Aspects of the Biology of *Brassica*/*Alternaria* Host/Pathogen Systems

By

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Dedication

For Catriona, with all my love.

Declaration

This thesis has been composed by myself and has not previously beem submitted for any degree. All sources of information and assistance have been specifically acknowledged.

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CONTENTS

Abstract

The main purpose of this study was to further an understanding of host-pathogen interactions and the role of phytotoxins in the host relationships of *Alternaria brassicae* (Berk.) Sacc. and *Alternaria brassicicola* (Schw.) Wilts., using microscopical, biochemical, and statistical approaches. A further aim was to assess the taxonomic positions of *A.brassicae* and *A.brassicicola* within the *Alternaria*, along with their attributes and behaviour patterns in relation to other members of the anamorph-genus.

On the leaf surfaces of host plants *A.brassicae* and *A.brassicicola* showed broadly similar patterns of development but with some features which distinguished them. The larger spores of *A.brassicae* typically produced two to three germ-tubes whereas *A.brassicicola* gave rise to only one. *A.brassicae* generally produced more extensive extra-matrical growth with hyphal branching and appressoria in intercalary as well as terminal positions; hyphae of *A.brassicicola* produced very few branches and usually terminated in appressoria. For both fungi appressoria were formed most frequently near to or over anticlinal walls of epidermal cells. Smaller numbers of appressoria were formed over periclinal walls and, in the case of *A.brassicae*, over stomata. The extra-matrical development of both species was more or less similar on contrasting host leaf surfaces, although *A.brassicae* showed somewhat reduced germ-tube numbers and stomatal penetrations on leaves with a pronounced waxy bloom. Marked differences in behaviour on different hosts or between hosts and non-hosts were evident only after penetration. In the case of *A.brassicae* unsuccessful penetrations were associated with only localised deposition of callose in the cell wall of the challenged cell, while with *A.brassicicola* unsuccessful penetration events were associated with cell wall responses which occurred in whole single cells.

In comparing *A.brassicae* and *A. brassicicola* with other *Alternaria* species varying in their host range and degree of parasitism, all exhibited essentially similar patterns of extra-matrical behaviour on leaves, culminating in the formation of appressoria. Distinctive host relationships were evident only in the post-penetration phase.

From successful penetrations *A.brassicae* produced a short sub-cuticular phase whereas *A.brassicicola* appeared to penetrate to intracellular position in the epidermis. Subsequent colonisation involved intercellular hyphal growth and extensive callose formation in host cell walls in response to both species.

Biochemical studies on different *Alternaria* species indicated that each produced a diverse range of metabolites which may exhibit fungitoxic and phytotoxic activity. In the present study cytotoxicity of the extracts was low. The studies failed to identify host-specific phytotoxicity, as only crude extracts were used, but the critical role of a host specific toxin, or resistance suppressor, is postulated in the initial establishment of infection.

In considering genetical variation within *A.brassicae* and *A.brassicicola*, there is little evidence of marked physiological specialisation in either species. However, in testing a small number of isolates against a dicarboximide fungicide, *A.brassicae* showed slight and *A.brassicicola* showed marked intraspecific variation in fungicide insensitivity. In testing for sensitivity to fungicides the isolates of *A.brassicae* used were found to exhibit distinctive colony growth characteristics.

Multivariate statistical techniques were applied to morphological, biochemical, and pathogenic characters to examine phylogenic relationships between selected species of *Alternaria*. Studies of the taxonomic relationships of the *Alternaria* species are constrained by the absence of teleomorphs, but certain genera within the Pleosporaceae are suggested to accommodate this group of toxigenic, leaf spotting, facultative parasites / saprophytes.

1. INTRODUCTION

Several species of *Alternaria* are recognised as pathogens of cruciferous plants, the two of the greatest practical significance in Britain being *Alternaria brassicae* (Berk.) Sacc. and *Alternaria brassicicola* (Schw) Wilts.. Studies have been made of both pathogens in this country for many years and interest intensified with the increased popularity of oilseed rape during the 1970s and early 1980s. There is still, however, only a limited understanding of the features which characterise their interactions with their host plants. This study considers host - pathogen interactions and the significance of phytotoxins in the host relationships of the two pathogens using microscopical, biochemical, and statistical approaches. The taxonomic positions of *A.brassicae* and *A.brassicicola* within the *Alternaria* and their attributes and behaviour patterns are also considered in comparison with other members of the anamorph-genus. A full understanding of the biology of plant pathogens can be of benefit in improving long-term strategies for their control. Understanding of the biology of host - pathogen interactions will become more important if sophisticated and less empirical crop improvement techniques, based around biotechnology, are to be successfully adopted.

The rest of this chapter gives a brief introduction to the *Alternaria* with particular reference to species recognised as pathogens of cruciferous plants, and considers their general biology, epidemiology, and control measures used against them. In Chapter 2 studies on the behaviour of *A.brassicae*, *A.brassicicola*, and other *Alternaria* species in association with hosts and non-hosts are described in an attempt to indicate features that are linked with fungal pathogenicity and plant resistance. An interest in the role of phytotoxins in pathogenicity is reflected in Chapter 3 which assesses the biological activity of culture extracts from different *Alternaria* species, including *A.brassicae* and *A.brassicicola*. The taxonomy and physiology of *A.brassicae*, *A.brassicicola* and other members of the anamorph-genus are examined in Chapter 4, including reference to features of their physiology and biochemistry which are not necessarily related to pathogenicity. Genetic variation both between and within species is of concern in crop protection in relation to pathogenicity and virulence, but may also be of practical importance with respect to resistance to fungicides. This aspect of *A.brassicae* and *A.brassicicola* is also briefly considered in Chapter 4.

The *Alternaria* belong to the class Deuteromycotina (Fungi Imperfecti), and are grouped in the dematiaceous Hyphomycetes with other fungi which produce naked conidia with pigmented walls on simple conidiophores arising from hyaline or pigmented hyphae. The conidia are generally elliptical or obclavate, euseptate porospores, arising through a pore in the conidiophore wall. Conidium chains are formed acropetally in species which produce them. Generally, while conidium morphology is of primary importance in the identification of *Alternaria* species, most species display a remarkable variation in cultural and morphological characteristics.

The deficiencies of the taxonomy of the Fungi Imperfecti have long been recognised (Kendrick, 1981a; b) and the apparent variability in the *Alternaria* might be better understood if teleomorphs were known. Currently the evidence suggests that the *Alternaria* are the conidial stages of ascomycete fungi belonging to the Pleosporaceae (Simmons, 1986c).

The two species of *Alternaria* which cause the greatest damage to brassica crops in the U.K., *A.brassicae* and *A.brassicicola*, are at opposite ends of the morphological spectrum within the *Alternaria*. *A.brassicae* produces large conidia (75 - 35(μm long) with prominent beaks, typically individually on conidiophores. *A.brassicicola* produces small conidia (18 - 130 μm long) which lack beaks, typically in long branched chains on conidiophores.

All of the crop species within the genus *Brassica* are susceptible to both pathogens, with only quantitative variation in resistance between and within species (Neergaard, 1945; Prasanna, 1984). *A.brassicae* and *A.brassicicola* also occur on cruciferous plants such as *Cheiranthus*, *Matthiola*, and *Sinapis* (Ellis, 1971), although less frequently than on brassicas. Other *Alternaria* species which cause similar symptoms, but which occur more rarely on brassicas, at least in Britain, are *A.alternata* (Fries) Keissler, *A.cheiranthi* (Lib.) Bolle, and *A.raphani* Groves & Skolko. *A.ethzedia* Barr & Simmons is known to occur on brassica leaf tissue (Simmons, 1986c), but the status of this species as a pathogen has not been determined. *A.alternata* is a plurivorous species which is commonly found as a contaminant of brassica seed and has been shown experimentally to infect brassica leaf tissue (Neergaard, 1945; Prasanna, 1984; McKenzie, Robb & Lennard, 1988). However, the contribution of this species to

Alternaria symptoms in the field remains unclear. *A.cheiranthi* is the cause of a leaf spot of *Cheiranthus cheiri* but also occurs on other crucifers (Ellis, 1971), while *A.raphani* is most frequently isolated from *Raphanus sativus* but can cause severe symptoms in brassicas (Ellis, 1971). In North America, *A.raphani* is a far more serious pathogen of oilseed brassica crops than it is in the U.K. and Europe, while *A.brassicicola* is rarely encountered (Degenhardt, Petrie, & Morrall, 1982). It has been suggested that this difference in the relative importance of the two species can be attributed to their respective temperature optima and the different climates of the two continents (Degenardt *et al.*, 1982).

A.brassicae and *A.brassicicola* are associated with warm humid conditions, although there is some variation between the results of various workers in relation to observed optimum conditions for the development of both species. For example, various temperature optima have been reported for *A.brassicae* and *A.brassicicola* (Degenhardt, 1978; Degenhardt *et al.*, 1982; Prasanna, 1984). The overall indication from these studies is that both species have temperature optima of approximately 20° C, with *A.brassicicola* tending to be favoured by slightly higher temperatures. Prasanna (1984) found that infection of leaf disks by *A.brassicae* and *A.brassicicola* could occur at relative humidities as low as 56%, while other studies have indicated that the presence of leaf surface water is required (Humpherson-Jones & Hocart, 1983).

Although *A.brassicae* and *A.brassicicola* are normally recognised as pathogens of the foliage and flowers/pods in brassica crops, all stages of crop growth may be affected. Indeed, both species are principally seed-borne and infected seed is the most common source of infection each season (Anon., 1984; Prasanna 1984). The pathogens are carried in infected seed either in embryo tissue or in the seed coat (Prasanna, 1984). The two fungi differ in their longevity in seed, *A.brassicae* remaining viable for up to 14 months (Neergaard, 1969), but *A.brassicicola* persisting for up to 12 years (Humpherson-Jones, Maude, & Ainsworth, 1980). In addition to inoculum from seed, the main source of primary infections in the early part of the growing season appears to be trash-borne mycelium (Anon., 1984). For *A.brassicae* survival in the soil may be aided by the production of microsclerotia and chlamydospores (Tsuneda & Skoropad, 1977).

Prasanna (1984) reviewed disease development for both *A.brassicae* and *A.brassicicola*. Seed-borne infection, or infection arising from soil-borne inoculum may kill seedlings before emergence or give rise to damping-off or wire stem symptoms. Spotting of the cotyledons may occur in less severe attacks, this type of symptom perhaps being related to whether the seed coat is shed or remains attached to the cotyledons until they expand. Conidia produced under favourable environmental conditions may be spread up the plant either in air currents or in rain splashes, producing secondary infections. In seed crops infection on the foliage provides a source of inoculum for infection of the pods and subsequently the seed. Infection of the pods under suitable conditions may result in premature desiccation, pod shatter, and loss of seed.

Inoculum of both species may be dispersed over considerable distances in air, although inoculum density decreases rapidly with increasing distance from its source. Humpherson-Jones & Maude (1982), for example, found viable conidia of *A.brassicicola* could be trapped in large numbers close to, and down wind from, *B.oleracea* seed crops during harvesting; conidia were dispersed for distances of up to 1800 m. The spread of *A.brassicicola* in cabbage crops has been modelled by Fontem, Berger, Weingartner, & Bartz (1991) who found that disease intensity declined dramatically within 7 m of disease foci, and suggested that *A.brassicicola* has a slow rate of epidemic development in comparison with other leaf spot fungi.

Following the prominence of *Alternaria* in oilseed rape in the U.K. during the early 1980's (Anon., 1984) the disease has declined in recent years. This reduction in the incidence of attacks has been due partly to the adoption of dicarboximide seed dressings and foliar applications which are highly effective against *Alternaria* and a range of other brassica pathogens (Anon., 1984). Despite the apparent ability for other species within the *Alternaria* to generate insensitivity to this group of fungicides (Pommer & Lorenz, 1987) there has been no reported field problem of insensitivity in either *A.brassicae* or *A.brassicicola*. In vegetable crops, where quality is important, application of iprodione or vinclozolin may be considered where there is a disease risk (Dixon, 1981). However, in oilseed rape, fungicide treatment to control leaf spotting is not recommended unless the disease is present in the upper foliage during flowering and seed filling (Anon., 1984). Husbandry can influence the severity of attacks by both *A.brassicae* and *A.brassicicola*. For example, careful disposal of crop trash by ploughing-in can reduce the carry-over of

inoculum from first to second crops.

With more vociferous public concern about the use of agrochemicals and increasing pressure on economic margins from rising product prices there will continue to be an interest in improving genetic resistance to *Alternaria* and other pathogens in brassicas. Currently however, there are no varieties which are recomended on the basis of improved resistance to *Alternaria* in any of the major brassica crops.

The most important host plants of *A.brassicae* and *A.brassicicola* are members of the *Brassica* genus. The six species of *Brassica* which are commonly grown for food or oil production are genetically interrelated, as shown in Figure 1.1. In Europe and North America the most important species are *Brassica campestris*, *B.napus*, and *B.oleracea*. Selective breeding within all of these species has produced a number of distinct morphological types which are grown for quite separate purposes. In Europe and North America the diploid *B.campestris* is grown either as an autumn or spring sown oilseed crop (principally in North America), or as turnip for human or livestock consumption. In Asia various leafy forms of *B.campestris* are important as vegetables (McNaughton, 1976a). Similarly, the allotetraploid *B.napus* is grown either as a spring or autumn sown oilseed crop (in North America and Europe), as swede for human or livestock consumption, or in a leafy form as fodder rape (McNaughton, 1976b). *B.oleracea* (diploid) has been bred into a diverse array of vegetable types including the cabbage group, cauliflower, brocolli and calabrese, Brussels sprout, and the kales (Thompson, 1976). The remaining three *Brassica* species, *B.carinata*, *B.juncea* and *B.nigra*, are grown principally as seed crops in Asia, Europe and North America for their use in mustard condiments. Of the brassica mustards, *B.juncea* is now the most widely grown, while *B.carinata* has historically been grown only in a small area in North Africa (hence its common name, Ethiopian mustard).

As a result of the diversity of crop types breeding of *Brassica* species has an extensive list of aims. There have been attempts to reduce the content of erucic acid and/or glucosinolates in most, if not all, species. In addition breeding objectives have included increase in the oil content of seed in oilseed crops, increased dry matter content in bulbs and stems of fodder crops, and better uniformity and winter

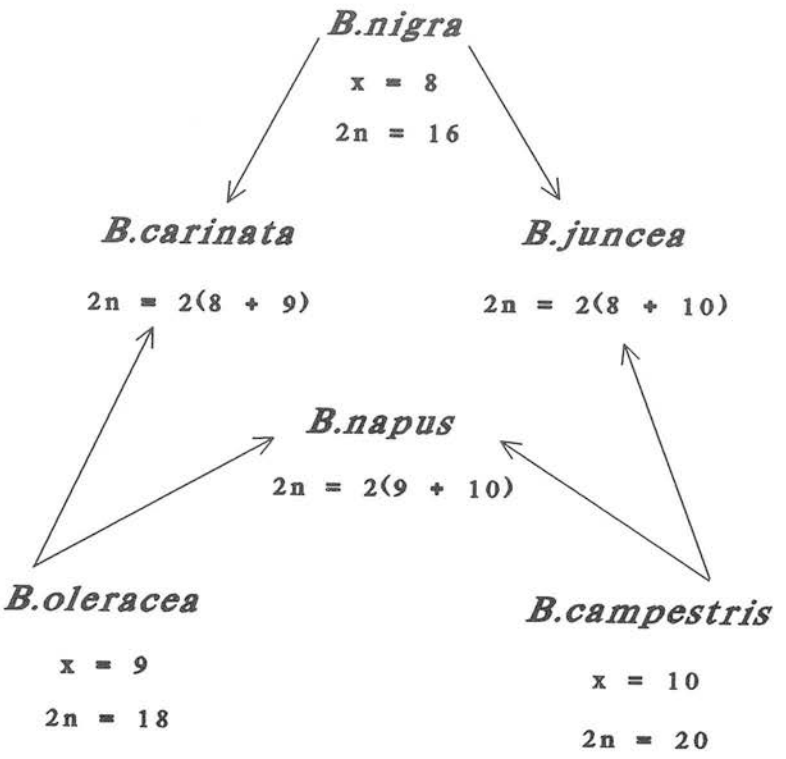


Figure 1.1: Inter-relationships between species within the *Brassica* genus.

hardiness in vegetable crops. In most species breeding for improved resistance to pests and diseases has also been conducted (Hemmingway, 1976; McNaughton, 1976a, b; Thompson, 1976).

Resistance to fungal pathogens has been increased steadily but not dramatically. Race specific (vertical and putatively oligogenic) resistance to important fungal pathogens has been found in brassicas: e.g., *Leptosphaeria maculans* (Mithen & Lewis, 1988), *Peronospora parasitica* (Moss, Crute, Lucas, & Gordon, 1988). However, it appears that acceptable levels of disease resistance may be achieved through the exploitation of race non-specific (horizontal and putatively polygenic) resistance. Indeed, despite the availability of race specific resistance it does not appear to have been widely used. Many of the fungal pathogens of brassicas are air-borne and polycyclic and it is unlikely that adoption of race specific resistance would be successful in the long term. The continued use of race non-specific resistance offers a more reliable alternative (Simmonds, 1991). Table 1.1 lists references which illustrate the quantitative nature of resistance to a number of important fungal pathogens of the *Brassica* genus.

Table 1.1: Sources indicating quantitative (polygenic) resistance to aerial fungal pathogens in *Brassica* species.

Pathogen	Type of experimental evidence	Source
Albugo candida	Inoculation of seedlings	Edwards & Williams (1987)
Alternaria brassicae	Inoculation of whole plants or excised plant parts	Humpherson-Jones & Hocart (1983) Prasanna (1984) Bansal, Seguin-Swartz, Rakow, & Petrie (1990)
A.brassicicola	Inoculation of whole plants or excised plant parts	Bravermann (1971; 1977) Prasanna (1984)
Erysiphe cruciferarum	Inoculation of whole plants and excised plant parts	Munro (1985)
Leptosphaeria maculans	Inoculation of seedlings and adult plants	Sherf (1968)
Peronospora parasitica	Observation of natural infection in field trials	Dixon (1981)
Pyrenopeziza brassicae	Inoculation of excised plant parts	Maddock, Ingram & Gilligan (1981)

The current evidence concerning the *Alternaria* suggests that race specific resistance is not available and that resistance is a polygenically determined character. A recent review of the genetics of horizontal resistance (Simmons, 1991) has suggested that selection in populations displaying horizontal resistance should produce cultivars with acceptable levels of durable resistance. In vegetable crops, however, selection of immunity may be more tempting since appearance is very important. Studies with *A.brassicae* have indicated that the non-brassica crucifers *Eruca sativa* (Tewari, 1986) and *Camelina sativa* (Jejelowo, Conn, & Tewari, 1991) may provide two sources of resistance which approach immunity. However, it is unclear whether these sources of resistance are race specific or race non-specific, and prospects for the transfer of resistance to brassicas are uncertain. In the case of *Eruca*, introgression of resistance may be possible *via* the fertile protoplast-derived hybrid genus *Erussica* which has recently been constructed (Sidar, Chatterjee, Das, & Sen, 1990).

The physiological basis of resistance and susceptibility in brassicas to *Alternaria* is not clearly understood but involves several factors, as might be inferred from the quantitative variation seen in resistance phenotypes. All of the following mechanisms may play some role in resistance: resistance to cellulolytic enzymes, resistance to phytotoxins, accumulation of phytoalexins, hypersensitivity, and structural changes in cell walls which limit ingress.

The degree of resistance to many fungal pathogens in the field apparently relies heavily on the interaction between physical features of the plant cuticle and environmental conditions. Interestingly resistance to several pathogens has been correlated to the nature of the cuticle or the epicuticular wax layer, including *A.brassicae* and *A.brassicicola* (Tewari & Skoropad, 1976; Skoropad & Tewari 1977; Prasanna (1984), *Peronospora parasitica* (Natti, Dickson, & Atkin, 1967); *Pyrenopeziza brassicae*, (Maddock *et al.*, 1981). With respect to the interaction between brassicas and their *Alternaria* pathogens, it is not known how important this type of pre-formed resistance barrier is compared with active resistance mechanisms which might operate after attempted penetration. However, it is known that disease spread and the severity of attack are related to leaf wettability and the availability of water.

Early studies of *A.brassicae* and *A.brassicicola* in common with most plant pathogens tended to concentrate on their ecology, epidemiology, and practical measures for their control. There has been a steady but limited amount of research on more fundamental aspects of their biology over the last 20 years, principally conducted by J.P. Tewari and his colleagues in Alberta. Although studies have therefore been made of the plant - pathogen interactions involving *A.brassicae* and *A.brassicicola*, we have little understanding of the fundamental aspects of these interactions.

The experimental studies and discussions in the following four chapters are intended to investigate several aspects of these host relations, and the biology and taxonomy of *A.brassicae* and *A.brassicicola* as representatives of a distinctive group of plant pathogens. One of the aims of the plant infection studies which are described in the following chapter was to identify crucial stages in fungal development when resistance factors might operate.

2. PLANT INFECTION STUDIES

2.1. INTRODUCTION

The Behaviour of Fungal Pathogens at Plant Surfaces.

The pre-penetration development of many plant pathogenic fungi has been clearly described. For example, Heath (1974) and Wynn (1976) have reported on the behaviour of different rusts, while Ellingboe (1972) has described the development of *Erysiphe graminis*. Similar studies have been made on a range of other fungi. The general subject area of pathogen behaviour on the aerial surfaces of plants has been reviewed by Preece (1976), while Aist (1981) and Wynn (1981) have also reviewed this stage of pathogen development in papers covering the entire infection process.

Table 2.1 lists methods of penetration which have been reported for several *Alternaria* species. Some variation in behaviour is noted in most cases, suggesting that *Alternaria* spores generally do not appear to have as rigid a post-germination development pattern as biotrophic leaf pathogens such as the rusts and powdery mildews. Some of the apparent variation reported in the behaviour of *Alternaria* species may be due to the comparatively small amount of work which has been conducted on these pathogens. Certainly their behaviour has never been subjected to the type of detailed analysis described by Ellingboe (1972) on *E.graminis*.

Considering *A.brassicae* and *A.brassicicola* in particular, the variation displayed in the pre-penetration growth occurs at several points. Thus, McKenzie *et al.* (1988) reported that conidia of both *A.brassicae* and *A.brassicicola* could produce one or more germ-tubes on brassica leaf disks. Tsuneda & Skoropad (1978) noted that *A.brassicae* germinated either normally by production of germ-tubes or, occasionally, by the production of secondary conidia. Production of secondary conidia has also been noted with *A.brassicicola* (McKenzie *et al.*, 1988).

The extent of hyphal growth prior to attempted penetration by *Alternaria* species has never been adequately described; this may be due in part to the great variation in behaviour displayed between conidia

Table 2.1: Reported penetration behaviour of *Alternaria* species

SPECIES	MEANS OF PENETRATION AT HOST SURFACE (HOST)	SOURCE
A.alternata	Stomatal or direct with or without appressoria (*Phaseolus*)	Saad & Hagedorn (1969)
	Stomatal (*Triticum*)	Dickinson (1981)
	Direct with appressoria (*Brassica*)	McKenzie *et al.* (1988)
A.brassicae	Direct with appressoria (*Brassica*)	Changsri & Weber (1963)
	Stomatal or direct with or without appressoria (*Brassica*)	Tsuneda & Skoropad (1978)
	Stomatal or direct with or without appressoria (*Brassica*)	Tewari (1986)
	Stomatal or direct with or without appressoria (*Brassica*)	McKenzie *et al.* (1988)
A.brassicicola	Stomatal or direct with appressoria (*Brassica*)	Changsri & Weber (1963)
	Direct with or without appressoria (*Brassica*)	McKenzie *et al.* (1988)
A.cucumerina	Direct with appressoria (*Cucumeris*)	Jackson (1959)
A.helianthi	Direct with appressoria or stomatal without appressoria (*Helianthus*)	Allen, Brown, & Kochman (1983)
A.longipes	Direct with or without appressoria (*Nicotiana*)	Von Ramm (1962)
A.porri	Stomatal (*Allium*)	Angell (1929)
A.raphani	Stomatal or direct with appressoria (*Brassica*)	Changsri & Weber (1963)
A.solani	Stomatal or direct (*Solanum*)	Harrison *et al.* (1965)
A.tagetica	Direct (*Tagetes*)	Cotty & Misaghi (1984)

from even one isolate on a single type of host surface. In addition, variation is also noted within *Alternaria* species in relation to the selection of sites of appressorium production and penetration. Tsuneda & Skoropad (1978) reported that *A.brassicae* produced appressoria prior to stomatal and direct penetration; in the case of direct penetration these workers found that *A.brassicae* did not display any apparent selection of sites on the leaf surface. Tewari (1986) and McKenzie *et al.* (1988) have found that *A.brassicae* or *A.brassicicola* may penetrate leaf surfaces directly without prior appressorium formation. In the case of *A.brassicae*, Tewari (1986) suggested that the pathogen's ability to penetrate directly without appressoria was due to localised cutinase activity because Transmission Electron Microscopy studies of penetration sites did not reveal any indentation of the cuticle around the penetrating hyphae.

A variety of factors may be involved in triggering the formation of appressoria by plant pathogenic fungi. Ellingboe (1972) has suggested that physical features of the epicuticular wax of barley are responsible for stimulating normal appressoria of *E.graminis*, but more recent work by Carver & Thomas (1990) has revealed that *E.graminis* forms typical appressoria at normal frequencies on barley leaves from which the epicuticular wax has been removed. Wynn (1976) showed that appressorium formation by *Uromyces phaseoli* over stomata relied on a precise tropic response by the pathogen to the shape of the guard cells. Considering the *Alternaria*, Akai, Fukutomi, Ishida, & Kunoh (1969) found that the epicuticular wax from onion leaves stimulated appressorium production by *A.porri*, but these workers noted that such stimulation was apparently a rare phenomenon amongst plant pathogens. It is not known if the physical features of brassica leaves affect the location of infection sites, or production of appressoria by *A.brassicae* or *A.brassicicola*.

Several workers have reported a correlation between levels of epicuticular wax on brassica foliage and resistance to *A.brassicae* or *A.brassicicola*. For example, in leaf inoculation studies, Prasanna (1984) found that species or varieties which exhibited greater waxiness or appeared to have high levels of epicuticular wax had greater resistance to both *A.brassicae* and *A.brassicicola*. Furthermore, rubbing leaf surfaces to remove the wax resulted in greater numbers of lesions on the rubbed areas. In more elaborate studies, Tewari & Skoropad (1976) and Skoropad & Tewari (1977) showed that the greater resistance of a cultivar of *B.napus* than one of *B.campestris* to *A.brassicae* was due to the thicker distal wax layer in the

former species. The resistance was considered to result indirectly from the reduction in surface wettability which was conferred by the thicker wax layer. However, the possibility that the wax presents a direct physical or chemical impediment to penetration by *A.brassicae* and *A.brassicicola* cannot be discounted, although the wax appears to have no direct inhibitory effect on fungal development since Conn & Tewari (1989) found that recrystallised wax from *B.napus* and *B.campestris* did not reduce germination of *A.brassicae in vitro*. There is still little evidence that variation in epicuticular wax characteristics have any influence on the post-germination development of *Alternaria* species.

In relation to breeding for resistance to *Alternaria* in the *Brassica* genus, exploitation of the effect of epicuticular wax may be of particular value. Wynn (1981) suggested that if particular characteristics of plant surfaces can confuse pathogen recognition systems, the resulting resistance is likely to be non-specific and to act by denying access to the plant completely, or to increase the latent period of infection. In considering this argument in relation to plant breeding, Wynn (1981) concluded that the only character which would allow exploitation of this form of resistance was wax production. Whether or not the epicuticular wax of *Brassica* species directly influences the behaviour of *Alternaria* species, the results of Skoropad & Tewari (1976) suggest that useful resistance to *A.brassicae* and *A.brassicicola* might follow indirectly from breeding to increase wax levels on brassica foliage.

Pathogen development after cuticular penetration

Aist (1981) considered that the development of many more pathogens than is recognised stops at a sub-cuticular phase. Several fungal pathogens develop an extensive sub-cuticular mycelium: for example, *Venturia inaequalis* on apple (Nusbaum & Keitt, 1938), *Rhynchosporium secalis* on barley (Jones & Ayers, 1974), *Pyrenopeziza brassicae* (Rawlinson, Sutton, & Muthaylu, 1978), and *Leptosphaeria maculans* (Hammond & Lewis, 1987), on brassicas. Tewari (1986) reported that *A.brassicae* developed a short sub-cuticular phase during colonisation of leaves of *B.napus*. However, in comparison with the pathogens listed above this phase of development is relatively short for *A.brassicae*.

Pathogens such as *Alternaria* spp. which do not form haustoria possibly obtain nutrients during tissue colonisation by damaging host cells and absorbing the leaked cell contents. During the sub-cuticular

development of *R.secalis*, permeability changes are induced in the underlying host cells by the pathogen (Jones & Ayers, 1972); Tewari (1986) has suggested a similar form of behaviour for *A.brassicae*, which is known to produce both phytotoxins (Degenhardt, 1978; Bains & Tewari, 1987) and cell wall degrading enzymes (Shohet, 1985) in culture. Tewari (1983) reported that hyphae of *A.brassicae* were not present in the green tissue surrounding necrotic or chlorotic lesions, suggesting that cell death in advance of the hyphae resulted from secreted phytotoxin(s). The action of pathogen-produced cell wall degrading enzymes (CWDE) may result in similar symptoms to those of cell damage from phytotoxins. Wood (1976) described the role of CWDE in host cell death: exposure of cells to CWDE results in cell separation which leads to cell collapse. Prior to cell collapse the cells become highly permeable to K+ ions.

The biochemical interactions between *A.brassicae* or *A.brassiciola* and host tissue are further complicated by the evidence of Suri & Mandahar (1984; 1985) that both pathogens produce cytokinins *in vivo* which may be responsible for the green islands which form round older lesions on host leaves. Suri & Mandahar (1984; 1985) have suggested that *A.brassicae* and *A.brassicicola* are hemibiotrophs. There is some indirect evidence that *A.solani* is able to exist in host tissue for several weeks without inducing symptoms (Harrison *et al.*, 1965), but the nutritional relationship between host and pathogen during this "latent" period has not been examined. In addition, Dickinson (1981) reported that *A.alternata* can form symptomless intercellular colonies in wheat leaves during the early stages of leaf senescence. However, there is no evidence that *A.brassicae* or *A.brassicicola* are able to colonise host tissue without symptom development. One further complication to any model of the nutritional relationship between *A.brassicae* and its host is evidence of its ability to produce abscisic acid (ABA) and other plant growth substances *in vitro* (Dahiya & Tewari, 1991). Abscisic acid is generally considered to promote senesence and ripening in plant tissues. Production of this compound at physiologically active concentrations by *A.brassicae in planta* would therefore agree with the general effects of the pathogen on its hosts, *i.e.* premature senesence of foliage and premature pod shatttering. However, there is no direct evidence that ABA is synthesised by *A.brassicae* during colonisation of plant tissues.

Although there has been some progress in studies of the interactions between *A.brassicae* and *A.brassicicola* and brassica crop plants there is no accepted model to explain the specificity of these two

Alternaria species to members of the *Cruciferae*. It is likely that any such model will have to take account of many interacting factors. Bains & Tewari (1987) have proposed that the specificity of *A.brassicae* is determined by the host-selective toxin destruxin B. However, there is good evidence that this factor alone does not determine the outcome of interactions between *A.brassicae* and plants which it might attempt to infect (Buchwaldt & Green, 1992).

The broad aim of the work in this chapter was to present a view of the interactions between *A.brassicae* and *A.brassicicola* and their host plants. In examining the variation in the behaviour of these two fungi on a variety of host and non-host plants, representing a range of susceptibilities, it was hoped to identify common features and differences in the development of the pathogens and to identify the stages at which compatibility is determined. Complementing studies of the two pathogens, a number of other *Alternaria* species, pathogenic or non-pathogenic to brassica, plants were also examined in order to establish common behaviour patterns displayed by members of the genus.

Three experiments were carried out. In Experiment 2.1 specific attention was given to possible effects of epicuticular wax on the behaviour of *A.brassicae*, *A.brassicicola*, and *A.alternata* following inoculation of lines of *Brassica oleracea* var. *gemmifera* with differing epicuticular wax characteristics. In Experiment 2.2 the interactions between a range of cruciferous species and three specific pathogens of this family, *A.brassicae*, *A.brassicicola*, and *A.raphani*, were examined, with particular attention being paid to the interactions occurring after attempted penetration. Experiment 2.3 involved an examination of the behaviour of a wider range of *Alternaria* species during their development on both host and non-host plants.

Throughout the studies multivariate analysis approaches which might be of value for the graphical representation of plant - pathogen interactions were utilised to supplement analysis of variance. An additional aim of this chapter was to evaluate the use of these techniques, which have been commonly used in ecological studies, in studies of plant - pathogen interactions.

2.2 MATERIALS AND METHODS

Experiment 2.1: The behaviour of *Alternaria brassicae*, *A.brassicicola*, and *A.alternata* on leaf disks of three epicuticular wax types of *Brassica oleracea* var. *gemmifera*.

Glasshouse-grown seedlings of three lines of *B.oleracea* var. *gemmifera* were supplied by Dr. T. Hodgkin (present address, I.B.P.G.R., Rome, Italy) from breeding material. The genotypes of these lines with respect to wax characteristics, are shown in Appendix 2.1, while the typical appearance of leaves and the physical structure of their epicuticular wax layer are illustrated in Plate 2.1. The plants were transplanted to 18 cm pots, one plant per pot, containing a peat based compost, and grown in a heated, illuminated glasshouse. Illumination conditions are described in Appendix 2.2. Leaf disks (1.5 cm diameter) were cut from just-expanded leaves at the crown of mature plants. The leaf disks were placed in 10 cm square dishes containing water agar with 80 ppm benzimidazole. Colonies of fungal isolates used in the study were grown on Corn Meal Agar (CMA) as described in Appendix 2.3. Inoculum from *A.brassicae*, *A.brassicicola*, and *A.alternata* was prepared by washing 3-week-old colonies of each of the isolates with a small volume of distilled water and agitating the colony surface with a glass spreader. Spore concentration in the inoculum suspension was adjusted to 5000/ml by assessment of spore numbers in 50μl drops of spore suspension spotted on filter papers, using a low power microscope. Inoculum was applied to leaf disks on the agar plates in a 50μl droplet by means of micropipette. Inoculated leaf disks were incubated at 20± 2°C and were sampled at 4, 8, 12, and 18 hours after inoculation. The experiment was arranged in three randomised replicate blocks, one block on each shelf of an illuminated incubator.

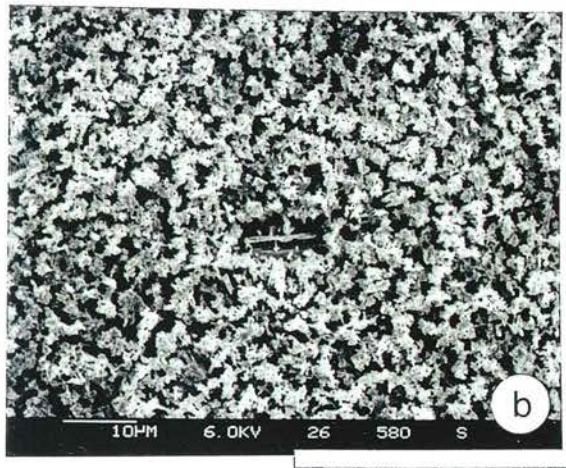
The leaf disks were prepared for fluorescence microscopy and stained with DAPI, ANS or Tinopal as described in Appendix 2.4. Observations were made on a Leitz Ortholux II microscope fitted with a 100W Mercury vapour lamp. Photomicrographs were taken with an Olympus OM2 camera with Kodak Ektachrome ASA 160 colour film. The development of each isolate was assessed by taking mean results for 30 conidia on each leaf disk at each sampling time. The following 12 variates were used to describe fungal development:

Plate 2.1: The appearance and epicuticular wax characteristics of leaves of three lines of *B.oleracea* var. *gemmifera* (descriptions are given on the facing page).

(a) The appearance of mature leaves of three lines of *Brassica.oleracea*. var. *gemmifera*. Left to right, Waxy, Intermediate, Glossy.

(b) - (d) The fine structure of the epicuticulr wax layer of the three leaf types shown in Plate 2.1a: (b) Waxy; (c) Intermediate; (d) Glossy. Note that the stoma visible in Plate 2.1b is almost obscured by the structure of the wax.


a


b
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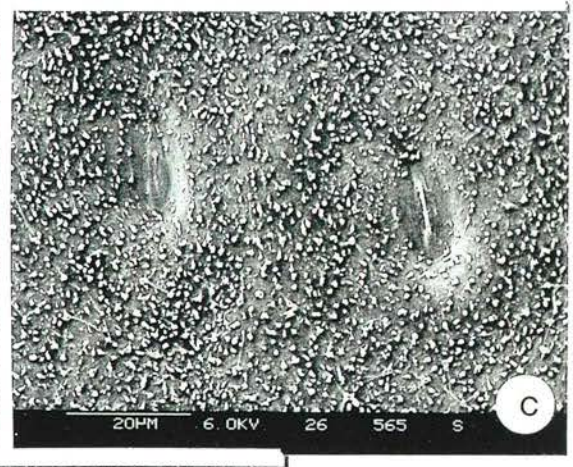
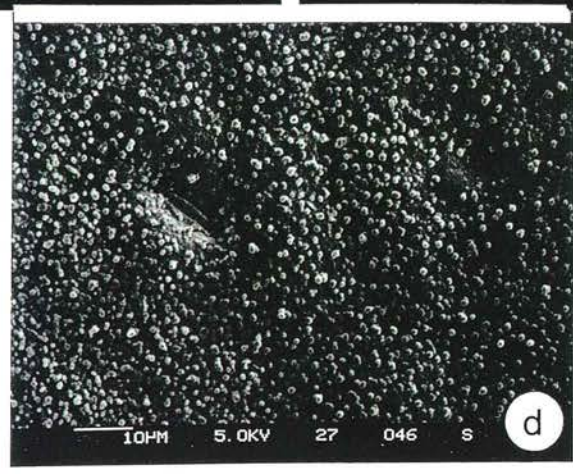

c
20µM 6.0KV 26 565 S


d
10µM 5.0KV 27 046 S

(1) Germination percentage. (Conidia were considered to have germinated if a positively stained germ-tube was visible under u.v. illumination.)

(2) Germ-tube number per conidium.

(3) The percentage of conidia germinating from their basal cell. *

(4) The percentage of conidia germinating from mid-body cells.

(5) The percentage of conidia germinating from terminal cells.

(6) The percentage of germ-tubes with at least one branch.

(7) Germ-tube length in μm

(8) The mean ratio of appressoria to germ tubes (per cent).

(9) The percentage of conidia which gave rise to a penetration event. (formation of appressorium or penetration without appressorium formation.)

(10) The percentage of conidia which gave rise to a penetration event at epidermal cell junctions. (anticlinal wall junctions)

(11) The percentage of conidia which gave rise to a penetration event over periclinal walls.

(12) The percentage of conidia which gave rise to a penetration event *via* stomata.

* In variates 3 - 12 percentages relate to germinated conidia only.

Experiment 2.2: Fungal behaviour and plant cell response in interactions between *Alternaria brassicae*, *A.brassicicola*, and *A.raphani*, and cruciferous hosts.

Plants of the following species were grown in peat based compost, three per pot, in 12 cm diameter pots in a Burkard isolation propagator: *Brassica napus* var. *oleifera* (Bn), *B.campestris* var. *campestris* (Bc), *B.oleracea* var. *gemmifera* (Bo), *B.carinata* (Ba), *B.juncea* (Bj), *B.nigra* (Bi), *Cheiranthus cheiri* (Cc), *Sinapis alba* (Sa). Cultures of isolates of *Alternaria brassicae* (Ab), *A.brassicicola* (Ac), and *A.raphani* (Ar), were grown on V8 Juice medium as described by Senior, Epton, & Trinci (1987), except that the incubation temperature was 20°C, and 2 g/l standard Davies Agar was added to the medium to increase the rate of dehydration. Adding the agar was also found to make harvesting of the conidia easier, as the liquid medium described by Senior *et al.* (1987) was often found to separate into a gelatinous fraction and a liquid fraction

after several days of fungal growth. Conidia adhered to the gelatinous colony surface on rinsing with distilled water and were difficult to remove. Addition of the agar prevented separation of the medium and facilitated harvesting of the conidia.

It was observed that the inoculation procedure employed in Experiment 2.1 led to clumping of conidia. Consequently an alternative method was devised to allow the application of dry inoculum. Conidia were washed from colonies in distilled water containing approximately 0.05% v/v Tween 20. The conidium suspension was centrifuged in 2 ml epindorfs in a haematocrit at 12,000 g for 1 minute, and the supernatant discarded. The pellet of conidia was resuspended in approximately 0.1 ml of 0.05% aqueous Tween 20 solution, and spread on to 4-5 microscope slides. The slides were then air dried at room temperature in the air stream of a fume cupboard for approximately 25 minutes. After drying the inoculum was brushed from the slides with a soft paint brush, and allowed to settle on leaf pieces which were to be inoculated. Inoculum density was estimated by observing the inoculated tissue under a dissection microscope.

Inoculated leaf disks were incubated in 25-compartment 10-cm-square cell culture dishes in which alternate rows of cells were filled with 80 ppm benzimidazole agar or warm tap water. The leaf pieces were placed on the agar and the dishes were sealed with parafilm. The wells filled with warm water provided a saturated atmosphere inside the dishes. The experiment was arranged in a randomised block layout with three replicate blocks, each composed of the dishes on one shelf of an incubator. Inoculated disks were incubated at $20 \pm 2°C$ for 24 hours before they were fixed for microscopic examination. Fungal development and plant cell response were assessed by light and fluorescence microscopy (Appendix 2.4).

Preliminary observations of hosts cell reactions indicated that three different types were distinguishable by 24 hours after inoculation. In the first type, fluorescence and thickening of the host cell wall was localised around the point of attempted penetration and hyphal growth was not visible in the cell; this was described as a localised callose deposition. In the second type, the contents of the attacked cell were fluorescent or granular, fluorescence and thickening were apparent around lateral cell walls, and hyphal growth was seen in the cell or cell wall but not in adjacent cells; this was described as a single cell reaction. These first two types were described collectively as confined penetrations. In the third type, all, or

most cells adjacent to the point of attempted penetration showed fluorescence or thickening around their walls, the inner surface of the walls often had a brown tint, and hyphal growth was readily visible in the attacked cells and intercellular spaces; this was described as an adjacent cell reaction or unconfined penetration. Twelve variates were used to describe fungal development and plant cell response; these were:

(1) Percentage germination.

(2) Mean number of germ tubes per germinated conidium.

(3) Mean germ tube length per conidium (μm).

(4) The mean ratio of appressoria formed per germ tube (percentage scale).

(5) The ratio of penetration events to germinated conidia (percentage scale).

(6) The percentage of penetrations which occurred at epidermal cell junctions.

(7) The percentage of penetrations which occurred over epidermal cell periclinal walls.

(8) The percentage of attempted penetrations *via* stomata.

(9) The percentage of attempted penetrations which produced a localised callose deposition.

(10) The percentage of attempted penetrations which produced a single cell reaction.

(11) The percentage of attempted penetrations which produced an adjacent cell reaction

(12) The percentage of germ tubes which induced no apparent host cell reaction.

In addition to these variates leaf disks infected with each of the pathogens were stained with phluroglucinol-HCl to test for the deposition of lignin or lignin aldehydes. Lignin or related compounds were not found in any of the interactions studied and no statistical analysis was conducted.

Experiment 2.3: Fungal behaviour and plant cell response in interactions between *Alternaria* species and host and non-host plants.

Plants of the following species and cultivars were grown, four per pot, in peat based compost in 12 cm pots in a Burkard isolation propagator in a glasshouse under unsupplemented daylight (day length was approximately 16 hours): *Brassica napus* var. *oleifera* (OSR) (cv. Lirradonna), *Lycopersicon esculentum* (TOM) (cv. Moneymaker), *Papaver rhoes* (PPY) (cv. Shirley, single mixed), *Triticum aestivum* (WHT) (cv. Armada). Growth of fungal cultures and production of inoculum was conducted as described for Experiment 2.2. The following fungal species were assessed: *A.alternata* (Aa), *A.brassicae* (Ab), *A.brassicicola* (Ac), *A.infectoria* (Ai), *A.raphani* (Ar), and *A.solani* (As). Inoculation procedure, growth conditions after inoculation, and allocation of inoculated leaf pieces to replicate blocks and experimental design followed that for Experiment 2.2. Fungal development and callose deposition in plant cells were assessed at 18 and 36 hours after inoculation by fluorescence microscopy (Appendix 2.4). In addition, Low Temperature Scanning Electron Microscopy (LTSEM) was used to observe the behaviour of the fungi on the leaf surfaces at 36 hours after inoculation (Appendix 2.5).

A similar range of descriptive variates was used to record fungal development and plant response as in Experiment 2.2, except that localised callose deposition and single cell reactions were not differentiated.

2.3 STATISTICAL METHODS

General approaches

Data for Experiments 2.1 - 2.3 were analysed initially by analysis of variance (ANOVA). In all three experiments a standard analysis of variance was used to perform hypothesis tests, while specific interactions between the various *Alternaria* isolates and the test plants were further examined by multivariate approaches. The Genstat 5 package running under VMS on the Edinburgh School of Agriculture VAX was used to perform all analyses.

Analysis of variance

In Experiments 2.1 and 2.3 the standard ANOVA was augmented by the examination of orthogonal contrasts and orthogonal polynomial contrasts to examine differences between levels of the treatment factors entailed in the *a priori* hypotheses underlying the experiments. The use of this type of extended ANOVA has been discussed by Gilligan (1986) and details of their inclusion in ANOVA in Genstat are given by the Genstat Committee (1987) and Digby, Galwey & Lane (1989).

The contrasts are set up to test a number of independent sub-hypotheses relating to differences between the levels of the factor under consideration. It is possible to test $n-1$ independent contrasts for a factor with n levels. However, Gilligan (1986) has emphasised that contrasts with no biological meaning should not be tested. In situations where only m from a possible $n-1$ contrasts are examined, the degrees of freedom remaining within the factor ($n-1-m$) are pooled and included in the ANOVA as a single term. In Genstat these are included as a "deviations" term which accounts for the remaining variance not accounted for by the specific contrast(s) examined. Each contrast then, employs one degree of freedom in the experiment and accordingly the corresponding hypothesis test is based on the F distribution for values of F with one degree of freedom in the numerator; in this sense such tests are unambiguous (Dawkins 1983; Gilligan, 1986).

Examination of orthogonal contrasts is of particular value for comparing the effects of factors whose levels differ qualitatively (Digby *et al.*, 1989). In the case of factors whose levels differ quantitatively (such as various sampling times) trends in data across levels of a factor can be revealed by breaking the factor down into orthogonal polynomial contrasts. The degrees of freedom within the factor are used to examine a number of specified regression analyses of the test variate over the levels of the factor. The regressions fitted are polynomials of sequentially increasing order. Thus, if a second order polynomial is fitted, a straight line is fitted first and the variance accounted for is presented along with its corresponding F probability; the extra variance accounted for by then fitting a quadratic curve is calculated and the corresponding F value given.

This approach allows sensitive examination of trends in data. Furthermore, it removes the

recurring problem in biological experimentation of how to present data from the interaction of a sampling time factor with other factors. In this situation, if the mean of the data shows an increase over time, the variance often increases with the mean. One possible approach is to present such data with the single s.e.d. calculated during the ANOVA for the interaction. This, however, is unsatisfactory since the pooled s.e.d. will probably be an underestimate of the true s.e.d. at later sampling times, and an overestimate of the true s.e.d. at early sampling times. An alternative is to examine each sampling time independently, allowing the calculation of individual s.e.d.s. Care must be taken with this approach when describing the data, to treat sampling times individually. The use of orthogonal polynomials relieves the difficulties with these approaches. It will detect whether levels of a factor differ in their response over all sampling times by testing whether there is evidence that a significant proportion of the variance can be accounted for by fitting different polynomials for each level of the first factor (e.g. isolates) over time. Thus, for each isolate all sampling times are treated together and the response over time is examined; this is arguably a more suitable approach to studies of the growth or development of organisms than examining the results of each sampling time individually. The application of orthogonal contrasts and orthogonal polynomials to each of the experiments in this section will now be briefly described.

In Experiment 2.1, involving a comparison of the pre-colonisation development of three *Alternaria* species on leaf disks of three lines of *B.oleracea* var. *gemmifera* with different epicuticular wax characteristics, the variation in the waxiness of the different lines used is a qualitative rather than quantitative factor. Total quantities of epicuticular wax do not differ significantly between plants of the different lines within a family, but the fine structure of the wax differs depending on the relative quantities of esters and secondary alcohols present (L.A. Berry, Edinburgh, pers. comm.). The underlying *a priori* hypothesis relating to this factor in Experiment 2.1 was:

> "Differences in the wax characteristics have no effect on the variates used to measure fungal development".

This hypothesis can be split into two contrasts between the three levels of wax:

1. A contrast betweeen the glossy and waxy types.
2. A test for linearity in response across the three levels of wax surface. This is a contrast between

the mean of the glossy and waxy types, and the intermediate type.

The resulting hypotheses are:

a. There is no difference in effect on fungal development between the least and most waxy surface types.

b. Response to differences in the waxiness of the surfaces is linear.

The corresponding coefficients supplied to Genstat in the ANOVA are shown in Table 2.2 below.

Table 2.2: Coefficients applied to three levels of the factor Leaf type in order to assess orthogonal contrasts between the levels in Experiment 2.1

Contrast tested	Leaf-Type		
	Glossy	Intermediate	Waxy
Glossy vs. Waxy	1	0	-1
Linearity of response	-1	2	-1

In the same experiment the time factor was subdivided into orthogonal polynomial contrasts. In this case, since there were four sampling times it would have been possible to fit a third order polynomial. However, the initial analysis was conducted with only linear and quadratic terms specified since a second order polynomial will often account for the majority of the variance in growth response curves. Where the deviations term was significant following this initial analysis the data were examined to assess the deviation from a quadratic response over time.

In Experiment 2.3, where a comparison was made of the development of six *Alternaria* species on leaf pieces of host and non-host plants, the *a priori* hypotheses were:

1. "There is no difference in development between the *Alternaria* species."
2. "There is no effect of plant substrate on the development of any of the *Alternaria* species."

No contrasts between specific *Alternaria* isolates were examined. However, the degrees of freedom within the plant factor were used to examine three specific orthogonal contrasts relating to the following hypotheses:

a. "There is no difference in fungal development on wheat as compared with the dicotyledenous plants tested."

b. "There is no difference in fungal development on oilseed rape as compared with tomato."

c. "There is no difference in fungal development on poppy (a non-host of all the *Alternaria* species used) as compared with oilseed rape and tomato (each hosts of at least one of the *Alternaria* species used)."

The coefficients applied to each of the levels of plant to obtain these contrasts are presented in Table 2.3 below.

Table 2.3: Coefficients applied to the factor plant to assess three orthogonal contrasts between levels of the factor in Experiment 2.3.

Contrast	Plant			
	OSR	poppy	tomato	wheat
wheat vs. dicots	-1	-1	-1	3
OSR vs. tomato	-1	0	1	0
poppy vs. OSR + tomato	-1	2	-1	0

Data from all three experiments for mean germ-tube numbers were treated to a square-root transformation (Cochrane, 1938) before initial analysis. After comparison with analysis of the original data, which showed that transformation had no effect on the significance of the analyses, only the analyses of the original data are discussed.

