasite Relationships* (Friend, J. & Threlfall, D.R., eds). Academic Press, London, New York & San Francicso. pp. 161-193.

Saad, S. & Hagedorn, D.J. (1969). Host-parasite relations in the initiation and development of bean *Alternaria* leaf spot. *Phytopathology* **59**, 1773-1774.

Saharan, G.S. (1988). Plant disease management in linseed. *Review of Tropical Pathology* **5**, 119-140.

Samuels, R.I., Charnley, A.K. & Reynolds, S.E. (1988). Application of reversed-phase HPLC in separation and detection of the cyclodepsipeptide toxins produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Chromatographic Science* **26**, 15-19.

Schäfer, W., Straney, D., Ciuffetti, L., Van Etten, H.D. & Yoder, O.C. (1989). One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. *Science* **246**, 247-249.

Scheffer, R.P. (1983). Toxins as chemical determinants of plant disease.. In: *Toxins and Plant Pathogenesis* (Daly, J.M. & Deverall, B.J., eds). Academic Press, Sydney. pp. 1-40.

Scheffer, R.P. & Briggs, S.P. (1981). A Perspective of Toxin Studies in Plant Pathology. In: *Toxins in Plant Disease* (Durbin, D.R., Ed.). Academic Press, New York. pp. 1-20.

Schmele, I. & Kauss, H. (1990). Enhanced activity of the plasma membrane localized callose synthase in cucmber leaves with induced resistance. *Physiological and Molecular Plant Pathology* **37**, 221-228.

Schneider, R. (1958). Untersuchengen über variabilität und taxonomie von *Fusarium avenaceum* (Fr.) Sacc.. *Phytopathologische Zeitschrift* **32**, 95-126.

Shahin, E.A. & Shepard, J.F. (1979). An efficient technique for inducing profuse sporulation of *Alternaria* species. *Phytopathology* **69**, 618-620.

Shimomura, N., Otani, H., Tabira, H., Kodama, M. & Kohmoto, K. (1991). Two primary action sites for AM-toxin produced by *Alternaria alternata* apple pathotype and their pathological significance. *Annals of the Phytopathological Society of Japan* **57**, 247-255.

Simmonds, N.W. (1991). Genetics of horizontal resistance to diseases of crops. *Biological Reviews* **66**, 189-241.

Simmons, E.G. (1967). Typification of *Alternaria, Stemphylium,* and *Ulocladium*. *Mycologia* **59**, 67-92.

Simmons, E.G. (1986). *Alternaria* themes and variations (22-26). *Mycotaxon* **25**, 287-308.

Simmons, E.G. (1992). Alternaria taxonomy: Current status, viewpoint, challenge. In *Alternaria: Biology, Plant Disease and Metabolites* (Chelkowski, J. & Visconti, A., eds). Elsevier Publishers, Amsterdam. pp. 1-35.

Singh, B.M. & Saharan, G.S. (1979). Inheritence of resistance to *Oidium lini* Skoric in linseed (*Linum usitatissimum* L.). *Euphytica* **28**, 531-532.

Singh, D., Mital, S.P. & Gangwar, L.C. (1956). Breeding for wilt resistance in linseed (*Linum usitatissimum* L.) in Uttar Pradesh. *Indian Journal of Genetics and Plant Breeding* **16**, 29-31.

Skipp, R.A., Harder, D.E. & Samborski, D.J. (1974). Electron microscopy studies on infection of resistant (*Sr6* gene) and susceptible near-isogenic wheat lines by *Puccinia graminis* f.sp. *tritici*. *Canadian Journal of Botany* **52**, 2615-2620.

Skoropad, P. & Tewari, J.P. (1977). Field evaluation of the role of epicuticular wax in rapeseed and mustard in resistance to *Alternaria brassicae*. *Canadian Journal of Plant Science* **57**, 273-279.