Multivariate approaches to examining plant-pathogen interactions

Two techniques, Principal Component (PCP) biplots and Correspondence Analysis (CPA), were applied to data from experiments 2.1 - 2.3 to assess interactions between plants and pathogens. The application of both techniques to units by variates data matrices has been discussed by Gower & Digby (1981). Both techniques allow the relationships between all levels of two factors which define a two-way table of data to be approximated in a two dimensional display. Gower & Digby point out that many data

sets can be considered as both two-way tables and as units by variates data matrices, allowing the application of both univariate and multivariate analyses.

In both PCP and CPA the data in a two-way table are treated as the coordinates of the units (rows) in an n dimensional space, where n is the number of columns (variables) in the table. PCP is used to approximate the relationships between the units in a smaller dimensionality by calculating new, hypothetical dimensions which are orthogonal linear combinations of the original data. Typically, more than 80% of the variation in the original data will be represented in the first two dimensions (Phillips & McNicol, 1986; Kempton & McNicol, 1990) meaning that a plot of the units in these first two dimensions will provide a reasonable approximation of the true relationships between the units.

The use of PCP has been extensively discussed by Gabriel (1971; 1981) who introduced the technique. The biplot allows the presentation of both units and variables in the same graphic frame. The units are represented by points while the variables are represented by vectors from the origin. The coordinates for the units are derived directly from the PCP, being the Principal Component scores. The vectors for the variables are described by the weights or coefficients of the columns of the original matrix used to determine the linear combinations resulting in the new principal components (Gower & Digby, 1981; Kempton & McNicol, 1990; Krzanowski, 1990).

An ANOVA which includes the interaction between two factors will produce a two-way table of means which can be subjected to PCP. Alternatively, the two-way table of interaction effects can be used for PCP to model the relationship between two factors. Estimates of interaction effects from an analysis of variance of two factors are the means of specific interactions between the levels of the factors under consideration, corrected for the overall mean of each of the levels of the factors and the grand mean of the data (Phillips & McNicol, 1986).

Where the estimated interaction effects are used as the data for PCP the resulting biplot has certain properties which make it an excellent descriptive tool for examining the interaction between the factors, as described by Gabriel (1981). In this type of biplot the squared lengths of the vectors for the

variables (or column factor) are the variances of each of the variables (or levels of the factor). Examination of the length of the vectors in the biplot gives an indication of the proportion of the total variance in the interaction which can be ascribed to each variable. Second, the cosine of the angle between the any two vectors gives an approximation of the correlation between the two variables. Where two variables lie 180° apart their correlation (with respect to the interaction) is zero. Generalising these two points, it can be said that any vectors which extend a relatively short distance from the origin represent variables which show little overall interaction, while vectors which lie close together represent variables which have similar effects on the units.

Certain generalisations can also be made about the plotted points representing the units (or levels of the row factor) in the biplot. Where a point lies close to the origin, the unit it represents shows little interaction with respect to the variables under examination. Units represented by points which occur close together on the plot are found to be similar in their overall interactions with the variables. The relationships between the units and any particular variable can be determined by examining the projections of the units on to the vectors representing the variables. The relative length of these projections provides an approximation of the ranking of the units with respect to the the variables. Where it is possible to make the projection of a unit on to one of the vectors only by extending the vector through the origin, the ranking of the unit is of reversed sign. Such units would be found to interact differently to that given variable than those whose projections were made on to the original vector.

In the situation such as in this section of work, where the data have already been examined by univariate tests, obtaining rankings, as described above, is likely to be of little importance since it will already be known which units differ significantly. However, comparison of the predicted ranking from the biplot with the results of the ANOVA, and a general appraisal of the distribution of units and vectors in the biplot will reveal where, if at all, it approximates relatively poorly to the interaction.

The underlying mathematics of biplot analysis are dealt with in detail by Gabriel (1971; 1981) and Krzanowski (1990). Digby & Gower (1981), Digby *et al.* (1989) and Kempton & McNicol (1990) provide brief discussions of the principles involved and the last two sets of authors also provide examples of the application of Genstat 5 to the technique. Previously, the PCP biplot has been used successfully by

Kempton (1984) to examine genotype by environment interactions in variety and nitrogen response trials in wheat, and by Phillips & McNicol (1986) to examine interactions between potato clones and nematode populations in the context of selection for improved nematode resistance. A diagramatical summary of the points noted above in relation to interpretation of biplots is presented in Figure 2.1.

The second multivariate technique which was employed diagnostically to plant pathogen interactions in this section was Correspondence Analysis (CPA). This technique also allows the variation in two-way tables to be represented in a smaller number of dimensions with the simultaneous plotting of points representing the different levels of both factors in the interaction. The technique is regularly applied to ecological data to simultaneosly assess, for example, similarities between sampling sites based on counts of species trapped (Digby & Kempton, 1987; Kempton & McNicol, 1990) and similarities between species based on how frequently they occur at certain sites. However, although CPA has special use in relation to counts data, where it can assist in analysis of contingency tables, it can be applied to any two-way table of non-negative data, for example percentage scores (Digby & Gower, 1981). Correspondence analysis employs an iterative process which finds ordinations for units and variables in a multidimensional Euclidean space so that the relative associations between them are preserved. Similarly to the principal components formed by PCP, the first few axes from CPA contain the majority of the variation among the units and variables.

The techniques of PCP and CPA have a similar result in terms of the graphical display achieved. Unlike a PCP biplot however, the normal procedure with CPA is to represent both the units and variables as points rather than as points and vectors respectively. The association between any unit and the variables in the plot is noted directly from the distance between the unit point and the variables' points. The plot similarly displays the relationships between units and between variables. Vectors can be employed in graphical displays from CPA to aid in the analysis of contingency tables. If the points for the units and variables resulting from the CPA of a two-way contingency table are plotted in two dimensions, vectors can be drawn from the origin to the points. Where the vectors for unit-points and variable-points lie close together, the corresponding cell in the two-way table is likely to have a greater value

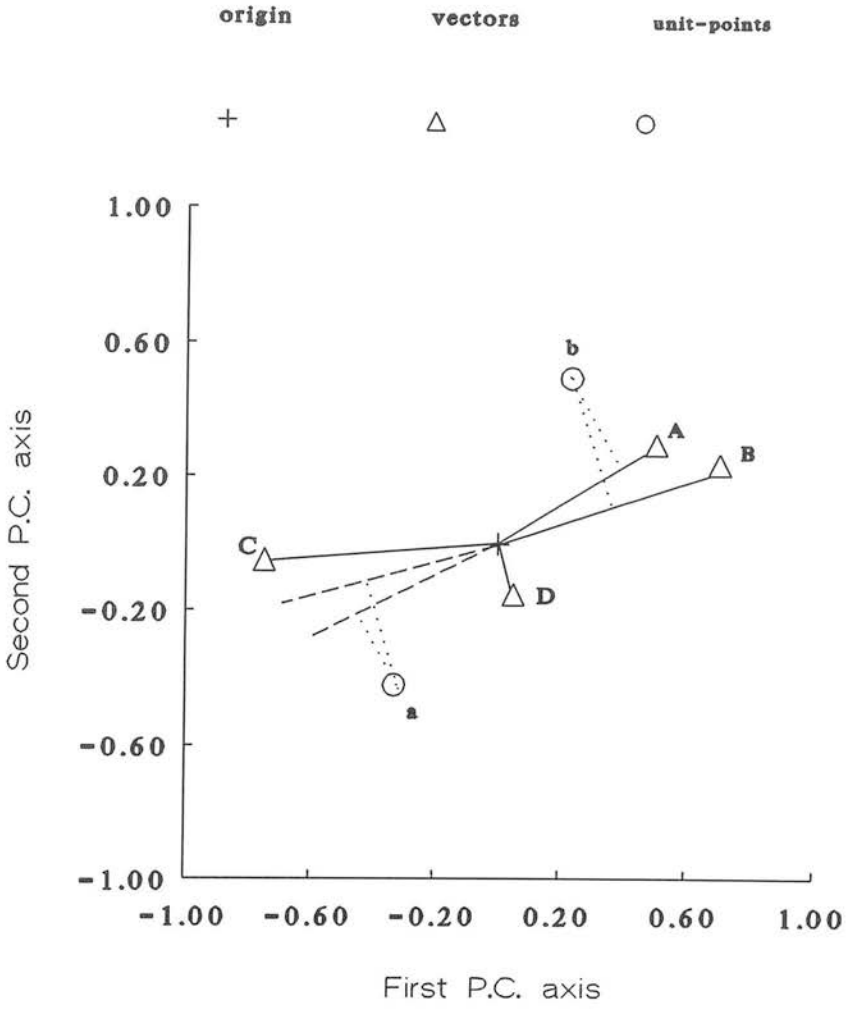


Figure 2.1: A hypothetical PCP biplot which illustrates features of the possible relationships between units and variables

Notes:

(1) A and B are highly correlated variables which are not clearly differentiated by their interactions with either a or b.

(2) a and b show different responses to all four variables having opposite signs in their projections on to the vectors.

(3) variable D is relatively unimportant in the interaction and unlikely to account for a significant interaction between the variables and units.

than would be predicted under unit by variable independence. Conversely, if the vectors are widely separated it is likely that the value in the corresponding cell of the contingency table will be lower than predicted from unit by variable independence.

Biplots were used in Experiments 2.1 to 2.3 to examine the interactions between fungi and plants. Here the data in the two-way tables were the tables of estimated treatment effects produced from ANOVA of the fungus by plant interaction for the unconfined penetration. In Experiment 2.1 CPA was used to assess association of particular penetration sites on the three leaf types with particular fungal isolates, while in Experiment 2.3 this approach was extended in conjunction with a chi-squared test to assess the deviation from a random distribution of penetration events displayed by each fungus on each plant surface.

Analyses of variance, PCP, and CPA for experiments 2.1 - 2.3 are summarised in Appendix 2.6

2.4 RESULTS AND DISCUSSION

Experiment 2.1: The behaviour of *Alternaria brassicae*, *A.brassicicola*, and *A.alternata* on leaf disks of three epicuticular wax types of *Brassica oleracea* var. *gemmifera*.

Results

The means for isolates and leaf types, representing the main effects of these factors on germination and germ-tube development, are presented in Table 2.4.

Germination and germ-tube development

Considering germination of the fungi, *A.brassicae* showed the highest mean germination percentage (90%), *A.brassicicola* the lowest (58%), while *A.alternata* was intermediate (75%). In the case of *A.brassicae*, there was no evidence that germination increased over sampling times; 85% of conidia had already germinated at 4 hours after inoculation while after 18 hours 93% had germinated. However, for both *A.brassicicola* and *A.alternata*, germination increased between 4 and 18 hours after inoculation. The increase was greater in the case of *A.brassicicola* for which germination rose from 41% to 80%, while in the case of *A.alternata* it increased from 66% to 85%. There was no evidence that germination differed between the glossy (78%) and waxy surfaces (75%), but on the intermediate surface (70%) it was lower than on the glossy surface (Table 2.4). There was no interaction between leaf type and sampling time with respect to germination.

In addition to differences in the rate of germination achieved by the isolates, there were differences in the numbers of germ-tubes produced. Conidia of *A.brassicae* produced more germ-tubes than those of *A.brassicicola* and *A.alternata* at all sampling times and on all leaf surface types. The average number of germ-tubes produced by conidia of *A.brassicae* was 2.2, while for *A.brassicicola* it was 1.1, and for *A.alternata* it was 1.2. However, there was a significant interaction between fungi and leaf types with respect to germ-tube number.

Table 2.4: Means for the main effect of fungus and leaf type on germination and germ-tube growth of three species of *Alternaria* grown on leaf disks of *B.oleracea* var. *gemmifera*.

	VARIATE [3]						
	Germ. (%)	NGT	BASE (%)	MID (%)	TERM (%)	GTL (μm)	BRCH (%)
SPECIES[1]							
A.brassicae	90	2.2	66	51	62	164	38
A.brassicicola	58	1.1	65	19	18	33	5
A.alternata	75	1.2	47	26	38	106	14
LEAF TYPE[2]							
Glossy	78	1.5	58	32	43	109	19
Intermediate	70	1.5	57	36	36	98	22
Waxy	75	1.4	63	28	38	95	16
s.e.d. (d.f. = 2, 60) ±	**3.8**	**0.05**	**4.3**	**3.7**	**3.8**	**10.5**	**3.0**

1 means of three leaf types and four sampling times

2 means of three fungi and four sampling times

3 Description of variates: **Germ.**, percentage germination; **NGT**, Number of germ-tubes per conidium; **BASE**, percentage of germinated conidia which germinated from basal cells; **MID**, percentage of germinated conidia which germinated from mid-body cells; **TERM**, percentage of germinated conidia which germinated from terminal cells; **GTL** germ-tube length; **BRCH**, percentage of germ-tubes with one or more branches

Thus, although conidia of *A.brassicae* produced more germ-tubes than those of *A.brassiciola* and *A.alternata* on all three leaf types, they produced fewer germ-tubes on the waxy surface than on the glossy and intermediate surfaces, while the number of germ-tubes produced by conidia of *A.brassicicola* and *A.alternata* did not vary between leaf types (Figure 2.2a).

The reduced number of germ-tubes by *A.brassicae* on the waxy leaf type is shown in more detail in Figure 2.2b. Examining the data for 18 hours after inoculation, far more conidia produced only a single germ-tube on the waxy surface (32%) than on the glossy (13%) and intermediate (15%) surfaces. Moreover, over 40% of conidia produced three or more germ-tubes on the glossy and intermediate surfaces while only 18% did so on the waxy surface.

The position of cells which gave rise to germ-tubes in relation to conidium morphology was found to vary. *A.brassicae* showed the greatest variation in distribution of germinating cells, *A.brassicicola*, the least, with *A.alternata* intermediate. In the case of *A.brassicae* 66% of

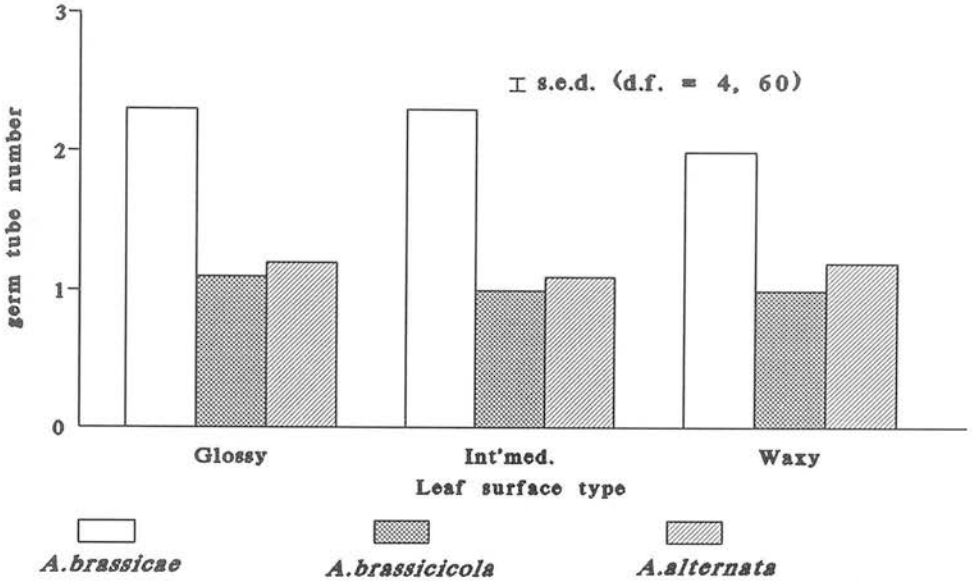


Figure 2.2a: Numbers of germ-tubes produced by germinating conidia of three *Alternaria* species on leaf disks of *B.oleracea* var. *gemmifera* of three different leaf surface types (average of four sampling times).

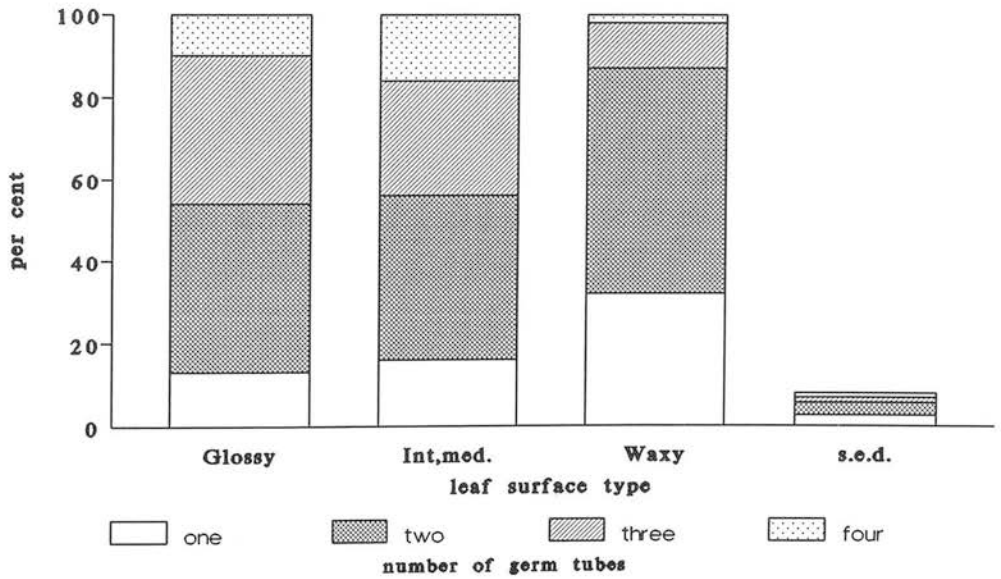


Figure 2.2b: The percentage of germinated conidia of *A.brassicae* which produced different numbers of germ-tubes on leaf disks of three leaf surface types of *B.oleracea* var. *gemmifera*, 18 hours after inoculation.

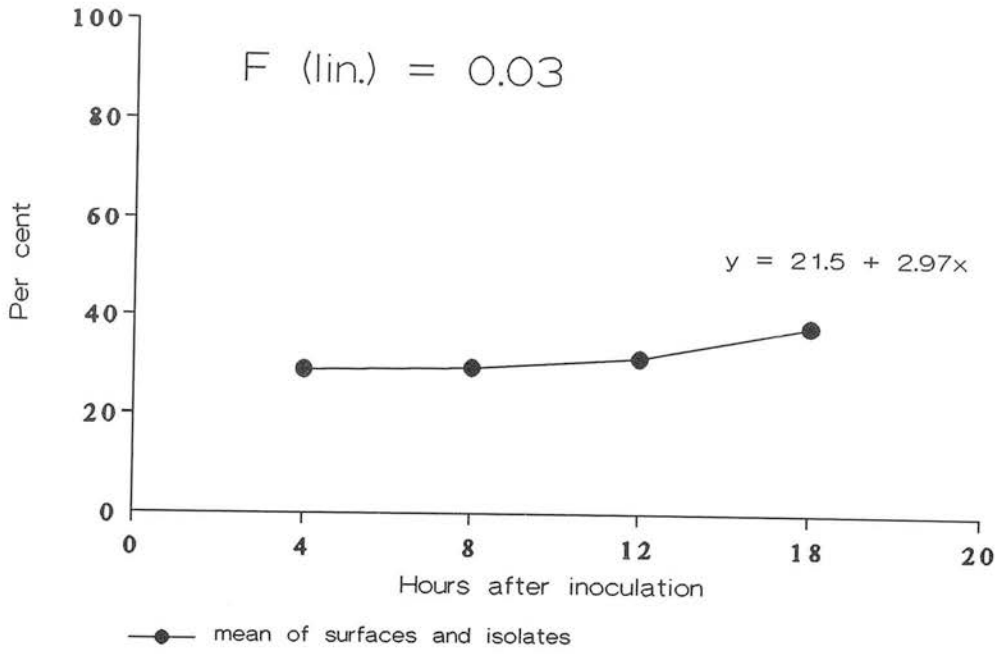


Figure 2.3: The percentage of germinated conidia which produced germ-tubes from mid-body cells, averaged over three species of *Alternaria* on leaf disks of three leaf types of *B.oleracea* var. *gemmifera*.

produced a germ-tube from their basal cells, while 51% did so from at least one mid-body cell and 62% of conidia germinated from their terminal cells. For *A.brassicicola*, a similar percentage germinated from basal cells (65%) but far fewer conidia germinated from mid-body cells (19%) or terminal cells (18%). With *A.alternata*, a lower percentage of conidia germinated from basal cells (47%) than with the other species, whilst in the case of mid-body (26%) and terminal cells (38%), *A.alternata* was intermediate between *A.brassicae* and *A.brassicicola*. Examining the change in the distribution of germ-tubes around the conidium body between 4 and 18 hours after inoculation, averaged over fungi and leaf types, there was no evidence that more basal cells or terminal cells germinated at later times than at earlier times. However, the percentage of mid-body cells which germinated increased linearly over time, from 29% to 38%, suggesting that where germination occurred after 4 hours, it did so from mid-body cells of conidia (Figure 2.3). The position of germ-tube formation on the conidia was not significantly affected by leaf surface type.

Hyphal growth following the extension of germ-tubes into hyphae varied between fungal species. Hyphae of *A.brassicae* grew faster than those of both *A.brassicicola* and *A.alternata*, with *A.alternata* intermediate in growth rate between the two brassica pathogens. Averaged over all leaf types, germ-tube length for *A.brassicae* increased from 35 μm to 326 μm between 4 and 18 hours after inoculation while increases for *A.brassicicola* and *A.alternata* were from 12 μm to 47 μm, and from 27 μm to 190 μm respectively (Figure 2.4a). Considering hyphal growth in relation to leaf type and sampling time, analysis of orthogonal polynomials showed that there were significantly different linear, but not quadratic, patterns of increase in germ-tube length between the glossy and waxy surfaces. Thus, although hyphal length was similar on all three leaf types between 4 and 12 hours after inoculation, between 12 and 18 hours after inoculation hyphal length increased for all three fungi more on the glossy surface than on the waxy surface, with the intermediate leaf type occupying a position between these two (Figure 2.4b).

In addition to differences in germ-tube length there were also differences between the isolates in the incidence of branching which occurred on germ-tubes. Considering the percentage of germ-tubes which produced at least one branch, averaged over leaf types and sampling times (Table 2.4),

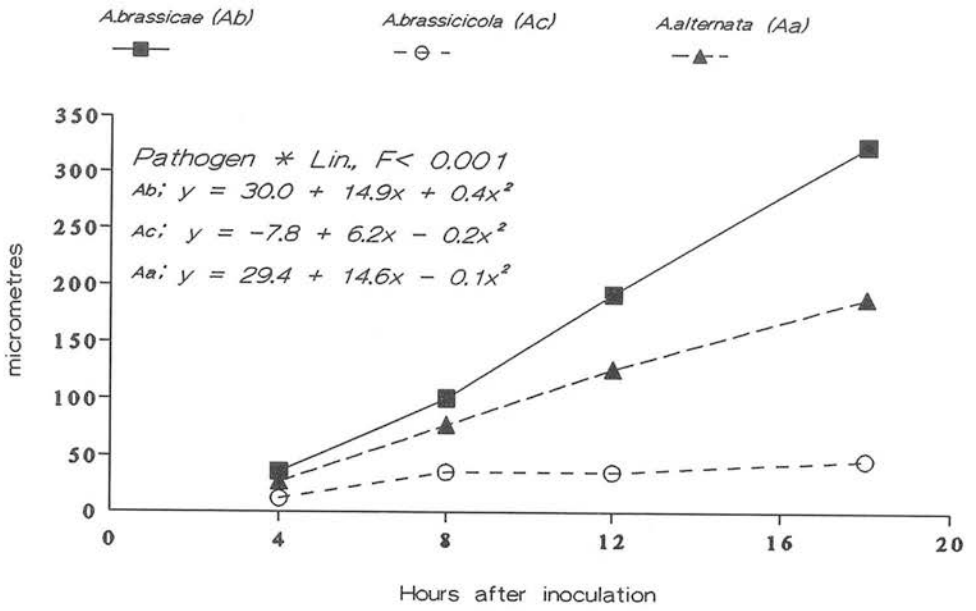


Figure 2.4a: Average germ-tube lengths of three *Alternaria* species grown on leaf disks of three leaf surface types of *B.oleracea* var. *gemmifera*.

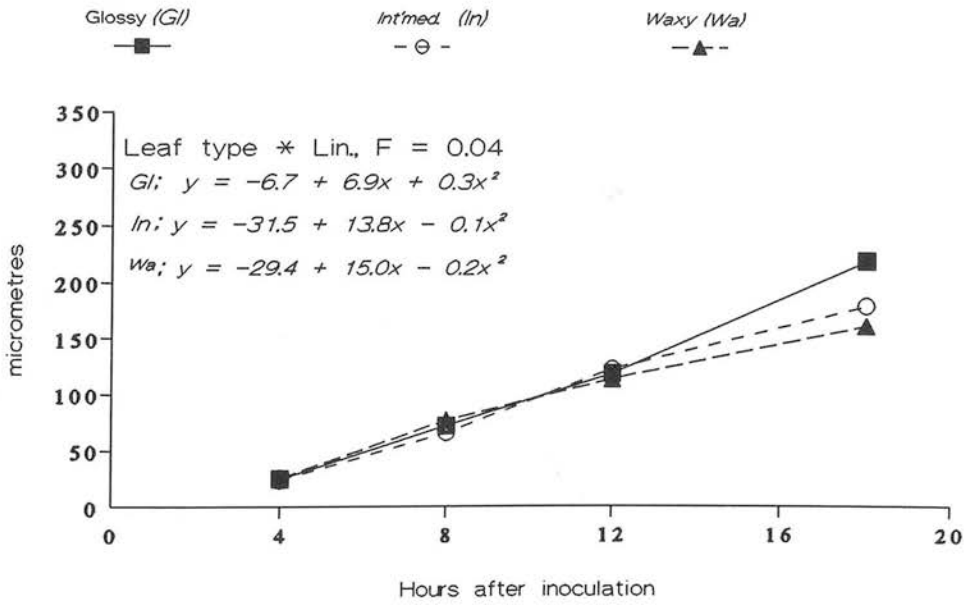


Figure 2.4b: The average of germ-tube lengths of three *Alternaria* species on leaf disks of each of three leaf surface types of *B.oleracea* var. *gemmifera*.

A.brassicae had the highest incidence (38%), *A.brassicicola* the lowest (5%), with *A.alternata* intermediate (14%). Examining change in the incidence of branching over time (Figure 2.5), with *A.brassicae*, the percentage of germ-tubes which showed at least one branch increased from 1% to 76% between 4 and 18 hours after inoculation, while with *A.brassicicola* the increase was from 1% to 9%, and with *A.alternata* it was from 0% to 29%. There was no evidence that the frequency of hyphal branching differed between leaf types, or of any interaction between leaf type and sampling time.

Apart from branching the only morphogenic change visible on hyphae of all three *Alternaria* species was the development of appressoria. The frequency of appressorium production varied between fungi and leaf types (Table 2.5), while there was also a significant component in the interaction between these factors. Germ-tubes of *A.brassicae* produced appressoria most frequently, *A.alternata* least frequently, with *A.brassicicola* intermediate. For *A.brassicae* the ratio of appressoria to germ-tubes increased from 0% to 29% between 4 and 18 hours after inoculation, with *A.brassicicola* it increased from 0% to 17%, while with *A.alternata* the ratio of appressoria to germ-tubes reached a maximum of 12% at 12 hours after inoculation, and decreased to 5% at 18 hours after inoculation (Figure 2.6a). For all three species the majority of appressoria were produced between 4 and 12 hours after inoculation.

In relation to leaf types, the ratio of appressoria to germ-tubes was higher on the intermediate surface (13%) than on both the waxy and glossy surfaces, where it was equal (9%) (Table 2.5). However, this difference resulted principally from the relatively high frequency of appressoria produced by *A.alternata* on the intermediate surface as compared with the glossy and waxy surfaces (Figure 2.6b).

Penetration

The higher percentage of conidia of *A.brassicae* which produced appressoria was reflected in a higher incidence of penetration by this species than by the other two. Averaged over leaf types and sampling times, the percentages of germinated conidia of each species which produced an infection event were: *A.brassicae*, 34%; *A.brassicicola*, 11%; *A.alternata*, 7% (Table 2.5).

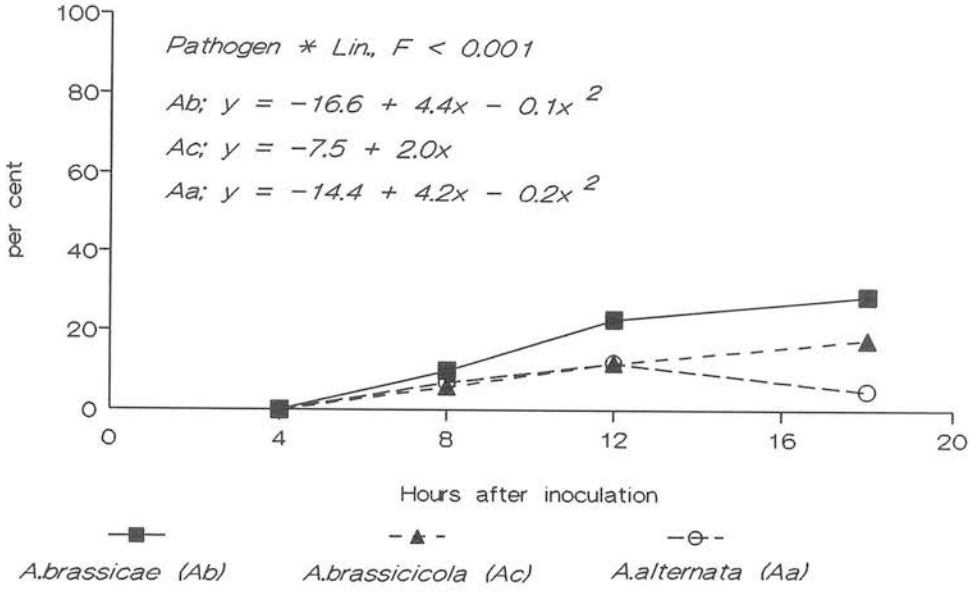


Figure 2.6a: The ratio of appressoria formed to germ-tubes for three *Alternaria* **species averaged over three leaf surface types of** *B.oleracea* **var.** *gemmifera*.

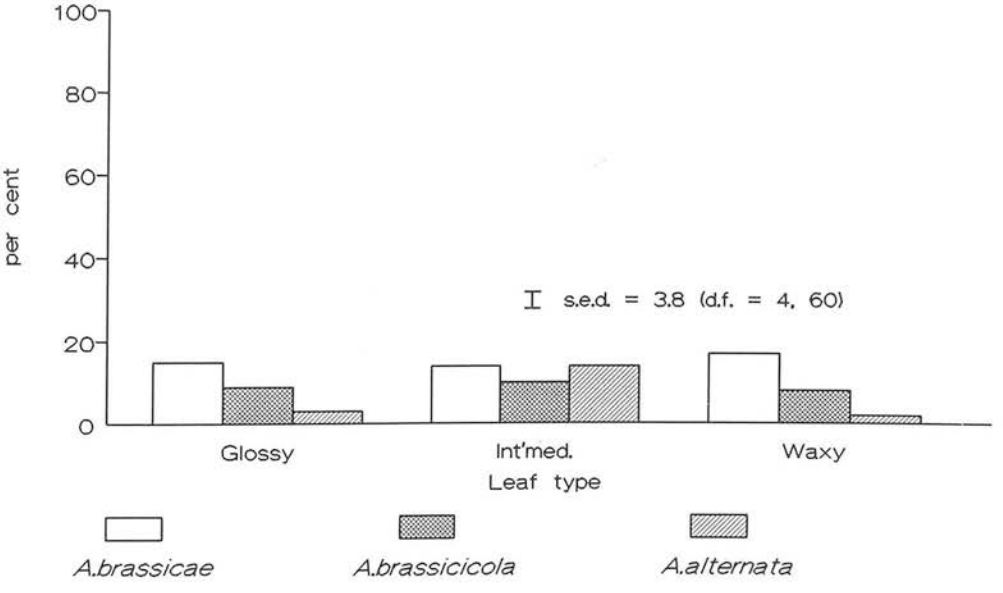


Figure 2.6b: The ratio of appressoria formed to germ-tubes on each of three leaf surface types of *B.oleracea* **var.** *gemmifera*, **for three species of** *Alternaria* **(averaged over four sampling times).**

Table 2.5: Means for the main effect of fungus and leaf type on the penetration behaviour of three species of *Alternaria* grown on leaf disks of *B.oleracea* var. *gemmifera*.

	VARIATE[3]				
	AP:GT (%)	**TPEN** (%)	**PECWJ** (%)	**PECPW** (%)	**PSTOM** (%)
SPECIES[1]					
A.brassicae	15	34	22	4	7
A.brassicicola	9	11	6	5	<1
A.alternata	6	7	4	2	<1
LEAF TYPE[2]					
Glossy	9	17	9	4	4
Intermediate	13	20	13	5	2
Waxy	9	14	10	3	1
s.e.d. (d.f. = 2, 60) ±	**2.2**	**3.4**	**2.5**	**1.6**	**0.8**

1 means of three leaf types and four sampling times

2 means of three fungi and four sampling times

3 Description of variates: **AP:GT**, ratio of appressoria to germ tubes; **TPEN**, percentage of germinated conidia which gave rise to a penetration event; **PECWJ**, percentage of germinated conidia which gave rise to a penetration event at epidermal cell wall junctions; **PECPW**, percentage of germinated conidia which gave rise to a penetration event over epidermal cell periclinal walls; **PSTOM**, percentage of germinated conidia which gave rise to penetration *via* stomata.

Penetration was rare at 4 hours after inoculation, but differences in the frequency of penetration were apparent between the fungi by 8 hours after inoculation (Figure 2.7). *A.brassicae* produced more infection events than the other species at each at this and all subsequent sampling times, with a final value of 62% at 18 hours after inoculation. The frequency of penetration by *A.brassicicola* and *A.alternata* was similar until 12 hours after inoculation, when the percentage of conidia which had produced a penetration event for *A.brassicicola* was 16% and for *A.alternata* it was 9%; at 18 hours after inoculation the figures were 19% and 11% respectively (Figure 2.7). There was no evidence that the frequency of penetration varied between leaf types or of any interaction between leaf type and fungus.

The fungi were found to penetrate leaf surfaces at three sites: junctions between epidermal cells, above periclinal walls of epidermal cells, and *via* stomata. The percentage of conidia which produced a penetration event at each of these sites was related to the total percentage of conidia which produced penetration events. However, in addition to the differences between isolates arising from differences in

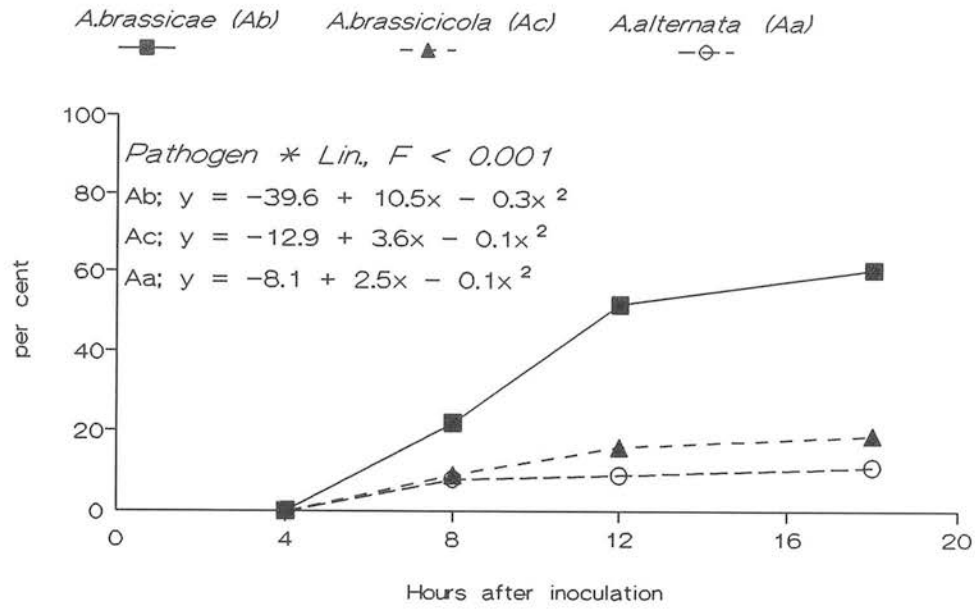


Figure 2.7: The percentage of germinated conidia of three *Alternaria* species which produced a penetration event on leaf disks of *B.oleracea* var. *gemmifera*, averaged over three leaf surface types.

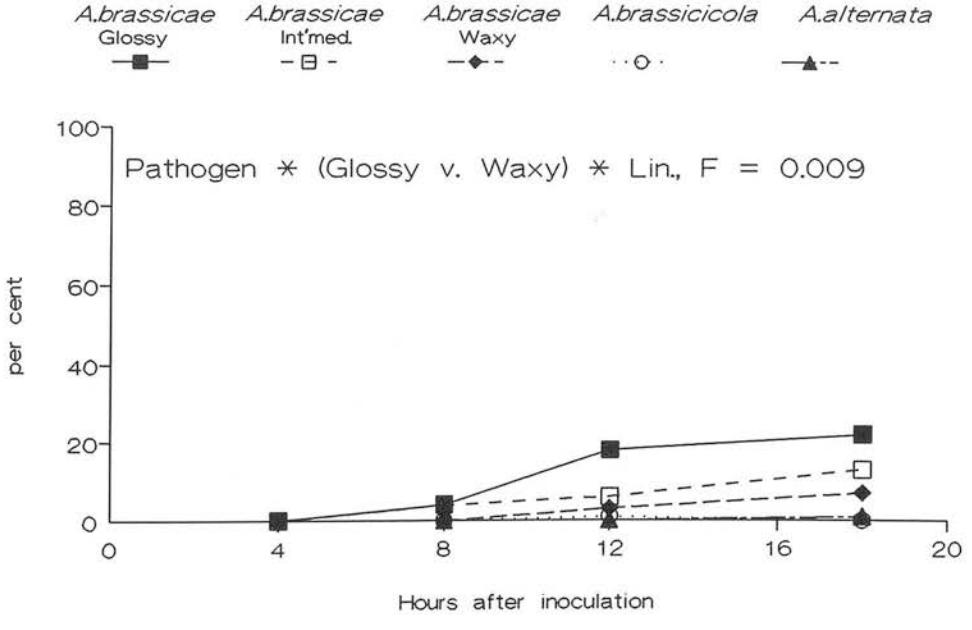


Figure 2.8: The percentage of germinated conidia of three *Alternaria* which gave rise to penetration *via* stomata on leaf disks of three leaf surface types of *B.oleracea* var. *gemmifera*.

their total incidence of penetration, there were also differences between the isolates in the relative frequency of penetration at each of the three sites. Table 2.5 shows the percentage of conidia which produced a penetration event at each of the three sites for each of the fungi.

A higher percentage of conidia of *A.brassicae* produced penetrations at epidermal cell junctions (22%) than over periclinal walls (4%) and stomata (7%), while with *A.brassicicola* the percentage of conidia which gave rise to penetration at cell junctions and periclinal walls was approximately equal (6% and 5%), but stomatal penetration was much less frequent (0.2%). In the case of *A.alternata*, penetration at cell junctions (4% of conidia) was twice as frequent as penetration through periclinal walls (2%), while penetration *via* stomata was rare (0.2%)

There was no evidence that the percentage of conidia which penetrated leaf surfaces at cell junctions or at periclinal wall sites varied between leaf types. However, there was an interaction between fungi, leaf type, and sampling time with respect to the percentage of penetrations which occurred *via* stomata. Thus, although stomatal penetration was greater for *A.brassicae* than for the other species, it was not equal for *A.brassicae* across the three leaf types. There was more stomatal penetration by *A.brassicae* on the glossy surface than on the waxy surface (22% and 7% respectively at 18 hours after inoculation), with stomatal penetration on the intermediate surface (13%) falling between these two (Figure 2.8).

The development of the pathogens as described previously is illustrated in plate 2.2 a - f.

Plate 2.2: Features of the development of *A.brassicae*, *A.brassicicola* and *A.alternata* on three leaf disks of three lines of *B.oleracea* var *gemmifera* with differing epicuticular wax characteristics

a. DAPI-stained germinating conidium of *A.brassicae*. The fluorescing germ-tube arising from the basal cell contains several nuclei.

b. Secondary conidia produced by *A.brassicae*, with nuclear material and the cytoplasmic connection between the conidia. DAPI stain.

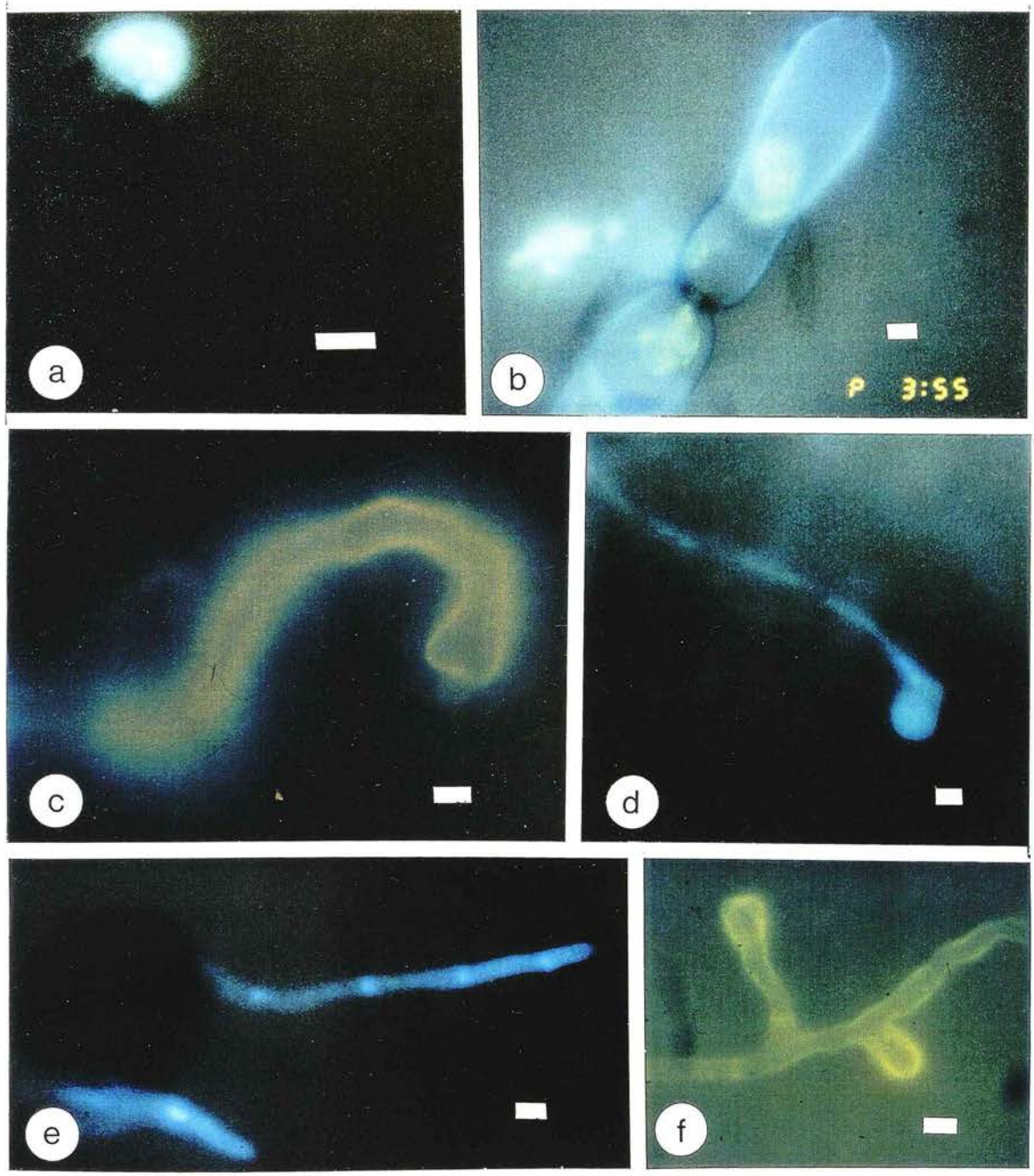
c. Germ-tube and terminal appressorium of *A.brassicae* 12 hours after inoculation. An infection hypha begining ingress, is visible beneath the appressorium. Tinopal stain.

d. Germ-tube and appressorium of *A.brassicicola* at 12 hours after inoculation. DAPI stain.

e. Germ-tube growth by *A.alternata* at 8 hour aftyer inoculation. The conidium has produced two germ-tubes. Uninucleate hyphal compartments are visible. DAPI stain.

f. Germ-tube growth by *A.alternata* at 12 hours after inoculation. Lateral branches are apparent and an appressorium has formed in one case. Tinopal stain.

Bar = approximately 5 μm in all cases.


a
b
P 3:55
c
d
e
f

Discussion

Emmett & Parberry (1975) suggested that pathogen development from spore germination to appressorium formation in a range of fungi is largely a predetermined, genetically programmed process which proceeds, with minor variations, in essentially the same way, independent of environmental factors provided a few basic requirements (e.g. suitable temperature or relative humidity) are met. The results of the present study indicate that this is true for all three *Alternaria* species examined here, although the predetermined pattern of development was found to differ between the species.

Some caution is necessary in attributing the observed differences in behaviour to different species characteristics as no account was made of intra-specific variation. Furthermore, although inoculum was prepared from vigorous, actively growing cultures, the occurrence of physiological variation in the inoculum must be considered. This has been discussed in relation to germination of *Erysiphe graminis* by Ellingboe (1972), and it may be noted that germination by *A.brassicicola* was lower than might have been expected from other studies.

A more comprehensive description of the developmental patterns of *A.brassicae* and *A.brassicicola* will be reserved until a later discussion when a wider range of experimental results can be considered. However, certain distinguishing features can be noted from the present study. The larger conidia of *A.brassicae* generally produced more germ-tubes than those of *A.brassicicola* and *A.alternata*, and resulting hyphae showed a higher growth rate, more profuse branching, and more extensive appressorium formation.

Selection of penetration sites appeared to differ between species. *A.brassicae* showed a preference for cell wall junctions and to a secondary degree, stomata. *A.brassicicola* penetrated mainly at cell junctions and cell periclinal walls, while *A.alternata* penetrated predominantly at cell junctions.

In considering possible environmental influences on the development of the fungi, previous studies of the behaviour of *A.brassicae* and *A.brassicicola* have suggested that fungal development is influenced by environmental factors such as leaf age, temperature, and relative humidity (Duek & Degenhardt, 1975; Degenhardt *et al.*, 1982; Prasanna, 1984) and leaf waxiness (Conn & Tewari, 1989). The work of Conn &

Tewari has particular importance since it indicates an underlying mechanism for the results several field and laboratory studies which have shown that *Brassica* species, and cultivars within species, which appear to have a more waxy leaf surface are more resistant to *A.brassicae* and *A.brassicicola* (Tewari & Skoropad, 1976; Skoropad & Tewari, 1977; Prasanna, 1984). Conn & Tewari's results may be summarised as follows:

1. Germination by *A.brassicae* was higher on *B.campestris* (relatively glossy) than *B.napus* (relatively waxy).
2. Removal of the epicuticular wax layer from both species increased germination.
3. Recrystallised wax did not inhibit germination by *A.brassicae in vitro*.
4. Germ-tube number was greater on leaves after removal of the wax layer.
5. Germination by *A.brassicae* at 6.5 hours after inoculation was between 40% and 70% in all treatments.

In the present study variation in the structure of the wax layer on leaf disks was not found to affect the germination of *A.brassicae*, *A.brassicicola*, or *A.alternata*, although differences were found in this experiment between the three species in their germination capacity. Spores of all three species were able to germinate and commence germ-tube growth within four hours of inoculation, revealing a capacity to respond rapidly to favourable environmental conditions. This was particularly obvious in the case of *A.brassicae*. In contrast to the low germination rate observed by Conn & Tewari, *A.brassicae* was found in the present study to achieve germination rates of 80% or higher within 4 hours of inoculation. However, although germination was not affected by variation in leaf waxiness, germ-tube number of this species was reduced on the waxy leaf type. Conn & Tewari (1989) suggested that a thick epicuticular wax layer might reduce germination and germ-tube number by impeding the flow of exudates from the leaf tissue to the leaf surface. Studies by Berry (1992) have shown that electrical conductivity in water droplets on leaf disks becomes greater over a period of 24 hours on the glossy type than on the waxy type, indicating that there is an effect of the structure of the epicuticular wax layer on the rate of diffusion of electrolytes to the leaf surface.

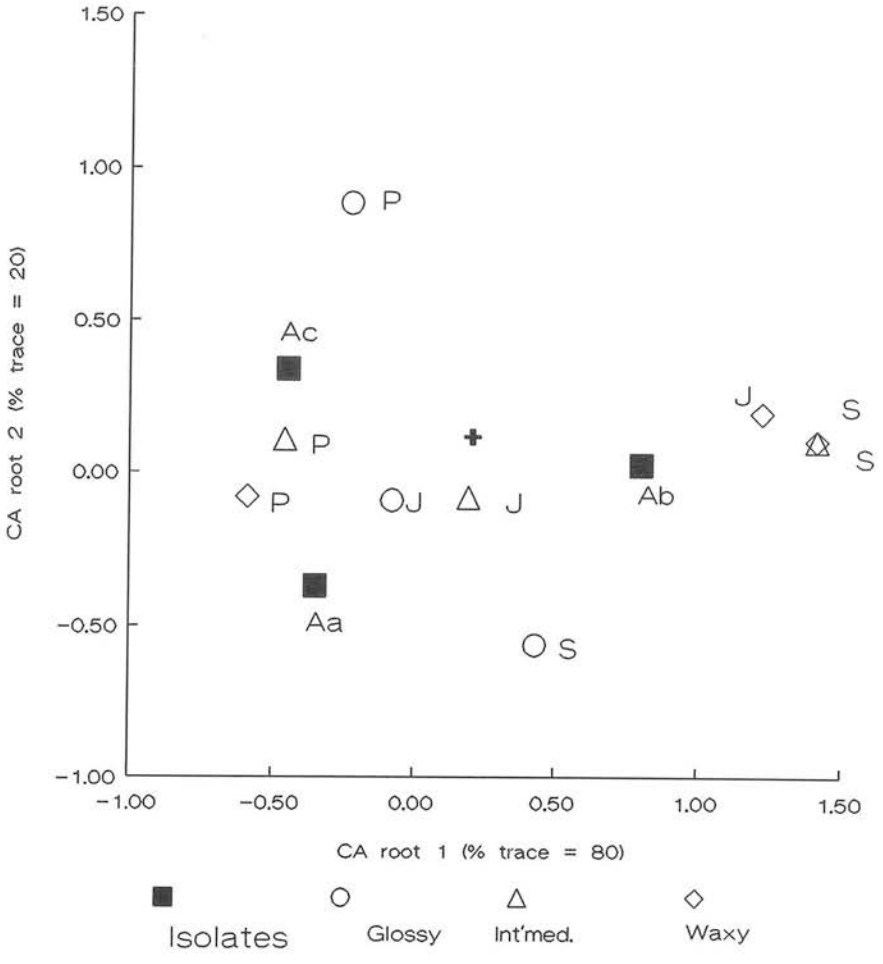
Conn & Tewari also suggested that conidia deposited from a spray might land on waxy leaf surfaces with no appreciable quantity of free water, this leading to reduced germination or reduced germ-

tube number. In the present study the inoculum was applied as a single large droplet which would reduce the chances of individual conidia being isolated without water. However, it is known that water droplets have lower contact angles with waxy surfaces than glossy surfaces (Berry, 1991) and under such conditions spores may be confined and crowding effects may occur (Prasanna, 1984). Thus, both in the work of Conn & Tewari, and the present study it is possible that the decrease in germ-tube number on the more waxy surfaces is due partly to the physical interactions between the wax and the water carrying the inoculum, in addition to the effect of the epicuticular wax on the availability of leachates at the leaf surface. In contrast to the results for *A.brassicae* no reduction in germ-tube number was noted on the waxy leaf type for either *A.brassicicola* or *A.alternata*, which produce fewer germ-tubes per conidum than *A.brassicae*. It is possible that in all three species the initial burst of germination is determined principally by the availability of water. Any **subsequent** production of germ-tubes perhaps being more susceptible to other environmental variables (*e.g.* availability of nutrients or self-inhibitors released during germination).

Although overall levels of appressorium production did vary between leaf types for *A.brassicicola* and *A.alternata* no direct relationship was apparent between the structure of the wax layer and the frequency of appressorium production. Thus the frequency of appressoria on germ-tubes was equal on the two most contrasting surfaces (glossy and waxy), but was higher on the intermediate type, which resembles more closely the glossy type than the waxy type. The lack of a clear relationship between wax structure and fungal behaviour was apparent in the relatively high frequency of penetration by *A.alternata* on the intermediate leaf type. There is no obvious explanation for this observation, but the high frequency on the intermediate type may have resulted from a favourable interaction of unidentified factors, for example a balance between the presence of inhibitory and stimulatory chemicals, which led to the occurrence of suitable conditions for appressorium formation on this particular leaf type. It should be noted that neither *A.brassicae* nor *A.brassicicola* showed a similar high level of penetration on this leaf type. Akai *et al.* (1969) found that epicuticular wax from onion stimulated appressorium production by *A.porri* both *in planta* and *in vitro*, but their studies were a comparison of the effect of presence or absence of the wax rather than of the effect of variation in physical structure as examined here.

With *A.brassicae* and *A.alternata* the majority of appressoria were formed over junctions between cell walls, perhaps in response to topographical or chemical stimuli, as has been suggested for several other pathogens (Preece, Barnes, & Bailey, 1967; Emmett & Parberry, 1975; Daniels, Lucas, & Peberdy, 1991). Tsuneda & Skoropad (1978) reported that *A.brassicae* formed appressoria over cell wall junctions and suggested that the pathogen might show an ability to select penetration sites. These authors, however, provided no numerical analysis of site selection. It is clear from the present study that while *A.brassicae* does produce appressoria at sites other than cell wall junctions, the pathogen displays selection in favour of this site for direct penetration. *A.brassicicola* was found to produce about as many appressoria over periclinal walls as over cell junctions. It might be considered that this still constitutes a selection of cell wall junctions as penetration sites, since the percentage of the leaf surface area composed of cell junctions might well be expected to be less than that represented by periclinal walls. Further studies incorporating assessments of the relative proportion of leaf area composed of each type of penetration site are required to allow clear analysis of this behaviour. However, it is possible that *A.brassicae* and *A.brassicicola* may respond to different stimuli at the leaf surface, giving rise to selection of two different, but overlapping populations of penetration sites.

A.brassicae was clearly differentiated from the other species by its selection of stomata as penetration sites. The difference in site selection for penetration by the three fungi is shown diagramatically in Figure 2.9. The biplot shows the relative importance to each pathogen of the three possible sites for penetration on each of the three leaf types, following Correspondence Analysis of the data for the percentage of conidia giving rise to penetration at each of the three sites. The points for stomatal penetration on the three leaf types occur together on the right with the point for *A.brassicae*, indicating the association of this species with this form of penetration. The points for *A.brassicicola* and *A.alternata* lie among all but one of the points for cell junction sites and periclinal sites toward the left of the plot. Associations between A.brassicicola and periclinal sites, particularly on the glossy surface, and A.brassicae and cell junction sites on the waxy surface were indicated by the CPA and should perhaps be investigated further. The small contribution cell junction sites on the glossy and intermediate surface types to the overall interaction is indicated by the proximity of the points for these sites near the origin of the biplot.



Description of sites within leaf types and isolates:

J: Cell junctions; **P**: Cell periclinal walls; **S**: Stomata

Aa: *A.alternata*; **Ab**: *A.brassicae*; **Ac**: *A.brassicicola*

Figure 2.9: A biplot representation of the association between three *Alternaria* species and three possible penetration sites on leaf disks of three lines of *B.oleracea* var. *gemmifera* with different epicuticular wax characteristics.

Although appressorium formation can be considered as an innate character, there are several reports which relate variation in the resistance of plants to the variation in the frequency of appressorium formation by fungal pathogens on different surfaces (Van Burgh, 1956; Flentje, 1957; Flentje, Dodman & Kerr, 1963). The broad pattern of development of *A.brassicae* and *A.brassicicola* on the leaf surface was similar to that of the weak pathogen *A.alternata*, but both, and in particular *A.brassicae*, were more successful in penetrating the leaf surface. It was indicated that surface variation may alter the behaviour of *A.brassicae* especially with respect to choice of penetration site.

This study and others (McKenzie *et al.*, 1988; Berry, 1991) have shown that *A.alternata* is able to penetrate leaf tissue of *Brassica* species. However, *A.alternata* is not reported as a common field pathogen of brassica crops. The term 'basic compatibility' (Heath, 1985) has been used in discussions of the interactions of biotrophic pathogens in gene-for-gene relationships with their host plants. It can be argued that *A.brassicae* and *A.brassicicola* have established a form of basic compatibility with *Brassica* species, but there is no evidence that this compatibility is based on a gene-for-gene relationship, indeed several studies have shown only a quantitative variation in resistance to both pathogens. The results of the present study have suggested that one of the factors which determines the interactions between *A.brassicae* and *A.brassicicola* and their brassica hosts is an adaption to features of their host plants' surfaces, associated with the induction of infection structures. However, this characteristic represents a quantitative rather than qualitative distinction from the weak pathogen *A.alternata*. Further interactions between *A.brassicae*, *A.brassicicola* and another *Alternaria* pathogen of cruciferous plants, *A.raphani*, and a range of different host species are considered in the next section.

Experiment 2.2: Fungal behaviour and plant cell response in interactions between *Alternaria brassicae*, *A.brassicicola*, *A.raphani*, and cruciferous hosts.

Results

Before dealing with quantitative assessments, a general description of the plant - pathogen interactions, based on observations of the development of the fungi and host cell response to infection is given and is illustrated in plates 2.3 - 2.5.

Germination by *A.brassicae* and *A.raphani* led to the production of up to four or five germ-tubes, but in the case of *A.brassicicola* typically only a single germ tube. By 24 hours after inoculation all three fungi had produced extensive extra-matrical growth, although this was more noticeable in the case of *A.brassicae* and *A.raphani* than with *A.brassicicola*. All three fungi elaborated appressoria prior to penetration. Germ-tubes of *A.brassicae* and *A.raphani* showed more variation in the number and position of appressoria than those of *A.brassicicola*. One appressorium was formed typically in a terminal position on germ-tubes of *A.brassicicola* while with *A.brassicae* and *A.raphani* appressoria were produced terminally and/or in intercalary positions. However, in the case of *A.brassicae* a higher proportion of germ-tubes produced no appressoria than with *A.raphani* and *A.brassicicola*.

Penetration by all three fungi occurred most often directly, at junctions between epidermal cells and to a lesser extent through cell periclinal walls. A proportion of penetrations occurred *via* stomata, notably in the case of *A.brassicae*.

Following penetration, the fungi established hyphae in the upper epidermal layer, although there was evidence of ramification through the leaf to the lower surface with all three pathogens. *A.brassicae* was found to exhibit a short sub-cuticular phase immediately after penetration in many cases, a feature that was not observed with *A.brassicicola* or *A.raphani*. In these species penetration was found to proceed to the epidermis, often to an intracellular position.

Plate 2.3: Features of the development of *A.brassicae* on different host plants.

a. Penetration of two stomata on *B.napus*. Callose depostion around the walls of the adjoining epidermal cells is indicated by the gaps in the fluorescent host cell reactions. Bar = 10 μm.

b. Single cell reaction by *B.oleracea*. Simple appressoria are visible with callose halos. Callose deposition around the walls of the challenged cell, which is partly necrotic, is also visible. Bar = 20 μm.

c. Direct penetration from a lobed appressorium and stomatal penetration on *B.napus*, with adjacent cell reactions and cell necrosis. Bar = 10 μm.

d. An appressorium with associated callose halo and sub-cuticular hyphae on *B.campestris*. Bar = 4 μm.

e. Intercellular development by *A.brassicae* in *B.nigra*. The epidermal cells appear normal. The fluorescence associated withthe hyphae may be localsied cellose deposition at points of attempted penetration. However, the fluorescence appears to be in an extra mural position and may be associated with the formation of an extra cellular by the pathogen. (Hancock & Huisman, 1981). Bar = 5 μm.

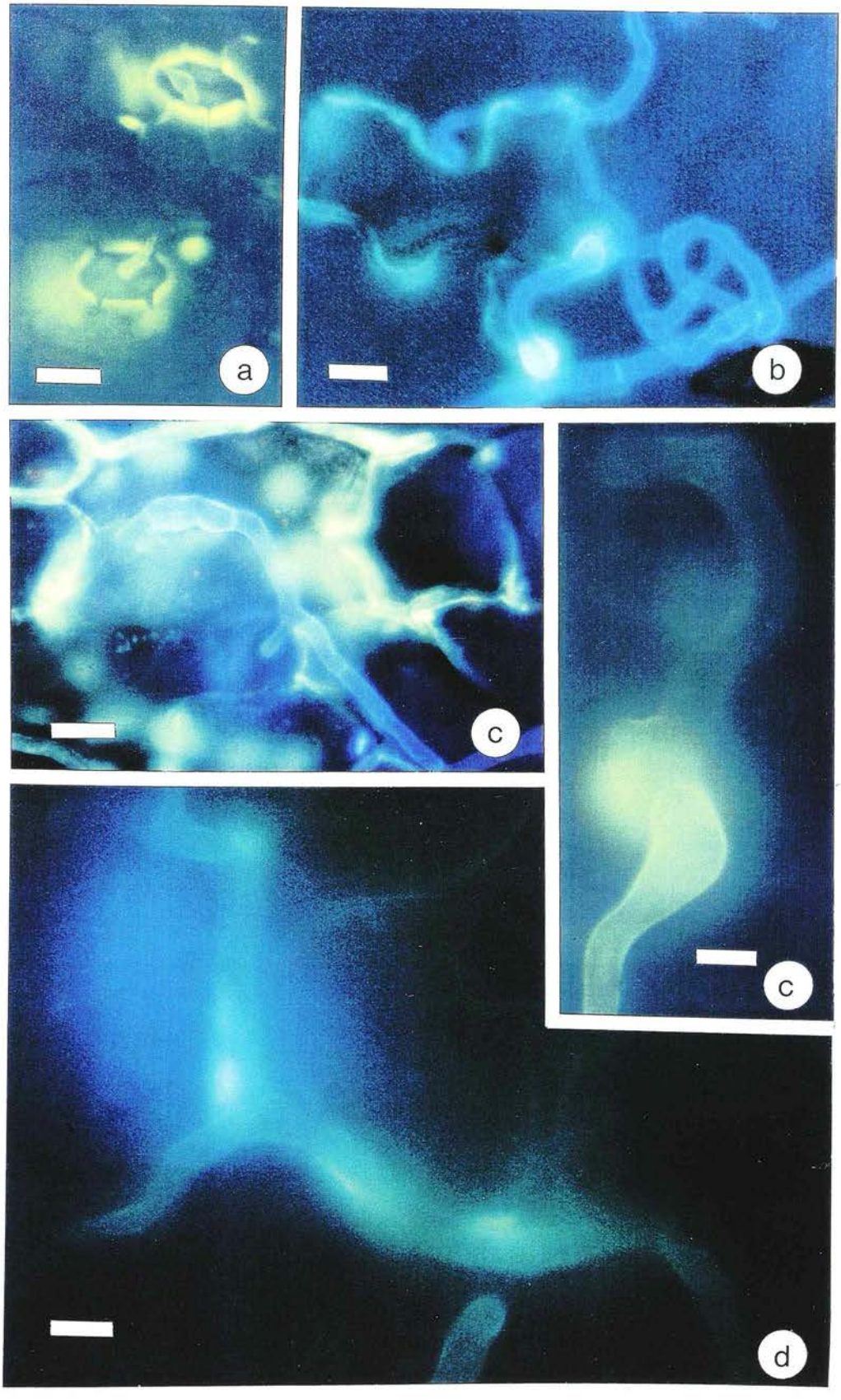

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Plate 2.4: Features of the development of *A.raphani* on different host plants.

a. A conidium with two germ-tubes, appressoria and necrotic epidermal cells. Limited callose reaction has occurred. Bar = 10 μm.

b. Localised callose deposition and intercalary appressoria on *C.cheiri*. Bar = 10 μm.

c. Localised plant cell reactions, cell wall fluorescence, appressoria and intracellular hyphae on *C.cheiri*. Bar = 7 μm.

d. Intercellular hyphae with associated localised callose deposition on *C.cheiri*. Bar = 5 μm.

e. Adjacent cell reactions associated with penetration on *S.alba*. Bar = 10 μm.

f. Intracellular hyphae in an epidermal cell, with cell wall fluorescence in *B.oleracea*. Bar = 5 μm.

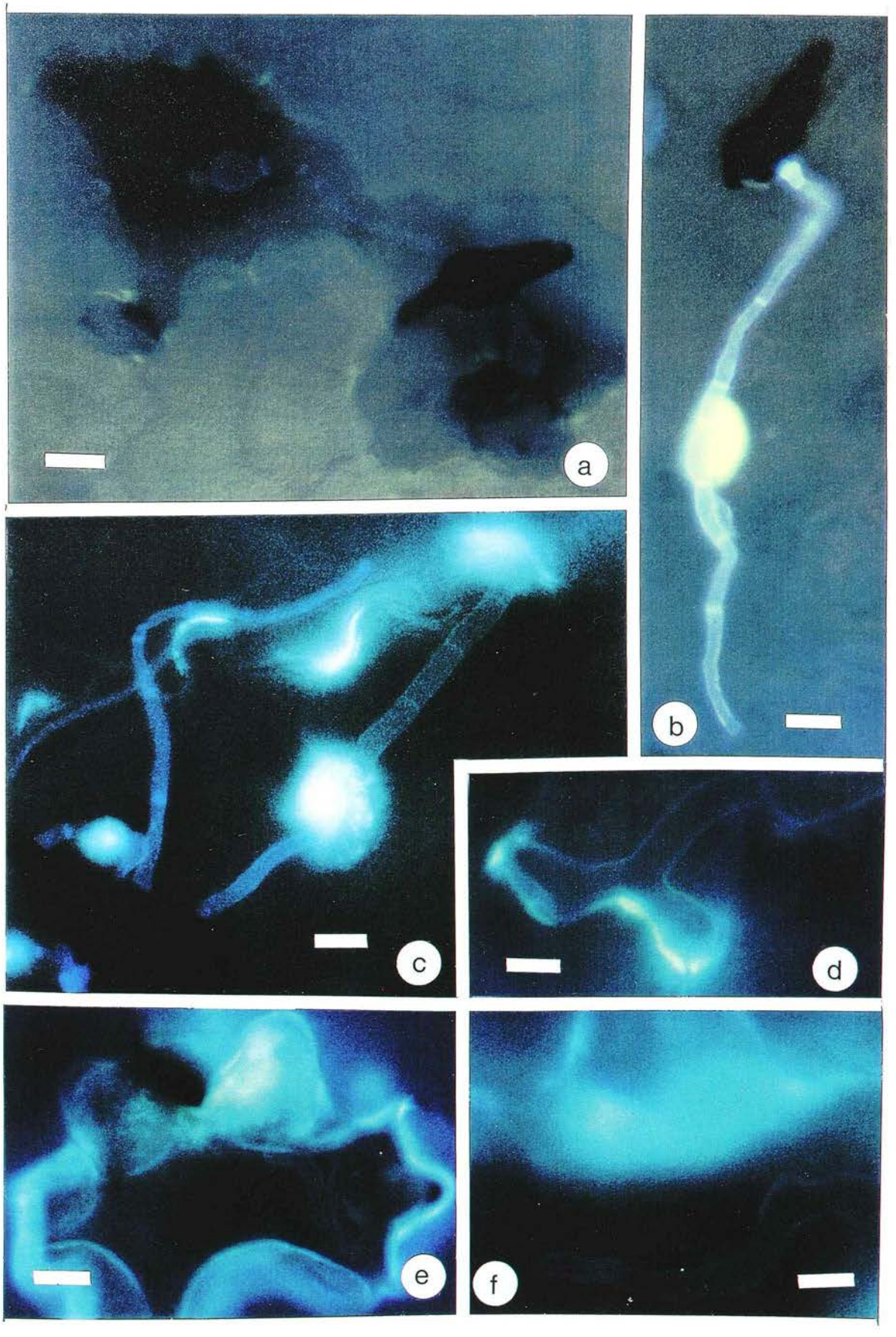
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Plate 2.5: Features of the development of *A.brassicicola* on different host plants.

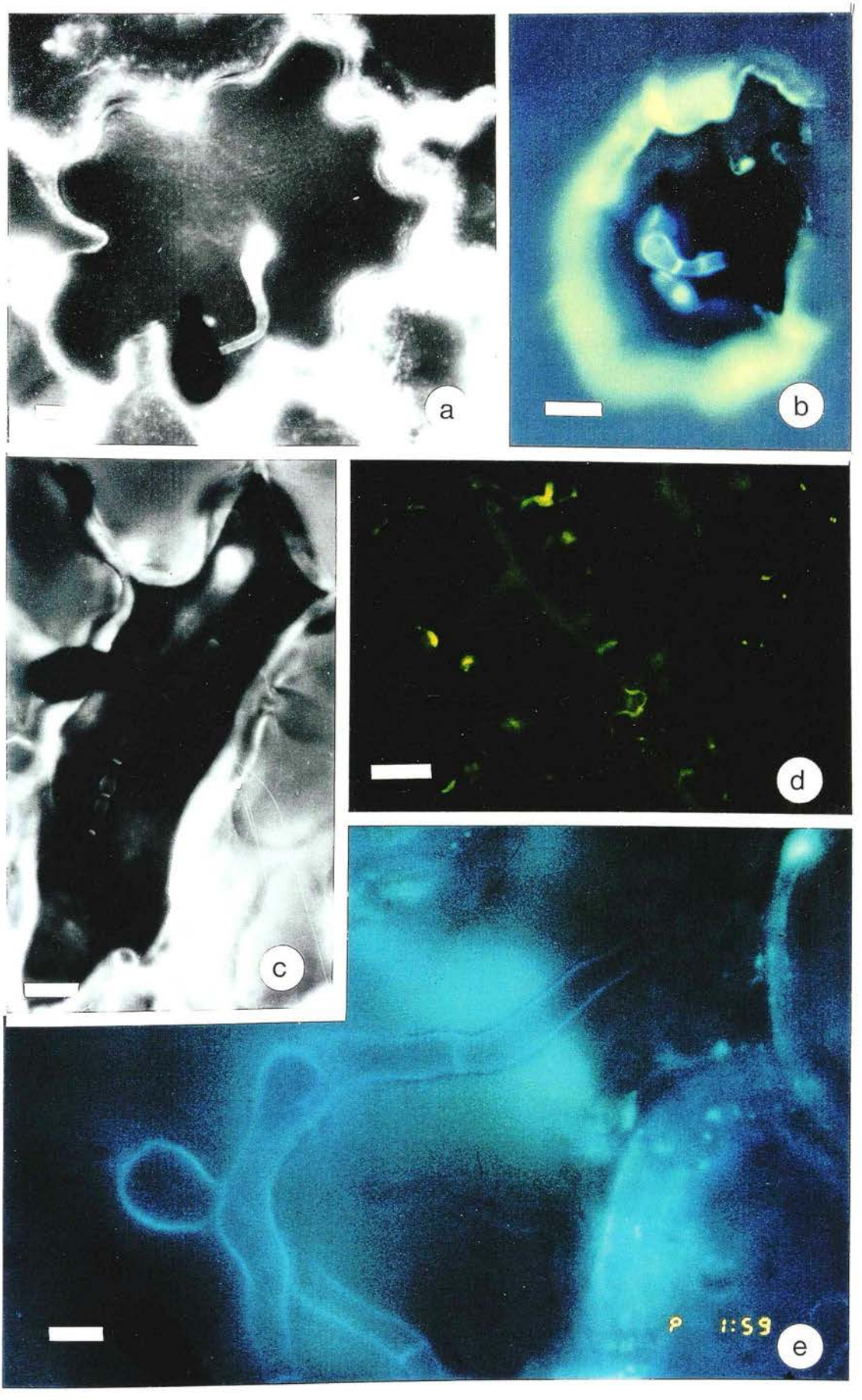
a. Periclinal penetration with associated callose halo and adjacent cell reaction on *B.campestris*. Bar = 10 μm.

b. Single cell reaction in response to penetration at an anticlinal wall junction on *B.oleracea*. Bar = 10 μm.

c. Periclinal penetration, host cell necrosis and intracellular hyphae on *C.cheiri*. Bar = 10 μm.

d. Cell reaction in the lower epidermis of *B.napus* due the presence of intercellular hyphae. Bar = 100 μm.

e. Intracellular hyphae and host cell wall detail in the upper epidermis of *B.oleracea*. Bar = μm.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Germination and germ-tube development

Data relating to pathogen germination and germ-tube development are summarised in Table 2.6. Significant differences were found between the isolates, but not between hosts, for germination and fungal development prior to penetration. Moreover, there was no evidence of interactions between pathogens and hosts with respect to germination or germ-tube growth (Appendix 2.6).

Table 2.6: Means for the main effects of pathogen and host on the germination and germ-tube growth of three *Alternaria* species on leaf disks of eight cruciferous plants at 24 hours after inoculation.

	VARIATE[3]			
	Germ. (%)	**NGT**	**GTL (μm)**	**AP:GT (%)**
Pathogen [1]				
A.brassicae	97	2.2	313	43
A.raphani	90	1.6	228	81
A.brassicicola	95	1.1	137	67
s.e.d. (d.f.= 2, 48)±	**1.9**	**0.07**	**20.0**	**6.1**
Host [2]				
B.napus	92	1.6	236	73
B.campestris	93	1.7	199	66
B.oleracea	98	1.8	218	64
B.carinata	94	1.6	269	67
B.juncea	97	1.7	198	60
B.nigra	91	1.5	279	70
C.cheiri	94	1.7	207	52
S.alba	93	1.7	204	58
s.e.d. (d.f.= 7, 48)±	**3.1**	**0.11**	**32.4**	**10.0**

1 Mean of eight hosts

2 Mean of three pathogens

3 **Description of variates: Germ.** Percentage germination; **NGT** mean number of germ-tubes per germinated conidium; **GTL**, mean germ-tube length; **AP:GT**, ratio of appressoria to germ-tubes as a percentage.

Germination by all three isolates was at least 90%. However, *A.raphani* (90%) had a lower germination than both *A.brassicae* (97%) and *A.brassicicola* (95%), which showed similar germination rates. In relation to germ-tube numbers, *A.raphani* occupied an intermediate position, producing mainly one or two germ-tubes per conidium in a range from one to four (Figure 2.10).

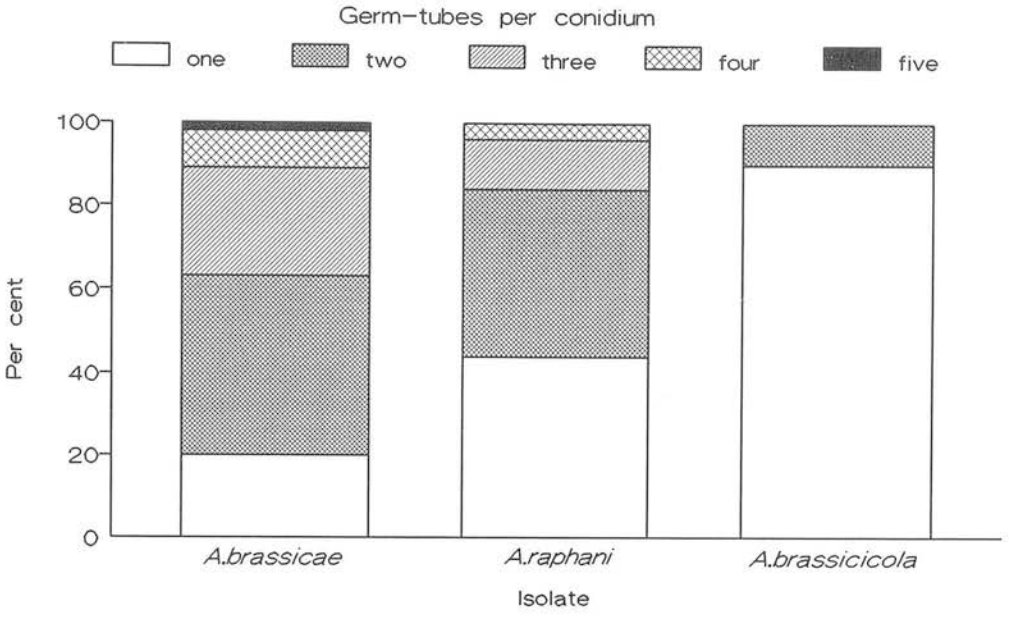


Figure 2.10: The percentage of germinated conidia of three *Alternaria* species with different numbers of germ-tubes (averaged for conidia on eight host species).

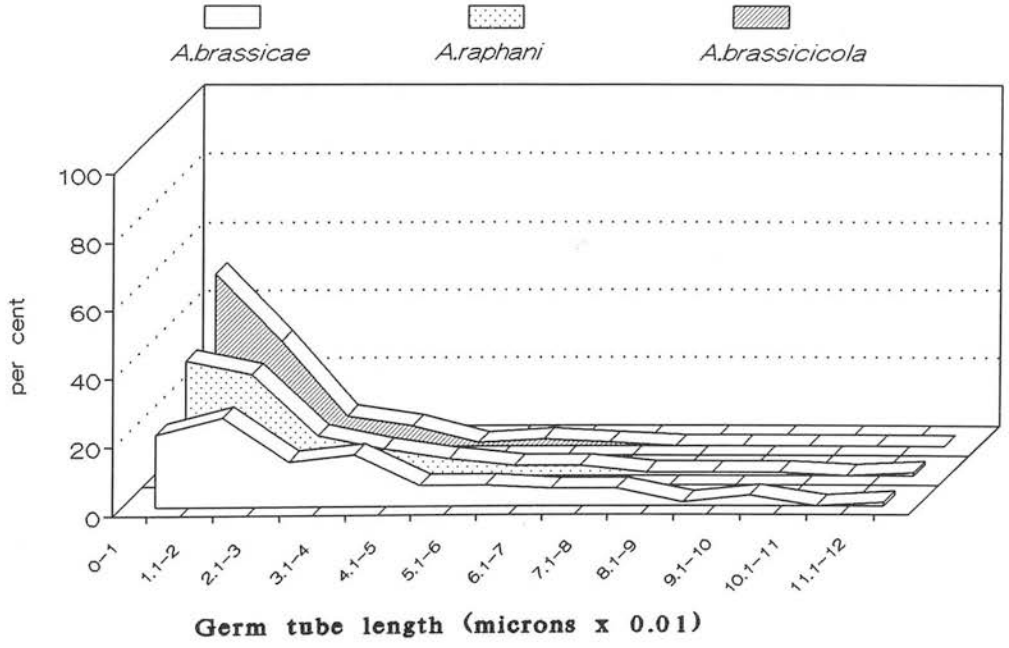


Figure 2.11: The percentage of germ-tubes in 12 length categories, for three isolates of *Alternaria* 24 hours after germination on host plant leaf disks (averaged for growth on eight host species).

Conidia of *A.brassicae* generally produced two or three germ-tubes within a range from one to five, while with *A.brassicicola*, most conidia produced a single germ-tube, or occasionally two.

Considering germ-tube length, *A.brassicae* produced the longest germ-tubes with a mean of 313 μm, a minimum of 19 μm, and a maximum of 1140 μm. *A.brassicicola* produced the shortest germ-tubes with a mean of 137 μm and a range of 11- 570 μm. *A.raphani* was intermediate with a mean germ-tube length of 228 μm and a range 19 - 1140 μm (Figure 2.11). The direction of germ-tube growth for all three species occurred without apparent topographic responses to the leaf surfaces, although the production of appressoria did not occur randomly on the leaf surface.

A.raphani had the highest ratio of appressoria to germ-tubes (81%), *A.brassicicola* was intermediate (67%), and *A.brassicae* had the lowest ratio of appressoria to germ-tubes (43%) (Table 2.6). As in Experiment 2.1, appressoria were found to be formed at three sites on the leaf surface, with some variation between the fungi in their selection of penetration sites.

Penetration

Data relating to the frequency of penetration events, and their distribution on the leaf surface are summarised in Table 2.7 (see also Appendix 2.6). On examining penetration rates from germinated conidia, differences were found between the fungi. *A.brassicae* and *A.raphani* produced more penetrations (105% and 124% of conidia respectively) than *A.brassicicola* (71% of conidia). However, there was no significant main host effect and no significant fungus - host interaction. Considering the occurrence of penetration events per germ-tube, proportionally more germ-tubes of *A.brassicae* (66%) failed to produce a penetration event than those of *A.brassicicola* (40%) and *A.raphani* (40%).

A similar percentage of penetrations (70% - 75%) occurred at cell wall junctions for all three fungi on all plants. With *A.brassicae* a slightly lower percentage of penetrations (17%) occurred over periclinal walls than with *A.raphani* (27%) and *A.brassicicola* (21%), the difference between *A.brassicae* and *A.raphani* being significant.

Table 2.7: Means for the main effects of pathogen and host on the penetration behaviour of three *Alternaria* species on leaf disks of eight cruciferous hosts at 24 hours after inoculation.

	VARIATE [3]				
	TPEN	**PNPGT**	**percentage of penetrations**		
	(%)	(%)	**ECWJ**	**ECPW**	**STOMATA**
Pathogen [1]					
A.brassicae	105	66	72	17	11
A.raphani	124	40	70	27	3
A.brassicicola	71	40	75	21	2
s.e.d. (d.f.= 2, 48) ±	**9.0**	**3.7**	**7.1**	**4.4**	**2.2**
Host [2]					
B.napus	106	50	70	27	9
B.campestris	114	45	84	26	3
B.oleracea	102	49	77	19	4
B.carinata	104	45	66	27	10
B.juncea	96	48	73	18	4
B.nigra	95	47	64	28	2
C.cheiri	82	59	70	12	2
S.alba	101	47	71	23	7
s.e.d. (d.f.= 7, 48) ±	**14.8**	**6.1**	**11.6**	**7.2**	**3.6**

1 Mean of eight hosts.

2 Mean of three pathogens

3 Description of variates: **TPEN**, percentage of penetrations per germinated conidium; **PNPGT**, percentage of germ-tubes not associated with a penetration event; **ECWJ**, percentage of penetrations which occurred at epidermal cell wall junctions, **ECPW**, percentage of penetrations which occurred at epidermal cell periclinal walls; **STOMATA**, percentage of penetrations which occurred *via* stomata.

A.brassicae showed a higher proportion of stomatal penetration (11%) than *A.raphani* (3%) and *A.brassicicola* (2%).

The occurrence of penetrations at cell junctions and over periclinal walls did not vary between hosts. However, there was a significant interaction between isolates and hosts with respect to frequency of stomatal penetration. *A.brassicae* showed some stomatal penetration on all hosts except *C.cheiri*, and stomatal entry by this fungus was especially frequent on *B.carinata* (31%) and *S.alba* (13%). Stomatal penetration by *A.raphani* was noted only on *S.alba*, *B.campestris*, and *B.napus* where it accounted for

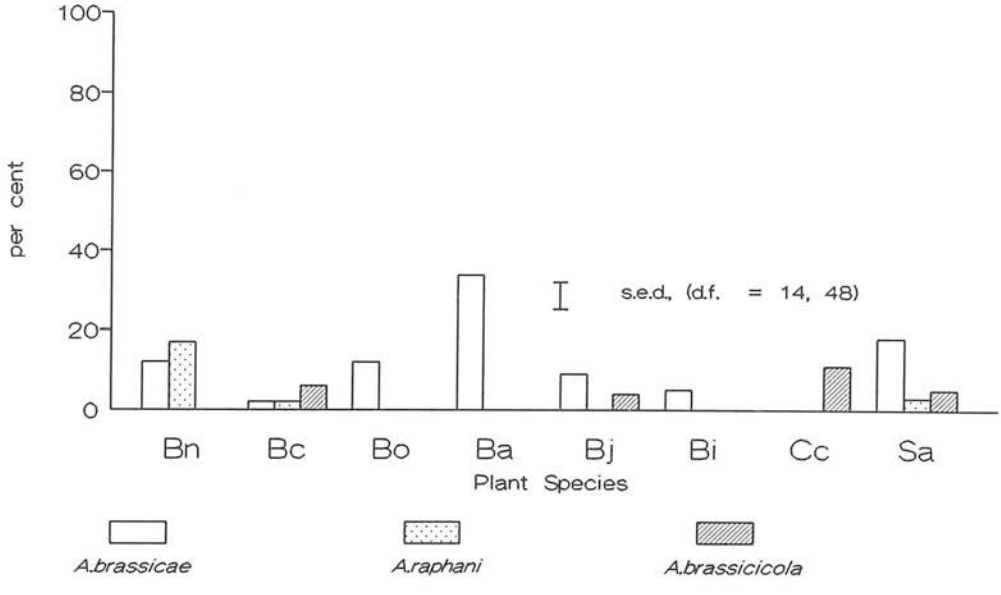


Figure 2.12: The percentage of germinated conidia of three *Alternaria* species which gave rise to stomatal penetration on leaf disks of eight host plants 24 hours after inoculation.

Hosts: Bn, *Brassica napus*; **Bc**, *C.campestris*; **Bo**, *B.oleracea*; **Ba**, *B.carinata*; **Bj**, *B.juncea*; **Bi**, *B.nigra*; **Cc**, *Cheiranthus cheiri*; **Sa**, *Sinapis alba*.

17% of all penetrations. With *A.brassicicola*, stomatal penetration was found on *B.campestris*, *B.juncea*, *C.cheiri* and *S.alba*, the highest incidence being on *C.cheiri* (11%) (Figure 2.12).

Host Cell Reactions

The main effects of pathogen and host on the incidence of each of the three types of host cell reaction noted are shown in Table 2.8 (see also Appendix 2.6).

Table 2.8: Means for the main effects of pathogen and host on host cell response to three *Alternaria* species grown on leaf disks of eight cruciferous hosts, at 24 hours after inoculation.

	Percentage of penetrations associated with		
	Localised callose deposition	**Single cell reactions**	**Adjacent cell reactions**
Pathogen[1]			
A.brassicae	51	4	45
A.raphani	26	21	53
A.brassicicola	19	33	48
s.e.d. (d.f.= 2, 48) ±	**4.4**	**3.8**	**4.1**
Host[2]			
B.napus	26	20	54
B.campestris	24	14	62
B.oleracea	30	18	52
B.carinata	20	19	61
B.juncea	36	20	45
B.nigra	28	31	42
C.cheiri	61	21	19
S.alba	31	14	55
s.e.d. (d.f.= 7, 48) ±	**6.7**	**6.1**	**7.2**

1 Mean of eight hosts

2 Mean of three pathogens

A higher percentage of penetrations were associated with localised callose deposition in the case of *A.brassicae* (51%) than either *A.brassicicola* (19%) or *A.raphani* (26%), for which the percentages of penetrations associated with localised callose deposition were similar. Over the eight hosts *A.brassicae* consistently had a higher incidence of localised callose deposition than *A.brassicicola* and *A.raphani*, but the

ranking of these two species varied between hosts. Considering differences between hosts, the percentage of penetrations associated with localised callose deposition was higher in *C.cheiri* than all other hosts, and was higher for *B.juncea* than *B.carinata*. No other differences between the means for hosts were significant. However, each host differed in its response to the pathogens. Thus, localised callose deposition occurred at a very high percentage of penetration sites of *A.brassicae* and *A.raphani* on *C.chieri* (90% and 72% respectively) (Figure 2.13). On the seven hosts other than *C.cheiri* the occurrence of localised callose deposition for *A.brassicae* ranged from 38% on *B.carinata* to 48% on *B.oleracea*: *A.raphani* displayed a greater variation than *A.brassicae* with a range of 5% (*S.alba*) to 33% (*B.juncea*). In contrast to *A.brassicae* and *A.raphani*, with *A.brassicicola* there were no differences between hosts with respect to the percentage of penetration sites which showed localised callose deposition, although the occurrence of this type of reaction was rather higher on *S.alba* than on the other plants. (Figure 2.13).

There were differences between the three fungi in the percentage of penetrations which were associated with single cell reactions. *A.brassicae* produced fewer single cell reactions (4% of penetrations) than either *A.raphani* (26%) or *A.brassicicola* (33%) which showed similar levels. No differences between hosts, or an isolate - host interaction were noted with respect to single cell reaction, with an range over hosts of 14 - 31% of all penetrations resulting in this type of cell reaction.

The percentage of penetrations which resulted in adjacent cell reactions were, on average, similar for the three fungi (45% - 53%), but there were significant differences between hosts and a significant interaction between pathogen and host with respect to the incidence of adjacent cell reactions. Thus, on *C.cheiri* there were far fewer adjacent cell reactions (19%) than on the other hosts, which ranged from 42% (*B.nigra*) to 62% (*B.campestris*). The low incidence of adjacent cell reactions on *C.cheiri* was due to the low values for *A.brassicae* (3%) and *A.raphani* (8%) only, the percentage for *A.brassicicola* being as high as 46%. In comparison with the other two species *A.brassicicola* showed less variation between hosts in the incidence of adjacent cell reactions which it induced, with levels ranging from 27% on *B.nigra* to 62% on *B.napus*. Excluding the results for *C.cheiri*, *A.brassicae* showed little variation in adjacent cell reactions,

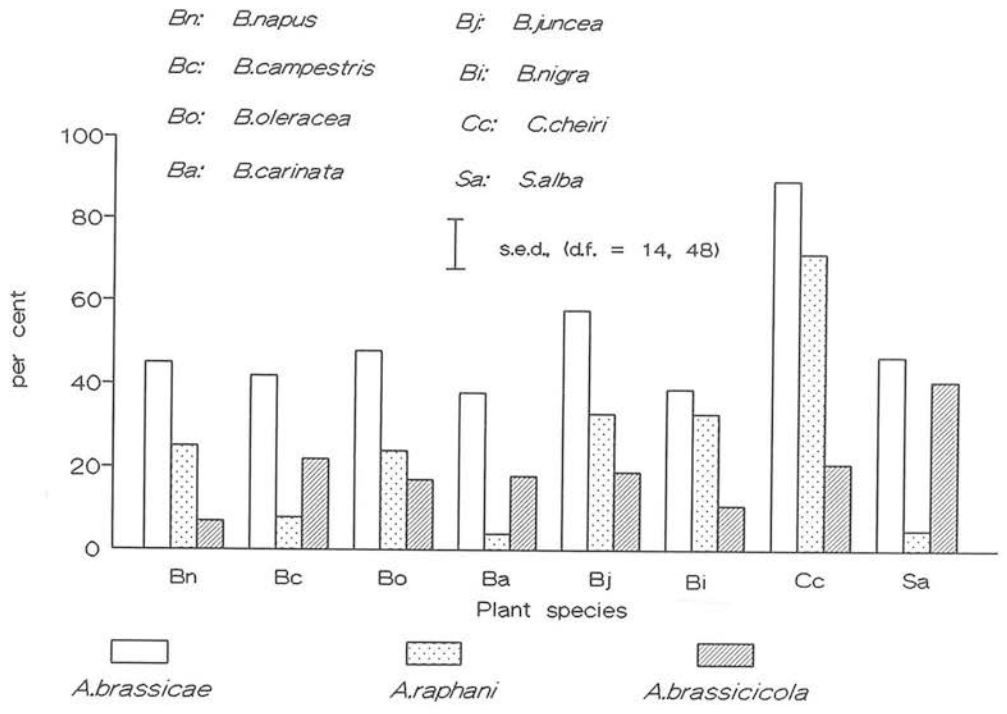


Figure 2.13: The percentage of penetrations by three *Alternaria* species which produced localised callose deposition during penetration on leaf disks of eight host plants 24 hours after inoculation.

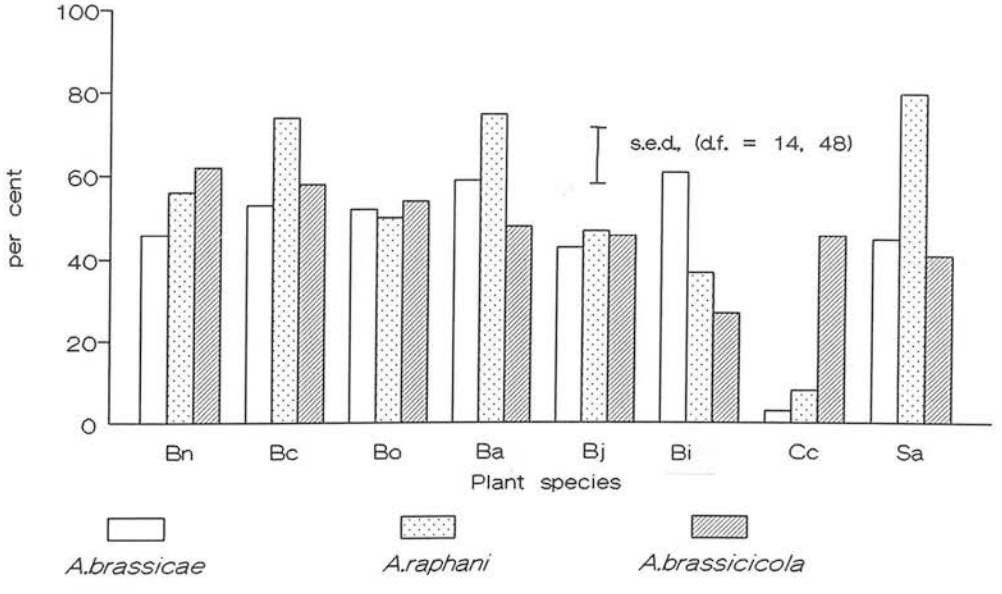


Figure 2.14: The percentage of penetrations by three *Alternaria* species which produced adjacent cell reactions on leaf disks of eight host plants 24 hours after inoculation.

with figures ranging from 43% (*B.juncea*) to 61% (*B.nigra*), while the variation for *A.raphani* was slightly greater varying from 37% (*B.nigra*) to 80% (*S.alba*) (Figure 2.14).

Discussion

Germination for *A.brassicae*, and more markedly for *A.brassicicola*, in the present experiment was higher than noted in Experiment 2.1. This may be related either to differences in the experimental protocol which reduced conidal clumping in the present experiment, to physiological differences between the isolates used (Ellingboe, 1972), as discussed previously, or partly due to the later sampling time used in the present experiment.

Confirmation of the differences in germination behaviour noted between *A.brassicae* and *A.brassicicola* in Experiment 2.1 was provided in this study. Thus, *A.brassicae* was found to produce more germ-tubes per conidium than *A.brassicicola*, the germ-tubes were found to have a faster growth rate, and extra-matrical growth was found to be more extensive. The behaviour of *A.raphani* was found to be intermediate with respect to these characters. It might be assumed, therefore, that germ-tube number and germ-tube length, although influenced by the environment, are innate characteristics of species, as suggested by Emmett & Parberry (1975) for many leaf infecting fungi. These characteristics might be partly related to conidium size, with the larger conidia of *A.brassicae* giving rise to more and longer germ-tubes than the other species.

There was no evidence in the present study that variation in host species influenced germination percentage, numbers of germ-tubes, or germ-tube length in the three *Alternaria* species, suggesting, as did the results of Experiment 2.1, that variation in leaf surface characteristics in themselves do not substantially influence the early development of *Alternaria* species. Furthermore, the present study indicated that there is no difference in the suitability of the leaf surface environment of the plants tested for germination and growth by the *Alternaria* species examined. Certainly, the results obtained do not support the hypothesis (Conn & Tewari, 1989) that germ-tube number for *A.brassicae* is modulated by leaf exudate availability on the leaf surface, since the host species used by these workers were included in the present study but no

differences were found between hosts in the extra-matrical development of the pathogen.

Surface hyphal growth by the three pathogens was not related to their success rate in establishing hyphae in the leaf tissue. Thus, for example, germ-tube lengths for *A.brassicae* and *A.raphani* were not significantly less on *C.cheiri* than that on other plants tested but the percentage of infections which gave rise to established intercellular mycelium was lower on this plant for both pathogens. On all plants examined, *A.brassicae* and *A.raphani* were found to produce germ-tubes which continued surface growth after appressorium formation, irrespective of whether the penetration attempt was successful. Von Ramm (1962) observed similar behaviour in *A.longipes* on tobacco. These observations raise the questions of how the behaviour of *Alternaria* species is controlled during pathogenesis, and of how nutrients derived from infected host tissue are partitioned in the developing mycelium. With *A.brassicicola*, in which appressoria were formed predominantly at terminal positions on the germ-tubes, surface growth did not continue in most cases after penetration had occurred. Thus, *A.brassicicola* was found, both in this experiment and in Experiment 2.1, to have a more determinate extra-matrical growth pattern than *A.brassicae*.

In comparing the ratio of appressoria to germ-tubes for *A.brassicae* and *A.brassicicola* in this and the previous experiment, it may be seen firstly that the ratios recorded were higher in the second study, probably due at least in part to the later sampling time. Figure 2.6a indicates that numbers of appressoria were still increasing at 18 hours after inoculation in the first experiment. It is also seen that whereas the ratios were higher in the first experiment for *A.brassicae* the position was reversed in the second study. A possible explanation to account at least partly for this result is the comparitively slow development shown by the isolate of *A.brassicicola* used in the first experiment.

Emmett & Parberry (1975) recognised two groups of pathogenic fungi, distinguishable by their tolerance to fluctuations in exogenous conditions which might affect appressorium production. The first of these groups shows little tolerance, requiring a fairly strict set of conditions to be present for successful appressorium production. The second group produces appressoria in less than optimal conditions and in response to a wider selection of stimuli. The results of this study indicate that *A.brassicae*, *A.brassicicola*, and *A.raphani* fall into the second of Emmett & Parberry's categories.

Physiological age of leaf tissue, chemical components of epicuticular wax, and the physical site of production have been noted to influence the location, frequency of production, and physical size of appressoria in other *Alternaria* species (von Ramm, 1962; Akai *et al.*, 1967; Saad & Hagedorn, 1969). The similarity of the distribution and number of appressoria formed by the isolates examined in this study implicates a general stimulus, common to all of the host/pathogen combinations tested. The majority of appressoria were formed over cell wall junctions, suggesting two possible types of stimulus; topographic and chemical.

A number of reviews (Emmett & Parbery, 1975; Hancock & Huisman, 1981; Wynn, 1981) have noted that appressorium formation above sub-cuticular cell wall junctions is a common feature in the leaf surface development of phytopathogenic fungi. The specific stimuli involved presumably vary between pathogens, although it is generally considered that the greater rates of exudation from the apoplast at cell wall junctions, and the topography of these areas are important factors. Appressorium production by the three pathogens examined in this study may be stimulated by a topographic recognition of position in conjunction with a stimulus provided by the difference in availability of exudates in the cell wall junction areas as compared with the periclinal surfaces of the epidermal cells. Von Ramm (1962) found that appressorium production by *A.longipes* was less site specific on older leaves than on young leaves. It would be expected that leaf exudates would be more readily available across the entire leaf surface in older leaves as the cells became senescent. The production of a proportion of their appressoria over periclinal sites by *A.brassicae*, *A.brassicicola*, and *A.raphani* may result from localised concentrations of exudates caused by thin points in the cuticle. However, it seems more likely that these appressoria result from a pre-programmed development pattern such as those suggested by Emmett and Parbery (1975) and occur at periclinal sites by chance. The more frequent production of appressoria over stomata by *A.brassicae* than by the other two species could then result from the increased probability of longer hyphae encountering stomata. Von Ramm (1962) concluded that *A.longipes* had no ability for specifically locating stomata, and that stomatal penetration by this species occurred by chance encounters of hyphae and stomata. There is some support for this hypothesis both from von Ramm's studies and the present work. In both cases hyphae of *Alternaria* species were noted to grow over stomata without entering or producing appressoria. However, Tsuneda & Skoropad (1978) found that penetration of leaves of *B.napus* by *A.brassicae* was

principally *via* stomata. There is no obvious reason why the results of the two studies should differ so greatly.

Some variation in the surface growth behaviour of *A.brassicae* and *A.brassicicola* was observed in Experiment 2.1 when these fungi were grown on leaves of *B.oleracea* with different epiculticular wax characteristics, and Conn & Tewari (1989) suggested that inter-specific variation in epicuticular wax characteristics may account in part for the lower susceptibility of some brassicas to *A.brassicae*. However, in earlier studies Tewari (1986) concluded that the difference in resistance of *B.napus* and *B.campestris* cultivars to *A.brassicae* was not determined by differences in the pre- and early post-penetration development of the pathogen on the two plants, and in the present experiment no significant variation was found in the surface development for *A.brassicae*, *A.brassicicola*, or *A.raphani* on the eight hosts tested here. However, there was evidence of significant variation between the pathogens and between hosts in relation to post penetration events which might account, at least partly, for differences in resistance between hosts.

A.brassicae, *A.brassicicola*, and *A.raphani*, on average, displayed similar success rates in establishing infections from attempted penetrations. As in previous reports on the behaviour of these and other *Alternaria* pathogens (Jackson, 1959; von Ramm, 1962; Tewari, 1986) it was found that all three *Alternaria* pathogens had an intercellular habit following penetration. Growth of the hyphae was accompanied by spreading areas of cell reaction both in the epidermis and in the upper layer of the mesophyll. Initial growth of all three pathogens was approximately parallel to the leaf surface. However, hyphae were commonly visible in the lower epidermis at sites immediately under unconfined penetrations. This suggests that an early stage growth by all three pathogens occurs transversely through the leaf. Jackson (1959) and Von Ramm (1962) reported a similar behaviour by *A.cucumerina* and *A.longipes*, noting that the cells between the upper and lower leaf surfaces collapsed to form necrotic lesions from which the fungi spread laterally.