Starratt, A.N. (1968). Zinniol: A major metabolite of *Alternaria zinniae*. *Canadian Journal of Chemistry* **46**, 767-770

Starratt, A.N. & White, S.A. (1968). Identification of some metabolites of *Alternaria cucumerina* (E. and E.) Ell.. *Phytochemistry* 7, 1883-1884.

Steele, J.A. & Mirocha, C.J. (1971). Identification of tenuazonic acid as an important toxic substance produced by *Alternaria* spp. *Phytopathology* **61**, 913 (Abstr.).

Stevenson, F.J. & Jones, H.A. (1933). Some sources of resistance in crop plants. Year Book, U.S. Department of Agriculture. pp. 192-216.

Stinson, E.E. (1985). Mycotoxins - their biosynthesis in *Alternaria*. *Journal of Food Protection* **48**, 80-91.

Stinson, E.E. & Moreau, R.A. (1986). Partial purification and some properties of an alternariol-o-methyltransferase from *Alternaria tenuis*. *Phytochemistry* **25**, 2721-2724.

Stoessl, A. (1980). Phytoalexins - a biogenetic perspective. *Phytopathologische Zeitschrift* **99**, 251-272.

Stoessl, A. (1981). Structure and biogenetic relations: fungal non-host specific. In: *Toxins in Plant Disease* (Durbin, D.R., ed.). Academic Press, New York. pp. 109-219.

Tammes, T. (1928). The genetics of the genus *Linum*. *Bibliographia Genetica* **4**, 1-36.

Templeton, G.E. (1972). *Alternaria* toxins related to pathogenesis in plants. In: *Microbial Toxins; Volume VIII Fungal Toxins* (Kadis, S., Ciegler, A. & Ajl, S.J., eds). Academic Press, New York. pp 169-192.

Templeton, G.E., Grable, C.I., Fulton, N.D. & Meyer, W.L. (1967). Tentoxin from *Alternaria tenuis*: its isolation and characterization. *Proceedings of the 1967 Mycotoxin Research Seminar*. U.S. Department of Agriculture, Washington, DC. pp. 27-29.

Tewari, J.P. (1983). Cellular alterations in the blackspot of rapeseed caused by *Alternaria brassicae*. *Phytopathology* **73**, 831.

Tewari, J.P. (1986). Sub-cuticular growth of *Alternaria brassicae* in rapeseed. *Canadian Journal of Botany* **64**, 1227-1231.

Tewari, J.P. (1991). Resistance to *Alternaria brassicae* in crucifers. *Bulletin OILB-SROP* **14**, 154-161.

Tewari, J.P. & Conn, K.L. (1993). Reactions of some wild crucifers to *Alternaria brassicae*. *Bulletin OILB-SROP* **16**, 53-58.

Tewari, J.P. & Skoropad, P. (1976). Relationship between epicuticular wax and blackspot caused by *Alternaria brassicae* in three lines of rapeseed. *Canadian Journal of Plant Science* **56**, 781-789.

Thomas, R. (1961a). Studies in the biosynthesis of fungal metabolites. 2, The biosynthesis of alternariol and its relation to other fungal metabolites. *Biochemistry Journal* **78**, 748-758.

Thomas, R. (1961b). Studies in the biosynthesis of fungal metabolites. 4, Alternariol monomethyl ether and its relation to other phenolic metabolites of *Alternaria tenuis*. *Biochemistry Journal* **80**, 234-240.

Tiburzy, R. & Reisener, H.J. (1990). Resistance of wheat to *Puccinia graminis* f.sp. *tritici*: Association of the hypersesitive reation with the cellular accumulation of lignin-like material and callose. *Physiological and Molecular Plant Pathology* **36** 109-120.

Tietjen, K.G., Schaller, E. & Matern, U. (1983). Phytotoxins from *Alternaria carthami* Chowdhury: Structural identification and physiological significance. *Physiological Plant Pathology* **23**, 387-400.

Toxopeus, H.J. (1956). Reflections on the origin of new races in *Phytophthora infestans* and the breeding for resistance in potatoes. *Euphytica* **5**, 221-237.