Differences between the pathogens in the ranking of host resistance, based on the occurrence of adjacent cell reactions were recorded in the present study. The interaction between the three *Alternaria* species and the eight hosts with respect to the occurrence of adjacent cell reactions is illustrated in biplot

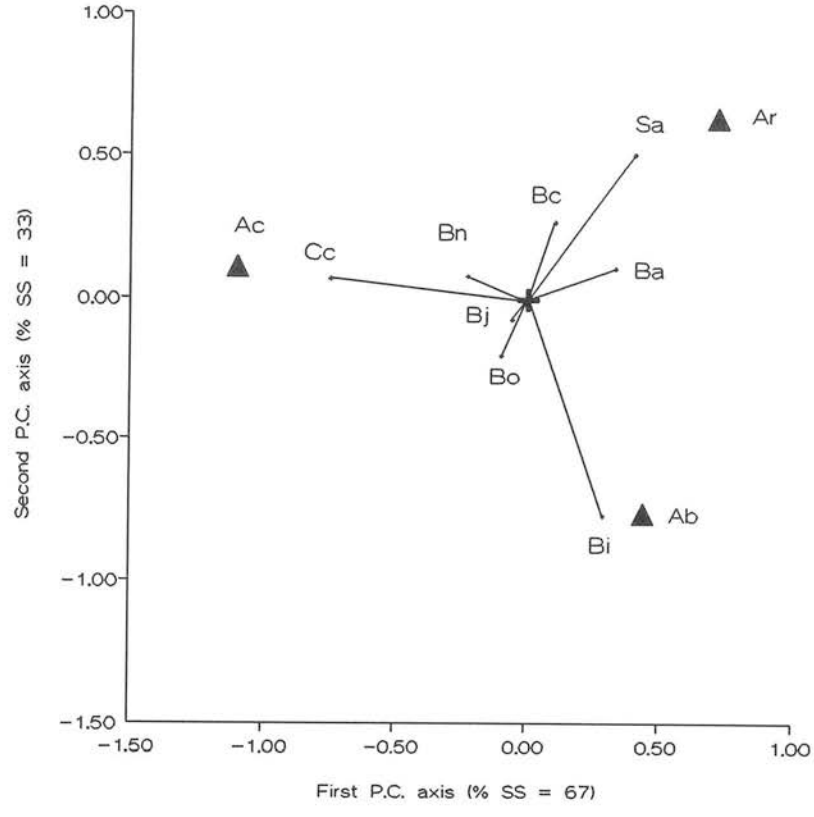
form in Figure 2.14.

B.napus, *B.carinata*, *B.campestris*, *B.juncea*, and *B.oleracea* were not clearly differentiated by the three pathogens; this is reflected by the relatively short vectors for these hosts and might be partly the result of features of the common cytogenetic background of these hosts (Hemmingway, 1976; Figure 1.1). The high incidence of adjacent cell reaction associated with *A.brassicae* on *B.nigra*, *A.brassicicola* on *C.cheiri*, and *A.raphani* on *S.alba* are all clearly indicated.

At confined penetration sites, where only a localised callose deposition or single cell reaction had occurred immediately under the point of penetration, hyphae were not observed in intercellular positions in the epidermis. These types of reaction might be assumed then to be associated with a successful resistance response on the part of the plant. In contrast, the adjacent cell reaction, in which callose deposition continued with advancing intercellular hyphae, is apparently associated with a susceptible condition in the plant.

Callose deposition is widely reported as a response to infection of plant cells by fungal pathogens,(Hinch & Clarke, 1982; Hachler & Hohl 1984; Munro, 1985; Tiburzy & Reisner, 1990). The significance of callose to resistance has been discussed by Aist (1976), and in many cases it appears that callose occurs as a non-specific response to cell damage and does not account for resistance. Perumalla & Heath (1989; 1991) for example, have shown that although callose deposition occurs in the non-host reaction of bean leaves to the cowpea rust fungus, it is not required for a successful resistance response. In contrast however, callose deposition appears to be the primary determinant of resistance in the reaction of certain barley cultivars to *Erysiphe graminis* f.sp. *hordei* (Aist & Gold, 1987).

In *Brassica* species Munro (1985) found that other resistance factors were probably more important than callose deposition in determining resistance to *E.cruciferarum*. However, in some cases in resistant plants callose deposition which continued after haustorium formation eventually led to encapsulation of haustoria. Callose deposition continued after penetration in the present study but was associated with susceptible responses and appeared to arise as successive cells came into contact with



ISOLATES: **Ab**, *A.brassicae*; **Ac** *A.brassicicola*; **Ar**, *A.raphani*
HOSTS: **Bn**, *B.napus*; **Bc**, *B.campestris*; **Bo**, *B.oleracea*; **Ba**, *B.carinata*; **Bj**, *B.juncea*; **Bi**, *B.nigra*; **Cc**, **C.cheiri**; **Sa**, *S.alba*

Figure 2.14: A biplot representation of the interactions between *A.brassicae*, *A.brassicicola*, *A.raphani*, and eight cruciferous hosts based on the occurrence of adjacent cell reactions.

advancing hyphae. These observations suggest that, as in many other cases, callose deposition occurred here as a general response to cell disruption concurrently with other cell responses which were effective in some cases in preventing fungal ingress.

That compounds other than callose are deposited at the site of penetration in response to *A. brassicae* was indirectly revealed by the results of Tewari (1986). Here, the author noted that an electron dense layer was deposited at sites of pentration. Callose is electron tanslucent and it seems likely that the material noted by Tewari is a polyphenol derivative such as lignin. Lignin and lignin aldehyde are known to be deposited in response to fungal infection in the cruciferae (Munro, 1985; Berry, 1991). However, although necrotic cells, and brown discolouration were noted in the present study in response to all three pathogens, no positive results for lignin deposition were recorded on staining with phloroglucinol-HCl. Munro (1985) found a low correlation between resistance and lignin or lignin aldehyde deposition in response to *E. cruciferarum* and concluded that these compounds are not involved directly in the resistance of crucifers to this pathogen. The results of this study provided no evidence that lignin was associated with cells responses to the pathogens examined.

Apart from forming a physical barrier to infection cell wall thickenings such as papillae may have other roles in resistance responses, *e.g.* restriction of nutrient movement from host cells, protection of carbohydrates from enzymatic degredation, reduction in the uptake of phytotoxins produced by the pathogen, or reduction in the diffusion of phytoalexins away from the site of penetration. It is relevant to this discussion that levels of resistance in a number of cruciferous species to *A. brassicae* have been correlated with the production of phytoalexins (Conn *et al.*, 1988), and that a hypersensitive response to *A. brassicae* has been reported in the cruciferous plant *Eruca sativa* (Conn & Tewari, 1986).

The hypersensitive response in plants is commonly, but not exclusively, associated with the presence of qualitative gene effects in interactions between biotrophic fungi and their hosts. However, it has been noted that hypersensitivity can occur in host-pathogen interactions in which there is apparently only quantitative variation in resistance (Crute, De Wit, & Wade, 1985) and also in compatible host-pathogen interactions (Asher & Thomas, 1983). Furthermore, there is good evidence that in many host -

pathogen systems hypersensitivity is accompanied by accumulation of phytoalexins and a range of other physiological and biochemical changes (Doke, Chai, & Kawaguchi, 1987).

Examining the resistance responses demonstrated by the host plants in the present study, the single cell reactions and localised callose deposition might be taken as forms of a hypersensitive response. Tomiyama (1982) categorised the various types of hypersensitivity which are known to occur. In the present case *A.brassicae* was more inclined to induce a disturbance in the cell wall only (category 1), than death of the cell at the point of penetration (category 2), which was more commonly induced by *A.brassicicola* and *A.raphani*. There is no clear explanation for the difference in the response of the plants to these pathogens although the tendency for *A.brassicae* to penetrate to a sub-cuticular position, while *A.brassicicola* and *A.raphani* penetrated predominantly to an epidermal position, may account in part for the difference. Alternatively, the variation in cell responses to the different pathogens may result from differences in sensitivity to host resistance factors in the *Alternaria* species.

Tomiyama (1982) also described forms of hypersensitivity in which more extensive but still localised cell responses occur. In this study, however, where necrosis or cell reaction was noted in cells adjacent to the point of penetration hyphae always appeared to extend into tissue without any apparent inhibition and such cases were concluded, as previously discussed, to represent failed resistance responses.

The eight host plants examined here showed broadly similar reactions to each of the pathogens. With *A.brassicicola* in particular there was little variation in the occurrence of successful penetration on the different hosts. However, in interaction with *A.brassicae* and *A.raphani*, *C.cheiri* was distinct in showing a much higher level of resistance than the other hosts. This difference may be partly related to host specialisation by these two pathogens and the relative phylogenetic distance between *C.cheiri* and the other plants examined. Variation in the reaction of a range of non-host species to these pathogens and other *Alternaria* species is considered in the next section.

Experiment 2.3: Fungal behaviour and plant cell response in interactions between *Alternaria* species and host and non-host plants.

Results

Germination and Germ-tube Development

Germination by all fungi averaged over sampling times and plant species was more than 90% and did not increase between the sampling times, except in the case of *A.infectoria* which showed a delay in germination. Germination for *A.infectoria* increased from 75% at 18 hours after inoculation, to 90% at 36 hours after inoculation (Table 2.9). *A.infectoria* was also exceptional in showing a variation in germination between the different plants, germination rates for this species averaging less on poppy (76%) and wheat (72%) than on OSR (93%) and tomato (86%) (Table 2.9, Figure 2.15, and Appendix 2.6).

Germinating conidia of each fungus produced a characteristic number of germ-tubes which was not affected plant species or sampling time. Species with larger conidia tended to produced more germ-tubes than those with smaller conidia. Conidia of *A.brassicae* produced the highest number of germ-tubes followed by those of *A.solani*, *A.alternata*, *A.raphani*, *A.infectoria*, and finally *A.brassicicola*. Mean germ-tube numbers for the fungi are presented in Figure 2.16a and frequency distribution of germ-tube number per conidium is shown in Figure 2.16b.

Germ-tube length was found to vary between fungi and plant species. There was a significant interaction between fungus and sampling time, but not between fungus and plant. At 18 hours after inoculation *A.solani* produced the longest germ-tubes (499 μm), with the remainder of the species ranging from 98 μm (*A.brassicicola*) to 299 μm (*A.infectoria*). At 36 hours after inoculation however, *A.infectoria* produced the longest germ-tubes, (769 μm) followed by *A.solani* (719 μm), while the other species ranged from 334 μm (*A.brassicicola*) to 544 μm (*A.alternata*). In examining the difference between the plants, it was found that at 18 hours after inoculation germ-tube lengths were similar an all four plants, but at 36 hours after inoculation germ-tubes on OSR (474 μm) and poppy (458 μm) were shorter than on tomato (578 μm) and wheat (620 μm).

Table 2.9: Germination and germ-tube for six *Alternaria* species at two sampling times after inoculation on leaf disks of four plant species.

	Germination per cent		Germ-tube number per conidium		Mean germ-tube length (μm)		Appressoria per germ-tube (%)	
	18	36h	18h	36h	18h	36h	18h	36h
Pathogen								
A. alternata	94	96	1.5	1.7	198	544	70	124
A. brassicae	92	93	2.3	2.4	173	359	24	16
A. brassicicola	98	94	1.2	1.3	98	334	54	94
A. infectoria	74	90	1.4	1.4	299	769	19	46
A. raphani	95	97	1.6	1.4	196	443	84	137
A. solani	100	94	1.9	1.8	499	719	73	98
s.e.d. (d.f. = 5, 85)	**±2.9**		**±0.12**		**±63.7**		**±10.9**	
Plant								
OSR	95	97	1.6	1.6	234	474	53	80
poppy	91	91	1.6	1.6	240	458	71	106
tomato	92	94	1.7	1.8	250	578	52	78
wheat	90	93	1.7	1.8	251	620	40	80
s.e.d. (d.f. = 3, 85)	**±2.3**		**±0.10**		**±51.2**		**±8.9**	

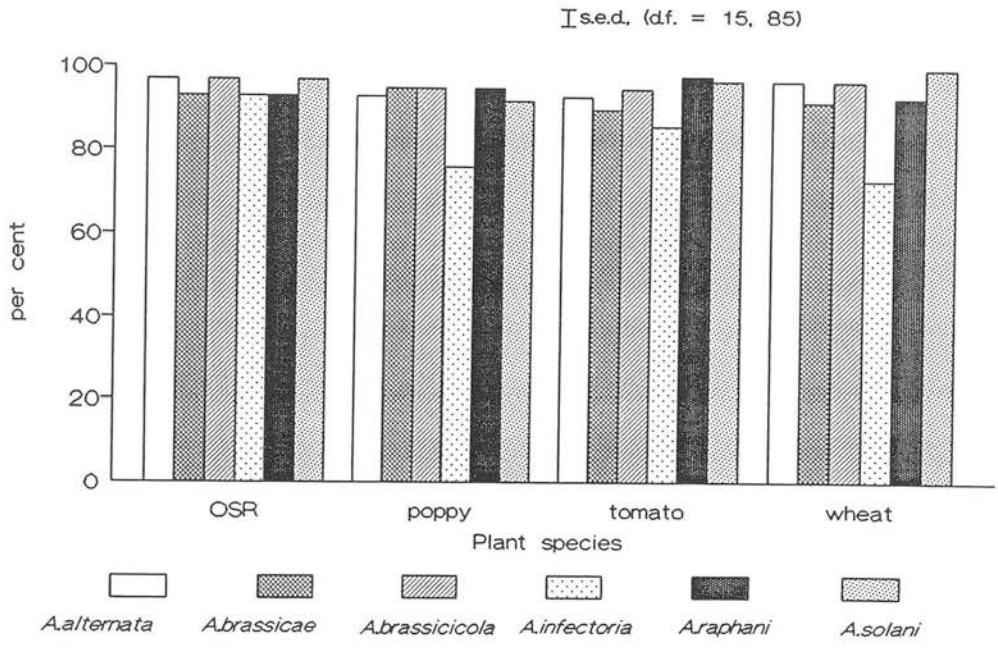


Figure 2.15: Germination percentage, averaged between 18 and 36 hours after inoculation for six *Alternaria* species on leaf disks of four plants.

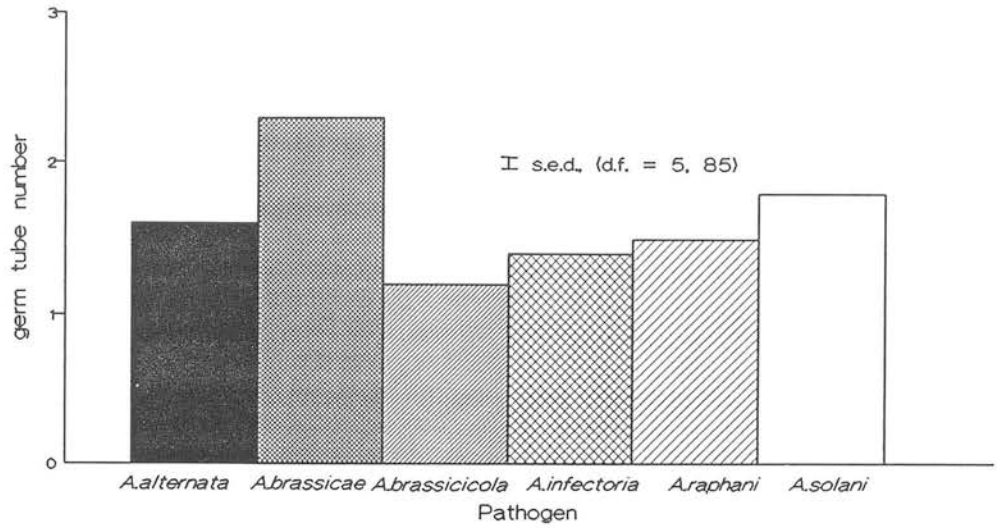


Figure 2.16a: Mean germ-tube number per conidium for six *Alternaria* species averaged between over four plant species and between 18 and 36 hours after inoculation.

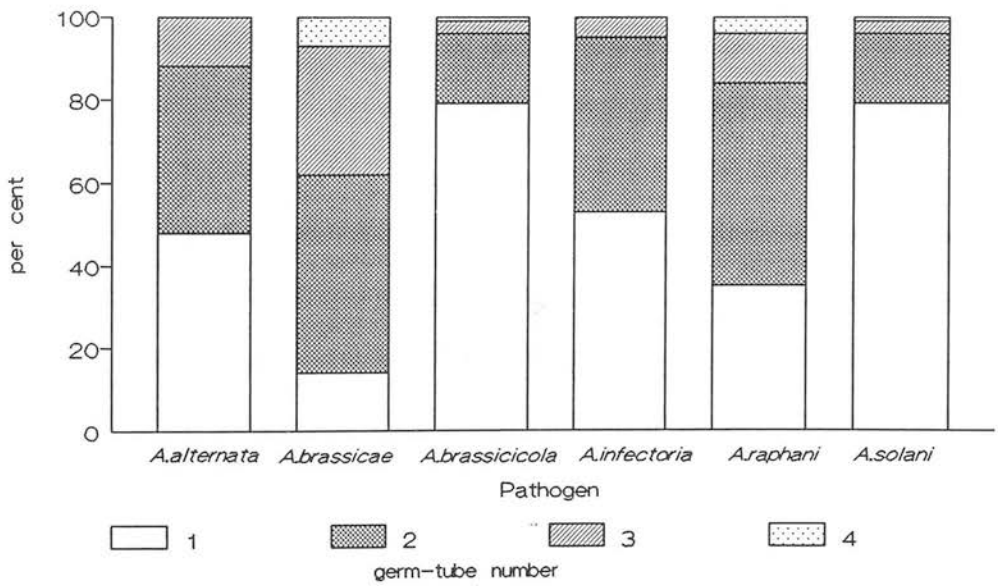


Figure 2.16b: Frequency distribution of germ-tube numbers produced by conidia of six *Alternaria* species, averaged over four plant species and between 18 and 36 hours after inoculation.

All fungi had formed appressoria by 18 hours after inoculation. Appressoria were formed at characteristic points on germ-tubes for each species, terminal positions for *A.brassicicola*, in terminal or intercalary positions for *A.brassicae* and *A.raphani*, and on lateral branches or terminally for *A.alternata*. *A.solani* was found to be similar to *A.brassicae* and *A.raphani* in forming appressoria terminally or in intercalary positions, while *A.infectoria* was similar to *A.alternata* and formed appressoria most frequently on lateral branches.

Averaged over plant species and sampling times, *A.brassicae* produced fewest appressoria per germ-tube and *A.raphani* the most. The ranking of the six species in descending order of average appressorium production was; *A.raphani*, *A.alternata*, *A.solani*, *A.brassicicola*, *A.infectoria*, and *A.brassicae*. Over all fungi and sampling times the the ratio of appressoria to germ-tubes was higher on poppy than on the other plants, where more or less similar ratios were found. However, there were significant interactions between fungi, plants, and sampling times. Considering the fungus/plant interaction, *A.brassicae* and *A.solani* showed a higher frequency of appressoria on their respective host plants (OSR and tomato) than on the other plants. For *A.alternata*, *A.raphani* and *A.infectoria* the incidence of appressoria was higher on poppy than on other plants, while in the case of *A.brassicicola* there was little variation in the frequency of appressoria between the plants.

With respect to the three way interaction between fungus, plant and sampling time, (Figure 2.17) the majority of the variance was accounted for in the interactions between fungi, time and the contrast between wheat and the dicotyledenous plants, and in the interaction involving the contrast between poppy, and OSR and tomato. Thus, *A.brassicicola* showed a rather large increase in appressorium to germ-tube ratio on wheat as compared to the other plants, and in comparison to the increases shown by the other *Alternaria* species on wheat. For *A.raphani* the appressorium to germ-tube ratio increased on wheat and poppy but not on OSR and tomato, while with *A.solani* increases were noted on OSR and tomato but not on poppy and wheat. In the case of *A.infectoria* the appressorium ratio increased over time only on poppy, while with *A.alternata* it increased on all plants, and with *A.brassicae* on none (Figure 2.17).

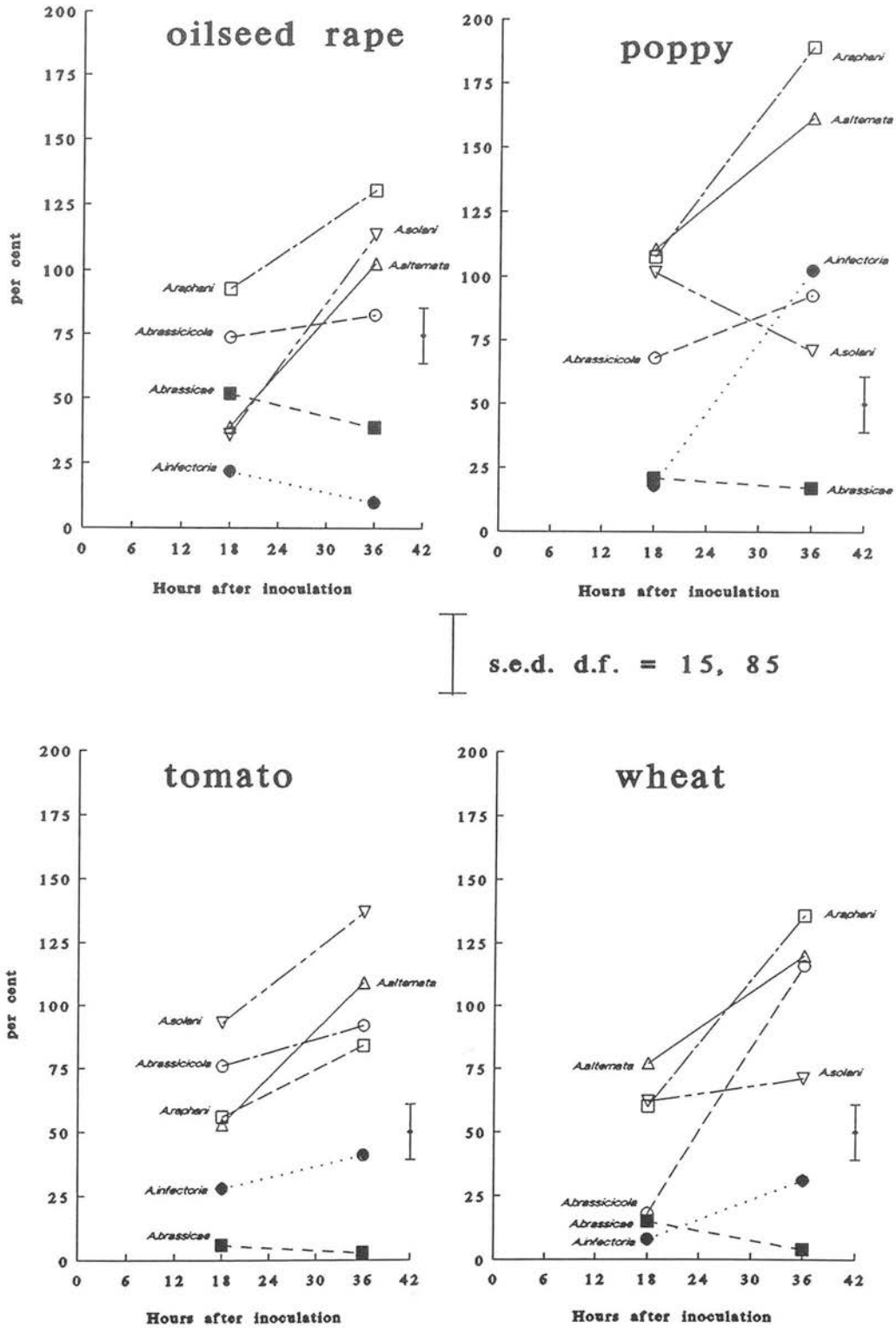


Figure 2.17: The ratio of appressoria to germ-tubes for six *Alternaria* species at 18 and 36 hours after inoculation on four different plant species.

Penetration

The average frequency of penetration by germinated conidia was closely correlated to appressorium production. Thus, averaged over sampling times and plant species, *A.alternata* (148%) and *A.raphani* (147%) produced the highest frequency penetrations per germinated conidium, followed by *A.solani* (132%) and *A.brassicicola* (84%), with *A.brassicae* (46%) and *A.infectoria* (44%) similar and significantly lower than the other species (Table 2.10).

Table 2.10: Ratio of penetration events to germinated conidia (as percentage) for six *Alternaria* species averaged between two sampling times after inoculation on leaf disks of four plants species.

Pathogen	**Plant species**				
	Oilseed rape	Poppy	Tomato	Wheat	**Mean**
A.alternata	109	177	148	157	**148**
A.brassicae	108	53	14	11	**46**
A.brassicicola	85	88	85	72	**84**
A.infectoria	14	77	57	30	**44**
A.raphani	158	192	113	127	**147**
A.solani	90	134	202	107	**132**
Mean	**94**	**120**	**103**	**85**	

s.e.d. Fungi (d.f. = 5, 85) ± 11.4
s.e.d., Plant (d.f. = 3, 85) ± 9.3
s.e.d., Interaction (d.f. = 15, 85) ± 22.9

Table 2.10 also shows the tendency for penetration, averaged over fungi and sampling times, to be most frequent on poppy and least frequent on wheat. However, there were significant interactions between plants and sampling time, while components of the three way interaction between the factors were also significant. *A.brassicae* and *A.solani* showed most penetration on their respective host plants (Table 2.10). The frequency of penetration by *A.brassicae* was higher on OSR (108% of conidia) than on other plants (11% - 53%). With *A.solani*, penetration occurred most frequntly on tomato (202%) and was significantly lower on other plants (90% - 134%). In the case of *A.brassicicola*, penetration occurred at approximately equal frequency on all plants. With *A.raphani* and *A.alternata*, the frequency of penetration was relatively high on all plants, but was especially high on poppy. However, these fungi appeared to differ in the

frequency of penetration on the other plants, with *A.alternata* tending to produce more penetration on tomato and wheat, while *A.raphani* gave rise to more on OSR. *A.infectoria* showed a comparatively low frequency of penetration on OSR (14%) and wheat (30%), and only moderate levels of penetration on tomato (57%) and poppy (77%) Penetration frequency for *A.infectoria* on tomato (57%) and on wheat (30%) were not significantly different to those of *A.brassicae* and *A.brassicicola* (Table 2.10).

Although the overall three-way interaction between isolates, plants, and sampling times was not significant, examination of orthogonal contrasts indicated that the pathogens differed in their change in incidence of penetration over time with respect to the contrast between poppy and OSR and tomato. Thus, *A.brassicae*, and *A.brassicicola* showed relatively little increase on these plants from 18 to 36 hours, while *A.alternata* showed an approximately equal and large increase on all three; with *A.infectoria* and *A.raphani* there was a marked increase in penetration on poppy but not OSR or tomato, but in contrast, penetration by *A.solani* showed a reverse trend (Figure 2.18).

Penetration occurred most frequently at anticlinal wall sites irrespective of pathogen or plant species, but was slightly lower at this site on wheat than on the dicotyledenous plants (Table 2.11). Penetration *via* periclinal walls did not differ between fungi or plant species, but unlike penetration *via* anticlinal walls it increased over time, from an overall average at 18 hours of 16% to 22% at 36 hours after inoculation. Stomatal penetration differed between pathogens, and there was a significant interaction between pathogens and plants (Table 2.12). Thus, *A.brassicae* and *A.solani* gave rise to more penetration *via* stomata than the other fungi, but with *A.brassicae* stomatal penetration occurred at high levels only on OSR and poppy, while with *A.solani* frequent stomatal penetration occurred on OSR, poppy, and wheat, but not tomato.

In order to assess deviation from a random distribution of penetration events on the leaf surface, the percentage of the leaf surface of each plant composed of each of the three sites was assessed as follows. On three replicate leaf pieces of each plant five longitudinal and five transverse 150 m transects were assessed by recording the type of leaf surface under successive 3 μm sections.

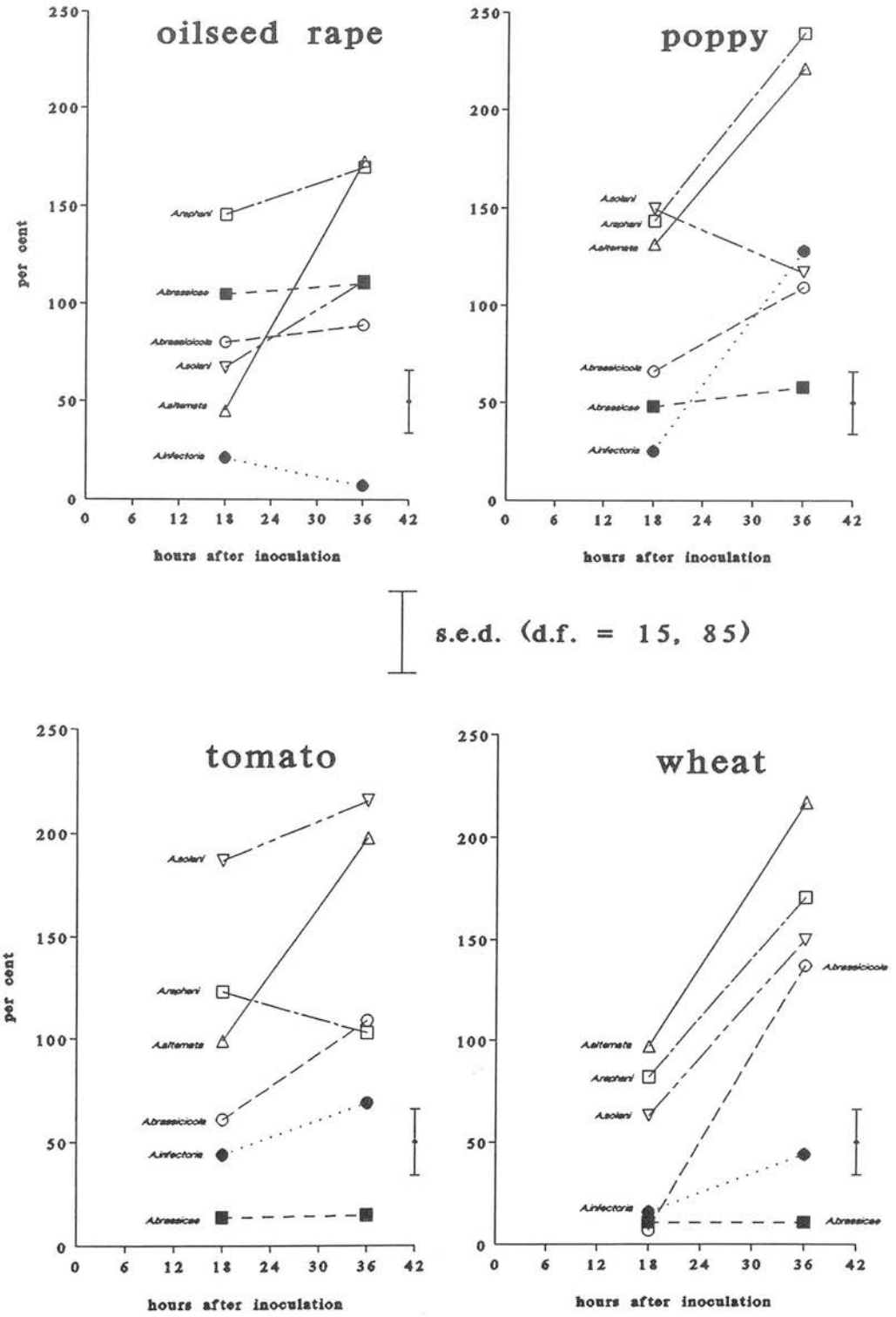


Figure 2.18: The ratio of penetration events to germinated conidia of six *Alternaria* species at 18 and 36 hours after inoculation on four plants.

Table 2.11: Percentage of penetration events which occurred at three sites on the leaf surface of leaf disks of four plants species inoculated with six *Alternaria* species (averaged between two sampling times).

Pathogen	Percentage of penetrations at:		
	Anticlinal walls	Periclinal walls	Stomata
A.alternata	74	18	7
A.brassicae	62	20	16
A.brassicicola	65	22	5
A.infectoria	65	8	7
A.raphani	73	20	7
A.solani	63	20	17
s.e.d. (d.f.= 5, 85) ±	**7.7**	**6.5**	**4.0**
Plant			
OSR	70	12	11
poppy	72	16	10
tomato	70	23	7
wheat	57	21	11
s.e.d., (d.f. = 3, 85) ±	**6.3**	**5.4**	**3.2**

Table 2.12: The percentage of penetrations which occurred *via* stomata for six *Alternaria* species averaged between two sampling times after inoculation on leaf disks of four plant species.

Pathogen	**Plant species**				
	Oilseed rape	Poppy	Tomato	Wheat	**Mean**
A.alternata	3	2	10	13	7
A.brassicae	33	23	8	5	**18**
A.brassicicola	5	5	6	3	**5**
A.infectoria	0	0	3	25	7
A.raphani	9	6	6	5	7
A.solani	18	21	7	21	**17**
Mean	**11**	**10**	**7**	**12**	

s.e.d. Fungi (d.f. = 5, 85) ± 4.0
s.e.d., Plant (d.f. = 3, 85) ± 3.2
s.e.d., Interaction (d.f. = 15, 85) ± 7.9

The percentage leaf area composed of each of the three types of site was calculated from these scores, and the percentages used to calculate expected appressorium numbers from the null hypothesis that, if appressorium formation occurred at random, the number of appressoria formed at each site would be proportional to the percentage of the leaf area represented by the site. Hypothesis testing employed the Chi-square statistic.

The tests indicated that the location of appressoria deviated significantly from a random distribution in most fungus/plant combinations. In the case of *A.brassicae* the observed distribution did not deviate from random on wheat or tomato, but in these cases the number of appressoria formed was low. The expected and observed ratios of appressoria at the three sites are shown in Table 2.13 with probabilities of the chi-square distribution with 2 degrees of freedom. On examining the results of these analyses in more detail, it is clear that the incidence of appressoria over cell junctions was higher in most cases than would be occur at random, while appressorium formation over periclinal walls and stomata was lower than would occur purely by chance, with the exception of stomatal penetration on OSR and poppy by *A.brassicae*, and on poppy and wheat by *A.solani*.

The percentage of germ-tubes which failed to penetrate differed between fungi and plants (Table 2.14), but the three way interaction between fungi, plants, and sampling times was also significant (Figure 2.19). *A.brassicae* produced the highest proportion of non-penetrating germ-tubes, followed by *A.infectoria* and *A.solani*, *A.brassicicola*, *A.alternata*, and finally *A.raphani*. In comparison with the dicotyledenous plants there were more non-penetrating germ-tubes on wheat.

With respect to the interaction between fungi and plants, *A.brassicae* and *A.brassicicola* produced relatively few non-penetrating germ-tubes on OSR, while the incidence for *A.brassicicola* was also low on poppy. *A.infectoria* and *A.solani* produced rather few non-penetrating germ-tubes on poppy and tomato, while *A.alternata* and *A.raphani* showed little variation in the frequency of non-penetrating germ-tubes between the four plants (Table 2.14).

Table 2.13: Chi-square values and associated probabilities for a test of goodness of fit to a proportional allocation of appressoria to three possible sites on the leaf surfaces of four plant species, for six species of *Alternaria* (data averaged between two sampling times).

		Observed ratio of sites			
SPECIES	**PLANT**	**ECJ***	**CPW**	**STO**	X^2
A.alternata		1:	0.15:	0.07	84.4***
A.brassicae		1:	0.16:	0.53	56.4***
A.brassicicola	OSR	1:	0.35:	0.08	34.7***
A.infectoria		1:	0.11:	0.00	16.2***
A.raphani		1:	0.19:	0.09	100.9***
A.solani		1:	0.47:	0.23	20.7***
Expected		**1:**	**1.76:**	**0.35**	
A.alternata		1:	0.24:	0.04	79.5***
A.brassicae		1:	0.18:	0.41	55.6***
A.brassicicola	Poppy	1:	0.39:	0.13	39.4***
A.infectoria		1:	0.19:	0.00	32.0***
A.raphani		1:	0.26:	0.08	150.1***
A.solani		1:	0.24:	0.24	108.1***
Expected		**1:**	**2.46:**	**0.16**	
A.alternata		1:	0.34:	0.18	49.1***
A.brassicae		1:	0.50:	0.25	3.0 NS
A.brassicicola	Tomato	1:	0.47:	0.11	43.0***
A.infectoria		1:	0.25:	0.05	21.4***
A.raphani		1:	0.36:	0.10	78.7***
A.solani		1:	0.23:	0.18	78.7***
Expected		**1:**	**1.72:**	**0.33**	
A.alternata		1:	0.39:	0.16	128.8***
A.brassicae		1:	1.00:	0.25	2.7 NS
A.brassicicola	Wheat	1:	0.38	0.14	58.6***
A.infectoria		1:	0.40:	0.00	28.3***
A.raphani		1:	0.26:	0.07	184.9***
A.solani		1:	0.84:	0.56	58.3***
Expected		**1:**	**3.59:**	**0.36**	

* **ECJ**, Epidermal cell junctions; **CPW**, Cell periclinal walls; **STO**, Stomata

X^2 with 2 d.f. = 13.8 ***: $P < 0.001$ NS: Non-significant

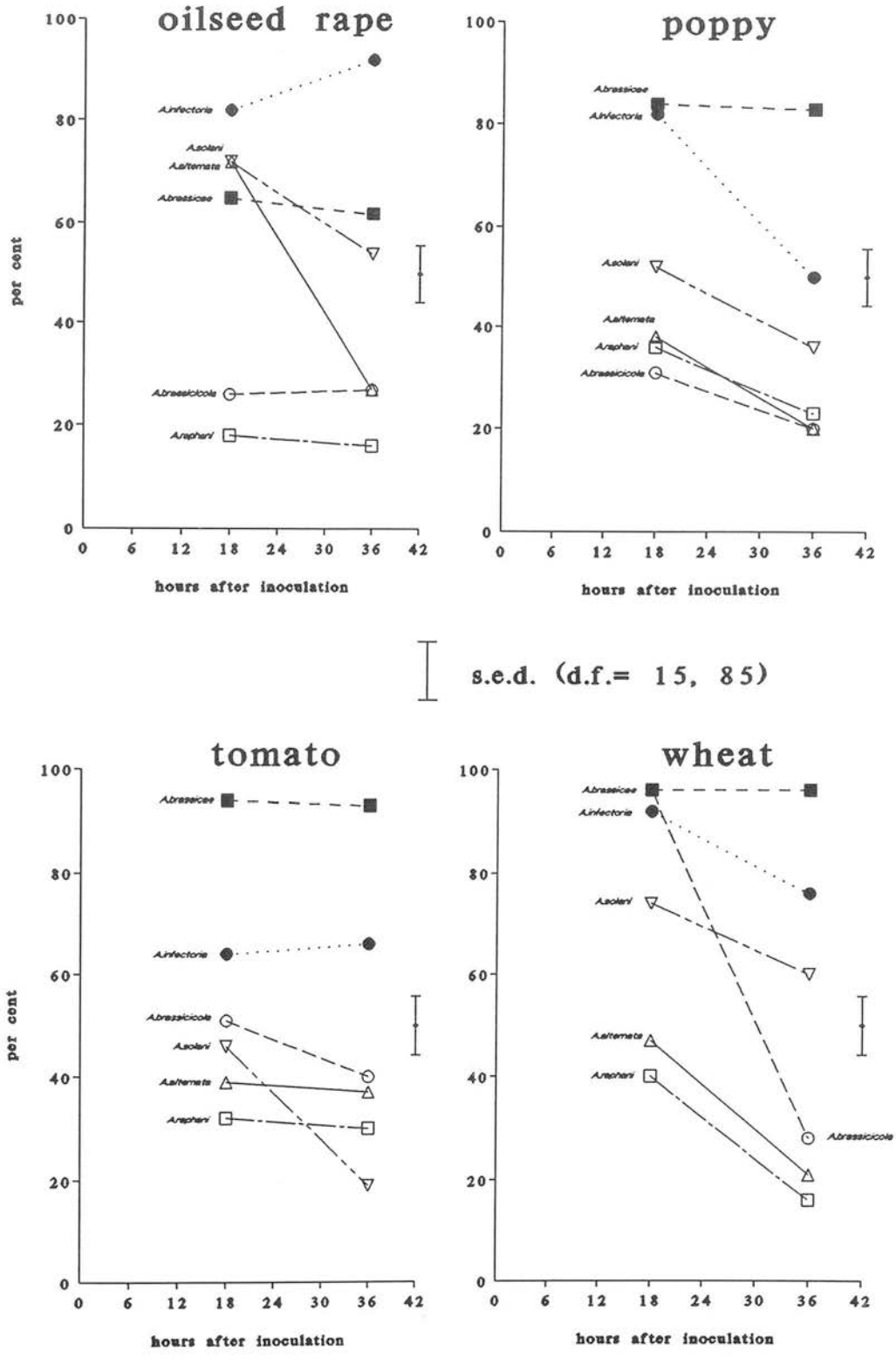


Figure 2.19: The percentage of germ-tubes of six *Alternaria* species which did not produce a penetration event on leaf disks of four plants at 18 and 36 hours after inoculation.

Table 2.14: The percentage of germ-tubes of six *Alternaria* species which did not produce a penetration event on leaf disks of four plants species, averaged between two sampling times after inoculation.

Pathogen	Plant species				
	Oilseed rape	Poppy	Tomato	Wheat	**Mean**
A.alternata	49	29	38	34	**38**
A.brassicae	63	83	93	96	**84**
A.brassicicola	26	26	45	62	**40**
A.infectoria	87	66	65	84	**75**
A.raphani	17	29	31	28	**26**
A.solani	63	44	32	87	**51**
Mean	**51**	**46**	**51**	**62**	
s.e.d. Fungi (d.f. = 5, 85) ± 3.3					
s.e.d., Plant (d.f. = 3, 85) ± 4.1					
s.e.d., Interaction (d.f. = 15, 85) ± 8.1					

Examining the interaction between fungi, plants, and sampling time (Figure 2.19) there was a general trend for the incidence of non-penetrating germ-tubes to decrease over time. At 18 hours after inoculation the incidence of non-penetrating germ-tubes was relatively high on wheat but at 36 hours after inoculation there was no difference between wheat and the other plants. Considering specific pathogen/plant combinations, the incidence of non-penetrating germ-tubes decreased significantly between 18 and 36 hours after inoculation in the following cases: *A.alternata* on OSR and wheat, *A.brassicicola* on wheat, *A.infectoria* on poppy, *A.solani* on tomato, and *A.raphani* on wheat.

Attempted penetration generally induced a plant cell response by 18 hours after inoculation. However, in a small number of cases with *A.brassicae*, *A.brassicicola*, and *A.infectoria* appressoria were observed without any associated cell reaction. These appressoria were designated uncompleted penetrations. The highest incidence of uncompleted penetrations occurred with *A.infectoria*, followed by *A.brassicicola* and *A.brassicae* (Figure 2.20a); there was no incidence of uncompleted penetrations with *A.alternata*, *A.raphani*, or *A.solani*. Appressoria with no associated cell reaction were more common on wheat than on the other plants, but only at the earlier sampling time (Figure 2.20b).

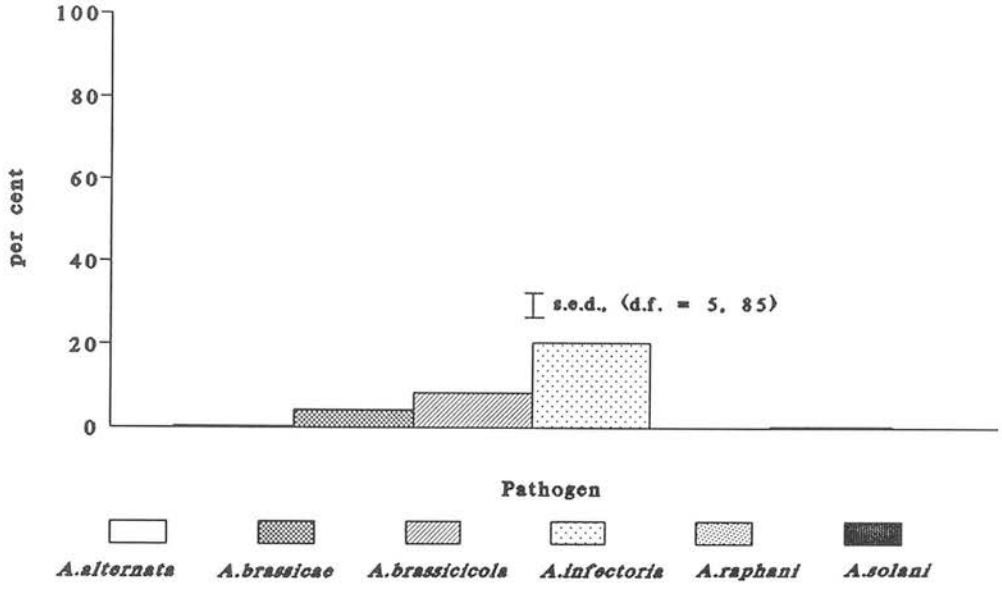


Figure 2.20a: The percentage of penetration attempts by six *Alternaria* species which were uncompleted, averaged over plant species and two sampling times.

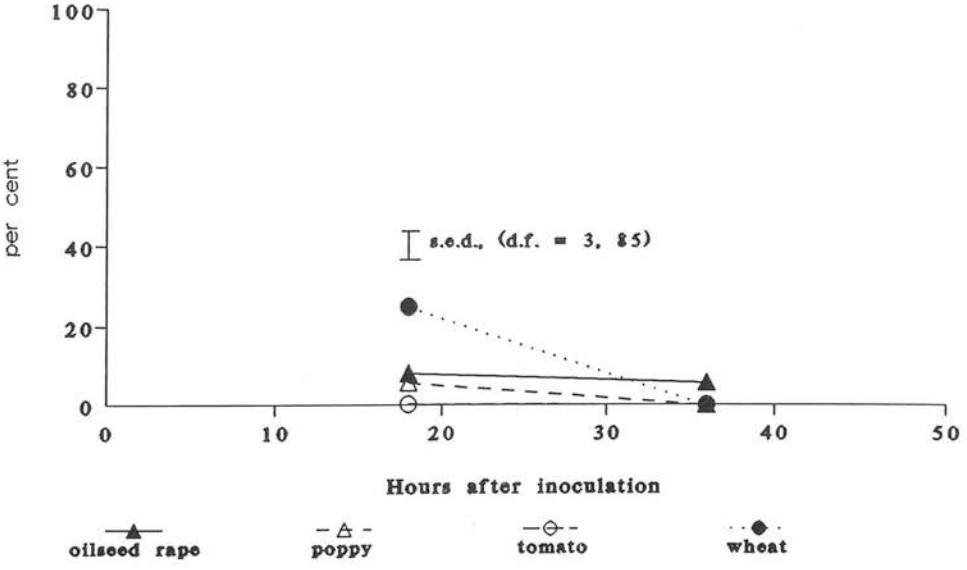


Figure 2.20b: The percentage of penetrations which were uncompleted on four plants at two sampling times, averaged over six *Alternaria* species.

Plant Cell Reactions

The frequency of confined penetration, where only localised callose deposition or a single cell reaction were noted, differed significantly between fungi and plants, and there was a significant interaction between these factors. However, there was no evidence of a significant three-way interaction between fungi, plants, and sampling time with respect to confined penetration, although there was an interaction between hosts and sampling times which was related to the change in the incidence of uncompleted penetrations noted above.

A.alternata showed the highest average frequency of confined penetration and *A.brassicicola* the lowest, while confined penetration was, on average, more common on wheat than on OSR and tomato but not poppy. Examining the interaction between plants and fungi (Figure 2.21a), *A.brassicae*, *A.brassicicola*, and *A.raphani*, showed a similar, relatively low, frequency of confined penetration on their host, OSR, with levels on the three other plants being higher, particularly for *A.raphani* on wheat. In the case of *A.solani*, confined penetration was lower on tomato than on the other plants. With *A.alternata* there was little difference in the incidence of confined penetrations between plants and at least 80% of penetrations were of this type, while with *A.infectoria* the incidence of confined penetration was high on tomato and poppy, and relatively low on OSR.

The frequency of confined penetration did not change significantly over time on any host except wheat where the frequency increased from 75% to 100% of penetrations between 18 and 36 hours after inoculation as the incidence of uncompleted penetrations decreased. (Figure 2.21b).

In relation to unconfined penetration, where hyphae ramified into tissue from the point of entry, the main effects of fungi, plants, and the interaction between these factors were significant. *A.brassicae*, *A.brassicicola*, and *A.raphani* showed their highest incidence of unconfined penetration on OSR (64 - 85%). There was no occurrence of unconfined penetration by these pathogens on wheat, and for all three the incidence of unconfined penetration was relatively low on poppy and tomato (17 - 31%). With *A.solani* the highest incidence of unconfined penetration occurred on tomato (68%), while similarly

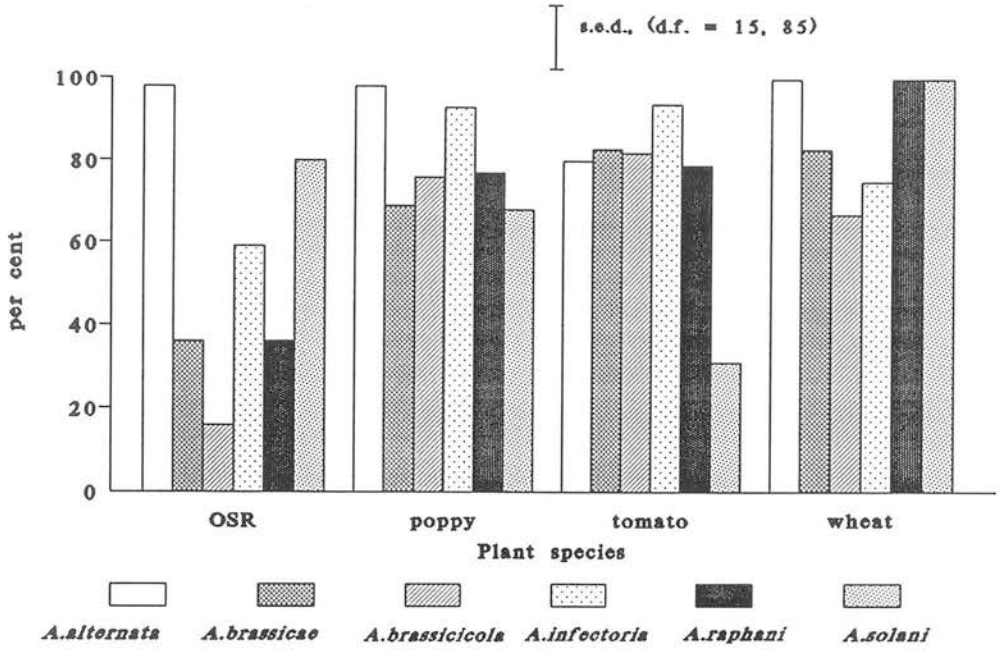


Figure 2.21a: The percentage of penetrations by six *Alternaria* species on leaf disks of four plant species which were confined, averaged over two sampling times.

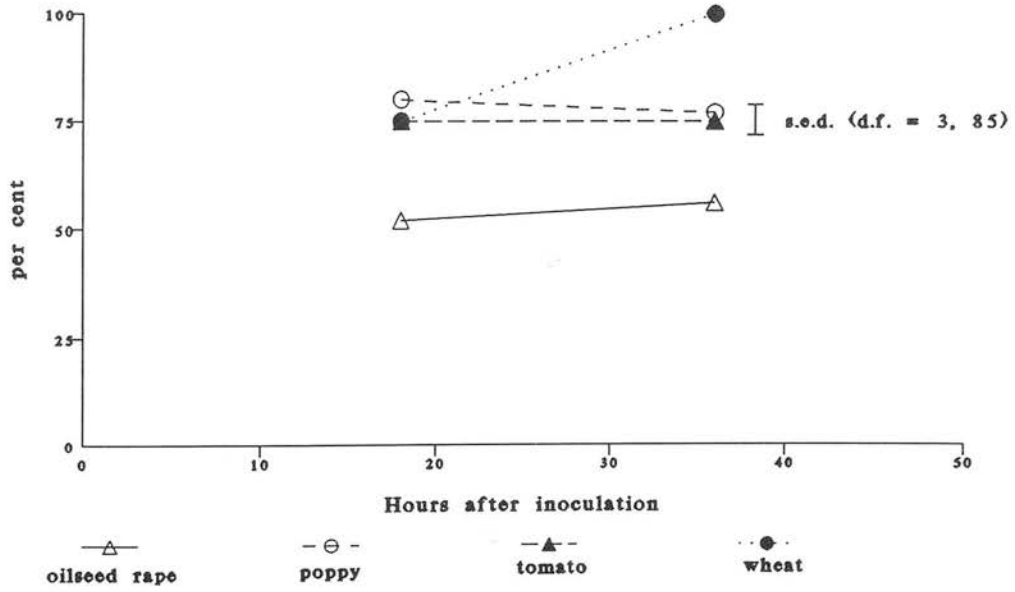


Figure 2.21b: The percentage of penetrations on leaf disks of four plant species which were confined at 18 and 36 hours after inoculation, averaged over six species of *Alternaria*.

to the brassica pathogens there was no unconfined penetration by this species on wheat; levels on poppy (32%) and OSR (20%) were low. For *A.alternata* and *A.infectoria* as noted above the majority of penetration attempts were associated with uncompleted penetrations or confined penetrations. However, a comparatively high frequency of unconfined penetration was noted for *A.alternata* on tomato, while with *A.infectoria* unconfined penetration was noted only on tomato and poppy and here at a low frequency (Figure 2.22).

Although the overall interaction between fungus, plant and sampling time was not significant, there was a significant interaction between these factors with respect to unconfined penetration in relation to the differences between the fungi on tomato and OSR (Figure 2.23). Thus the frequency of unconfined penetration was lower for *A.brassicae* on tomato at 36 hours as compared to 18 hours, but overall levels of penetration in this interactions was low. In contrast the occurrence of onconfined penetrations by *A.brassicicola* on tomato increased significantly between 18 and 36 hours after inoculation. Levels of unconfined penetration for other fungus/plant combinations did not change significantly over time (Figure 2.23).

Features of the development of each fungus and plant cell responses are illustrated in plates 2.6 - 2.10. *A.brassicae*, *A.brassicicola*, and *A.raphani* were found to behave similarly in the present experiment as in experiment 2.2. Although *A.brassicae* could be found to produce a short sub-cuticular growth phase on OSR, comparison of this behaviour with that of *A.solani* on tomato (Plates 2.6 and 2.10) suggested that the capacity for sub-cuticular development in *A.brassicae* is limited, even in comparison with closely related fungi.

In association with non-hosts, penetration was most frequently found to stop before the cell wall was completely penetrated, or to give rise to hyphae confined in an intracellular position (Plates 2.7 and 2.8).

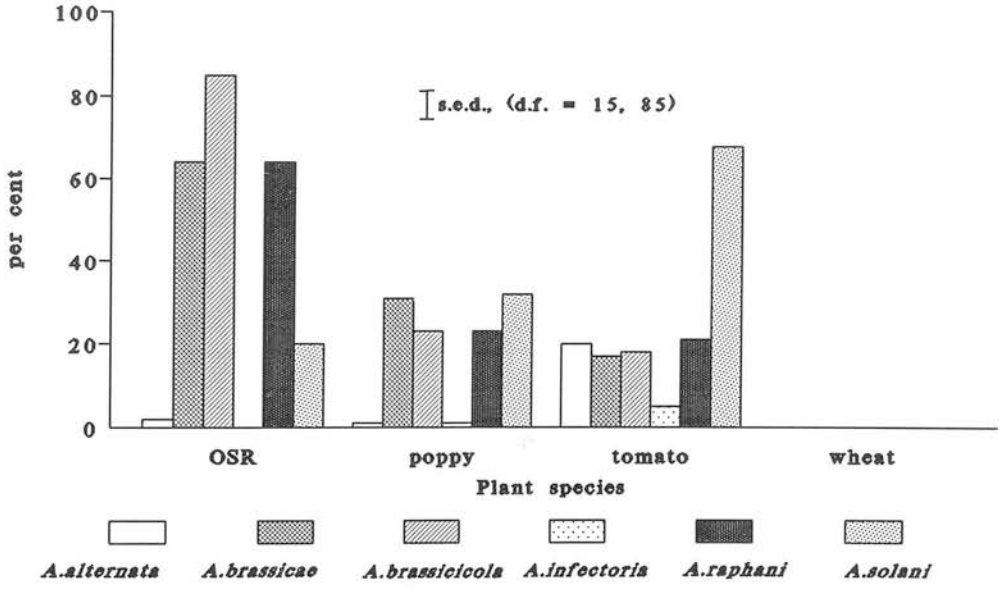


Figure 2.22: The percentage of penetrations which were unconfined for six *Alternaria* species on leaf disks of four plants, averaged between 18 and 36 hours after inoculation.

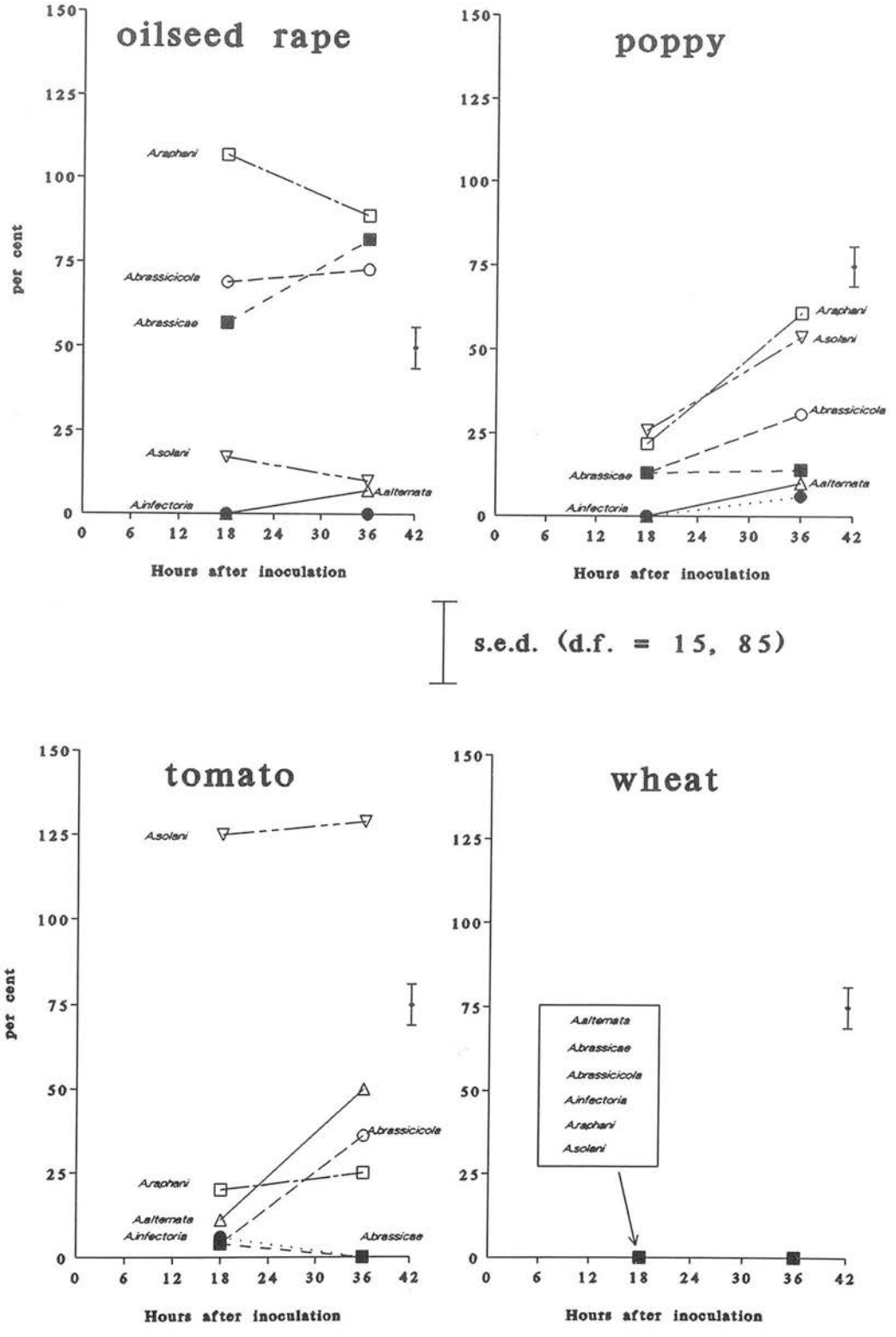


Figure 2.23: Percentage of penetrations which were unconfined for six *Alternaria* species on leaf disks of four plants at 18 and 36 hours after inoculation.

Plate 2.6: Features of the behaviour of *A.brassicae* in association with host and non-host plants.

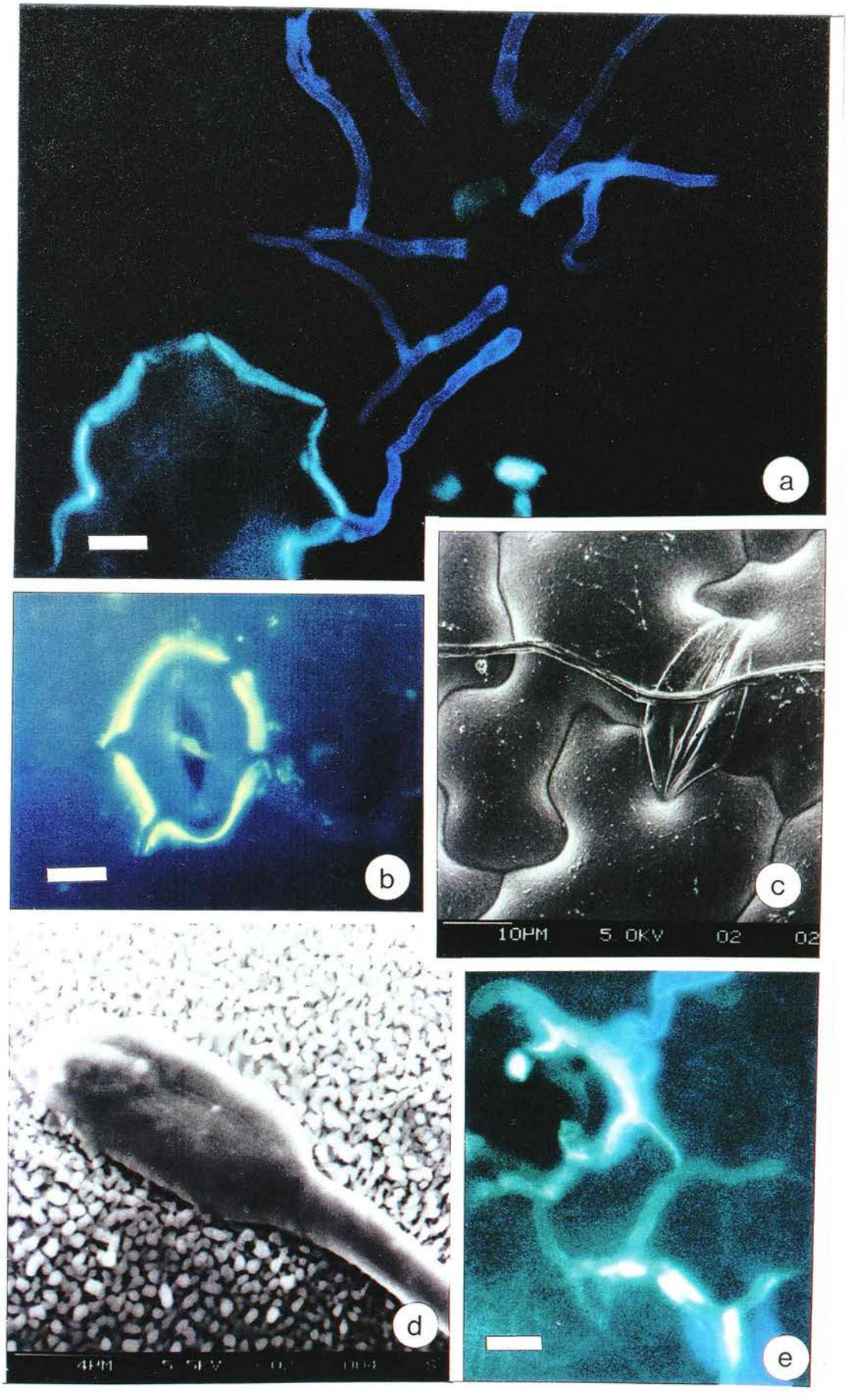
a. Germination by a conidium of *A.brassicae* resulting in the production of several germ-tubes. An appressorium has formed at an anticlinal cell wall junction, resulting in single cell reaction and some autofluorescence. Bar = 15 μm.

b. Stomatal penetration on poppy without appressorium formation. Localised cell reaction is visible in the cell walls of adjoining epidermal cells. Bar = 10 μm.

c. Extra matrical growth in tomato with apparent lack of recognition of a stomata. Scale given on plate.

d. Terminal appressorium formation on wheat, but no evidence of degredation of the epicticular wax or adhesion of the hypha to the leaf surface. Scale given on plate.

e. Intercellular growth on *B.napus*. Localised extramatrical fluoresence is apparent but the host cells appear normal. Bar = 10 μm.


a
b
c
10PM 5 0KV 02 02
d
4PM
e

late 2.8: Features of the behaviour of *A.brassicicola* in association with host and non-host plants.

a. Conidium, germ-tube, and appressorium on *B.napus*. The conidium is standing on the leaf surface with the pore in the terminal cell clearly visible. Scale is given on the plate.

b. Appressorium at an anticlinal cell wall junction and associated localised callose deposition on poppy. Bar = 15 μm.

c. Intracellular growth in poppy. The hyphae apparently following the cell wall in a necrotic cell with adjacent cell walls fluorescing brightly. Bar = 10 μm.

d1. Appressorium formation and adjacent cell reaction on *B.napus*. The appressorium is visible with a lobed vesicle inside the challenged cell. Bar = 10 μm.

d2. Intracellular hyphae originating from the vesicle viewed in plate d1. Hyphae are visible growing from both lobes of the vesicle. Bar = 10 μm.

e. Appressorium formation on wheat at an anticlinal cell wall junction and over a stoma. The right hand appressorium has shrunk and the smooth cucticle surface is visble indicating adherence of the hyphae to the plant cell surface. Scale is given on the plate.

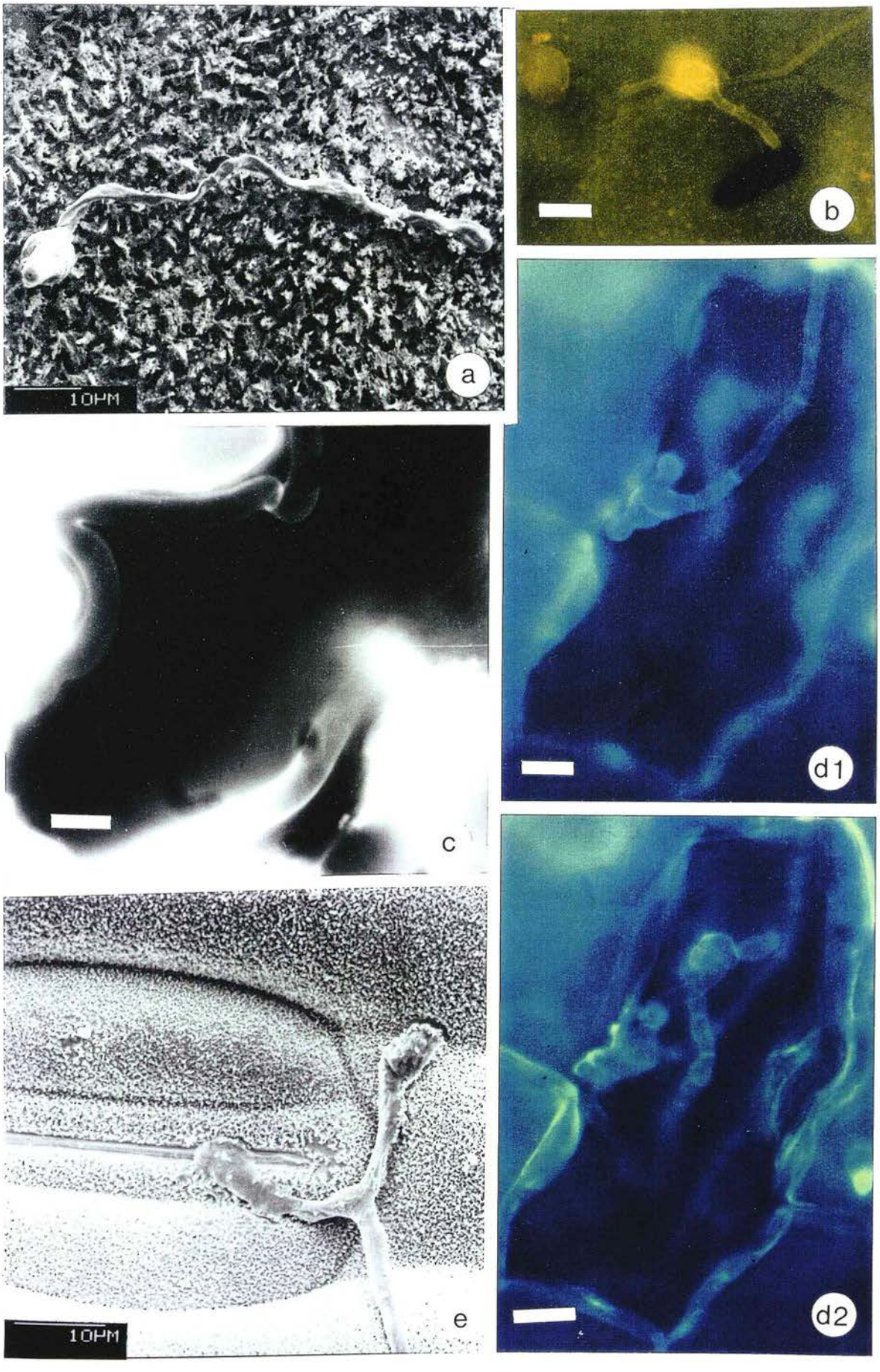

10μm
a
b
c
d1
d2
e
10μm

Plate 2.8: Features of the behaviour of *A.raphani* in association with host and non-host plants.

a. Appressorium formation and associated adjacent cell reaction on *B.napus*. Bar = 10 μm.

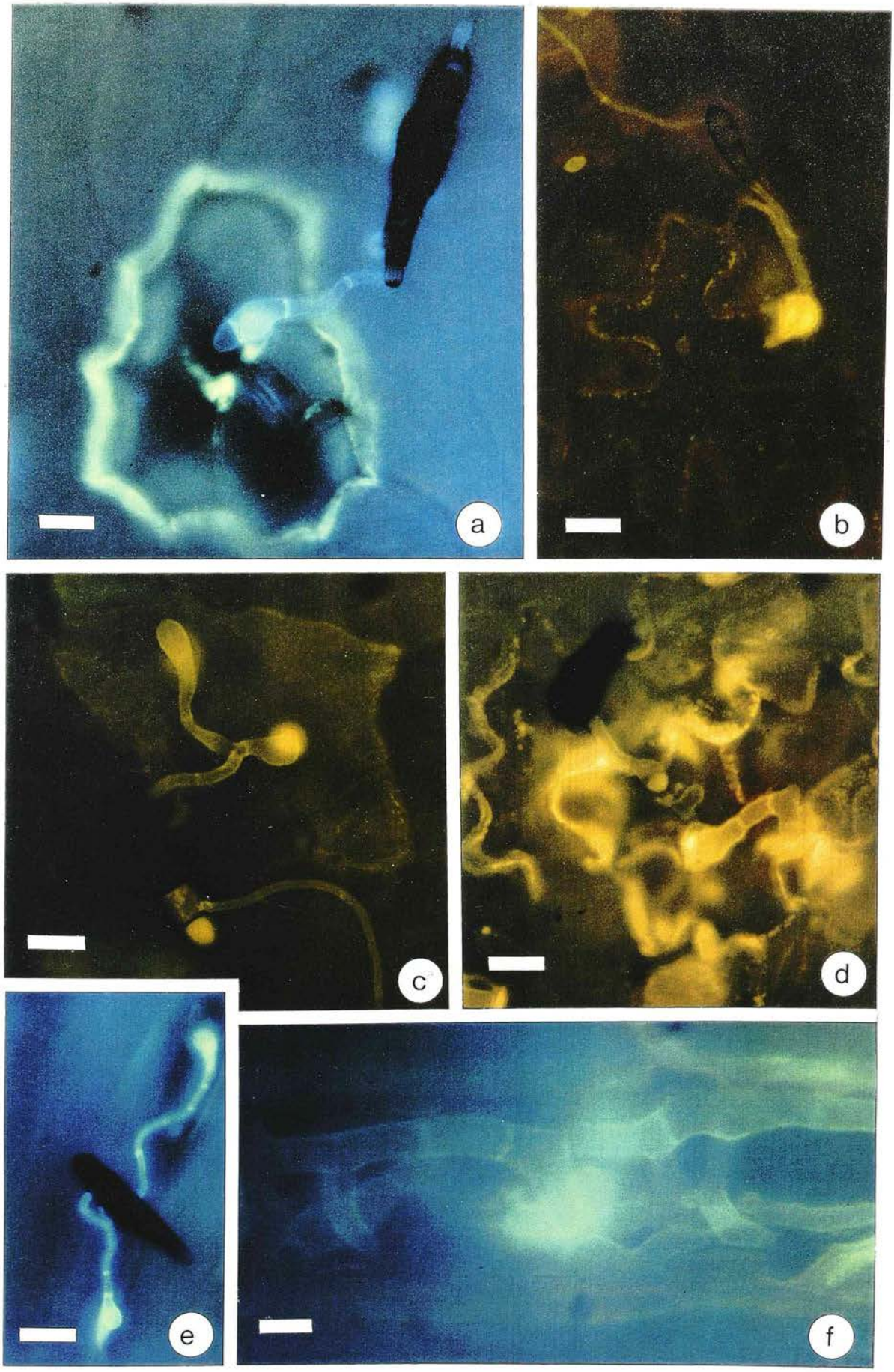
b. Appressorium formation and associated single cell reaction on tomato. Bar = 30 μm.

c. Appressorium formation and associated callose halo and single cell reaction on poppy. Bar = 12 μm.

d. Adjacent cell reaction, intracellular hyphae, hyphal egress, and second penetration on tomato. Bar = 12 μm.

e. Local cell reactions on wheat associated with appressoria. Bar = 30 μm.

f. Intracellular hyhae displaying coiling, confined in a single epidermal cell on wheat. Bar = 5 μm.


a
b
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d
e
f

Plate 2.9: Features of the behaviour of *A.solani* in association with tomato.

a. Sub-cuticular growth and hyphal egress. Scale is given on the plate.

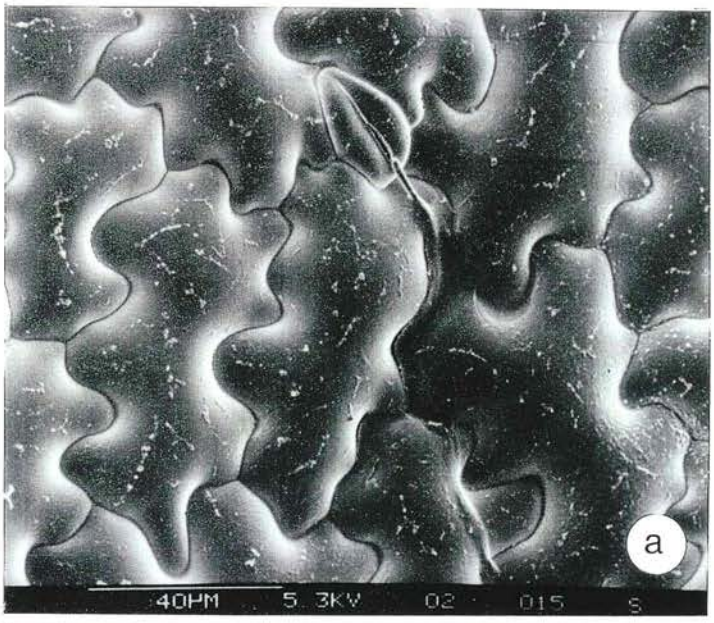
b. Magnified view of the sub-cuticular hypha shown in plate **a.** Scale is given on the plate.

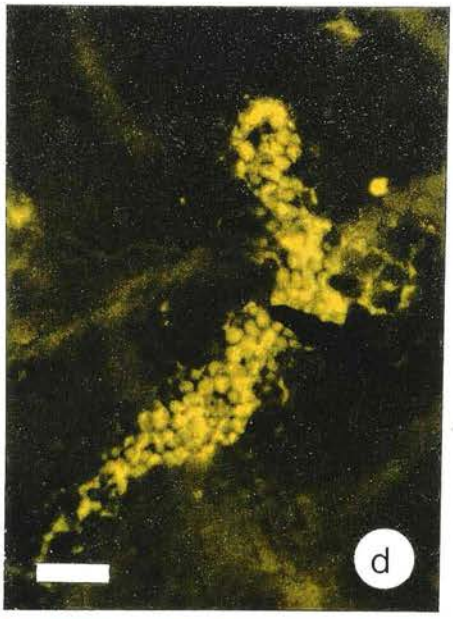
c. Magnified view of the hyphal tip shown in plate **a.** adjacent to a stomatal pore. Scale is given on the plate.

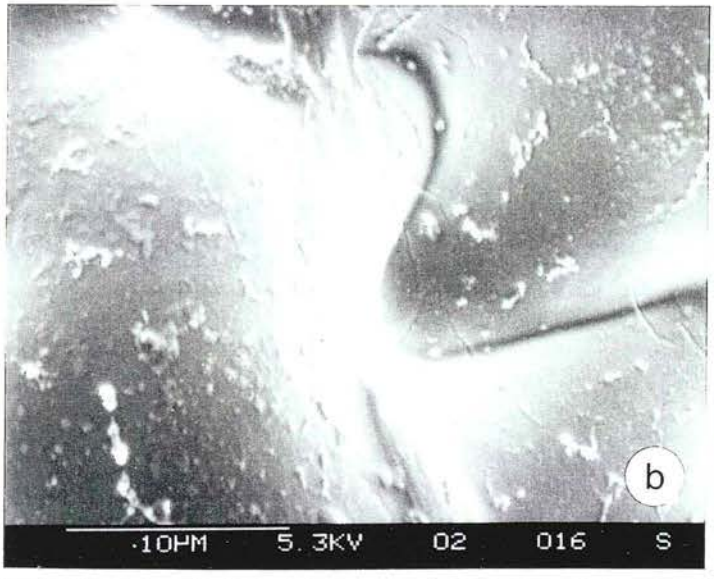
d. A conidium and zones of reacting cells associated with sub-cuticular growth. Bar = 50 μm.

e. Hyphal egress from a sub-cuticular position. Bar = 10 μm.

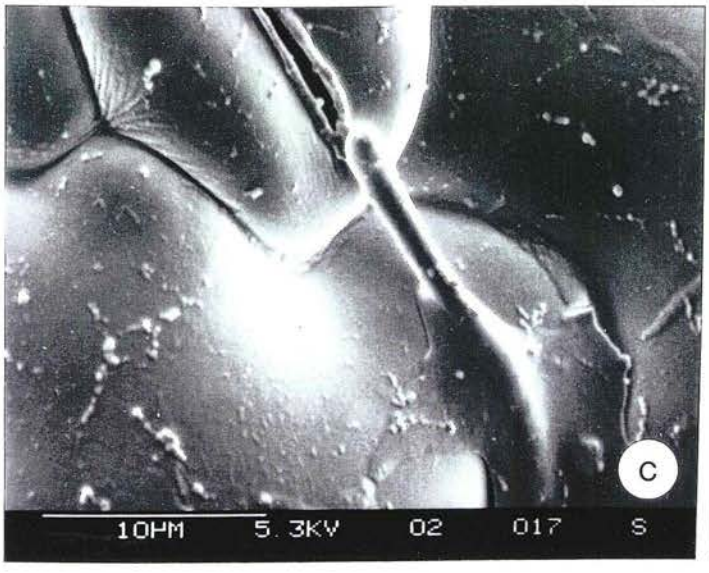
f. Egress and repenetration. Bar = 10 μm.


40UM 5.3KV 02 015 S
a


d


10UM 5.3KV 02 016 S
b


e


10UM 5.3KV 02 017 S
c


f

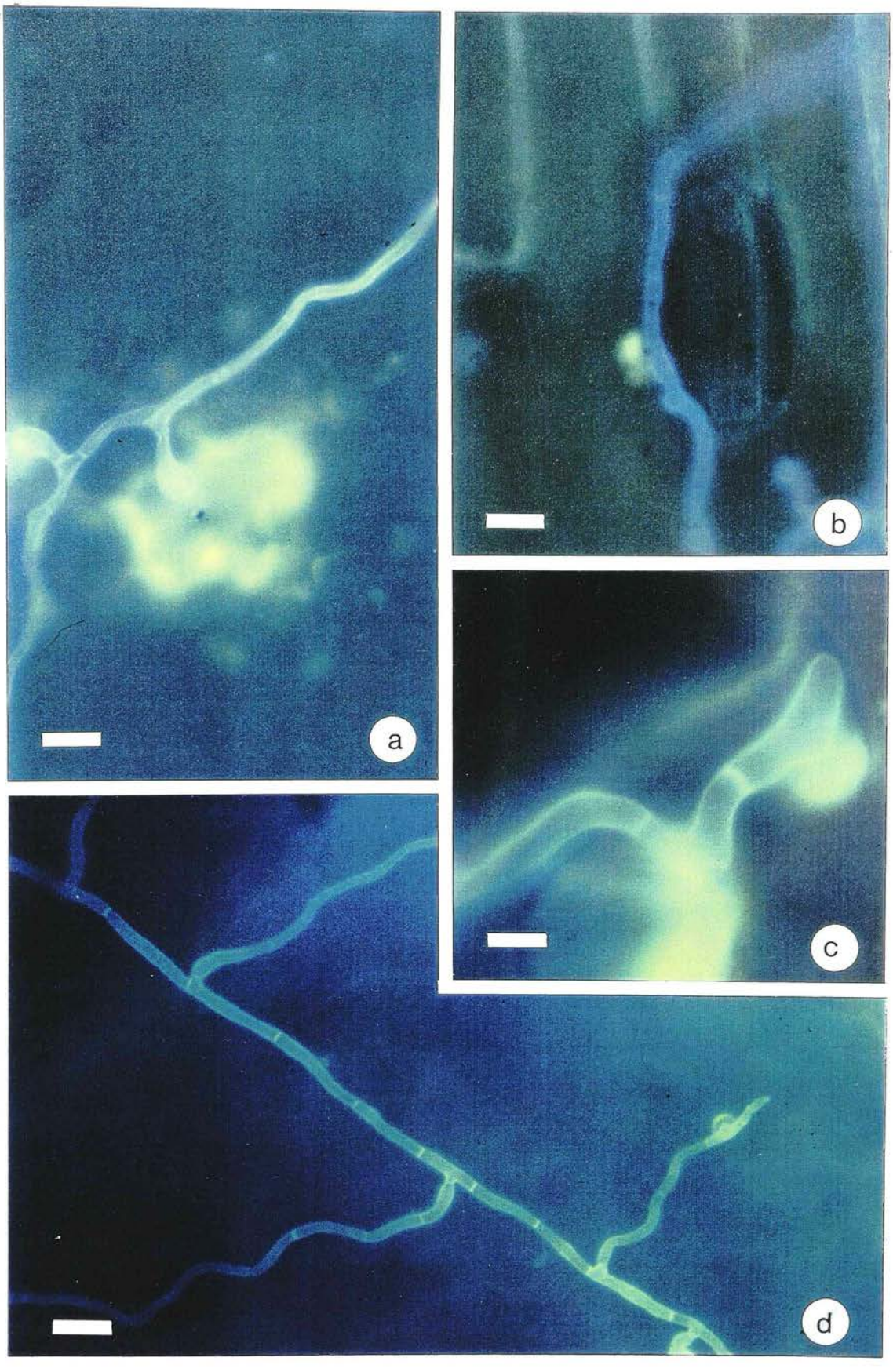
Plate 2.10: Features of the behaviour of *A.alternata* and *A.infectoria* in association with host and non-host plants.

a. Adjacent cell reaction on tomato associated with penetration by *A.alternata*. The appressoria on lateral branches are visible. Bar = 10 μm.

b. Localised callose deposition associated with attempted penetration from an intercalary appressorium produced by *A.alternata* on wheat. Bar = 10 μm.

c. Localised cell reactions by tomato in response to attempted penetration by *A.alternata*.

d. Extra matrical growth by *A.infectoria* and a localised cell reaction on *B.napus*. Bar = 10 μm.


a
b
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d

Discussion

In this third experiment the number of *Alternaria* species examined was extended and the range of plants expanded to include non-hosts of the brassica pathogens. Ride (1985) discussed studies by several workers which dealt with components of non-host resistance to fungal pathogens. Predictably, few generalisations could be drawn from the work which Ride reviewed regarding the timing or mechanisms of non-host resistance in relation to fungal development. In this study there was no evidence that germination was impaired for any of the *Alternaria* species on any of the plants. This finding further supported the suggestion from the two previous experiments that germination by conidia of *Alternaria* is relatively independent of leaf surface characteristics. Similar results have been reported in non-host situations with rust fungi (Heath, 1977) and with *Botrytis* spp. (Mansfield & Hutson, 1980). In contrast to these finding, Johnson (1977), cited by Bushnell (1979)) noted that germination by *Erysiphe cichoracearum* was significantly reduced on some non-host plant families. Thus, there is no clear generalisation which can be made about the role of germination inhibition in non-host resistance, even in separating specialised biotrophic fungi from necrotrophs or hemibiotrophs. The lower germination rate recorded for *A.infectoria* than for the other species in this study may reflect a difference between this species and the more specialised pathogens in their rapidity of response to suitable conditions. However, similar reservations to those expresssed about the significance of the low germination recorded for *A.alternata*, *A.brassicicola* and *A.raphani* in the previous experiments, other than demostrating physiological variation in the inoculum, must be made here about germination by *A.infectoria*.

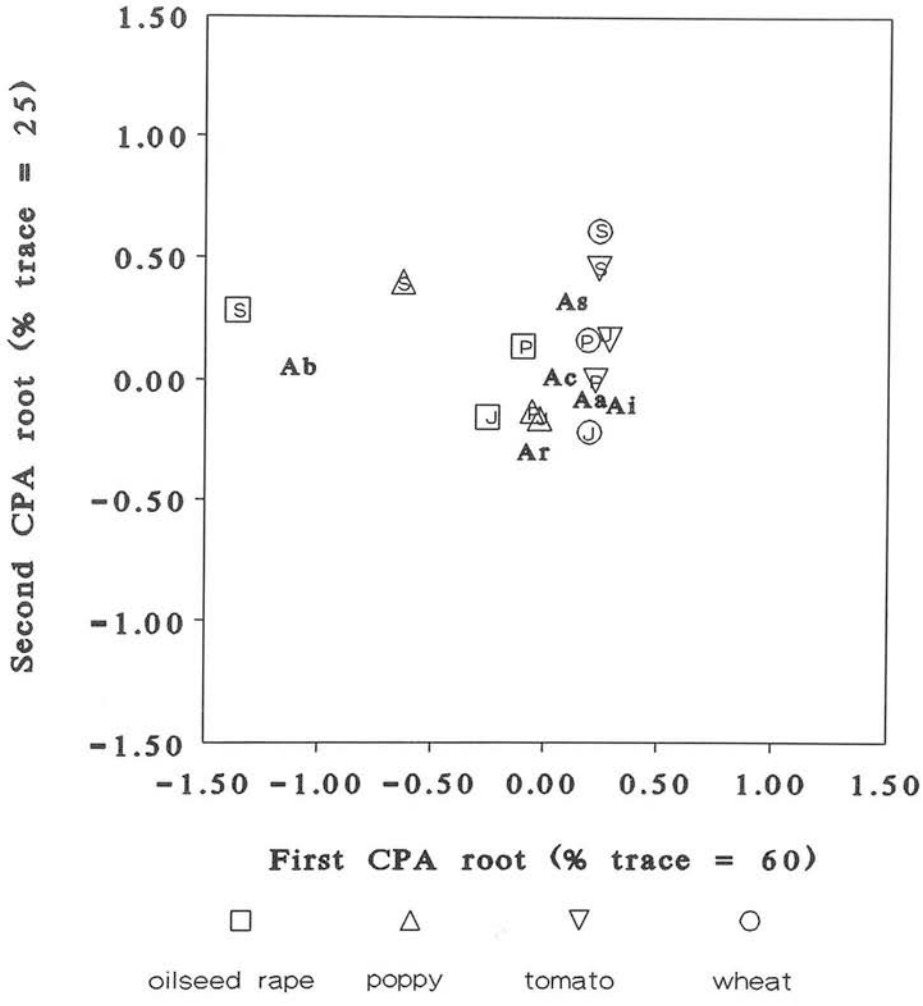
Germ-tube number of the *Alternaria* species examined was not found to be affected by plant species on which spores germinated, but was again shown to be predominantly an innate characteristic of each fungal species. The two species with relatively large conidia (*A.brassicae* and *A.solani*) produced the highest number of germ-tubes. Unfortunately the present study provided no information about the mechanism which determines the germination capacity of individual conidium compartments in *Alternaria* conidia. Conidia of *Alternaria* are initially single-celled (Simmons, 1967; Chapter 4) and all conidium compartments are derived from the initial cell by division. It is interesting to speculate whether those compartments which will germinate are determined during conidium maturation, and also to consider whether conidium compartments are functionally isolated, operate from a pooled food reserve in a

predetermined non-competitive way, or are in competition during spore germination.

Despite the apparently random direction of growth of their germ-tubes after germination, a notable feature of the development of all six *Alternaria* species was the selection of anticlinal cell wall junctions as sites for appressorium production. The formation of appressoria preferentially at this site on four species from different plant families supports the theory that appressorium production is stimulated by general chemical and topographic signals since the topography and physiology of the plants used varied considerably.

There was further evidence that *A.brassicae* differs from the other *Alternaria* brassica pathogens examined because of its apparent ability to recognise stomata as sites for penetration. Dickinson (1981) found that successful penetration of wheat leaves by unspecialised isolates of *A.alternata* occurred almost exclusively *via* stomata, evidence which may suggest that stomatal penetration and host specificity are not always highly correlated. However, Tsuneda and Skoropad (1978) found that entry by *A.brassicae* on *B.napus* was predominantly stomatal, while in the present study penetration by *A.brassicae*, although occurring *via* stomata relatively frequently, was found to be predominantly direct. Although this study has produced quantitative data there is a need for further investigation of the signals which stimulate penetration attempts at different sites. Berry (1992) examined the production of appressoria by *A.brassicicola* on a range of *Brassica oleracea* wax mutant types. The overall ratio of appressoria formed at the three sites was 1:0.29:0.14 (cell junctions:periclinal walls:stomata) which is similar to the distribution found for this species on the four plants examined here. Berry's results and those from the present study suggest that the signals for appressorium formation at cell junctions in *A.brassicicola* are general; this may be true of the other *Alternaria* species examined here.

A biplot of the association of each fungus with each of the three penetration sites on the four plant species, following Correspondence Analysis, is shown in Figure 2.24. In the case of appressoria formed over stomata, the relatively high selection by *A.brassicae* and *A.solani* for this site compared with the other fungi is clearly demonstrated by the location of these species/sites toward the top of the plot. The distinctness of



Aa: *A.alternata*; **Ab**: *A.brassicae*; **Ac**: *A.brassicicola*; **Ai**: *A.infectoria*; **Ar**: *A.raphani*; **As**: *A.solani*

J: Cell junctions; **P**: Cell periclinal walls; **S**: Stomata

Figure 2.24: A biplot representation of the associations between six *Alternaria* species and three possible sites of appressorium formation on leaf surfaces of four plant species.

A.brassicae from the other species is also clear, as is the small interaction between the other isolates in their choice of cell junction and periclinal sites; i.e. all of relevant points are clustered close to the origin.

Germ-tubes were longer on wheat than on any other plant, but did not differ significantly in length on the three dicotyledonous plants. There was, therefore, no evidence that non-host resistance in the plants examined was expressed by suppression of germ-tube growth. A number of factors may account for the relative increase in germ-tube length on wheat. There was no successful establishment by any of the fungi on wheat, and the longer germ-tubes may have resulted from continued surface growth following failed penetration events. Gees &Hohl (1988) noted that germ-tubes of *Phytophthora infestans* recommenced growth after forming abortive appressoria at unsuitable sites on potato leaves, and often produced futher appressoria. Alternatively, it is possible that the formation of appressoria and penetration were delayed on wheat because it lacks surface stimuli which trigger these events. This would result in longer germ-tubes, or a delay in penetration. The lower average number of penetration events recorded on wheat than on the other plants, partly supports this idea, although the differences in frequency were not significant between wheat and oilseed rape and tomato. With all of the fungi except *A.brassicae* the theory is further supported by the observation that the percentage of non-penetrating germ-tubes decreased over time more on wheat than it did on plants on which the fungi were able to establish intercellular hyphae. Although *A.brassicae* also produced longer germ-tubes on wheat than on other plants, there was no increase in the percentage of penetrating germ-tubes on wheat over time with this species.

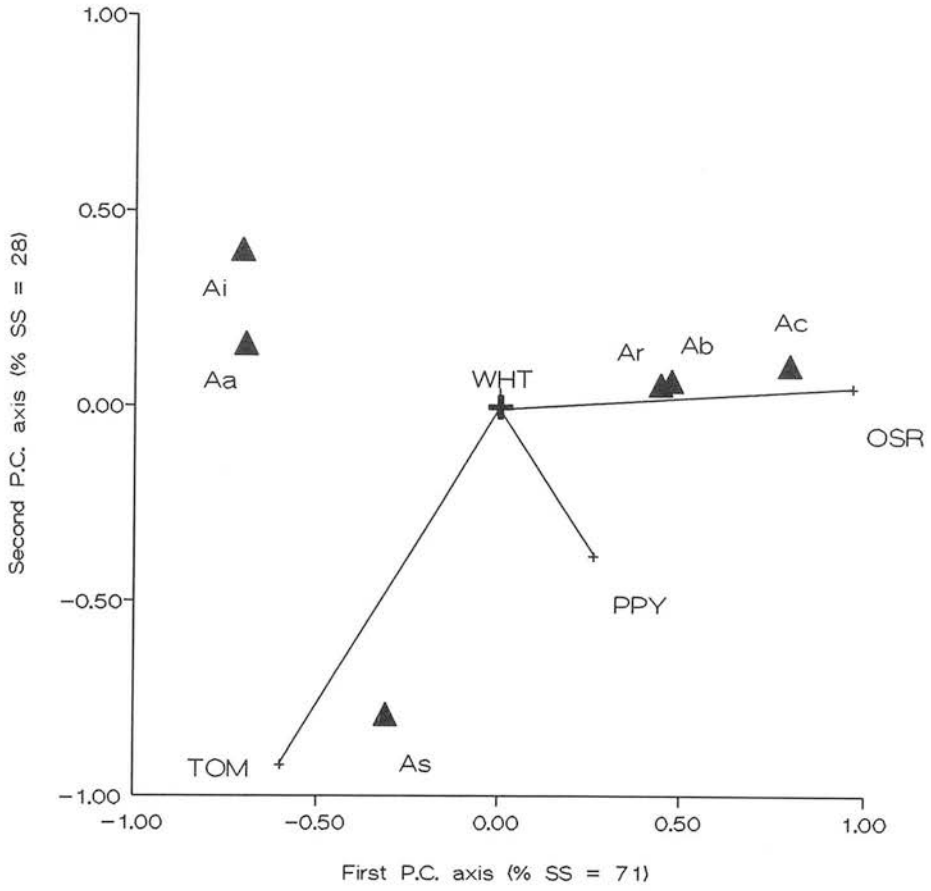
Of the specialised pathogens examined, *A.brassicae* showed the most pronounced sensitivity to the difference between host and non-host tissue. Thus, unconfined penetration increased with this pathogen only on its host. Taken together these observations suggest that with all of the species examined successful penetration occurs earlier on host plants than on non-hosts. This is in keeping with one of the few generalisations which Ride (1985) was able to draw from his review of similar studies. An increased specificity on the part of *A.brassicae* and *A.solani* as compared with *A.brassicicola* and *A.raphani* was also indicated by the fact that total penetration events for *A.brassicae* and *A.solani* were highest on their host plants, whereas for the other *Alternaria* species examined the total rate of penetration either did not differ significantly between the plants or was greater on non-host plants than on their hosts. Host specificity in

several rusts and in *Botrytis* has also been correlated to attempted penetration (Heath, 1974 ; 1977; Mansfield & Hutson, 1980).

A.brassicae differed from the other fungi examined here in consistently producing fewer appressoria on non-host plants than on its host. With *A.brassicicola* and *A.solani* appressorium formation was slightly delayed on non-hosts, but incidence at 36 hours was approximately equal on all plants (Figure 2.17). With *A.alternata*, *A.brassicicola*, *A.infectoria*, *A.solani*, and *A.raphani*, the incidence of non-penetrating germ-tubes decreased between sampling times on plants on which there was no unconfined penetration, but not on those on which unconfined penetration was frequent (Figure, 2.19). Furthermore, the incidence of uncompleted penetrations decreased on wheat but not on other plants suggesting that the penetration process was slower on average for on this plant (Figure 2.20b). These findings indicate that components of non-host resistance in some of the interactions examined act during the early stages of fungal development after inoculation. However, that such components do not have equal importance in all cases is shown by the fact that there was no difference in the incidence or timing of appressorium production by *A.raphani* or *A.alternata* between plants.

Although components of non-host resistance caused a reduction in attempted penetration by *A.brassicae* and delayed penetration by the other species, the most dramatic difference between compatible and incompatible interactions was evident after penetration by fungi had been attempted. This is displayed diagramatically in the biplot in Figure 2.25 which is based on the incidence of unconfined penetrations by each pathogen on each plant. The association of the three brassica pathogens with OSR is obvious, as is the association of *A.solani* with tomato, and separation of these two hosts by their respective successful pathogens.

It appears therefore, in the interactions examined here, that the primary determinant of host specificity operates after pathogen and host cell walls have come into contact. One further interesting feature in the behaviour of *A.brassicae* and *A.solani* on host as opposed to non-host tissue was the establishment of sub-cuticular hyphae. The inability of the fungi to establish sub-cuticular growth on



Description of Plants and Fungi:

OSR: oilseed rape; **PPY**: poppy; **TOM**: tomato; **WHT**: wheat
Aa: *A.alternata*; **Ab**: *A.brassicae*; **Ac**: *A.brassicicola*; **Ai**: *A.infectoria*;
Ar: *A.raphani* **As**: *A.solani*

Figure 2.25: A biplot representation of the relationship between six *Alternaria* species and four species of plant, based on the incidence of unconfined penetration by the fungi on leaf disks of the plants.

non-hosts suggests the absence of a required trigger which stimulates this behaviour, or the presence of resistance factors which inhibit it. The sub-cuticular phase after penetration may provide these pathogens with the oppportunity to accumulate the required inoculum potential to allow further colonisation of their hosts' tissue. One plausible theory is that the early growth of the pathogen allows it to release toxins which precondition the underlying tissue to subsequent infection. If insufficient fungal biomass accumulates at the site of penetration it may be phytotoxins do not reach a biologically active concentration.

Wheat was found to be the most complete non-host of all the fungi examined, with no successful establishment recorded. In all but one case, ingress halted before the plant cell wall had been penetrated, and resulted in limited callose deposition. The reaction of wheat to non-pathogenic fungi has been previously examined by Ride & Pearce (1979) who found that lignified papillae formed as early as 12 hours after inoculation in challenged wheat leaves. These papillae were, in addition, found to be highly resistant to degradation, a character attributed to the presence of lignin. There was no evidence in the present study that lignin was deposited at penetration sites, although Young (1926) detected the presence of lignified papillae in wheat leaves inoculated with *Alternaria*. The failure of hyphae in the present study to penetrate wheat is considered unlikely to result purely from the deposition of callose since this was deposited in compatible interactions on host plants where ingress was not halted.

Two possible classes of a resistance mechanism are implicated in the non-host resistance of wheat in this case. The first of these groups might be considered 'positive' or 'active' mechanisms and may include phytoalexin accumulation, the activity of chitinase and/or glucanase enzymes, or detoxification of phytotoxins. The second class of resistance mechanism, 'passive' or 'negative' mechanisms, might include low susceptibility of the cell wall to degradation, unavailability of essential nutrients, or low sensitivity of wheat to phytotoxins produced by the fungi. Although both *A.brassicae*, and *A.brassicicola* produce cell wall degrading enzymes (Shohet, 1985), Ride (1985) notes that there is probably not enough chemical difference between the cell walls of different plants to cause any real difference in susceptibility to enzymes from different pathogens. However, in the case *A.brassicae* at least, it is known that host-selective phytotoxins are produced *in vivo* (Buchwaldt & Jensen, 1991). One of the features of interest in the non-host resistance of wheat was the confinement of hyphae of *A.raphani* within a single cell at the point of penetration. A

similar form of resistance was described by Comstock & Scheffer (1967) in the non-host resistance of maize to *Helminthosporium avenae*. However, Comstock & Scheffer (1967) found that the addition of the host-specific toxin from *H.carbonum*, a related pathogen of maize, suppressed the defence reaction allowing *H.avenae* to colonise maize tissue.

The results of the present study do not allow a precise identification of the factors involved in non-host resistance in the plants examined. However, the need for some kind of biochemical compatibility between *A.brassicae*, *A.brassicicola* and their host plants is suggested by the decreasing success rate in colonisation from more to less closely host-related non-hosts; i.e. both OSR and poppy belong to the Papavarales while tomato is in the Solanales, and wheat is in the Poales. The fact that successful penetration does not stop completely when these fungi are challenged with non-cruciferous plants is not unexpected, since a portion of the inoculum even of more specialised pathogens will successfully establish in non-host tissue (Heath, 1977). However, the pattern of decreasing success rate of the *Alternaria* pathogens on non-hosts suggests that a number of 'pathogenicity factors' are involved in pathogenicity; these need not be the same for each pathogen. Increasing phylogenetic distance between the challenge plant and the host of the pathogen would lead to decreasing compatibility between the pathogenicity factors and their targets in the plant, and thus to a progressive reduction in success rate for the pathogen.

In conclusion, the present study indicated that *A.brassicae* displays a greater host specificity than *A.brassiciciola* and *A.raphani*. Particularly in the case of *A.brassicae*, the fungal behaviour was affected by factors at the leaf surface, and attempted penentration was lower on non-host plants. However, resistance factors which operate after attempted penetration appear to be more important than those at the leaf surface in determining compatibility. In cross inoculations with *A.solani* on OSR, a similar pattern of interactions was noted as for the brassica pathogens on tomato. These findings are consistent with those of several other workers who have provided evidence that non-host resistance is multi-component and may involve the same mechanisms as host resistance (Ride, 1985).

2.5 GENERAL DISCUSSION

The main objectives of the experimental work of this chapter were to further: (1) an understanding of the infection behaviour of *A.brassicae* and *A.brassicicola*; and (2) an understanding of the factors underlying the host specificity of these two fungi. During the course of these studies statistical techniques, which are not commonly used in this research field have been employed to help in the visualisation of host - pathogen interactions, and the results allow an appraisal of the scope of application of such methods to further studies of this nature.