Tsuneda, A. & Skoropad, W.P. (1978). Behaviour of *Alternaria brassicae* and its mycoparasite *Nectria inventa* on intact and excised leaves of rapeseed. *Canadian Journal of Botany* **56**, 1333-1340.

Turner, J.A. (1987). *Linseed Law: A Handbook for Growers and Advisors*. BASF U.K. Ltd., Hadleigh, UK. 356 p.

Ueno, T., Nakashima, T., Hayashi, Y. & Fukami, H. (1975). Structures of AM-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria mali*. *Agricultural and Biological Chemistry* **39**, 1115-1122.

Van der Plank, J.E. (1963). *Plant Diseases: Epidemics and Control*. Academic Press, New York. 349 p..

Van der Plank, J.E. (1978). *Genetic and molecular basis of plant pathogenesis*. Springer-Verlag, Berlin. 167 p.

Van Dyke, C.G. & Trigiano, R.N. (1987). Light and scanning electron microscopy of the interaction of the biocontrol fungus *Alternaria cassiae* with sicklepod (*Cassia obtusifolia*). *Canadian Journal of Plant Pathology* **9**, 230-235.

Van Etten, H.D., Matthews, D.E. & Matthews, P.S. (1989). Phytoalexin detoxification: inportance for pathogenicity and pratical implications. *Annual Review of Phytopathology* **27**, 143-164.

Van Etten, H.D., Matthews, D.E. & Smith, D.A. (1982). Metabolism of phytoalexins. In: *Phytoalexins* (Bailey, J.A. & Mansfield, J.W., eds). Blackie, Glasgow and London. pp. 181-212.

Vassil, I.K. (1990). The realities and challenges of plant biotechnology. *Biotechnology* **8**, 296-301.

Vidhyasekaran, P., Borromeo, E.S. & Mew, T.W. (1992). *Helminthosporium oryzae*toxin suppresses phenol metabolism in rice plants and aids pathogen colonization. *Physiological and Molecular Plant Pathology* **41**, 307-315.

Vloutoglou, I. (1994). Epidemiology of *Alternaria linicola* on linseed (*Linum usitatissimum* L.). PhD thesis, University of Nottingham, Nottingham, UK.

Vloutoglou, I., Fitt, B.D.L. & Lucas, J.A. (1995). Survivial and seed to seedling trasmission of *Alternaria linicola* on linseed. *Annals of Applied Biology* **127**, 33-47.

Von Ramm, C. (1962). Histological studies of the infection by *Alternaria longipes* on tobacco. *Phytopathologische Zeitschrift* **45**, 391-398.

Walker, J.C. (1952). *Diseases of Vegetable Crops*. McGraw-Hill, New York. 529 p.

Webster, J. & Dix, N.J. (1960). Succession of fungi on decaying cocksfoot culms. III. A comparison of the sporulation and growth of some primary saprophytes on stem, leaf blade and leaf sheath. *Transactions of the British mycological Society* **43**, 85-99.

Wei, C, -I. & Swartz, D.D. (1985). Growth, and production of mycotoxins by *Alternaria alternata* in synthetic, semi-synthetic, and rice media. *Journal of Food Protection* **48**, 306-311.

Wheeler, H. (1974). Cell wall and plasmalemma modifications in diseased and injured plant tissues. *Canadian Journal of Botany* **52**, 1005-1009.

Wood, R.K.S. (1967). Physiological Plant Pathology. In: *Botanical Monographs* (James, W.O. & Burnett, J.H., eds.) Volume 6. Blackwell Scientific Publications, Oxford, Edinburgh. pp. 55-87.

Wynn, W.K. (1976). Appressorium formation over stomates by the bean rust fungus: Response to a surface contact stimulus. *Phytopathology* **66**, 136-147.

Wynn, W.K. (1981). Tropic and taxic responses of pathogens to plants. *Annual Review of Phytopathology*, **19**, 237-255.

Yoder, O.C. (1980). Toxins in pathogenesis. *Annual Review of Phytopathology* **18**, 103-129.