All infection studies were conducted on excised leaf disks rather than whole plants. This approach had several advantages relating to the control of environmental conditions, economy of space and materials, and reduction in the risk of cross contamination. Accepting these advantages, it is recognised that variation may occur between excised and intact leaf tissue in the development of leaf infecting fungi and in plant responses. However, there are several indicators that the use of a leaf disk system for the present studies provided a reasonable representation of the interactions between the fungi and plants examined. First, the behaviour of the fungi was similar to that reported by previous workers who have used intact or excised whole leaves (Changsri & Weber, 1963; Tsuneda & Skoropad, 1978; Tewari, 1986). Secondly, in the 'host' infection study (Experiment 2.2) and the 'non-host' infection study (Experiment 2.3), the pattern of susceptibility of the plant tissue correlated well with previous whole-plant studies which have been made with these fungi (Neergaard, 1945; Degenhardt, 1978; Prasanna, 1984). In work with other fungi and hosts, for example *Erysiphe graminis - Avena sativa* (Jones & Hayes, 1971), a good correlation between the results of excised leaf piece infection experiments and field trials has been found.

Comprehensive studies by Munro (1985) allowed the construction of schematic models of the development of *Erysiphe cruciferarum*. The development of the *Alternaria* species examined here was found to be inherently more variable than that of *E.cruciferarum* and so any diagramatic representation of their development must necessarily be a poorer summary of their actual behaviour. This notwithstanding, a diagramatic view of the development patterns for *A.brassicae* and *A.brassicicola* on susceptible host plants is attempted in Figure 2.26.

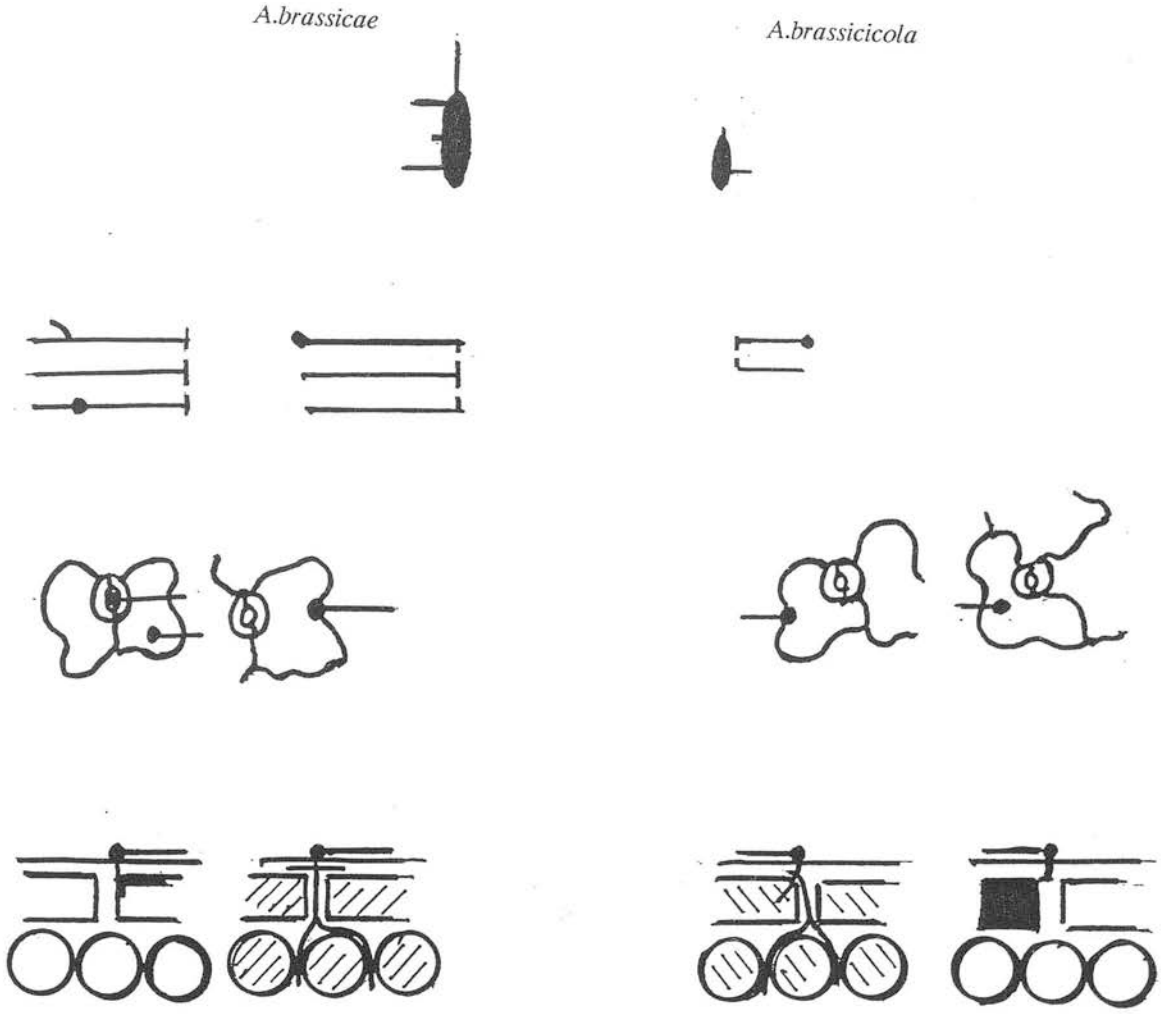


Figure 2.26: Generalised development patterns of *A.brassicae* and *A.brassicicola* on host plants.

Examining the development of the fungi after spore deposition, *A.brassicicola* is characterised by the production of a single germ tube from each conidium. The germ-tube may reach a length of 100 - 200 μm by 18 - 24 hours after inoculation. The germ-tubes elaborate (in most cases) terminal appressoria, typically at the edges of epidermal cells near junctions between anticlinal cell walls, or less frequently over periclinal walls, and direct penetration follows. Penetration was found to occur predominantly to an intracellular position in the epidermis in this study, although other workers have noted a sub-cuticular phase for *A.brassicicola* (McKenzie *et al*., 1988; Berry, 1992). Penetration directly into the lumen of epidermal cells is not commonly reported for fungi other than *Erysiphe*, although it has been recorded previously in the interactions between *Colletotrichum trifolii* and *Medicago sativa* (Mould, Boland, & Robb, 1991), and *Septoria nodorum* and *Triticum aestivum* (Karjalainen & Lounatmaa, 1986), and was illustrated by Comstock & Scheffer (1967) as the mode of entry for *H.carbonum* on maize. Ramification occurs intercellularly through the mesophyll to the lower epidermis from the point of penetration. There is a relatively small amount of concurrent lateral, intercellular development in the upper epidermis. Cell reactions in host plants typically involve callose deposition and necrosis. Where penetration is unsuccessful, cell reactions are limited to a localised callose deposition in the cell wall beneath the appressorium, or to a necrotic hypersensitive response in the attacked cell. Successful penetration and establishment is associated with extensive cell necrosis and callose deposition.

A.brassicae produces typically 2-3 germ-tubes per conidium. These reach a length of 250 - 350μm by 18 - 24 hours after inoculation and elaborate appressoria either terminally or in intercalary positions. Appressoria are formed less frequently by individual germ-tubes of *A.brassicae* than those of *A.brassicicola*. The low frequency of appressoria on germ-tubes, however, is to some extent off-set by the greater number of germ-tubes produced per conidium. With *A.brassicae* appressoria are formed predominantly at cell edges or less frequemtly over stomata, and over cell periclinal walls. Direct penetration occurs either to an intracellular position in the epidermis, or to a sub-cuticular position. Penetration *via* stomata occurs either to an intercellular position or into the guard cells. Ramification following penetration is intercellular proceeding both laterally in the upper epidermis and simultaneously through the mesophyll, and then laterally in the lower epidermis. Where infection fails to become established after penetration colonisation stops at an early stage with the production of callose localised in cell walls. As with *A.brassicicola* successful

colonisation by *A.brassicae* is associated with extensive cell reaction.

It would appear that the processes leading to the establishment of infection by populations of spores of biotrophic pathogens such as *E.cruciferarum* comprise a series of synchronised events occurring over a limited period. In contrast, infection by the *Alternaria* is more protracted; the elaboration of infection structures and penetration occurring over a prolonged period, due in part to variation in the physiological condition of the spores. In *Erysiphe*, where unicellular conidia are rapidly produced, Ellingboe (1972) was able to develop techniques which allow the production of a standardised inoculum which is synchronised for germination and subsequent development. However, the production of multinucleate conidia such as those found in the *Alternaria* with thickened, pigmented walls is a more complex process and the extended time for development allows a greater chance for physiological variation at the time of release. Furthermore, the multinucleate condition of *Alternaria* spores and hyphal compartments allows the possibility of heterokaryotic conidia. Although inoculum was produced and prepared carefully to produce a high population of viable spores, morphological and physiological variation was evident in the inoculum of all species used.

In contrasting the behaviour of *Erysiphe* and *Alternaria* in ecological terms it is suggested that the strategies of these pathogens are different. With *Erysiphe* very large numbers of spores are produced and can subsequently establish infections and produce further spores in a rapid cycle, with weight of numbers ensuring a high infection rate, representing an r-type strategy (Thresh, 1978). In the case of *Alternaria*, however, spore numbers produced are fewer, but these are able to withstand adverse conditions, are capable of extended extra-matrical growth before establishing a parasitic relationship with plant tissue, and the latent period of infection is relatively long, thus representing an intermediate position in the r-K continuum (Zadocks & Schein, 1979).

Comparing *A.brassicae* and *A.brassicicola*, the balance between r and K strategies varies between the species: *A.brassicae* produces fewer and larger spores, but *A.brassicicola* shows a greater capacity for survival in association with seed (Neergaard, 1969), while its requirements for growth and sporulation in axenic culture are less demanding than those of *A.brassicae* (Neergaard, 1945; Changsri & Weber, 1963).

With respect to their ecologies, *A.brassicae* appears to be more host-dependent than *A.brassicicola*, which shows less variation in its infection behaviour between host and non-host plants.

In Experiments 2.1 and 2.2 the development of the fungi was found to be little affected by variation in leaf surface characteristics. Specifically, in Experiment 2.1, although there was a slight reduction in the number of germ-tubes produced by *A.brassicae* on the most waxy surface, variation in leaf waxiness was found to have little overall effect on surface development. However, on the waxy surface there was a reduction of stomatal penetration by *A.brassicae*, perhaps because the fungus was unable to recognise the topography of the stomatal complex. The results suggest that the surfaces of typical members of the *Brassica* genus are similar enough to allow normal or typical development by both *A.brassicae* and *A.brassicicola*. Furthermore, it was found in Experiment 2.2 that the growth of another *Alternaria* pathogen of the Cruciferae, *A.raphani*, was not significantly affected by variation in leaf surface characteristics between the host plants tested. However, in examinations of the growth of the fungi on the surfaces of non-host plants, differences were found between *A.brassicae* and *A.brassicicola*. *A.brassicicola* was not affected by plant species with respect to attempted penetration, and was in this respect similar to the less specialised species, *A.alternata*. In contrast, penetration attempts by *A.brassicae* were less frequent on non-host plants than on OSR, suggesting that *A.brassicae* may be a more specialised pathogen than *A.brassicicola*. It is interesting in this context that *A.brassicae* is less amenable to axenic culture than *A.brassicicola*, tending to lose its capacity for sporulation and to degenerate faster (Neergaard, 1945) as already indicated.

The success rate with which *A.brassicae*, *A.brassicicola*, *A.raphani*, and *A.solani* achieved intercellular growth on test plants appaered to be related to the phylogenetic distance between the plant and the normal host of the fungus. Although under field conditions differences in the level of resistance encountered here might well produce qualitative differences in the host/non-host status of the plants, at the microscopic level differences between OSR, poppy, and tomato were found to be quantitative. Qualitative difference in the success of fungal penetration occurred between OSR and wheat, however. Taken together the results of these studies suggest that for all of the *Alternaria* species examined, irrespective of their apparent degree of host specificity, host/non-host relationships are determined by an array of interactions.

Table 2.15: A summary of the infection behaviour characteristics of six *Alternaria* species

Characteristic[1]	Species					
	A.brassicae	*A.brassicicola*	*A.raphani*	*A.solani*	*A.alternata*	*A.infectoria*
Germ tube number >1	+	-	±	+	±	-
Extensive extra-matrical growth	+	-	+	+	+	+
High appressorium : germ-tube ratio	-	+	+	+	+	-
High percentage of non-penetrating germ-tubes on;						
OSR	-	-	-	±	±	+
poppy	+	-	-	±	±	+
tomato	+	-	-	-	±	+
wheat	+	+	+	+	+	+
Frequent anticlinal wall penetration	+	+	+	+	+	+
Frequent periclinal wall penetration	±	±	±	±	±	-
Frequent stomatal penetration	±	-	-	+	-	-
High penetration rate on;						
OSR	+	+	+	-	+	±
poppy	±	+	+	+	+	±
tomato	±	+	+	+	+	±
wheat	±	+	+	+	+	±
High unconfined penetration rate on;						
OSR	+	+	+	-	-	-
poppy	-	-	±	±	-	-
tomato	-	-	-	+	-	-
wheat	-	-	-	-	-	-

1 + character shown to a high degree; ± character shown to an intermediate degree; - character shown to a low degree or absent. In order to derive the data for Principal Coordinates Analysis the data were tansformed to a qualitative numerical scale on which + = 3, ± = 2, and - = 1.

Significantly, in all cases resistance was at least partly, and usually mostly, determined after or during attempted penetration. A summary of behavioural features of *A.brassicae*, *A.brassicicola* and other *Alternaria* species is shown in Table 2.15. The data used to derive the scores in Table 2.15 were subjected to Principal Coordinates Analysis to reveal groupings in the six *Alternaria* species examined. A scatter plot of

the relative association of the species is presented in Figure 2.27. The disimilarity of *A.brassicae*, to *A.brassicicola*, and *A.raphani*, and the similarity of *A.alternata*, *A.brassicicola*, are both revealed. The use of this technique to examine similarities between species using characters not directly related to pathogenicity is examined in more detail in Chapter 4.

The application of multivariate techniques to the data from these plant infection studies had two primary objectives:

1. Clear graphical presentation of plant-pathogen interactions with respect to several variables.
2. Visual inspection of host-pathogen interactions with respect to single variables.

In Experiments 2.2 and 2.3 PCP biplots were found to produce accurate summaries of the plant - pathogens interactions, as defined by the occurrence of unconfined penetrations. Examining the interactions in Experiment 2.2, the biplot identified the most susceptible hosts (*B.nigra*, *C.cheiri*, and *S.alba*, respectively) of the three pathogens *A.brassicae*, *A.brassicicola*, and *A.raphani*. Thus, the combined length of the vectors in the plot for these three plants suggested that they accounted for 65% of the variance, with the other five hosts each accounting for between 3% and 10% of the variance, while the first two PCP axes accounted for 100% of the interaction sum of squares (Figure 2.14). It can be seen then, that the biplot technique summarised the original 3 * 8 data matrix in 3 points and 8 lines; an analogous summary of the interaction by more traditional methods would require 24 separate bars, or a table with 24 entries.

The biplot of the corresponding interaction from Experiment 2.3 accounted for 99% of the interaction sum of squares. In this case the accuracy of the biplot resulted from the strength of the host specificity displayed by the brassica pathogens and *A.solani* (Figure 2.25). Thus, the first PCP axis separated plants and pathogens mainly on the basis of their pathogenicity to OSR, while the second PCP axis separated *A.solani* and its host tomato from the other species. Relating these features of the biplot to the ANOVA of the interaction, the intercation of the pathogens with the orthogonal contrast between OSR and tomato acounted for 78% of the interaction sum of squares alone.

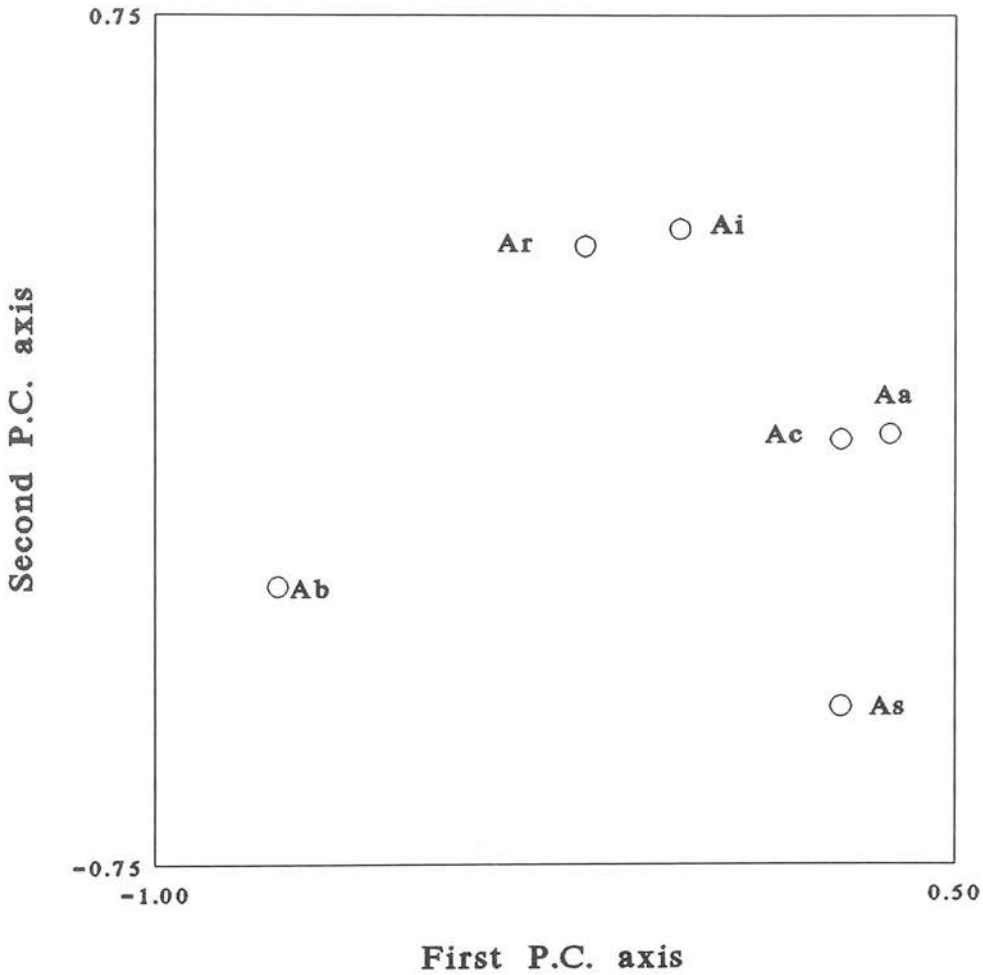


Figure 2.27: A scatter plot of the relative associations between 6 *Alternaria* species based on general features of their pathogenic behaviour in Experiments 2.1 - 2.3.

In fact the biplot slightly over-estimated the variance due to OSR and tomato since the combined lengths of the vectors for these two plants accounted for 80% of the total in the biplot.

Phillips & McNicol (1986) found that the biplot approach was able to distinguish the difference in interactions between cyst nematode populations and potato clones determined by qualitative resistance factors from those determined by quantitative resistance factors. The technique was found to provide a suitable means of summarising interactions in the present study whether these involved variations in quantitative resistance (Experiment 2.2) or more qualitative resistance (Experiment 2.3).

Whereas the data subjected to PCP analysis in experiments 2.2 and 2.3 were two-way tables of single variates, the data used for CPA in Experiments 2.1 and 2.3 were multivariate in nature. Although penetration at three sites on the leaf surface was examined by ANOVA, since penetrations occurring at each site were components of the total incidence of penetration, it was desirable to examine the interaction between plants simultaneously with respect to all three sites. Correspondence Analysis allowed this approach by summarising the two-way contingency table formed with pathogens as rows and plant/site combinations as columns. Furthermore, in Experiment 2.3, where expected incidence of penetrations under fungus/plant independence were calculated, the biplot from the CPA provided visual inspection of contingency testing conducted with the chi-square test. A weakness of the chi-square test is that it concentrates on specific components within complex interactions such as those under consideration here. Correspondence Analysis allows a full picture of the interaction and its components, while retaining information about the important sources of deviance.

Examining the CPA biplot from Experiment 2.1 (Figure 2.9), as noted in section 2.4, the selection of stomatal sites by *A.brassicae* was highlighted, as was the preference for the anticlinal wall site on the waxy leaf type for this pathogen. With respect to the initial analysis of variance, there was no evidence of a significant plant * pathogen interaction effect in relation to the incidence of penetration at anticlinal sites. However, while there were slightly fewer penetrations at anticlinal sites on the intermediate and waxy surfaces for *A.alternata* and *A.brassicicola* than on the glossy surface, with *A.brassicae*, the incidence of penetration at anticlinal sites did not vary between surfaces; this difference is pinpointed in the biplot.

In Experiment 2.3 the CPA biplot highlighted the preference for stomatal sites in the case of *A.brassicae* and *A.solani* as compared with the other species. However, the individual chi-square tests indicated that all six pathogens showed positive selection of anticlinal wall sites, and the biplot illustrated that, apart from the high incidence of stomatal penetration by *A.brassicae* and *A.solani*, there was little differentiation between the fungi with respect to selection of penetration sites on the four plants.

It has been possible to conclude from these studies that the biplot techniques examined may be of general use to plant pathologists involved in examining a range of plant - pathogen interactions. Although the numbers of interacting variables in the present study was relatively low, producing simple biplots, there are no theoretical reasons why more complex data sets cannot be treated in a similar fashion (Gabriel, 1981; Greenacre, 1981; Digby *et al.*, 1989). In this respect Anderson, Beute, Wynne, & Wongkaew (1990) showed the ability of PCP biplots to identitify, from a set of 14 parameters, those which allowed simultaneous selection of increased resistance to a two-fungus disease complex in a group of 55 peanut genotypes.

The results of the present study indicated clearly that the important factors determining compatibility in the interactions examined occur during, or after, attempted penetration. Canadian researchers have made a number of discoveries with respect to the factors which determine host specificity in *A.brassicae*. It is possible to unite much of their work with current theories about the role of cytosolic calcium ions in triggering papillum formation (Aist & Gold, 1987) to produce a model which can explain qualitative and quantitative variation in resistance to *Alternaria* species. Such a model is presented below (Figure 2.28, text) but it is stressed here that although individual components of the model are known to exist, the model itself is theoretical.

Known factors of relevance to the resistance model:

A.brassicae and *A.brassicicola* are known to produce cell wall degrading enzymes (Shohet, 1985).

A.brassicae and *A.brassicicola* are known to produce phytotoxic metabolites, in the case of *A.brassicae* these contain a host selective component (Bains & Tewari, 1987).

Infection structures

Penetration

Elicitor

HYPERSENSITIVITY

High $[Ca^{2+}]_{cyt}$?

PHYTOALEXIN + +

No colonisation

Suppression of HR (HST)

LOW $[Ca^{2+}]_{cyt}$?

PHYTOALEXIN -

Initial colony growth

Lesion development, (HST or nHST)

Stable nutritional base established

Induction of sporulation, reduced colony expansion

Lesion restriction, resistance factors able to confine fungal growth

Colonisation

HR = Hypersensitive reaction

HST = Host specific toxin

nHST = non Host-specific toxin

Figure 2.28: Model of possible cellular interactions determining specificity in interactions between *Alternaria* species and potential host plants.

Resistance to *A.brassicae* in cruciferous plants can involve the production of phytoalexins (Conn *et al*, 1988).

Resistance responses to both *A.brassicae* and *A.brassicicola* involve the accumulation of callose in the walls of attacked cells, in resistant plants this is more localised.

Cytosolic calcium ion concentration is thought to be important in determining the rate and extent of callose accumulation in papillae in response to penetration by *E.graminis* in barley (Aist & Gold, 1987)

A.brassicae is reported to sequester calcium during penetration (Tewari, 1990).

In incompatible interactions fragments from cell wall degredation elicit a hypersensitive response, papillum formation and phytoalexin accumulation. Any compounds produced by the pathogen have no effect on the permeability of the cell membrane, so that Ca^{2+} is not lost. The accumulation of papillae in the cell wall slows pathogen growth and the associated accumulation of phytoalexins inhibits it. Pathogen ingress is halted.

In compatible interactions the activity of cell wall degrading or chitinase or glucanase enzymes during penetration liberates plant cell wall fragments or fungal cell wall fragments which may elicit hypersensitive resistance responses (Heath, 1985). The fragments stimulate phytoalexin accumulation and Ca^{2+} mediated callose deposition. In toxin-sensitive plants, phytotoxin(s) released by the fungus increase membrane permeability causing an efflux of cytosolic Ca^{2+} which is enhanced by sequestrating activity by the pathogen. Papillum formation is reduced or delayed and pathogen development is not slowed. Phytoalexins either do not accumulate to sufficient concentration to inhibit pathogen development because of the increased permeability of the cell membrane, or the more rapid growth of the pathogen due to the lower accumulation of callose is sufficient for it to outgrow the other defence reactions. The initial phase of colony growth is successful allowing the pathogen to accumulate sufficient mass to release toxins which suppress further defence reactions by the plant; colony growth occurs.

Quantitative variation in any or all of the pathogen or host factors, possibly coupled with pathogen - host surface environment interactions would acccount for quantitative expression in resistance. Similarly

then, qualitative variation in any important factor would probably lead to qualitative variation in resistance, this being seen most generally at the non-host level. Although all of the potential factors identified would be of importance in the interactions the proposed role of the phytotoxins in suppressing the resistance response by inducing membrane damage may be pivotal to the success of the pathogen. Furthermore, host specificity would be dependent on the selective toxicity of the toxin, at least at the early stages of infection, if similar defence responses occurred in both host and non-host plants.

The results of this chapter have indicated broad similarities in the infection behaviour of *A.brassicae* and *A.brassicicola*. In both species interactions between fungal cells and plant cells have been found to be important in determining resistance. A model of the initial phase of pathogen - plant interactions places a high importance on the activity of phytotoxins produced by the pathogens in determining host specificity. The purpose of following chapter will be an examination of the biological activity of metabolites produced by the fungi in relation to pathogenesis.

3. PHYTOTOXIN RELATED STUDIES

3.1. INTRODUCTION

Table 3.1 lists *Alternaria* species which have been shown to produce biologically active secondary metabolites, and the reported activities of the metabolites. There is a wide spectrum of activity across these compounds which include antibiotics, fungitoxins, insecticides, mutagens, mycotoxins, phytotoxins, and plant growth substances.

Examining the ecological significance of some of the compounds, production of antibiotics and insensitivity to them may be important factors in the ability of soil fungi to compete as saprophytes (Garrett, 1970). Although the *Alternaria* are commonly considered as pathogens of the aerial parts of plants (Ellis, 1971) chlamydospore production has been reported in *A.brassicae* (Tsuneda & Skoropad, 1977) and with both *A.brassicae* and *A.brassicicola* it is known that some disease carry over is possible in crop trash in the soil. With *A.solani* chlamydospores are an important source of inoculum in early blight of tomatoes (Patterson, 1991), and may play a similar role in the case of *A.brassicae*. In relation to microbial antagonism, *A.brassicicola* produces at least one metabolite with antibacterial and antifungal activity (Harvan & Pero, 1976) which might be involved in competitive interactions in the soil or on the leaf surface.

Mycotoxins are commonly produced by storage fungi but do not determine the ability of the fungi to colonise their substrate, the significance of mycotoxins in plant material being determined by whether the plant is intended for consumption. Mycotoxins produced by *Alternaria* species have been detected in both fresh and processed food (Bruce, Stack, & Mislevic, 1984 ; King & Schade, 1984); much of the toxicological work has been reviewed by King & Schade (1984). Tenuazonic acid (TeA) has been considered as the most important mycotoxin of the *Alternaria* (King & Schade, 1984) and its presence has been reported from a number of foodstuffs (Meronuck, Steele, Mirocha, & Christensen, 1972; Mislivec, Bruce, Stack, & Bandler, 1986). However, other commonly occurring *Alternaria* metabolites have received some attention due to the possibility of their toxic, carcinogenic, or teratogenic effects (Chu & Bennett, 1981; Pollock, Di Sabatiano, Heimsch, & Hilbelink, 1982; King & Schade, 1984).

Table 3.1: ***Alternaria*** **species reported to produce biologically active metabolites, and their reported activities**

SPECIES	ACTIVITY OF METABOLITES
A.alternata	antibacterial, antifungal, antiviral, insecticidal, mycotoxic, mutagenic, phytotoxic
A.brassicae	antibacterial, teratogenic, cytotoxic, insecticidal, phytotoxic, plant growth regulation
A.brassicicola	antibacterial, cytotoxic, teratogenic, phytotoxic, mutagenic, plant growth regulation
A.carthami	antimicrobial, cytotoxic
A.cheiranthi	antibacterial, teratogenic, mycotoxic, phytotoxic mutagenic
A.chrysanthemi	phytotoxic
A.cinerariae	antifungal
A.crassa	cytotoxic, phytotoxic
A.cucumerina	phytotoxic
A.dauci	antibacterial, antimicrobial, cytotoxic, phytotoxic
A.helianthi	phytotoxic
A.infectoria	antibacterial, cytotoxic, teratogenic, phytotoxic mutagenic
A.macrospora	antimicrobial, phytotoxic
A.porri	antifungal, phytotoxic
A.radicina	phytotoxic
A.raphani	antibacterial, teratogenic, mycotoxic, mutagenic, phytotoxic
A.solani	antibacterial, antifungal, cytotoxic, phytotoxic mutagenic
A.tagetica	antimicrobial, phytotoxic
A.tenuissima	antimicrobial, phytotoxic
A.triticina	phytotoxic
A.zinniae	antimicrobial, phytotoxic

In cruciferous crops there has been little work on the occurrence of *Alternaria* metabolites in oil, vegetables, or animal feed. Mycotoxin production by *A.brassicae* and *A.brassicicola* was examined by McKenzie *et al.* (1988) who found that both species produced metabolites *in vitro* which were toxic to human epithelial cells (HEp2). However, they found no relation between levels of *Alternaria* contamination in seed samples of oilseed rape and subsequent cytotoxic activity in extracts from the seed. Bruce *et al.*

(1984) reported low levels of *A.brassicae*, *A.brassicicola*, *A.cheiranthi*, and *A.raphani* in samples of wheat, barley and rye in the U.S.A. These isolates were tested for the production of TeA, alternariol (AOH) and alternariol methyl ether (AME) and most were found to produce at least one of the toxins. The single isolate of *A.brassicae* tested produced only TeA, while the single isolates of *A.raphani* and *A.cheiranthi* produced all three toxins. In the case of *A.brassicicola* (which was isolated more frequently than the other species), two of the seven isolates tested produced all three toxins, two produced only AOH and AME, one produced only TeA, and two produced none of the toxins.

Insecticides, cytotoxins, and phytotoxins may play some role in the pathogenicity of the producing organism. *Alternaria* species are not commonly reported as pathogens of invertebrates. However, *A.brassicae* is reported to produce destruxin b (Ayer & Pena-Roriguez, 1988a) a compound which is also produced by *Metarhizum anisopliae*, a pathogen of silkworms. There is no direct evidence that cytotoxins are involved in the colonisation of animal tissue by *Alternaria* species, and there is little evidence for the involvement of cytotoxins in human disease caused by the *Alternaria*. However, *Alternaria* spores are a known cause of allergic reactions in humans and *Alternaria* species have also occasionally been reported to cause bronchial disorders and skin infections (Schlueter, Fink, & Hensley, 1972; Pederson, Mardh, Hallberg, & Jonsson, 1976).

Both *A.brassicae* and *A.brassicicola* have been found to produce metabolites with plant growth regulator activity. Suri & Mandahar (1984; 1985) showed that the pathogens were able to synthesise cytokinins *in vitro* and *in planta*, and postulated that these compounds were responsible for the green islands which form around lesions on infected leaves (Prasanna, 1984; Suri & Mandahar, 1984; 1985). More recently, Dahiya and co workers have purified and identified four plant growth substances produced by *A.brassicae* in culture (Dahiya, Tewari, & Woods, 1989; Dahiya & Tewari, 1991). One of these compounds was found to be a cytokinin related to zeatin while the other three, one of which was identified as abscisic acid, were found to have a range of negative effects on growth such as reduction in cholorphyll content of tissue and promotion of senesence.

In the present study interest is primarily in phytotoxic activity of metabolites of *A.brassicae* and

A.brassicicola in pathogenesis. A tentative summary of current information on the chemical nature and mode of action of known *Alternaria* phytotoxins is shown in Table 3.2. based largely on the review papers of King & Schade (1984) and Kohmoto, Otani, & Nishimura (1987). Although the whole group comprises a diverse range of chemical types there is a tendency for certain types to predominate. Many of the compounds are polyketides (dibenzopyrones) or have chemical structures related to them. A second group comprises three cyclic peptides, which display some specificity in their host range; a fourth host specific toxin(s), as yet only partially characterised (the ATs), is/are also ninhydrin positive, indicating the presence of a peptide group (Nishimura, 1987).

Stinson (1985) described the biosynthetic pathways which have been proposed for a number of phytotoxic metabolites which are produced by *Alternaria* species; these were mainly polyketides, including TeA, but also the anthraquinone altersolanols, along with their possible derivative zinniol. Of the polyketides produced by *Alternaria* species, alternariol (AOH), alternariol methyl ether (AME), alternaric acid (AcA), and TeA have been shown to have phytotoxic activity (King & Schade, 1984). Synthesis of AME is known to occur in *A.alternata via* AOH and S-adenosyl methionine; it might also be synthesised from altersolanol A in *Alternaria* species which do not produce AOH (Stinson, 1985).

Stoessel (1981) has discussed possible pathways for synthesis of AcA. The compound is proposed to be the product of the fusion of two polyketide chains, with the likely components being 14 C and 4 C chains. AcA is of interest in the present study as it has been shown to be phytotoxic to members of the *Cruciferae*, (Brian, Curtis, Hemming, Unwin, & Wright 1949; Templeton, 1972) but its production by *A.brassicae* or *A.brassicicola* has not been assessed.

King and Schade (1984) stressed the importance of TeA as a toxic metabolite of the *Alternaria*, and on the evidence of Kinoshita, Renbutsu, Khan, Kohmoto, & Nishimura (1972) suggested that its occurrence was probably widespread in the *Alternaria*. The compound is a potent phytotoxin and

Table 3.2: Characteristics of phytotoxins produced by *Alternaria* species

TOXIN	CHEMICAL GROUP	HST* ?	SITE or MODE OF ACTION	SPECIES
Alternariol	Á-pyrone	NO	undefined: causes chlorosis	*A.alternata*
Alternariol methyl ether	Á-pyrone	NO	undefined: causes chlorosis	*A.cucumerina*
				A.dauci
				A.solani
				A.tenuissima
Alternaric acid	dihydro 1, 4 pyrone	NO	undefined: causes chlorosis	*A.porri*
				A.solani
Brefeldin	polyketide	NO	undefined: causes chlorosis	*A.carthami*
Dihydrbrefeldin	polyketide	NO	undefined: not reported	
Culvularin	heterocyclic	NO	undefined: not reported	*A.cinerariae*
Dihydroculvularin	heterocyclic	NO	undefined: not reported	*A.infectoria*
Radicinin	heterocyclic	NO	undefined: not reported	*A.chrysanthemi*
				A.radicina
Radicinol	heterocyclic	NO	undefined: not reported	*A.chrysanthemi*
Deoxyradicinin	heterocyclic	NO	undefined: not reported	*A.helianthi*
Tenuazonic acid	teramic acid	NO	inhibits nucleic acid and protein synthesis	*A.alternata*
				A.crassa
				A.solani
				A.tenuissima
				A.brassicae

* Host specific toxin

Table 3.2 (cont'd): Characteristics of phytotoxins produced by *Alternaria* species

TOXIN	CHEMICAL GROUP	HST ?	SITE or MODE OF ACTION	SPECIES
Tenuazonic acid	tetramic acid	NO	inhibits nucleic acid and protein synthesis	*A.brassicicola* *A.cheiranthi* *A.rapahni*
Tentoxin	cyclic depsipeptide	NO	chloroplast membrane	*A.alternata*
Zinniol	anthraquinone derivative	NO	undefined: causes chlorosis	*A.dauci* *A.macrospora* *A.porri* *A.solani* *A.tagetica*
Not named	peptide	NO	undefined: mimics pathogen symptoms	*A.raphani*
Not named	not determined	NO	undefined: mimics pathogen symptoms	*A.triticina*
Not named	not determined	NO	undefined: causes chlorosis and necrosis	*A.brassicicola*
Destruxin-B	cyclic depsipeptide	NO	plasma membrane	*A.brassicae*
AF	trienoic acid	YES	plasma membrane	*A.fragariae*
AK's	trienoic acids	YES	plasma membrane	*A.kikuchiana*
AM's	cyclic depsipeptide	YES	chloroplast and plasma membranes	*A.mali*
ACR	Ã-pyrones	YES	mitochondria	*A.citri*
ACT	not determined	YES	plasma membrane	*A.citri*
AT	peptide	YES	mitochondria	*A.longipes*

mycotoxin, which has been shown to inhibit both protein and nucleic acid synthesis, and to cause chlorosis and death in a range of plants including *Brassica* species (Templeton, 1972; King & Schade, 1984). Studies by Gatenbeck & Sierankiewcz (1973) on the biosynthesis of TeA showed that specific analogues were produced by cultures of *A.alternata* when they were provided with either L-valine or L-isoleucine. Stoessel (1981) discussed the possible pathways for the production of TeA; the compound is assumed to normally be produced by a condensation reaction between L-isoleucine and acetoacetic acid.

With respect to peptide-containing phytotoxins, although a considerable effort has been made to determine the structures and sites of action of polypeptide toxins, very little is known of their production *in vivo*. However, in common with the polyketides AOH and AME (Haggblom, 1987), the polypeptide HSTs are synthesised *de novo* in germinating spores (Nishimura & Kohmoto, 1983b; Hayashi, Tanabe, Nishimura, Kohmoto, & Otani, 1991). Haggblom suggested that enzyme systems required for the production of polyketides were transported into the developing conidia from conidiophores, since protein biosynthesis inhibitors did not prevent the production of AOH and AME in conidia, indicating that *de novo* enzyme production was not a prerequisite for polyketide production. A similar situation may also apply to the enzymes responsible for the synthesis of the polypeptide toxins.

The AM toxins, which confer pathogenicity to isolates of *A.mali* to susceptible apple cultivars, appear to have their primary site of action at the plasma membrane causing rapid loss of electrolytes (Kohmoto *et al.*, 1987). Secondary effects are noted in the chloroplast membranes, but the precise mode of action remains undetermined. A similar pattern of action sites has been reported for toxins produced by *A.brassicae* in brassica leaf tissue (Tewari, 1983). The AM toxins and destruxin b are very similar in structure to tentoxin, which is host selective but not host specific. All three compounds contain secondary amino acids, N-methylalanine in the case of the AM toxins and tentoxin, and proline in the case of destruxin b. Structure-activity studies on analogues of the three toxins have indicated that both common features and unique features of the molecules determine their toxicity (Nishimura & Kohmoto, 1983b; Ayer & Pena-Rodriguez, 1987a; Edwards, Lax, Lillehoj & Boudreaux, 1987). It has been shown in all three toxins that activity is lost or reduced in linear analogues suggesting that their cyclic nature is important in determining

activity. With tentoxin and the AMs it has also been suggested that the unsaturated $C=CH_2$ in the dehydroalanine moiety imparts some of the activity as a toxin. Edwards *et al.* (1987) suggested that the cyclic nature and N-methylation in tentoxin may be important for preventing degradation of the compound and thus contribute to toxic activity indirectly *in vivo*. Specifically in the case of tentoxin, the cis-trans-cis-trans conformation of the four amino acids in the ring is important for maximum activity (Edwards *et al.*, 1987). The similarity in structure of these three peptide toxins and the similarity in their sites of action raises interesting questions about the similarity in metabolism in the different *Alternaria* species and the evolution (or conservation) of toxin binding sites in their hosts.

A cyclic depsipeptide HST (HC toxin) is produced by another of the Pleosporaceae, *Cochliobolus carbonum*. However, in this case toxicity is thought to be determined by the unusual amino acid, 2-amino-8-oxo-9,10 epoxydecanoic acid (AOE) (Macko, 1983). Macko noted that AK toxin produced by *A.kikuchiana* also contains an epoxy ring. It has since been found that AF toxin produced by *A.fragariae*, has a highly similar molecular structure to the AK toxins including an identical decatrienoic acid moiety containing the epoxy ring. These *Alternaria* toxins have substantially the same host range (Nishimura, 1987). Hydrolysis of the epoxy ring in HC toxin has been shown to remove toxic activity (Macko, 1983).

Compared with the volume of work concerning the HSTs produced by small-spored *Alternaria* anamorphs there has been relatively little research on the involvement of phytotoxins in the pathogenesis of *Alternaria* pathogens of the *Cruciferae*. *A.brassicae* has been the subject of the most detailed studies conducted to date. Ayer & Pena-Rodriguez, (1987a) and Bains & Tewari (1987) described the isolation and host specific phytotoxic activity of destruxin B. It is worth noting that Bains and Tewari (1987) used different bioassay techniques to test the phytotoxicity of destruxin B to hosts and non-hosts. The toxin was applied in aqueous solution directly to the leaf surface of hosts, but was administered *via* the vascular system to non-hosts. Such differences in technique might well be expected to determine the symptoms expressed in bioassays. Indeed, more recent studies by Buchwaldt & Jensen (1991) and Buchwaldt & Green (1992) have shown that although destruxin B is produced in infected brassica leaves by *A.brassicae*, and the toxin is released by germinating spores and germ-tubes, when it was tested for phytotoxic activity against both host and non-host leaves by a droplet application method, it was found to induce symptoms in

both hosts non-hosts. The toxin showed highest activity against brassica plants, with activity decreasing as taxonomic relatedness between the challenged plants and *Brassica* decreased. Phytotoxic symptoms were detectable in non-hosts of *A.brassicae* such as *Chenopodium*, *Solanum*, and *Triticum*. Buchwaldt & Green (1992) concluded that destruxin B is host selective rather than host specific.

Tewari (1983; 1986) and Bains & Tewari (1985) have suggested that the role of destruxin B in the pathogenicity of *A.brassicae* is to predispose tissue to colonisation by inducing electrolyte leakage in advance of growing hyphae. In cases of reported HST production by *Alternaria* species (except AT production by *A.longipes*) the toxins are released from the germinating conidia and appear to allow penetration of the susceptible host tissue. In these cases the toxins act as the primary determinants of disease. For example, it has been hypothesised that AK toxins allow the colonisation of susceptible tissue by suppressing early defence reactions (Kohmoto *et al.*, 1987), although more recent studies have indicated that the aggressiveness of any particular toxin-producing isolate may not be related quantitatively to the concentration of toxin produced during germination (Kohmoto, Akimitsu & Otani, 1991).

In the case of *A.brassicae*, Buchwaldt & Green (1992) concluded that individual conidia do not produce sufficient destruxin B during germination and penetration for the toxin to determine the success of penetration attempts. However, this analysis was based on the time at which macroscopic symptoms first appeared after treatment of brassica leaf tissue with the toxin (36 - 72 hours after inoculation), and the concentration of toxin required to induce these symptoms. It is possible that destruxin B may have significant effects at the cell level at an early stage of the interaction between pathogen and host which determine the success of the primary infection, but do not lead to the immediate expression of phytotoxic symptoms. Buchwaldt & Green (1992) supported their theory with results from studies of a sterile isolate *A.brassicae* which did not produce destruxin B in culture. Buchwaldt & Green suggested that the isolate probably did not produce destruxin B during penetration when mycelium plugs were used to inoculate brassica leaves. Following this procedure typical symptoms of *A.brassicae* infection were produced and it was concluded that destruxin B does not act as a pathogenicity factor (*sensu* F.B.P.P., 1973) but as a virulence factor (*sensu* F.B.P.P., 1973). However, destruxin B could be detected in chlorotic tissue produced by infection by the sterile isolate three days after inoculation. The authors did not comment on

when the pathogen apparently regained its ability to synthesise the toxin. It is possible, if the presence of host tissue acted as a trigger for the production of destruxin B, that this could have occurred soon after the fungus and plant tissue were brought into contact. Indeed, Buchwaldt & Green's finding that conidia of *A.brassicae* germinating in leaf extracts produce destruxin B within one hour supports the theory that toxin synthesis may be rapidly stimulated by some component of the host tissue. There is a need for detailed study of the effects of destruxin B on host cells at a short time interval after inoculation in order to clarify whether the toxin has a role in the initial establishment of the pathogen in its host's tissue.

Some research has also been conducted on the other major *Alternaria* pathogens of brassicas. Degenhardt (1978) isolated and partially purified two groups of unidentified, ninhydrin positive, high molecular weight phytotoxins, from *A.raphani* and *A.brassicae*. These did not show host specific activity, but interacted synergistically, producing increased toxicity on *Brassica* species. McKenzie *et al.* (1988) found that chloroform-soluble extracts from cultures of *A.brassicicola* inhibited root elongation in oilseed rape seedlings and induced non host specific necrotic spotting on leaf tissue. Comparable extracts from *A.brassicae* were found to produce similar effects, but the phytotoxicity of the extracts of both of these species was less than that of extracts from cultures of *A.alternata*.

The published reports of research on *A.brassicae* (Degenhardt, 1978; Bains & Tewari, 1985; 1987; McKenzie *et al.*, 1988; Buchwaldt & Green, 1992) have shown that as extracts from cultures have been increasingly purified host specificity in the extracts has increased. It has already been noted that *A.brassicae*, *A.brassicicola*, and *A.raphani* can produce TeA *in vitro* (Bruce *et al.*, 1984). The presence of NHST's such as TeA, in crude extracts from cultures of these species may account for the observed lack of host specificity in bioassays of crude extracts and, although the production of these compounds has not been shown *in vivo*, the ability to produce compounds with toxicity to a wide host range raises questions about the relative importance in pathogenicity of any HSTs these species may produce.

The production of a phytotoxin by a pathogen, whether the toxin acts as a determinant of virulence or pathogenicity, may effect selection for resistance in host crops. Normal selection for resistance to leaf pathogens is conducted in field plots with naturally occurring or artificially introduced inoculum. These

techniques may be supplemented by the use of glasshouse trials or, in some cases, detached plant parts maintained in culture (Russell, 1978). However, where a pathogen produces a toxin it may be possible to select for disease resistance through the use of the toxin in the absence of the pathogen. There is considerable interest in the use of plant tissue culture for such work as it is theoretically possible to screen a high volume of plant material relatively quickly. Some success has been reported from this type of approach to resistance breeding. For example, maize lines resistant to *Cochliobolus maydis* have been produced following exposure of tissue cultures to purified T toxin produced by the fungus (Geggenbach & Green, 1975; Brettel, Ingram & Thomas, 1980). Similarly, resistance to AT (*A.longipes*) toxin in tobacco has been detected using a plant protoplast bioassay system (Nishimura, 1987).

In relation to selection for resistance to *Alternaria* in brassica species, McDonald & Ingram (1985) reported the selection of lines of *B.napus* which showed increased resistance to *A.brassicicola* following regeneration of secondary embryoids which had been exposed to culture extracts from the fungus while in tissue culture. However, selection of regenerated plants which showed resistance to *A.brassicicola* was also possible from secondary embryoids which had not been exposed to extracts from the fungus while in culture, indicating that resistance may result as a response to the culture environment rather than to the presence of the toxin. Studies with *A.brassicae* which used callus cultures also failed to provide evidence that selection for resistance to this pathogen was possible using crude culture extracts (Williams & Pink, 1987). However, with the use of destruxin B it may be possible in the near future to produce a more specific selection pressure on tissue cultures of *Brassica* species. A recent review by Nishimura (1987) suggests that selection for disease resistance to *Alternaria* species which produce HST's is not at an advanced stage, despite the relatively high interest in these host-pathogen systems. A more general review of the use of tissue culture for selection of disease resistant plants by Daub (1986) noted significantly that no variety had been released as a result of *in vitro* selection for increased toxin resistance; the same situation was reported more recently by Vassil (1990).

For host-pathogen systems in which an HST is the primary determinant of pathogenicity, cultivar-race specificity often occurs. This is illustrated by the high susceptibility of homozygous ASC^- recessive tomato lines to isolates of *A.alternata* f.sp. *lycopersici* which are able to synthesise AL toxins. These isolates

are non-pathogenic to lines of tomato which do not carry the ASC^- allele (Clouse & Gilchrist, 1987). However, it has been noted previously (section 2.1) that there is little evidence of race specificity in interactions between *A.brassicae* or *A.brassicicola* and *Brassica* species (Humpherson-Jones, Hocart, 1983; Prasanna, 1984). In several of host-pathogen combinations in which *Alternaria* species produce HST's the genetic basis of susceptibility is understood; typically a small number of alleles is involved. This information is summarised in Table 3.3. The genetic basis of susceptibility to destruxin B is unknown, but the results of inoculation studies by the workers noted above suggest that control over susceptibility is multigenic. Unfortunately deriving the precise nature of susceptibilty will require a number of host lines of different susceptibilities but with similar and known genetic background. That this approach might prove successful is suggested by the fact that Bains & Tewari (1987) found that destruxin B was able to detect susceptibility differences between three cultivars of *B.campestris*.

Table 3.3: The genetic basis of susceptibility to HSTs produced by *Alternaria* species

HOST	TOXIN (pathogen)	GENETIC BASIS OF SUSCEPTIBILITY
apple	AM *A.mali*	Three or more multiallelic genes
pear	AM *A.mali*	unknown
pear	AK *A.kikuchiana*	Single dominant gene
strawberry	AF *A.fragariae*	Single diallelic gene with additive recessive susceptibility
tomato	AL *A.alternata* f.sp *lycopersici*	Single diallelic gene with additive recessive susceptibility

The cultivar-race specificity which characterises interactions involving HSTs has been compared with the cultivar-race specificity which is displayed in classic gene-for-gene systems (Ellingboe, 1976;

Scheffer, 1983; Heath, 1985). It appears that a common impression of HST pathogens, the impression expressed in Heath's (1985) discussion for example, is that HST producing pathogens are unusual pathogens which exist otherwise as saprophytes. Heath considered that the production of HSTs by pathogens circumvents otherwise durable resistance in their host plants by killing cells, thus allowing the fungi to feed necrotrophically. Scheffer (1983), however, had previously pointed out that there is actually little evidence to support this point of view. Significantly, Scheffer refuted the generalisation that the HST-producing pathogens always kill cells in advance of colonisation and feed necrotrophically. More recently, Kohmoto *et al.* (1987) have suggested that HSTs from *Alternaria* species inactivate active defence mechanisms in susceptible pear tissue and that this leads to colonisation by pathogenic isolates. Although electrolyte leakage from host cells occurs in these cases about 30 minutes after exposure to the toxin it appears that the pathogen colonises live tissue. Considering *A.brassicae* and *A.brassicicola* in this respect, there is no evidence that host cells are killed by toxins produced by the fungi prior to penetration.

Nishimura & Kohmoto (1983a; b) have suggested that HST producing isolates of *A.alternata* are derived from a saprophytic population, although as noted above this does not entail the pathogenic isolates feeding as necrotrophs. Nishimura & Kohmoto have pointed out that isolates of *Alternaria* which are able to synthesise both AK and AM toxins can be collected from previously apparently saprophytic populations by sequential inoculation of toxin-sensitive cultivars of pear and apple. Drawing together the evidence provided by Scheffer (1983), Nishimura & Kohmoto (1983a; b), and Heath (1985) it seems reasonable to suggest that HST-producing fungi are able to live saprophytically in the absence of suitable hosts, but as hemibiotrophs in their colonisation of susceptible plants.

The work of Nishimura & Kohmoto (1983a) has highlighted how little is known about the genetics of toxin production in *Alternaria*, and about the maintenance of the toxin producing genotypes in the environment. It is true to say that although little is known of these processes in other *Alternaria* species even less is known in the case of *A.brassicae* and *A.brassicicola*.

Knowledge of the involvement of phytotoxins in the pathogenicity of *A.brassicae* and *A.brassicicola* is still either sketchy or lacking. Previous reports have suggested that both *A.brassicae* and *A.brassicicola* produce a number of secondary metabolites in culture yet this range of metabolites has received little attention in relation to their virulence or pathogenicity. This section of work is an attempt to examine the nature and biological activity of such compounds and the possible role of non host specific toxins in the pathogenicity of *A.brassicae* and *A.brassicicola* to brassica plants. In the first of three experiments two other *Alternaria* species, *A.alternata* and *A.solani* were included along with *Leptosphaeria maculans*, a pathogen of brassicas which causes leaf spot and sterm canker symptoms. The work in this section can be considered under the following headings:

1. Experiment 3.1: Tests for fungistatic, cytotoxic and phytotoxic activity in extracts from cultures of *Alternaria* species and *Leptosphaeria maculans*.

2. Experiment 3.2: Examination of *A.brasssicae* and *A.brassicicola* for the production of tenuazonic acid and alternaric acid.

3. Experiment 3.3: Metabolites of *A.brassicae* and *A.brassicicola* produced in leaf disks of *B.napus*.

3.2 MATERIALS AND METHODS

Experiment 3.1: Tests for fungistatic, cytotoxic, and phytotoxic activity in extracts from cultures of *Alternaria* species and *Leptosphaeria maculans*.

Culture growth

The following species and isolates were used in the study: *A.alternata* (Aa4, Aa5), *A.brassicae* (Ab3, Ab4, Ab5), *A.brassicicola* (Ac4), *A.solani* (As1), and *Leptosphaeria maculans* (Lm1). Still cultures were grown in 100 ml of LCM (Appendix 3.1) in 250 ml conical flasks. Cultures were seeded with three 1.5 cm plugs taken from the edge of 2-week-old cultures of each isolate grown on PDA. The flasks were maintained at 20± 2° C in constant darkness in water baths for 10, 20 or 30 days. Three flasks were harvested for each isolate at each sampling time. Intra- and extra-cellular metabolites were extracted together.

Extraction of metabolites

At each sampling time the liquid and mycelium from the three cultures for each isolate were pooled by decanting into 500 ml beakers and the pH determined using a dip electrode. The mycelium and culture liquid were homogenised for one minute in a food blender and the resulting slurry filtered sequentially, using a vacuum filter, through Whatman No.1 and GF/C filters to remove cell debris. The filtrate was transferred to 500 ml separating funnels and extracted first with 50 ml and then three 10 ml aliquots of chloroform which was collected through Whatman 1PS papers containing anhydrous K_2SO_4 to remove aqueous contaminants. The resulting chloroform extract was dried *in vacuo* at 40° C on a rotary evaporator and the residue taken up in 1 ml of chloroform, transferred to a pre-weighed sample vial, then dried under nitrogen. The dry weight of the residue was determined at this stage and it was then redissolved in 0.1 ml of chloroform to give the final extract for TLC analysis and TLC bioassay of anti-fungal activity.

Qualitative TLC

Qualitative TLC was conducted on 0.25 mm thick, foil-backed silica gel plates (Merk, Art. 5553/4) either with or without fluorescent background. The plates were developed in toluene/ethyl acetate/formic

acid (6/3/1 v/v) at room temperature and viewed under short or long wave u.v. (254 nm or 360 nm). Tenμl of the extract from each isolate was run on each plate. Three pure known *Alternaria* metabolites were available for comparison, Alternariol methyl ether (Sigma), Tentoxin (Sigma), Tenuazonic acid (Royal Institute of Technology, Stokholm, Sweden).

Antifungal activity

TLC bioassay of antifungal activity was conducted using the same TLC system as described for qualitative TLC. After plates had been developed they were dried overnight at room temperature and were then sprayed with 10 ml of a spore suspension of *Cladosporium sp*. in a glucose based nutrient solution (10 g glucose, 5 g KH_2PO_4, 5 g KNO_3, 0.5 g $Mg_2SO_4.12H_2O$/l). The plates were incubated at 20° C in a sealed plastic box lined with damp paper towels, until the plate surface was covered with the inoculated fungus.

Cytotoxicity test

Cytotoxic activity in the extracts was tested using 5μl of each extract, after the extracts had been dried under nitrogen and resuspended in ethyl acetate. The ethyl acetate extract was added to microtitre wells containing cultured pig liver cells. The cells were incubated with the extracts for 48 hours after which the growth medium containing the extracts was poured off and the cells were fixed with 70% ethanol. After fixing the wells were filled with 0.1% v/v aqueous Gurr's improved Giemsa stain. Following 24 hours staining, cytotoxicity was assessed using a 0 - 4 scale, where a score of 0 was equal to no visible cell damage, and a score of 4 indicated 100% cells disrupted. All extracts were tested in triplicate.

Phytotoxicity tests

Leaf disk bioassay for phytotoxicity was conducted using leaf disks or pieces cut from first leaves of glasshouse-grown plants of oilseed rape (cv. Lirradonna) and wheat (cv. Armada). The leaf disks or pieces were supported in 80 ppm benzimidazole agar in 10 cm square petri dishes. Fiveμl of each extract was added to a 5μl capacity paper wick and the chloroform was allowed to evaporate. The wicks were placed on the surface of the leaf pieces and 10μl of distilled water was added. The leaf disks were incubated at 20° C for 3 days and were then scored on a 0 - 5 scale for phytotoxic effects. Three replicates for each extract/plant species were tested.

For microscopic examination of the effects of extracts from *A.brassicae* on leaf tissue, additional leaf disks were treated with extracts from Ab4 exactly as described above. After incubation with the extracts the wicks were removed from the leaf disks which were then prepared for freeze fracture LTSEM (Appendix 3.2) and tissue at the site of extract application was examined.

Data transformation and analysis

For statistical analysis the scores from the cytotoxicity and phytotoxicity bioassays were transformed according to the method of Cochrane (1938) for small whole numbers (Section 2.2). Untransformed data were also analysed and where transformation was found to have no effect on the outcome of the analyses only original data are reported. Data were examined by analysis of variance (Appendix 3.3).

Control treatments for all of the assays in the experiment consisted of uninoculated culture liquid treated exaclty the same as the fungal cultures.

Experiment 3.2: Examination of *A.brassicae* and *A.brassicicola* for the production of tenuazonic acid and alternaric acid.

Culture growth

Still cultures of four isolates of *A.brassicae* (Ab1, Ab2, Ab3, Ab4) and five isolates of *A.brassicicola* (Ac1, Ac2, Ac3, Ac4, Ac5) were grown in the dark in 500 ml LCM (Appendix 3.1) in 1000 ml laboratory bottles (Schott) with screw-on teflon caps in water baths at 20° C for 30 days.

Extraction of metabolites

The culture liquid was acidified to pH 2.5 with 1N HCl and was extracted with 100 ml and three 50 ml aliquots of chloroform. The chloroform was divided into two equal volumes and was dried as described in Experiment 3.1 The residue from one of the chloroform extracts was taken up in 1 ml of methanol, dried under nitrogen, and the residue taken up in 0.1 ml of methanol which was used for TLC to test for the

presence of tenuazonic acid in the extracts. The residue from the second chloroform extract was dissolved in hot CCl_4 in an extraction hood. The CCl_4 was allowed to cool to room temperature. Alternaric acid in such extracts appears as white needle-like crystals (Brian *et al.*, 1951).

Phytotoxicity test

Leaf disk bioassay was conducted as described above with leaf disks of *B.napus* (cv. Lirradonna) and differences between species and isolates were examined by analysis of variance (Appendix 3.4).

Experiment 3.3: Metabolites of *A.brassicae* and *A.brassicicola* produced in leaf disks of *Brassica napus*.

Plant growth and inoculation

Brassica napus var. *oleifera* plants (cv. Lirradonna) were grown in 12 cm pots as described in Experiment 2.2. First leaves were excised when fully expanded and cut into 1.5 cm disks with a sterile cork borer. The disks were placed 20 per dish in 10 cm square petri dishes and were inoculated with 50μl of a spore suspension of either *A.brassicae* (Ab4) or *A.brassicicola* (Ac5) containing 5000 conidia per ml. Spore suspensions were prepared as described in Experiment 2.1 except the fungi were grown as described in Experiment 2.2. The inoculated leaf disks were incubated at 20± 2° C in a perspex bench-top incubator under a natural day length of approximately 16 hours for five days. Sixty leaf disks were inoculated with each pathogen. Control leaf disks were inoculated with distilled water and incubated with pathogen-treated leaves.

Detection of fungal metabolites

All leaf disks for each pathogen or the control treatment were ground for approximately 2 minutes with 10 ml of iso-octane in a ceramic pestle and mortar. The resulting slurry was transferred to 250 ml 'quickfit' conical flasks and a further 40 ml of iso-octane was added. The flasks were stoppered and shaken on a rotary shaker at 100 cycles per minute for 1 hour. After extraction the iso-octane (containing fats) was discarded and 50 ml of ethyl acetate was added to each flask. The flasks were shaken for a further hour and the ethyl acetate then collected through Whatman 1PS papers. The ethyl acetate was divided into

two fractions which were then prepared as described above (Experiment 3.2) to test for the presence of tenuazonic acid and alternaric acid.

In addition to analysis of the TLC plates for the production of standard *Alternaria* metabolites and bands matching those produced by the isolates *in vitro*, the plates were also sprayed to detect the presence of sesquiterpenoid metabolites. The phosphomolybdic acid / ceric sluphate (PACS) spray reagent was as follows: 5% w/v phosphomolybdic acid in 5% v/v aqueous H_2SO_4 with one to two grains of ceric sulphate addded. Plates were sprayed then heated to 150° C for five minutes. A positive reaction is indicated by the production of a bright blue colour (Ayer & Pena-Rodriguez, 1987a).

3.3 RESULTS AND DISCUSSION

Experiment 3.1: Tests for fungistatic, cytotoxic, and phytotoxic activity in extracts from cultures of *Alternaria* species and *Leptosphaeria maculans*.

Results

The pH of culture liquids and the dry weight of residue harvested are shown in Figures 3.1 and 3.2 respectively. It was found that the pH of cultures generally increased slightly from approximately 5.4 to between 5.5 and 6.0 over the incubation period. Examining the changes in culture pH over time in more detail, it was found that pH increased in all cases except with Ab5 and Aa5. In the case of Ab5 there was little change in pH over time while with Aa5 pH decreased very slightly between 20 and 30 days incubation. Considering the other isolates, the *A.brassicicola* isolate was distinct in that pH increased more rapidly between 10 and 20 days than it did between 20 and 30 days incubation; with all other isolates pH increased more rapidly between 20 and 30 days. This increase was generally least in the case of *A.brassicae*.

In contrast to culture pH, there was no clear relationship between incubation period and total yield of extracted residue. With four of the eight isolates (Ab3, Ab4, Lm1, and As1) the dry weight of extract harvested was lower at 20 days than at both 10 and 30 days, while with Ab5, Ac4, and Aa5 the weight of extract harvested decreased with increasing incubation period. Finally, with Aa4 the weight of extract harvested was greater at 20 days than at 10 and 30 days after inoculation.

As expected from preliminary observations (McRoberts, unpublished data) there was a considerable variation between species in the number, R_{FS}, and colours of metabolites separated by TLC (Figures 3.3 and 3.4). With all isolates there was a tendency for the number of fluorescent bands to increase with increasing culture age. Thus, for example with *A.solani*, six distinct bands were visible in extracts from 10-day-old cultures while in 20-day-old cultures twelve bands were visible. There was also a trend with all isolates, except *A.brassicicola*, for variation in band colour to increase with increasing culture age, this again suggesting a greater diversity of metabolites in older cultures.

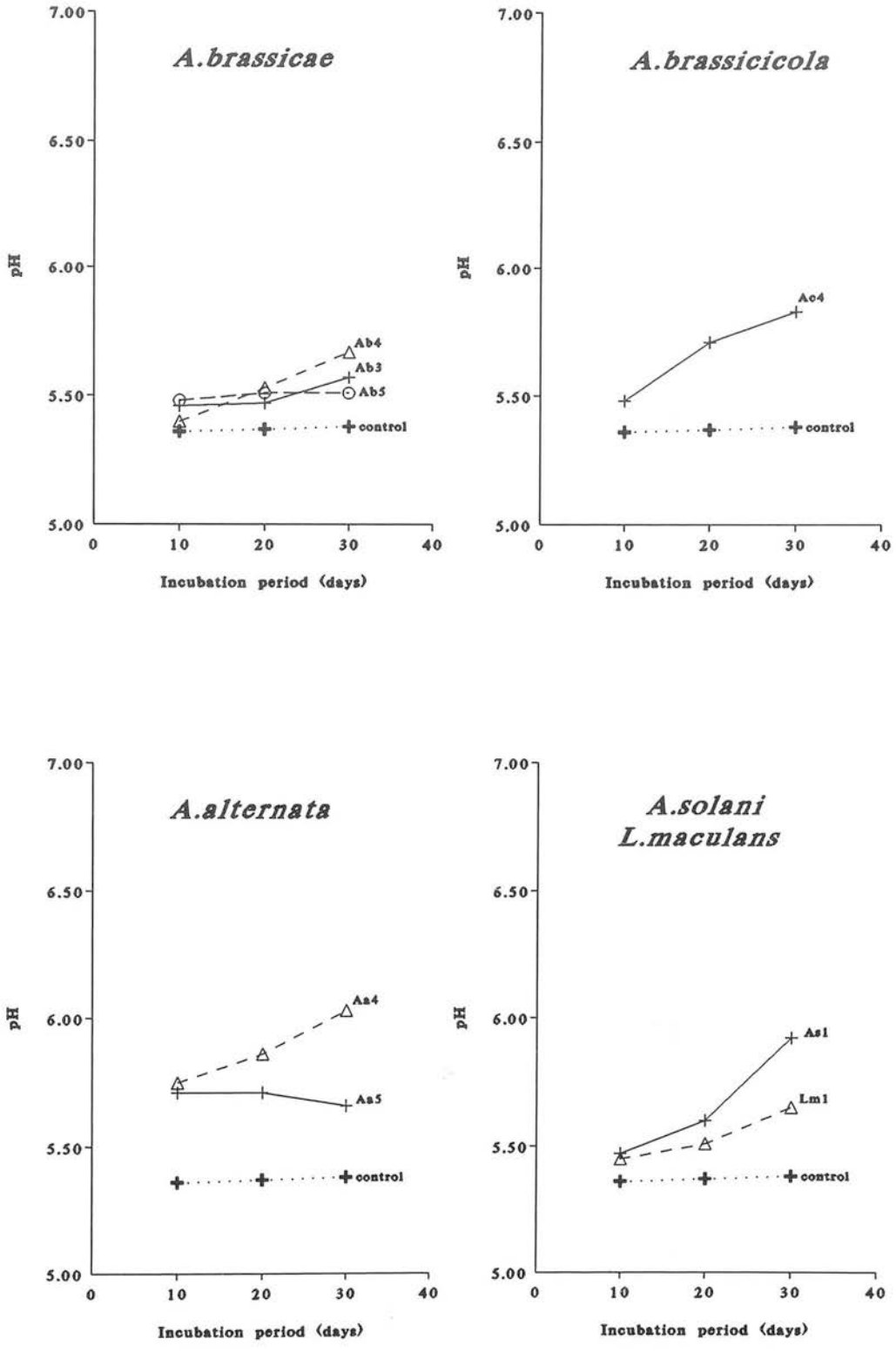


Figure 3.1: Change in culture pH over time for still-grown cultures of *Alternaria* **and** *Leptosphaeria maculans*.

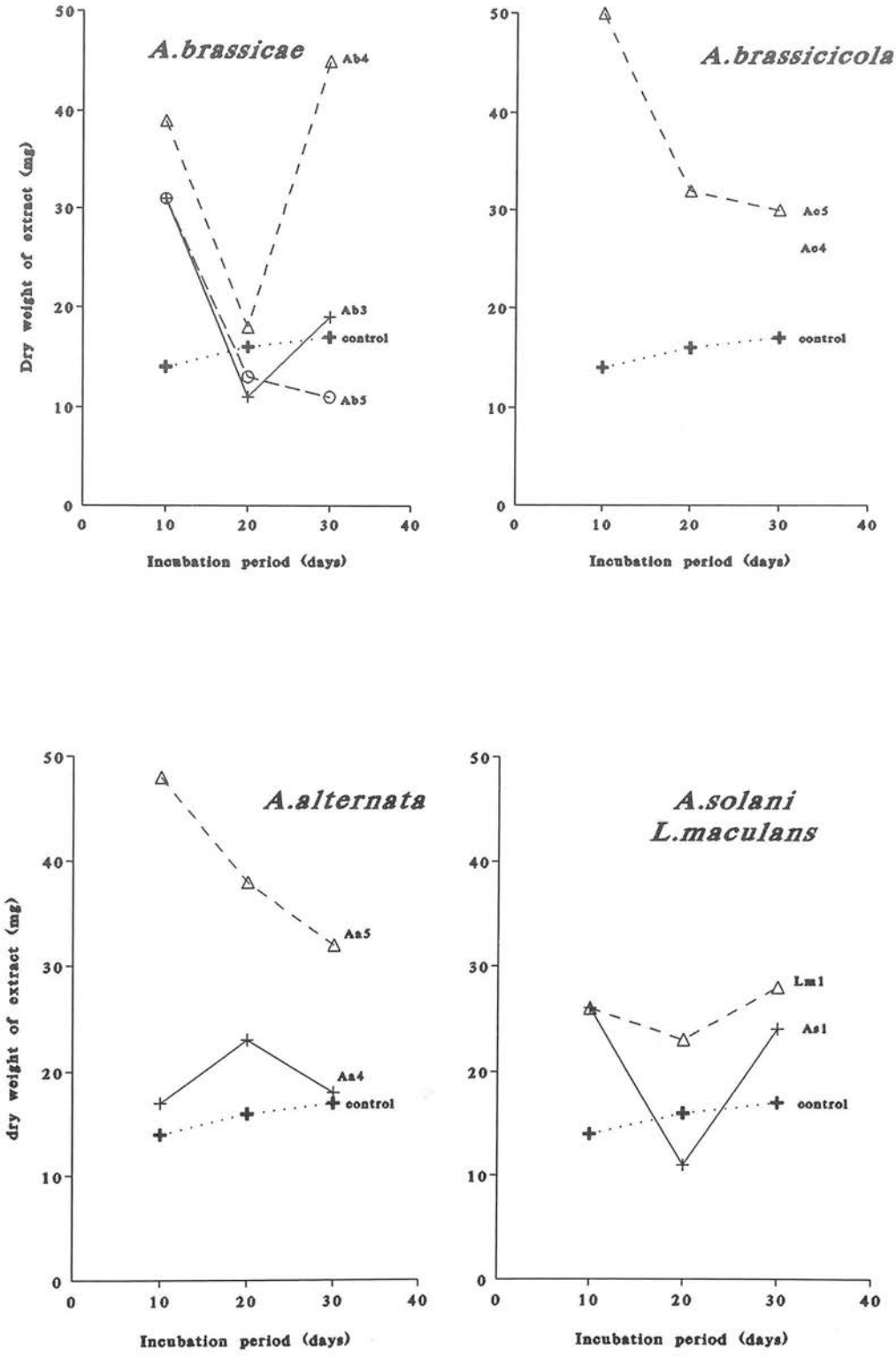


Figure 3.2: Change in the dry weight of extract from still-grown cultures of *Alternaria* **and** *Leptosphaeria maculans*.

In addition to an increase in the diversity of metabolite colours over time, there was also a change in the colours of the bands which predominated in the extracts. Thus, for *A.brassicae*, *A.brassicicola*, *A.solani*, and *L.maculans* 10-day-old cultures contained a majority of blue-fluorescing bands. However, after 20 and 30 days incubation these had been largely replaced by green bands, and blue-fluorescing bands were found only in *A.brassiace* at 30 days.

Examining the production of specific metabolites, none of the isolates was found to produce tenuazonic acid or tentoxin, but extracts from 10-day-old cultures of *A.solani* and *A.brassicicola*, and 20-day-old cultures of Ab4 and Ab5 contained AME. All isolates produced a green band at R_F 0.58 - 0.63 in extracts from 10-day-old cultures which also appeared in extracts from all isolates, except Ac4 and Lm1, at 20 and 30 days. Another green band at R_F 0.66 - 0.73 was produced by all isolates in 30-day-old cultures and by As1 and Lm1 at 20 days.

Certain groups of metabolites characterised individual species. *A.alternata* produced a group of orange and brown bands between R_F 0.30 and 0.45 while in approximately the same zone *A.brassicae* isolates produced a mixture of blue and green bands which showed some variation over time and between isolates. In 30-day-old cultures all three *A.brassicae* isolates produced a unique blue band at R_F 0.18 - 0.21. *A.brassicicola* was characterised by a relative lack of metabolites, while *A.solani* produced the most complex pattern of metabolites including several green bands in common with *A.brassicae* and one brown band at R_F 0.45 which it shared with *A.alternata*. The *A.alternata* isolates produced a yellow fluorescing band which was in the correct position on the plates for it to be altertoxin-II, a minor mycotoxic metabolite produced by some isolates of this species (Chu, 1981). However, in the absence of a standard positive confirmation of the identity of the metabolite was not possible. *Leptosphaeria maculans* produced green and blue fluorescing bands which were common to some *Alternaria* isolates and also a distinctive pale band at R_F 0.4 - 0.5 in 30-day-old cultures.

With respect to u.v.-quenching bands, there was less variation between isolates than was present in fluorescing metabolites. Furthermore, with all isolates apart from *L.maculans*, there was little evidence

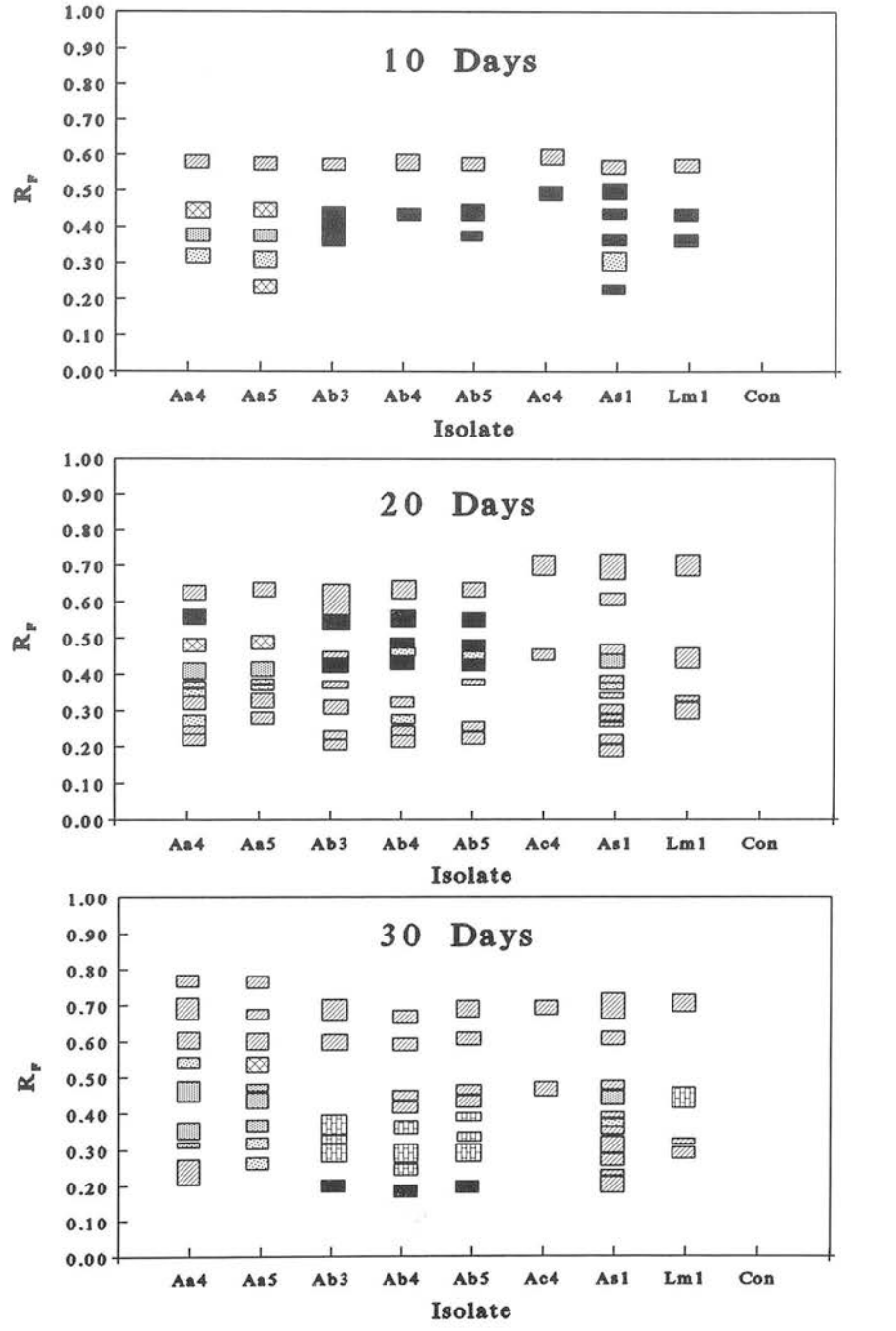


Figure 3.3: A diagramatic representation of R_F positions of fluorescing bands in extracts from still-grown cultures of *Alternaria* spp. and *Leptosphaeria maculans* after development in one dimensional TLC and observation under u.v. illumination.

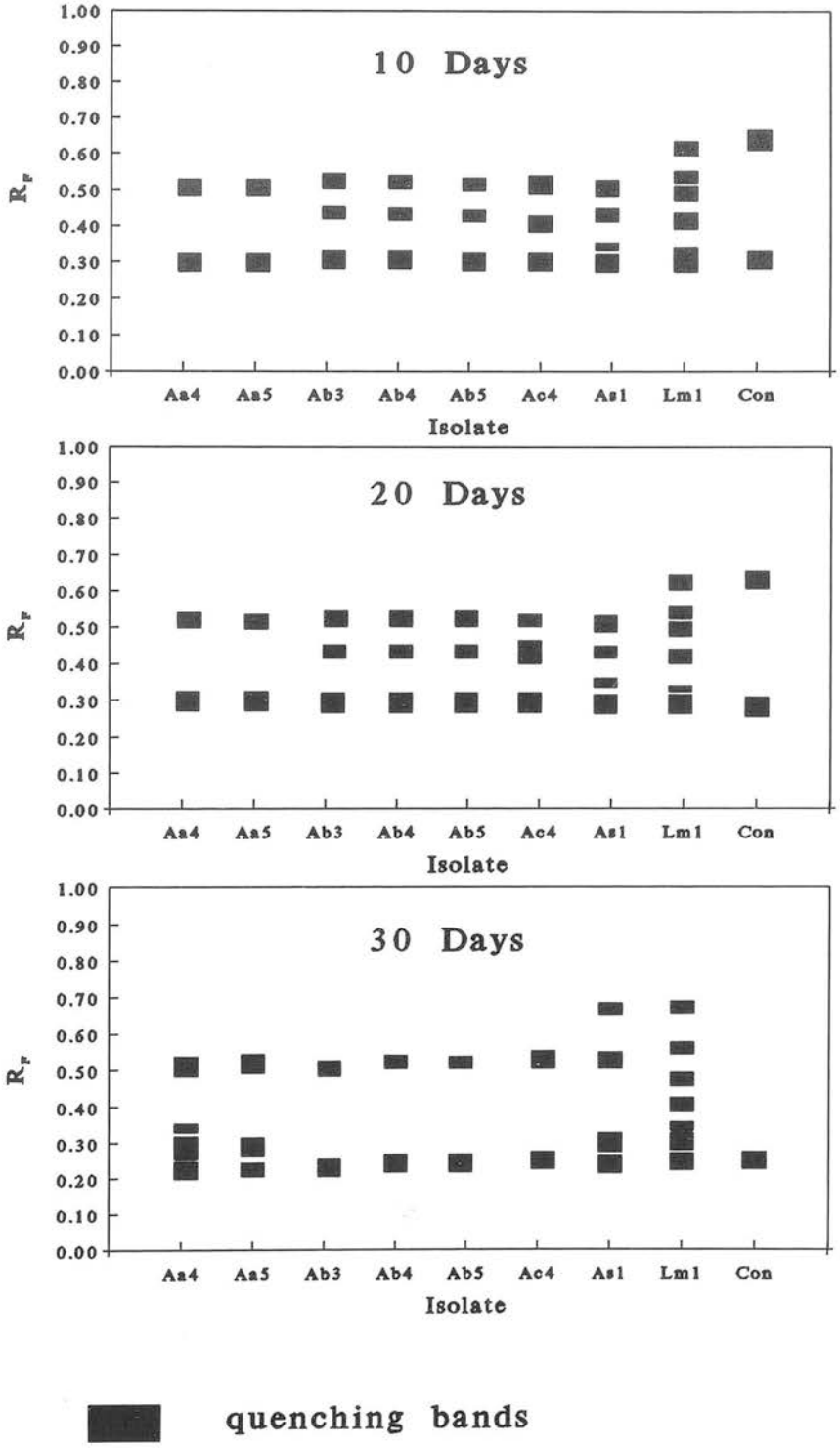


Figure 3.4: A diagramatic representation of R_F positions of shortwave u.v. quenching bands in extracts from still-grown cultures of *Alternaria* spp. and *Leptosphaeria maculans* after development in one dimensional TLC and observation under u.v. illumaintion.

of an increase in numbers of u.v.-quenching metabolites over time in contrast to the position for fluorescing metabolites: *L.maculans* had the most complex profile of quenching metabolites and was distinct from the *Alternaria* species in this respect. Extracts from all isolates and the control medium contained a quenching band at R_F 0.30, while another band at R_F 0.65 was present in extracts from the control medium and Lm1, but not in the extracts from any of the *Alternaria* isolates. All fungal isolates produced a quenching band at R_F 0.50 in extracts at all sampling times. In addition *A.brassicae*, *A.brassicicola*, *A.solani*, and *L.maculans*, but not *A.alternata*, produced a band at approximately R_F 0.45 in extracts from 10 and 20 day-old cultures. In 10 and 20 day-old cultures *A.solani* and *L.maculans* produced a band at approximately R_F 0.33 which was not produced by the other fungi at these times. However, at 30 days after inoculation cultures of *A.alternata* were also found to contain a metabolite at this position.

Antifungal activity

The positions of inhibitory bands resulting from the TLC bioassay for fungistatic activity are shown in Figure 3.5. Inhibition zones were noted only on plates developed with extracts from 30-day-old cultures. Differences were found between the various cultures in the position of inhibitory bands produced; isolate Ab4 did not produce any inhibitory bands, while all other isolates produced at least one zone of inhibition. Isolates Aa4, Aa5, and Ab5 produced a single inhibitory band with an R_F of 0.11 - 0.19. Isolates Ac4, As1, and Lm1 produced an inhibitory band with R_F value of 0.2 - 0.3. Isolate Ab3 produced three inhibition zones with R_F values 0.04 - 0.09, 0.1 - 0.2, and 0.3 - 0.4; The last of these bands was shared with a second band produced by Lm1. Of all inhibition zones, those occurring at R_F 0.30 - 0.40 for Ab3 and Lm1, and at R_F 0.23 - 0.30 for As1 corresponded to either fluorescing bands or quenching bands on qualitative TLC plates (Table 3.4).

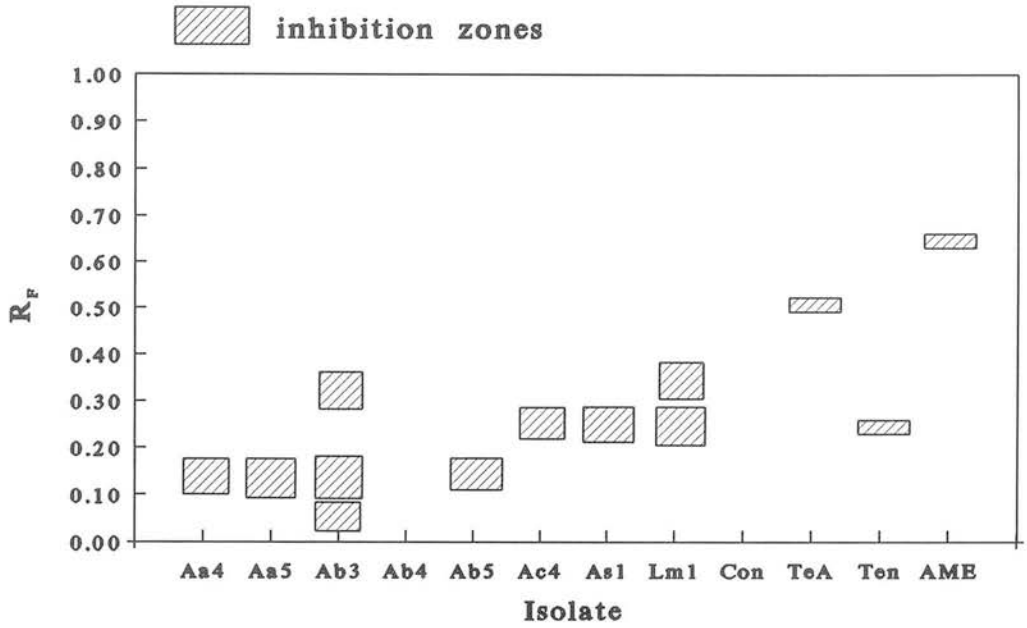


Figure 3.5: Zones of anti-fungal activity produced from extracts of still-grown cultures of *Alternaria* and *Leptosphaeria maculans* after development on TLC plates.

Table 3.4: A comparison of the R_F of zones of antifungal activity and metabolites from qualitative TLC plates for extracts from cultures of *Alternaria* and *Leptosphaeria maculans*.

ISOLATE	R_F of antifungal bands	Characteristics of corresponding metabolite(s)
Aa4	0.11 - 0.19	none
Aa5	0.11 - 0.19	none
Ab3	0.04 - 0.09	none
	0.10 - 0.20	none
	0.30 - 0.40	pale fluorescence
Ab5	0.11 - 0.19	none
Ac4	0.11 - 0.19	none
As1	0.23 - 0.30	green fluorescence and u.v. quenching
Lm1	0.20 - 0.30	green fuorescence and u.v. quenching
	0.30 - 0.40	green fluorescence and u.v. quenching

Phytotoxic activity

The results of the leaf disk bioassay are summarised in Figure 3.6 (see also Appendix 3.3). Generally, where symptoms were produced, the form of the symptoms was similar for extracts from different culture ages and isolates, but the severity differed between plants. Thus necrosis and chlorosis occurred on both plants (Plate 3.1a) but were clearly more severe on oilseed rape than on wheat. Older cultures gave the most severe symptoms on oilseed rape, but there was little effect of culture age on the severity of symptoms noted on wheat (Figure 3.6a). With respect to the interaction between fungus and plant (Figure 3.6b), it was found that all isolates except Ac4 produced distinctly more severe symptoms on oilseed rape than on wheat. With Ac4, the phytotoxicity index was not significantly different between plant species. Both isolates of *A.alternata* produced significantly higher phytotoxicity indices than the other fungi tested. *A.brassicae* produced the next most severe symptoms, while As1, Lm1 and produced similar phytotoxicity indices on oilseed rape which were higher than the index produced by Ac4.

Microscopic symptoms induced on oilseed rape and wheat by the extract from Ab4 are shown in Plate 3.1 b-g. Damage to cells appeared to be localised to the area immediately under the wick and a sharp division was obvious between the treated and untreated areas (Plates 3.1a and d). However, there were clear differences in the degree of damage on oilseed rape and wheat. On oilseed rape the epidermal cell layer was found to have collapsed in the treated area, and the upper mesophyll layer was also compressed so that the overall depth of the treated area was considerably reduced as compared with the untreated area (Plates 3.1b and c). In wheat there was less disruption to the epidermal cell layer, and, although some cells showed signs of compaction, others retained a typical round cross section. There was little sign of damage in the mesophyll layers of treated wheat tissue and the treated areas of leaves were similar in depth to untreated areas (Plates 3.1e and f).

Cytotoxic activity

With respect to cytotoxicity tests, there was no evidence that any of the isolates had cytotoxic activity against pig liver cells at the concentration at which they were tested.

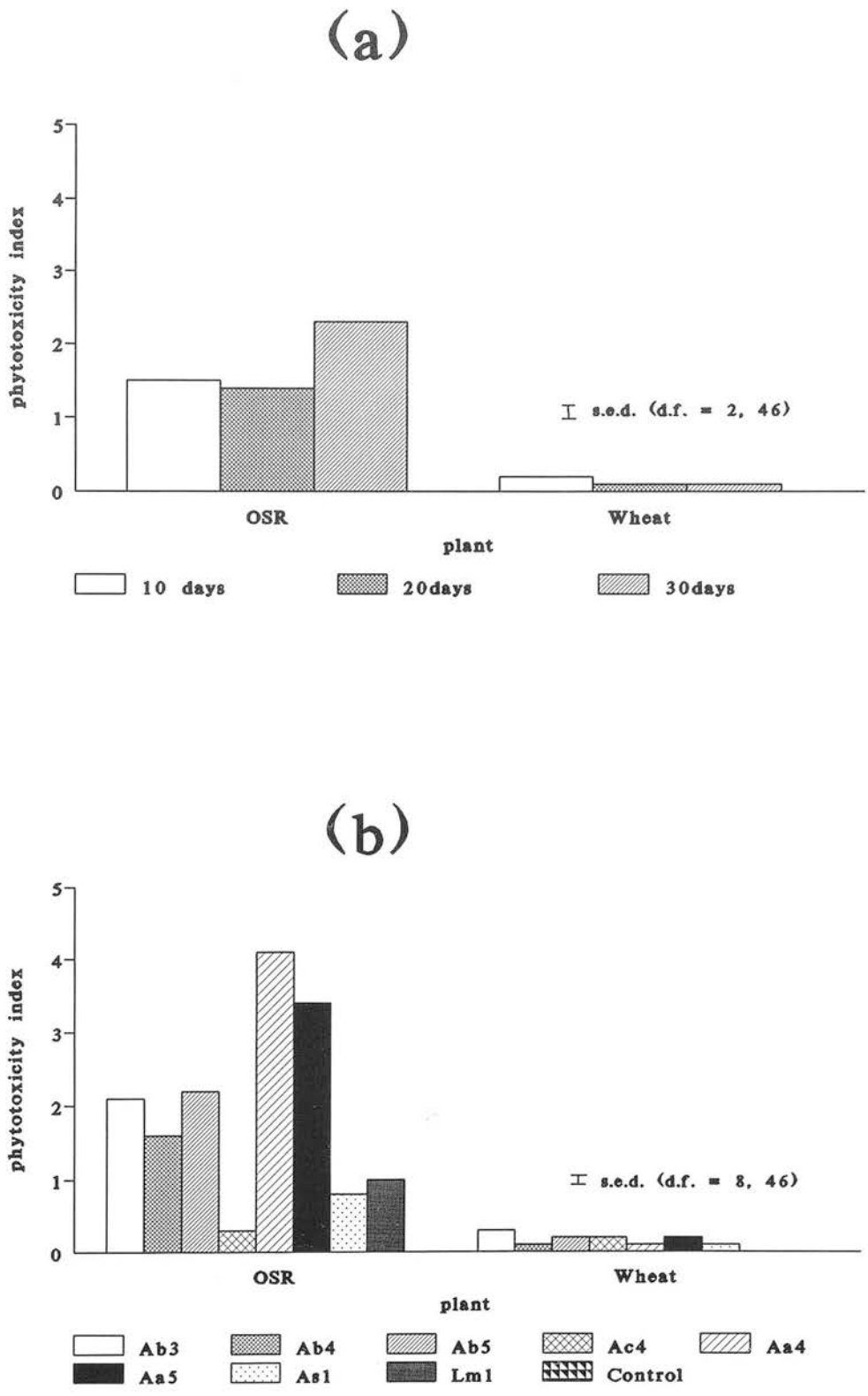


Figure 3.6: Phytotoxicity indices for leaf pieces of oilseed rape and wheat treated with extracts from cultures of different ages of *Alternaria* and *Leptosphaeria maculans*: (a) effects of culture age averaged over fungal species; (b) effects of fungal species averaged for culture age.

Plate 3.1: Symptoms and cell damage in leaf tissue of oilseed rape and wheat treated with organic extracts from 30-day-old cultures of *A.brassicae* (Ab4).

a. Symptoms produced by the application of culture extract from *A.brassicae* (Ab4) to leaf tissue of *B.napus* cv. Lirradonna. The magnification is approximately 15 times.

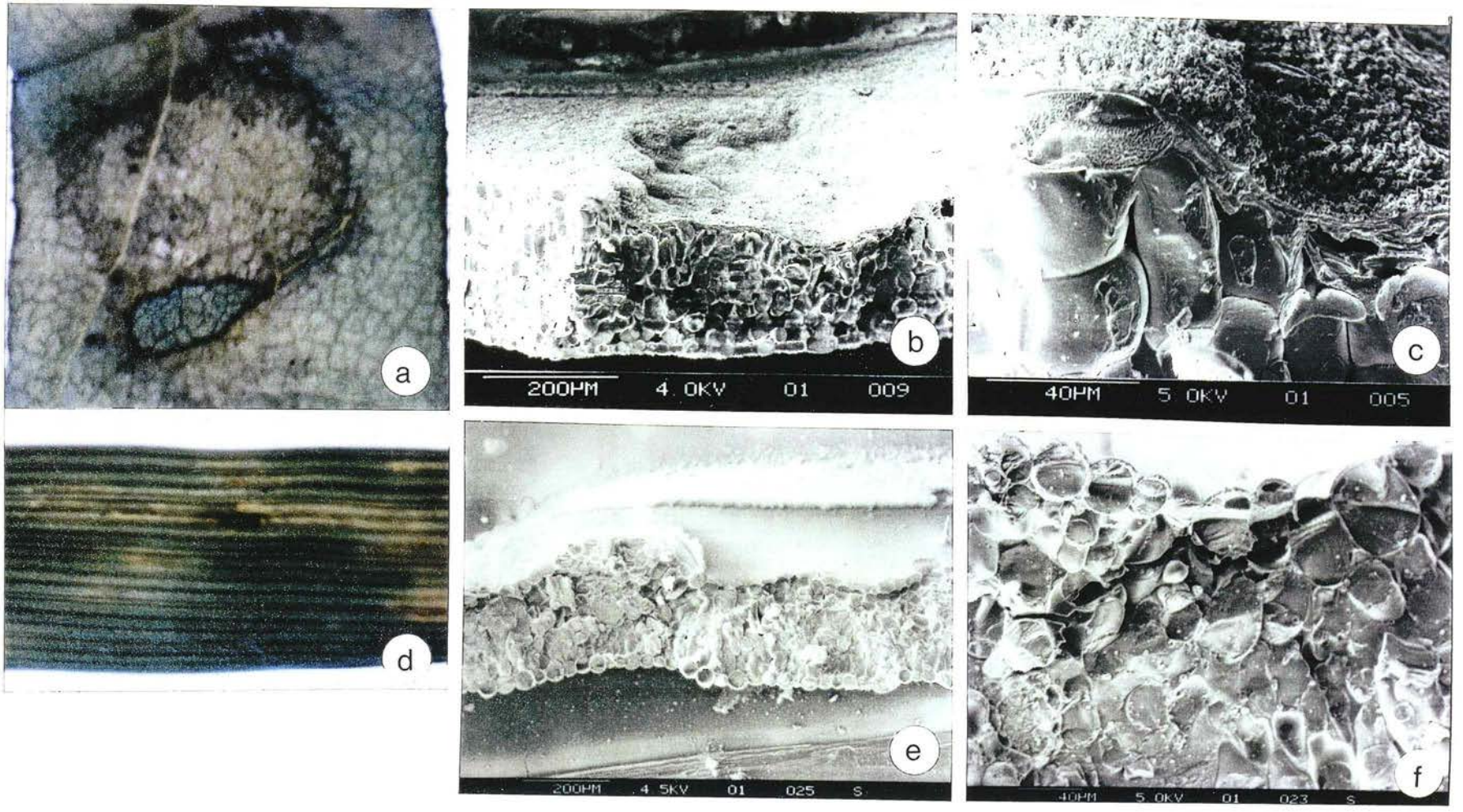
b. An area of *B.napus* leaf tissue showing similar symptoms to those shown in plate **a.**, following freeze fracture through the treated area, as seen by LTSEM. Scale is given on the plate.

c. The boundary between the treated area and untreated area shown in plate **b.** The damage to the epidermal cells in the treated area is visible.

d. Symptoms produced by the application of culture extract of *A.brassicae* (Ab4) to leaf tissue of *Triticum*
aestivum cv. Armada. The magnification is approximately 15 times.

e. An area of *T.aestivum* leaf tissue treated as for the *B.napus* leaf tissue in plate **b.**

f. A view of wheat leaf cells corresponding to the *B.napus* tissue shown in plate **c.** In contrast to the *B.napus* tissue it can be seen that the epidermal cells in the wheat tissue have mostly retained their shape.


a
b
200PM 4.0KV 01 009
c
40PM 5.0KV 01 005
d
e
200PM 4.5KV 01 025 S
f
40PM 5.0KV 01 023 S

Discussion

During the 30 day incubation period there was a general tendency for culture pH to increase slightly while qualitative TLC revealed, especially in the case of fluorescent metabolites, that the metabolite profile of each isolate altered during the culture period. In contrast however, there was no common pattern of change over time with respect to the dry weight of extract obtained from cultures. The results may indicate that maximum accumulation of metabolites occurred by 10 days after inoculation.

Although there was generally no overall increase in the dry weight of extracts, the number of metabolites present increased over time with all isolates except *A.brassicicola*. *L.maculans* was also exceptional in that it produced comparatively few fluorescing metabolites and a large number of u.v.-quenching metabolites. The number of u.v.-quenching metabolites generally did not increase with culture age.

Of the standard *Alternaria* metabolites which were available for comparison, only AME was found to be produced. Although production of AME is common in *A.alternata* isolates (King & Schade, 1984) neither of the *A.alternata* isolates tested here produced AME. *A.solani* and *A.brassicicola* produced detectable amounts of AME at 10 days but not later in the culture period, a finding similar to that of Wei & Swartz (1985) who found that maximum production of AME by *A.alternata* occurred in synthetic liquid culture at 10 - 14 days. In contrast, detectable levels of AME were produced by *A.brassicae* only at 20 days, indicating a difference in the behaviour of this species in culture from the others.

Considering the overall profiles of the fungi, *A.brassicicola* was distinguishable from the other species in that the isolate examined produced few metabolites, compared with the other species. In comparing *A.alternata* and *A.brassicae*, which were represented by more than one isolate each, it was noted that certain metabolites were characteristic of each species, which may indicate fundamental differences in metabolism between these species. In a previous comparison of several *Alternaria* species (Cotty & Misaghi, 1984) *A.brassicae* and *A.brassicicola* were found to differ from *A.solani* in that they did not produce zinniol in culture, perhaps indicating the existance of metabolic pathways in *A.solani* which are not present in the brassica pathogens.

Several *Alternaria* species have previously been reported to inhibit the growth of other fungi. In *A.cinerariae*, *A.tomato*, and *A.solani* this activity has been correlated to the production of antifungal metabolites (Brian *et al.*, 1951; King & Schade, 1984), while Wu & Lu (1984) found that one of four isolates of *A.alternata* was antagonistic to *A.brassicicola*, although the effect was not necessarily the result of antifungal metabolites produced by *A.alternata*. The present study identified an interesting diversity within and between the species examined with respect to antifungal metabolites. With three of the isolates which produced zones of antifungal activity the R_F of inhibition zones corresponded to the R_F of metabolites separated by TLC, implicating compounds in these bands as the active components. However, in all of the isolates which inhibited growth of *Cladosporium* sp. there was a zone of inhibition which did not contain any metabolites visible by longwave or shortwave u.v. illumination. It may be the case that the active component in the inhibition zones which did show either flourescence or quenching is not the compound responsible for the reactions to illumination. In order to determine whether the toxicity in each band is associated with a metabolite which reacts to u.v. illumination it would be neccessary to separate each band in 2 dimensional TLC and repeat the bioassay. Although there is no strict relationship between the behaviour of compounds under u.v. illumination, there is a tendency for those which quench u.v. to have biological activity (T. Simpson, University of Bristol, pers. comm.). The possibility that the active compounds are general cell toxins or self inhibitors is suggested by the fact that antifungal activity was detected only in extracts from 30-day-old cultures. Thus, either the compounds responsible for inhibition did not occur in the culture fluid and actively growing mycelium at 10 or 20 days, or they had not accumulated to sufficient concentrations at these times to produce an inhibitory effect in the TLC bioassay.

The results of the phytotoxicity test also suggested that at least part of the toxic activity in the extracts might have resulted from general cell toxins which accumulated in the idiophase of culture. This is suggested by the increase in phytotoxicity noted for most isolates in extracts from 30 day old cultures as compared with extracts from younger cultures. However, obvious phytotoxic symptoms were evident in extracts from younger cultures and a number of potent phytotoxins which have been reported to be produced in culture by *Alternaria* species occur after similar periods of culture to those used in the present study (Brian *et al.*, 1951; Janardhanan & Husain, 1983; Bains & Tewari, 1987). It is therefore possible that the toxicity of the extracts resulted from compounds which have some involvement in pathogenesis. The

phytotoxicity of *A.brassicae* extracts to wheat leaf pieces noted here is in keeping with the results of previous workers (Degenhardt, 1978; Bains & Tewari, 1985; McKenzie *et al.*, 1988) who have reported non host specific toxicity from crude extracts of this species. The compounds which give rise to this toxicity have neither been identified nor isolated, and their production by the fungus *in vitro* does not provide direct evidence that they are involved in pathogenesis. Recent studies by Buchwaldt & Green (1992) have shown that destruxin b, which is considered to be a virulence factor in *A.brassicae* is not host-specific in its activity.

Experiment 3.2: Examination of *A.brassicae* and *A.brassicicola* for the production of tenuazonic acid (TeA) and alternaric acid (AcA).

Results

Tenuazonic acid

All of the isolates tested produced a shortwave u.v.-quenching band at approximately the correct R_F position for TeA in one dimensional TLC (Plate 3.2). However, when extracts were developed in two dimensional TLC only Ac5 was found to produce a band which matched the R_F position of TeA in the second phase and gave compatible colour reactions with the ethanolic ferric chloride and 2-4 DNP reagents. The R_F positions and reaction colours of metabolites and the TeA standard are shown in Table 3.5.

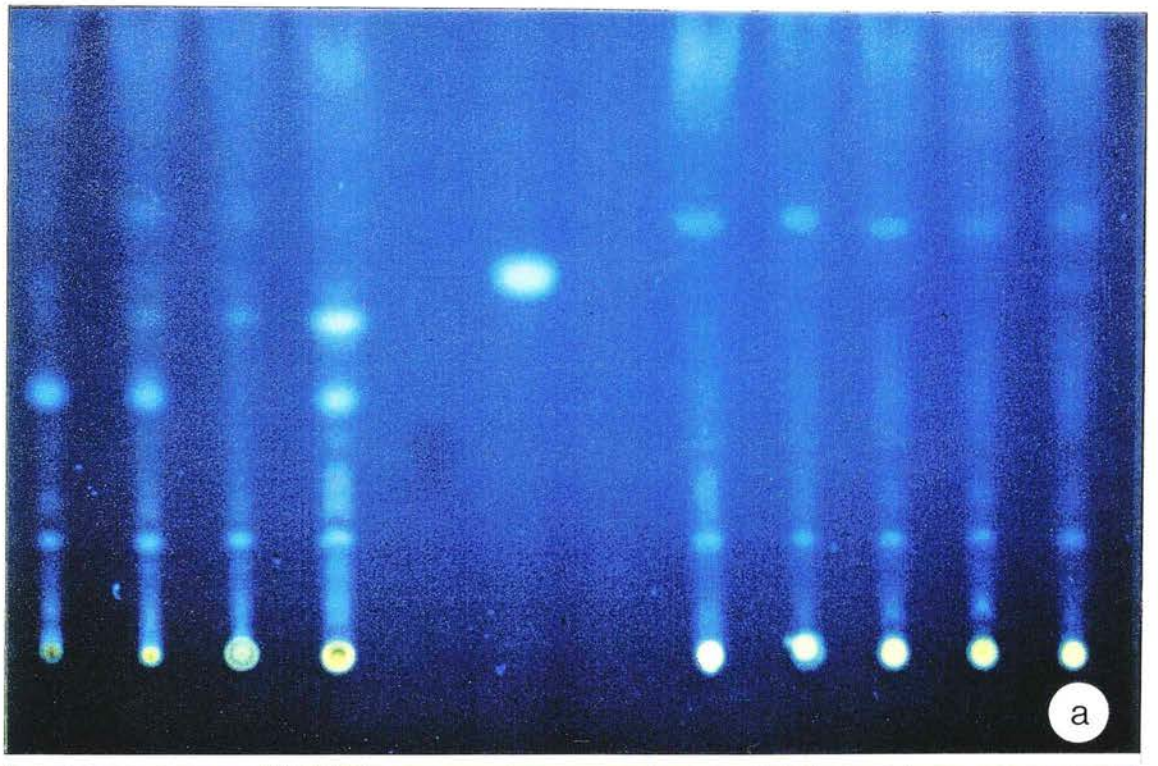
Table 3.5: R_F positions and reaction colours for metabolites from *A.brassicae* and *A.brassicicola* following two dimensional TLC of cholorform extracts of culture liquid.

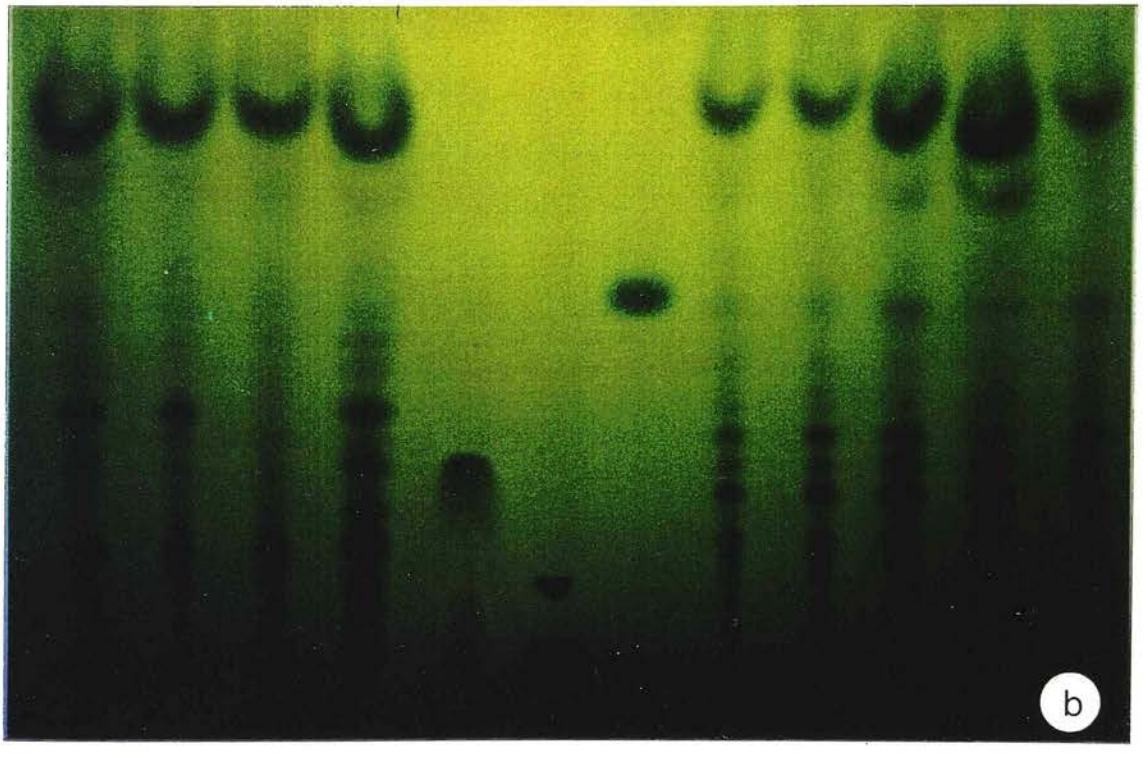
ISOLATE	R_F of possible TeA-matching bands		colour in reaction to plate spray reagents	
	1st phase	2nd phase	EtOH/FeCL3	2-4 DNP
Ab1	0.39 - 0.41	0.30 - 0.33	light brown	grey/purple
Ab2	0.39 - 0.42	0.30 - 0.33	light brown	grey/purple
Ab3	0.40 - 0.42	0.30 - 0.32	light brown	grey/purple
Ab4	0.38 - 0.41	0.30 - 0.33	light brown	grey/purple
Ac1	0.40 - 0.42	0.61 - 0.62	no reaction	red
Ac2	0.40 - 0.42	0.60 - 0.61	no reaction	red/orange
Ac3	0.41 - 0.43	0.61 - 0.62	no reaction	red
Ac4	0.40 - 0.42	0.61 - 0.63	no reaction	red
Ac5	0.40 - 0.42	0.50 - 0.51	orange	yellow
TeA	0.41 - 0.42	0.50 - 0.51	orange	yellow

Alternaric acid

All isolates produced a viscous red or brown residue after chloroform extraction of acidified culture liquids, as described by Brian *et al.* (1949; 1951). However, crystalised deposits of AcA could not be recovered from the hot CCl_4 residue for any isolate.

Plate 3.2: Fluorescing and u.v.-quenching bands produced by four isolates of *A.brassicae* **(Ab1,Ab2, Ab3, Ab4) and five isolates of** *A.brassicicola* **(Ac1,Ac2,Ac3,Ac4,Ac5) after one dimensional TLC.**


a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Metabolite profile

The metabolite profiles of each of the isolates are shown in Plate 3.2. Considering fluorescing metabolites, there was a high degree of intra-specific homogeneity in *A.brassicae* and *A.brassicicola*, but little interspecific similarity. However, both species produced a green fluorescing metabolite between R_F 0.58 and 0.62. In keeping with the findings of the previous experiment, *A.brassicae* was found to produce a more complex array of fluorescing metabolites than *A.brassicicola*. With respect to u.v.-quenching metabolites, a number of bands were found to be common to both *A.brassicae* and *A.brassicicola*. The majority of these metabolites were concentrated between R_F 0.3 and 0.6, although all isolates except Ab4 produced a large band between 0.68 and 0.82. In addition to a similarity between the species with respect to their u.v.-quenching metabolites, they showed a high degree of similarity with respect to metabolites which gave a positive reaction to PACS spray reagent. The majority of PACS positive metabolites were found between R_F 0.05 and 0.4, at lower R_F values than the fluorescing and u.v.-quenching metabolites. Six of the nine PACS-positive bands found were shared by eight of the nine isolates. Ab2 was the most individual isolate, having only only one PACS-positive band, which, moreover, did not match those produced by any of the other isolates (Figure 3.7). The PACS reagent detects a wide range of metabolites but with respect to the *Alternaria*, positive reactions may be indicative of the presence of metabolites produced concurrently with the destruxins; destruxin B is difficult to reveal directly in TLC (Ayer & Pena-Rodriguez, 1987a).

Phytotoxicity Test

Observed phytotoxicity indices for the isolates are shown in Figure 3.8 (see also Appendix 3.4). Examination of orthogonal contrasts indicated that the fungal extracts, on average, had a higher phytotoxic index than the control treatment ($F < 0.001$), and the *A.brassicae* isolates had a significantly higher average index than the *A.brassicicola* isolates ($F < 0.001$). Furthermore, there was less intraspecific variation in the sample of *A.brassicae* isolates than between the *A.brassicicola* isolates. Thus, the phytotoxicity index for *A.brassicae* had a minimum of 2.0 and a maximum of 3.3, while with *A.brassicicola* the range was from 0.3 to 2.5.

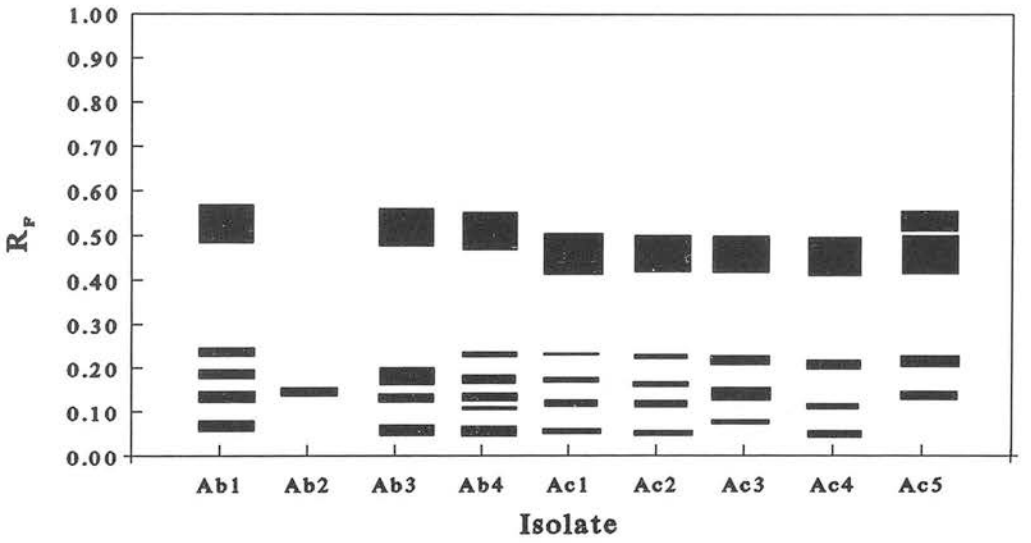


Figure 3.7: Positive bands produced by four isolates of *A.brassicae* (Ab1, Ab2, Ab3, Ab4) and five isolates of *A.brassicicola* (Ac1, Ac2, Ac3, Ac4, Ac5) on TLC plates after developing with PACS spray reagent.

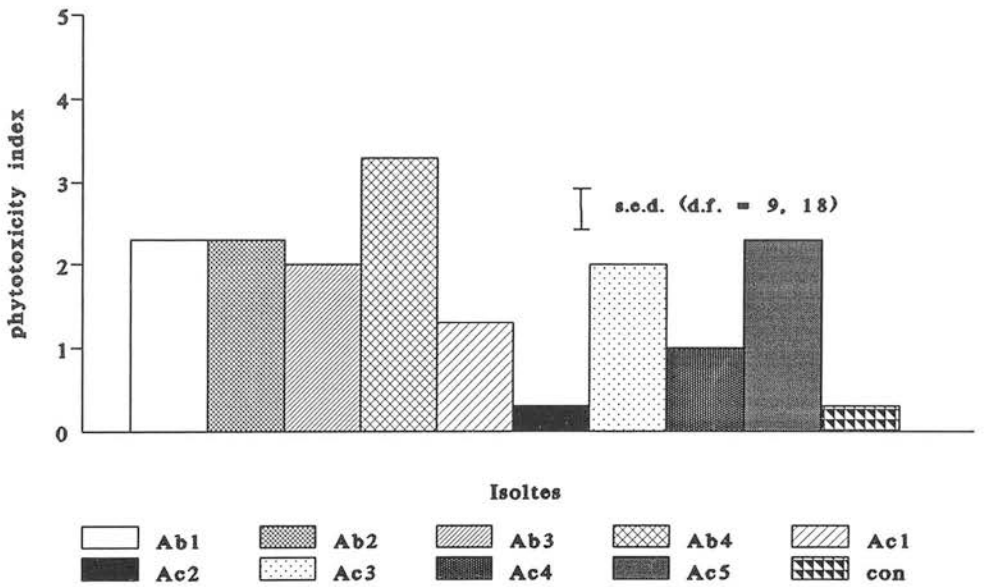


Figure 3.8: Phytotoxicity indices for four isolates of *A.brassicae* (Ab1, Ab2, Ab3, Ab4) and five isolates of *A.brassicicola* (Ac1, Ac2, ac3, Ac4, Ac5) on leaf disks of *Brassica napus*.

Discussion

There have been few studies of the production of common *Alternaria* metabolites by *A.brassicae* and *A.brassicicola*. Tests for their ability to produce AcA have not previously been reported, and although the present study examined only a few isolates of each species the results suggest that the ability to synthesise this compound may not be widespread in these species. Brian *et al.* (1951) found that AcA apparently accumulated with increasing culture age, at least until 30 days after initiation in cultures of *A.solani*. However, these workers also found that production of AcA (as estimated by a fungal growth inhibition bioassay) was greater at higher sucrose concentrations than those used in the present study. This notwithstanding, measureable concentrations of AcA were produced by *A.solani* in media with the same sucrose content as that used here. Tenuazonic acid has previously been found to be produced by *A.brassicae* and *A.brassicicola* (Bruce *et al.*, 1984) and was produced by one isolate of *A.brassicicola* in the present study.

Some similarity in the metabolite profiles, particularly with respect to the PACS-positive bands of from *A.brassicae* and *A.brassicicola* was of interest. Ayer & Pena-Rodriguez (1987b) isolated a number of novel non-phytotoxic sesquiterpenoid metabolites from *A.brassicae*; one of the major components of the group, desmethyldestruxin b, was identified initially by its reaction with PACS on TLC plates. The results of this study indicate that *A.brassicae* and *A.brassicicola* may share a metabolic pathway which gives rise to such metabolites. Although the compounds apparently have no phytotoxic activity they may be important to these species in some respect other than their common pathogenicity to *Brassica* species.

The phytotoxicity of extracts of *A.brassicae* in this experiment was similar to that in the previous experiment. The simultaneous production of toxic effects on leaf tissue and the lack of TeA and AcA in the extracts from *A.brassicae* suggest that these toxins are not involved in pathogenesis by this species. Confirmation of this suggestion will require the large scale screening of isolates of *A.brassicae* and studies of the occurrence of these metabolites in infected tissue. Interestingly, the single isolate of *A.brassicicola* which did produce TeA had a higher phytotoxic index than the other isolates. Among the other four

A.brassicicola isolates there was a considerable variation in phytotoxic activity, with Ac3 having a phytotoxic index almost as high as that of the TeA-producing isolate, AC5. A primary role for TeA in pathogenesis by *A.brassicicola* therefore seems doubtful. However, in isolates which produce the compound it may contribute to aggression or act as a secondary determinant of pathogenicity. As with *A.brassicae*, the elucidation of the precise role of TeA in pathogenesis of *A.brassicicola* will require large scale studies of the occurrence of the compound in infected tissue.

Experiment 3.3: Metabolites produced by *A.brassicae* and *A.brassicicola* in leaf disks of *Brassica napus*.

Results

TeA and other metabolites visualised by TLC

Table 3.6 shows the R_F positions and colours of the bands present in the extracts from leaf disks infected with each fungus. Neither TeA (R_F = 0.40 - 0.42, u.v. quenching) nor AME (R_F = 0.63 - 0.65, blue fluorescence)) was found in extracts from the infected leaf material for either pathogen. However, a fluorescing band at R_F 0.28 - 0.31, which showed brown fluorescence, matched the position of a fungistatic band produced by *A.brassicicola* in Experiment 3.1 (Figure 3.5). Of the bands which were detected in extracts from *A.brassicae*-infected leaf material, three of the four fluorescing bands matched metabolites previously found in extracts from this isolate grown *in vitro* (Figure 3.3). A green fluorescing band at R_F 0.34 - 0.36 matched a fungistatic band produced by *A.brassicae* in Experiment 3.1.

Alternaric acid

As in Experiment 3.2, although a red/brown residue was obtained from drying the chloroform extract, AcA could not be recovered when recrystallisation from hot CCl_4 was attempted.

Table 3.5: Comparison of R_F and colours of compounds produced in leaf disks of *Brassica napus* infected with *A.brassicae* and *A.brassicicola*.

ISOLATE	R_F and colour of fluorescing bands	R_F of quenching bands
A.brassicae (Ab4)	0.18 - 0.20 green *	0.23 - 0.25
	0.26 - 0.28 pale green *	0.27 - 0.29
	0.28 - 0.29 blue	0.36 - 0.37
	0.34 - 0.36 green *	
A.brassicicola (Ac5)	0.19 - 0.21 green	0.23 - 0.24
	0.25 - 0.27 pale green	
	0.28 - 0.31 brown *	
	0.33 - 0.35 green	
	0.35 - 0.37 brown	
TeA	**0.41 - 0.42**	

* Bands which matched R_F and colour of metabolites produced *in vitro*.

Discussion

A number of bands visible after development of extracts from infected leaf tissue by TLC resulted from the presence of the *Alternaria* species in the tissue. In the case of *A.brassicae*, three of the bands detected may have been produced by the pathogen since they matched bands produced by the same isolate *in vitro*. With *A.brassicicola*, one band was detected in infected leaf tissue which was also present in extracts from the axenic cultures of the pathogen. It cannot be determined from the results of this study whether the *Alternaria* metabolites detected in the infected material have a role in pathogenicity or are simply compounds present in detectable quantities, but which have no toxic effect. The remaining bands detected in extracts from infected tissue may contain compounds produced either by the pathogens, the plant, or which resulted from the interaction of the host and pathogen. However, these bands did not correspond to metabolites produced by either isolate *in vitro*.

Rudolph (1976) discussed some of the possible reasons for the difficulty encountered in isolating phytotoxins from infected plant material. One of the most plausable explanations is that the toxic compound is physiologically active at concentrations which are too low to be detected by standard

extraction and chromatography procedures. This may explain the negative results obtained in the present study for the presence of TeA in extracts from *A.brassicicola*-infected tissue. Considering the production of metabolites *in planta* by *A.brassicae*, recent studies which utilised HPLC have shown that destruxin b and other destruxins are produced in infected plant tissue (Buchwaldt & Jensen, 1991).

With respect to the identity of the unknown metabolites which were produced in infected material, several phytoalexins are known to be produced by *B.napus* in response to infection by *A.brassicae* (Conn *et al.*, 1987). These compounds, however, all show characteristic bright blue fluorescence under u.v. illumination, and none of the bands produced in this study showed this type of fluorescence.

3.4 GENERAL DISCUSSION

The *Alternaria* are known to produce a diverse range of secondary metabolites. While products of the polyketide pathway are produced in many species, the diversity of chemical structures found in phytotoxic metabolites alone suggests that there is considerable variation between species in the detail of their secondary metabolism (King & Schade, 1984; Stinson, 1985). Few species have been examined with a view to characterising their metabolite profiles; studies have predictably concentrated on the production of biologically active metabolites. In this section the biological activity of *Alternaria* secondary metabolites has been considered in relation to their possible roles as mycotoxins, antibiotics with fungicidal activity, and phytotoxins of hosts and non-hosts. Some of the practical, ecological, and pathological implications of the results may now be considered.

It is likely that the low cytotoxicity of the extracts examined in the present study indicated the absence of mycotoxins in the extracts since the bioassay method is reputedly more sensitive than TLC in the detection of toxins in contaminated feedstuffs (Robb & Norvall, 1985). Generally *Alternaria* mycotoxins have a lower toxicity than mycotoxins produced by other toxigenic genera such as *Penicillium*, *Aspergillus* (King & Schade, 1984) and *Fusarium* (McKenzie, Robb & Lennard, 1987). The lack of cytotoxicity in the *Alternaria* has no obvious ecological significance for the species under investigation since the group is not associated parasitically with animals. In relation to their presence as contaminants of animal feedstuffs, the results are in keeping with previous findings that they do not present a cytotoxicity risk, although McKenzie *et al*. (1988) did demonstrate some cytotoxic activity from fungal extracts.

The results of the study also provided no evidence that antibiotics play a significant role in the ecology of the *Alternaria* species examined. Fungitoxic activity was noted only in extracts from older cultures and was presumably associated with the production of staling products in the stationary phase of culture. It should be stressed however, that only one test organism, *Cladosporium*, was assessed.

Production of destruxins *in planta* by *A.brassicae* (Buchwaldt & Jensen, 1991; Buchwaldt and Green 1992) provides evidence for the importance of phytotoxins in the pathogenicity of *Alternaria* species. The present study examined *A.brassicae* and *A.brassicicola* for the production of common non host specific

phytotoxins, TeA and AcA, since it has been reported that semi-purified extracts from both species cause non host-specific necrosis in leaf tissue bioassays. No evidence was found in these studies that either of the phytotoxins was present in leaf tissue infected by either pathogen. However, McKenzie *et al.* (1988) showed that crude culture filtrates from *A.brassicae*, *A.brassicicola*, and *A.alternata* had a non host specific activity. They also suggested, from the germination responses of brassica pollen, using the TLC bioassay technique of Hodgkin and MacDonald (1986), that the three *Alternaria* species produced different toxins.

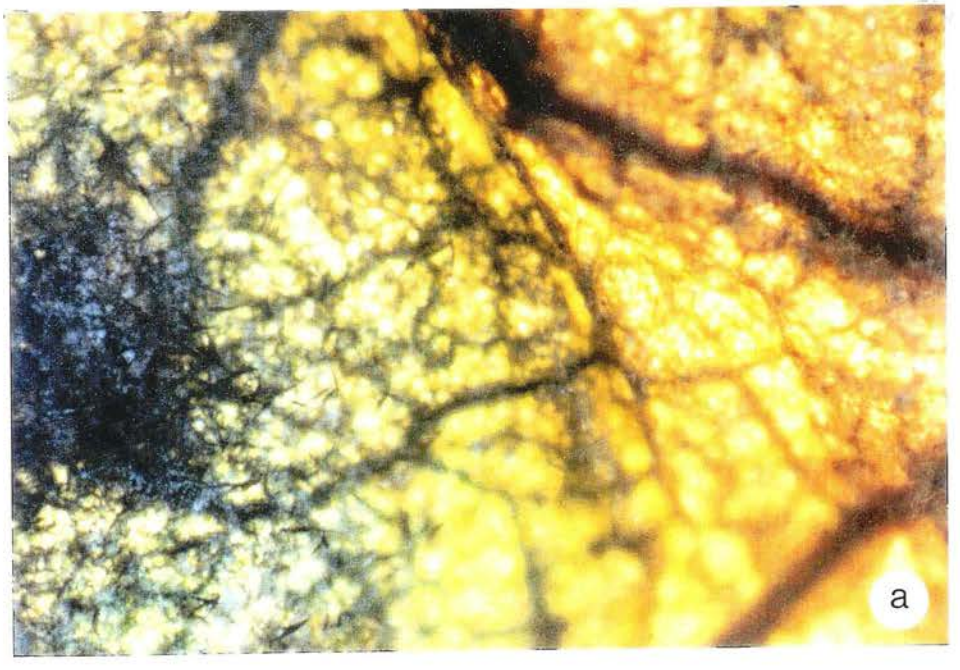
Qualitative TLC examination of *A.brassicae* and *A.brassicicola* in the present study has revealed both differences and similarities between these species in the metabolites which they produce *in vitro*, particularly with respect to compounds which quench U.V. radiation, and those which give a positive reaction to PACS spray reagent. This finding, when viewed in the context of the similarity in host range of the two species, suggests the need for detailed examination of *A.brassicicola* in order to establish whether it produces phytotoxins related to the destruxins produced by *A.brassicae in vitro* and *in planta* (Ayer & Pena-Rodriguez, 1987a; Buchwaldt & Jensen, 1991). Ayer & Pena-Rodriguez (1987b) found that the major non-phytotoxic metabolites produced by *A.brassicae* were sesquiterpenoids rather than polyketides. The difference in metabolism in *A.brassicae* from, for example *A.alternata* which produces mainly polyketide metabolites, may be related to the adaptation of *A.brassicae* to a particular host range. It would be desirable in this respect for the secondary metabolism of *A.brassicicola* to be examined in similar detail.

The higher phytotoxicity indices found for extracts from *A.brassicae* as compared with *A.brassicicola* in the present study may be related to a number of possible causes. For example, the difference in toxicity may have arisen because the compounds produced by the species are different (McKenzie *et al.*, 1988), or alternatively *A.brassicicola* may produce less toxin *in vitro*. Without a more detailed comparison of these two species, utilising purified toxins, it is impossible to discriminate between these possiblities. This unanswered question highlights one of the major problems in the use of crude culture filtrates in phytotoxin studies.

Research on the role of host specific toxins produced by the *Alternaria* and other members of the

Pleosporaceae has for many years indicated their involvement at an early stage in the interaction between the fungi and their hosts as suppressors of active defence (Comstock & Scheffer, 1973; Nishimura & Kohmoto, 1983a). Studies with AK toxin have resulted in the production of a theoretical model based on experimental observation which considers the toxin in such a role (Kohmoto *et al.*, 1987). Recent advances in the understanding of the importance of simple divalent cations, particularly Ca^{2+}, in a variety of cell functions including resistance responses (Kunoh, 1991) have complemented the development of this proposed role for HSTs. It appears that in some cases HSTs may suppress resistance by inducing electrolyte leakage which, in turn, may lead to a reduced resistance response for a number of secondary reasons (Kunoh, 1991). The proposed role for HSTs then, is more subtle than earlier suggestions that the electrolyte leakage induced in the cells made nutrients available to the developing fungal hyphae, and that the toxins killed cells in advance of colonisation to allow necrotrophic feeding. If the HSTs are not to cause widespread cell damage it would expected that they would be localised around growing hyphae and have low mobility within plant tissue. The freeze-fracture LTSEM studies conducted here suggest that toxins present on the leaf surface migrated only as far as the epidermis and upper mesophyll, supporting the hypothesis that they would act locally to hyphae invading the tissue. Buchwaldt and co-workers reported that destruxin B is produced by germinating conidia of *A.brassicae* (Buchwaldt & Green, 1992) and is present in the uncolonised tissue in advance of fungal hyphae (Buchwaldt & Jensen, 1991). This pattern of toxin and pathogen distribution was also recorded by Tewari (1983), who suggested that the *A.brassicae* was not present in the green tissue surrounding lesions. However, observations made in the present study during the isolation of naturally infected *B.oleracea* leaves, indicated that the pathogen may be present and sporulate in the green tissue close to lesions (Plate 3.3). There is a need for accurate microscopic examination of infected tissue to determine the exact physical relationship between the pathogens and the most remote tissue from the point of inoculation which shows signs of toxin damage. It is, however, tentatively suggested that there is a requirement for an initial period during growth in the host plant when resistance responses are supressed to allow colonisation of tissue. The role of the HST in this behaviour would be to block resistance responses such as phytoalexin accumulation.

Plate 3.3: Sporulation by *A.brassicae* **in green tissue around a naturally occurring lesion on** *Brassica oleracea*.


a

Buchwaldt & Green (1992) published an examination of the production of destruxin-B during spore germination and germ-tube growth of *A.brassicae*. Production of HSTs by germ-tubes and germinating conidia has been emphasised as an essential step if pathogenesis is to successfully rely on the suppression of resistance by the toxin. Buchwaldt & Green concluded that the concentration of the toxin produced by individual conidia was too low to induce the formation of visible lesions. However, there is circumstantial evidence that compounds produced by germinating spores and germ-tubes of *A.brassicae* interact with potential host plants. Thus, Jejelowo *et al.* (1991) found that phytoalexins accumulated in leaf surface inoculum droplets containing conidia of *A.brassicae* following inoculation of the resistant crucifer *Camelina sativa*, even though penetration was almost absent. It is possible that destruxin b acts as an elicitor of defence responses because of a low toxin sensitivity of the target cells. Buchwaldt & Green (1992) found that *C.sativa* had a lower sensitivity to destruxin B than *Brassica* species in a droplet application assay. A low level of damage in the challenged cells may allow them to respond by the production of phytoalexins and the stimulation of phytoalexin production in neighbouring cells. In plants which are more sensitive to the toxin, although the toxin may still act as an early elicitor of defence reactions, the defence reactions are also suppressed by the toxin. Bailey & O'Connell (1989) have indicated that phytotalexin production occurs when living and dead plant cells are in contact and for many pathogens it is the timing and extent of cell death which are crucial in determining the success of colonisation. At one extreme a successful hypersensitive response allows surrounding tissue to respond quickly and for a considerable time, whereas in an expanding lesion living cells may begin to respond but resistance is suppressed before the response becomes fully active.

In the model of the possible biochemical interactions between *Alternaria* species and their host cells presented in section 2.4 it was suggested that the toxin may act as a determinant of pathogenicity. The toxins may act by inducing membrane leakage which leads to suppressed structural and biochemical resistance responses. For example, from studies of cultured safflower cells Titjen & Matern (1984) proposed that Brefeldin A, produced by *A.carthami*, may act as a determinant of pathogenicity by suppressing phytoalexin production in safflower. These workers used an elicitor purified from the walls of *A.carthami* (a pathogen of safflower) and a similar elicitor from cell walls of *Phytophthora megasperma* (a non-pathogen of safflower) to elicit the production polyacetylenic phytoalexins in safflower cells.

Production of the phytoalexins was inhibited by the addition of Brefeldin A, produced by *A.carthami*.

In the simplest model of interactions between *A.brassicae* and *A.brassicicola* and their hosts the toxin(s) could act as the elicitor of resistance in both resistant and susceptible tissue but have a dual role as a suppressor of resistance in susceptible tissue only, as described above. However, the results of Chapter 2 suggest that non-pathogens elicit similar defence reactions as the brassica pathogens implying that resistance may not be elicited by toxins produced by *A.brassicae* and *A.brassicicola*. It is currently unclear how resistance is triggered in host plants of *A.brassicae* and *A.brassicicola*. However, it is known that phytoalexins accumulate in both susceptible and resistant plants. The interaction between toxins produced by the pathogens and plant cells needs to be examined in more detail.

The majority of toxin producing fungal pathogens, including the *Alternaria*, belong to the family Pleosporaceae, or to anamorph-genera which are probably connected to this family. These fungi are ubiquitous group of facultative pathogens or facultative saprophytes, many of which (similarly to *A.brassicae* and *A.brassicicola*) cause serious leaf spot diseases in important crop plants, while others are almost obligate saprophytes. Scheffer (1989) has proposed that these opportunistic fungi have some of the attributes of more specialised pathogens, including the ability to penetrate living tissue, and has indicated examples of such fungi that have become highly virulent when they aquire new or recombined genetic material. The results of this study support Scheffer's proposal. Thus in Chapter 2 a number of *Alternaria* species with different host ranges and degrees of specficity were all found to produce appressoria and to attempt to penetrate leaves irrespective of whether the leaves were of host or non-host tissue. Phytotoxins would apppear to be implicated as determinants of pathogenicity or virulence. The evolution of specialised pathogens within the group may be due to changes in the secondary metabolism of ancestral forms and further study of the secondary metabolism of members of the group may reveal some idea of potential and disposition for adaptation to pathogenicity. Heiny & Gilchrist (1991) recently reported that *Stemphylium botryosum*, *S.versicarium*, and *Alternaria alternata* f.sp. *lycopersici*, but not *Ulocladium* sp. produce stemtoxin, a non host-specific peptide toxin, giving some indication of a similarity in metabolism between the related species. In the next chapter the taxonomic relationships of a number of *Alternaria* species are explored with particular emphasis on secondary metabolites produced by *A.brassicae* and *A.brassicicola*.

4. TAXONOMY

4.1 INTRODUCTION

A discussion of the taxonomic position of the Alternaria *anamorph-genus.*

The early nomenclature and the establishment of the type material for the *Alternaria* anamorph-genus have been discussed by Wiltshire (1933) and by Simmons (1967). The name *Alternaria* was introduced by Nees in 1817. The new genus which contained a single species, *Alternaria tenuis*, was not recognised by Fries, who grouped it with *Torula* in *Systema Mycologicum* in 1832. Early confusion of nomenclature resulted from the lack of clear descriptions for *A.tenuis* and the various species of *Macrosporium* which Fries established in 1832. The confusion continued when species were erroneously allocated to the genera *Ulocladium* and *Stemphylium*. Despite the availability publications by, Wiltshire (1933; 1938) and Neergaard (1945) which clarified the situation, it was not until Simmons (1967) discussed the relative merits of the names *A.tenuis* and *A.alternata*, indicating that the latter is the more suitable for the type species of the "genus", that *Alternaria* obtained a type species proper. The name proposed by Simmons for the type species is:

Alternaria alternata (Fries) Keissler.

Simmons (1967) also illustrated the general features of conidium formation and highlighted the major differences between the conidia of the type species from *Alternaria*, *Stemphylium*, and *Ulocladium*. A tentative list of common *Alternaria* species published to date is given in Table 4.1, along with an indication of the normal host family for each species.

Neergaard (1945) discussed the morphological and developmental differences which separate *Alternaria* from *Stemphylium* emphasising the pattern of conidium development and the presence of a true or pseudo beak as valuable taxonomic characters. Simmons (1986c) has stressed the difficulties of using this type of morphological character to distinguish *Alternaria* species from each other, and has suggested that the pattern of septation during conidium development is probably of more use for differentiating species.

Table 4.1: ***Alternaria*** **species grouped alphabetically by host family**

A.gomphrenae	Amaranthaceae 1	*A.macrospora*	Malvaceae 12
A.multirostrata	Araceae 2	*A.papaveris*	Papaveraceae 13
A.dianthi	Carophyllaceae 3	*A.passiflorae*	Passiflorae 14
A.dianthicola		*A.sesami*	Pedalaceae 15
A.carthami	Compositae 4	*A.alternata*	Plurivorous 16
A.cinerariae		*A.longissima*	
A.denisii		*A.infectoria*	
A.helianthi		*A.tenuissima*	
A.helianthificiens		*A.anagallidis*	Primulaceae 17
A.leucanthemi		*A.fragariae*	Rosaceae
A.tagetica		*A.kikuchiana*	
A.zinniae		*A.mali*	
A.brassicae	Cruciferae 5	*A.citri*	Rutaceae 18
A.brassicicola		*A.chlamydospora*	Saprophytic 19
A.ethzedia		*A.limaciformis*	
A.raphani		*A.mouchacce*	
A.cucumerina	Cucurbitacea 6	*A.phragmuspora*	
A.granulosa		*A.conjuncta*	Scrophulariaceae 20
A.pepinicola		*A.photistica*	
A.angustiovoidea	Euphorbiaceae 7	*A.alternata* f.sp. *lycopersici.*	Solanaceae 21
A.euphorbia		*A.crassa*	
A.euphorbiicola		*A.longipes*	
A.ricini		*A.solani*	
A.padwickii	Gramineae 8	*A.cichorii*	Umbelliferae 22
A.triticina		*A.dauci*	
A.triticola		*A.petroselini*	
A.cassiae	Leguminoseae 9	*A.radicina*	
A.eureka		*A.ramulosa*	
A.porri	Liliaceae 10	*A.molesta*	Phoceneae 23
A.linicola	Linaceae 11		

The type of detailed developmental study proposed by Simmons (1986c) may be a requirement for differentiating species in which the mature populations of conidia are so heterogeneous in their morphology that differentiating them on the basis of the appearance of mature spores is problematical. Within the *Alternaria* anamorph-genus the most common problems of taxonomy and identification relate to the large number of prevalent, plurivorous anamorphs which resemble *A.alternata* (Neergaard, 1945; Simmons, 1986c; 1990). For example, Simmons (1981) discussed the difficulties in distinguishing *A.longipes* from *A.alternata* in relation to the occurrence of both species on tobacco; Simmons was emphatic that the two

species were distinct. Japanese research into the ecology of toxin-producing *Alternaria* anamorphs has suggested that previously differentiated species (including *A.citri*, *A.mali*, and *A.longipes*) may be pathotypes or, *formae speciales*, of *A.alternata*, although the isolates in question have not been subjected to rigorous morphological examination (Nishimura & Kohmoto, 1983a; b). However, Simmons (1990) has criticised this approach and has suggested that *A.citri* is quite distinct from the *A.alternata* group species which are also found on citrus species.

Nishimura & Kohmoto (1983a; b) have advanced an interesting hypothesis on the evolution of *formae speciales* of *A.alternata* which are distinguished by the production of various host specific toxins. The concept of the *forma specialis* (f.sp.) is well established in plant pathology. *Formae speciales* occur in many of the important groups of fungal pathogens, from the biotrophic rusts and powdery mildews, to the facultative pathogens such as species of *Fusarium* and *Verticillium* which are closer in their ecological behaviour to *Alternaria*. In the biotrophic pathogens, the concept of the f.sp. is used to distinguish between morphologically identical forms which differ with respect to host range. The genetic control of compatibility (as far as this is understood) seems to be similar between pairs of ff.sp and hosts; for example *Erysiphe graminis* f.sp. *hordei*/Barley Mla, Mlg loci, and *E.graminis* f.sp. *tritici*/Wheat Pm loci (Hiura, 1978; McIntosh, 1978). As a consequence, the physiology and biochemistry of pathogenicity/resistance is similar for different f.sp./host combinations.

Arguably, the ff.sp found in anamorph-genera such as *Fusarium*, *Verticillium* and those proposed for *Alternaria* are of a very different nature to those described in the rusts and powdery mildews. The genetic differences between different toxin producing *A.alternata*-like isolates are not known. The toxins, which certainly determine host specificity, are a diverse group of chemical types, and have an equally wide range of sites and modes of action (Nishimura, 1987). Presumably resistance is determined by diverse genetic means in the various hosts (Table 3.2). However, the isolation of strains of "*A.alternata*" which are able to synthesise two host specific toxins suggests either these may well be ff.sp. of a single species, or anamorphs of different species which are able to achieve genetic recombination. Interestingly, different species of the related genus *Bipolaris* are able to mate. *Bipolaris carborum* and *B.victoriae* are specific pathogens of maize and oats respectively; pathogenicity in both cases is determined by the production a host

specific toxin. Experiments conducted in the 1960s established that offspring from sexual crosses between these species gave rise to a number of isolates which produced both toxins. These isolates, however, had a reduced general fitness (Scheffer, Nelson, & Ulllstrup; 1967). A similar relationship may exist between toxin producing isolates of *Alternaria*. However, sexual recombination has never been recorded even within these putative ff.sp., far less between them, either in nature or under laboratory conditions. Nevertheless, suitable recombination events could occur between toxin producing strains in the absence of sexual recombination, through the formation of heterokaryons or by a parasexual cycle. The uncertainties about the identity of *A.alternata*-like toxin-producing isolates are likely to remain until extensive, comparative morphological and/or biochemical studies are conducted. Simmons (1990) has recently examined this question with reference to *Alternaria* species isolated from citrus hosts. Simmons found that *A.citri* was a morphologically distinguishable species which occurred relatively infrequently on citrus hosts. In adddition to *A.citri*, and of more common occurrence, were a number of species which belonged to the *A.alternata/A.tenuisssima* complex.

Identification of most *Alternaria* species, outwith the *A.alternata* group, is relatively simple provided the source substrate is known and the isolated *Alternaria* species can be induced to sporulate. This notwithstanding, there was considerable confusion over distinguishing *A.brassicae* from *A.brassicicola*, during the last century and the first few decades of this century. Neergaard (1945) reviewed many of the early reports of the two pathogens and gave a brief discussion of their separation into two clearly described species. Some of the confusion appears to have resulted purely from lack of convention on nomenclature. For example, it is clear from his description that *A.brassicicola* was the causal agent in Weimer's (1924) report of damage to cauliflower, which he attributed to an *Alternaria* species named as *A.brassicae.* Two years later Weimer (1926) reported damage to cruciferous plants by a different *Alternaria* species which he gave as *A.herculea* (the pathogen in question being *A.brassicae*). Neergaard (1945) noted that this difference in the naming system was still common in the U.S.A when his monograph of Danish species of *Alternaria* and *Stemphylium* was published in 1945. The name *A.brassicicola* (Schweinitz) Wiltshire was established by Wiltshire in 1947. Wiltshire (1947) also provided the final version of the name *A.brassicae*; *i.e. A.brassicae* (Berkshire) Saccardo. Neergaard (1945) had dealt with *A.brassicicola* under the name *A.circinans* (Berk. & Curt.) Bolle, and suggested *A.brassicae* (Berk.) Bolle for *A.brassicae*.

The problems which surround the classification of the *Alternaria* anamorph-genus are shared by many of the Fungi Imperfecti, the essential difficulty being that morphological similarity does not necessarily entail a close genetic relationship between two species; this point has been discussed by Kendrick (1981b). It is also illustrated in the context of anamorph genera related to *Alternaria* with reference to Figure 4.1: It can be seen from Figure 4.1 that, on the basis of teleomorphs, *Alternaria* and *Phoma* are more closely related than *Alternaria* and *Ulocladium*. However, on the basis of anamorph morphology *Alternaria* and *Ulocladium* appear very similar, while *Phoma*, which produces pycnidia rather than naked conidia, is morphologically distinct. The taxonomic distiction between pycnidial and conidial fungi has been questioned, however. For example, Sutton (1981) has suggested that the mechanisms by which the conidia are produced are more important as taxonomic guides than the presence or absence of a wall around the conidiophores.

The true identity of an anamorphic fungus can be established only when the identity of the teleomorph is known. Two lists were published in 1979 which documented reported connections between Fungi Imperfecti and the Basidiomycetes (Kendrick & Watling, 1979), and the Fungi Imperfecti and the Ascomycetes (Kendrick & Di Cosmo, 1979). Table 4.2 gives teleomorphs which have been reported to have *Alternaria* anamorphs, and was compiled from the second of these lists and the references shown at the foot of the table.

All teleomorphs which have been reported to produce *Alternaria* conidial states are in the *Pleosporaceae*, many being segregates of *Pleospora*. The family *Pleosporaceae* has generated considerable disagreement among taxonomists on the subject of the number of segregates which should be recognised (Wehmeyer, 1961). Simmons (1986c) concentrated on a collection of morphologically similar *Alternaria* anamorphs which were derived from ascospore cultures, with one exception in which a conidium-derived culture produced mature ascomata. Simmons placed these teleomorphs in a new genus, *Lewia* Barr & Simmons, and discussed the difficulties in the nomenclature of the group of teleomorphs which have been found to produce *Alternaria* and *Stemphylium* anamorphs. He suggested that the name *Pleospora* should be

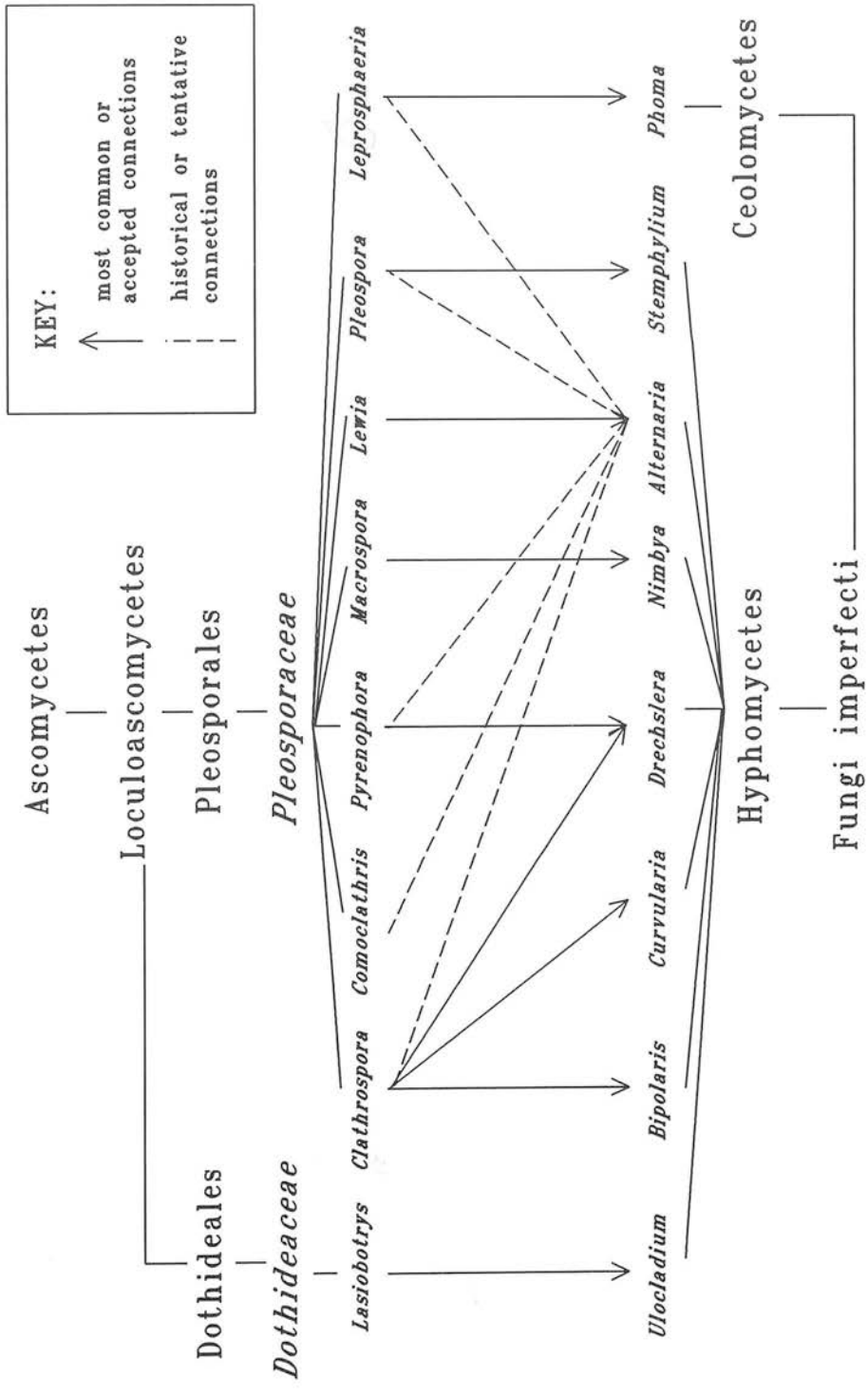


Figure 4.1: A representation of reported connections between genera in the *Pleosporaceae* and anamorph-genera.

Table 4.2: Reported teleomorph-anamorph connections involving *Alternaria* species

Teleomorph	Anamorph
Clathrospora diplospora	*A.alternata* (a)
C.elyne	
Comoclarthis planispora	*Alternaria* sp. (b)
Leptosphaeria heterospora	*A.alternata* (a)
Lewia conjuncta	*A.conjuncta* (c)
L.ethzedia	*A.ethzedia* (c)
L.eureka	*A.eureka* (c)
L.infectoria	*A.infectoria* (c)
L.photistica	*A.photistica* (c)
Pleospora flavo-fusca	*Alternaria* sp. (d)
P.infectoria (L.infectoria)	*A.alternata* (e)
	Alternaria sp. (d,f)
*P.orbicularis**	*Alternaria* sp. (d)
*P.scirpicola**	*Alternaria* sp. (g)
*P.scrophulariae**	*Alternaria* sp. (d)
*P.valesiaca**	*Alternaria* sp. (g)
Pyrenophora alternarina	*Alternaria* sp. (h)

(a) Simmons (1952) **(b)** Arx & Muller (1975) **(c)** Simmons (1986c)
(d) Kendrick & Di Cosmo (1979) **(e)** Bilgrami (1974) **(f)** Ellis (1971)
(g) Ellis (1976) **(h)** Whitehead & Dickson (1952)

* see text

applied to teleomorphs which produce *Stemphylium* anamorphs, and that maintenance of a number of segregates as separate genera would help in making the identity of *Pleospora* as compact as possible.

The species marked with an asterix in Table 4.2 have commonly been reported as anamorph states of *Pleospora* species (Wehmeyer, 1961; Ellis, 1976). Simmons (1989) erected a new anamorph genus, *Nimbya* to hold these, and separated their teleomorphs from *Pleospora* into the new genus *Macrospora*; utilising a defunct synonym of *Pleospora*. Conidia of *Nimbya* species are distinct from *Alternaria* in being distoseptate while those of *Alternaria* are euseptate. Figure 4.1 shows the current position of *Pleospora*, and several of its segregates with reported anamorphs, within the Ascomycetes, and the position of these anamorphs within the Fungi Imperfecti.

To date no teleomorphs have been reported for either *A.brassicae* or *A.brassicicola*. However, this situation does not represent a dilemma for pathologists as the two species are easily distinguished by colony growth characteristics and conidium morphology. For practical purposes a lack of knowledge about the phylogenic relationship between *A.brassicae* and *A.brassicicola* does not limit our ability to control them.

There has apparently been no published work which has dealt directly with the elucidation of phylogenic relationships between *Alternaria* species. Neergaard (1945) constructed three morphological sections within the anamorph-genus on the basis of conidium chain length and beak length, which remain as standard points of reference today. The Neergaard sections were:

(1) *Longicatenatae*: (*e.g. A.alternata*). Species which typically produce beakless or short-beaked conidia in long chains.
(2) *Brevicatenatae*: (*e.g. A.cheiranthi*, or *A.raphani*). Species which typically produce short chains of 3-5 conidia which have short to long beaks.
(3) *Noncatenatae*: (*e.g. A.solani*). Species which typically produce solitary conidia which have long beaks.

Ironically even this broad-brush approach to internal classification in the *Alternaria* was not without exceptions, and Neergaard placed *A.brassicae* as intermediate between the *Brevicatenatae* and *Noncatenatae*. *A.brassicicola* was placed in the *Longicatenatae*. More recently, Simmons (J. David, IMI Kew, pers. comm.) has suggested that *A.brassicicola* forms a distinct species group within the *Longicatenatae* due to the phragmosporous appearance of the conidia and their distinctive apical cell.

Neergaard (1945) stressed that he did not intend to imply any phylogenic relationships in his morphological groupings. However, one of the recurring themes of his subsequent discussions of the individual species seems to imply such relationships between morphologically similar species; this perception has become established with little real evidence to support it.

It has already been noted above that similarity in morphology does not entail similarity of genotype, the converse also being true (Kendrick, 1981b; Simmons, 1986c). Based on the limited number of teleomorph connections which have been made (Table 4.2) there is no evidence that *Alternaria* species of any of the three conidial types have been connected to teleomorphs outwith the *Pleosporaceae*. However, as only a small number of *Alternaria* species have been connected with a teleomorph at all, this approach offers little help in establishing phylogenic relationships within the anamorph-genus.

Ellis (1971; 1976) described many of the most common *Alternaria* species and arranged them in order, according to the abruptness of the transition from the conidium body to the beak. In common with Neergaard, Ellis was careful to stress that he did not intend this ordering to imply any phylogenic relationship between species, indicating (Ellis, 1976) that the idea had proved workable for identification as an alternative to keying the species out. Examination of Ellis's illustrations reveals the degree of variation in conidium morphology which exists across the *Alternaria*, while revealing the degree to which the steps between the extreme types of conidium morphology are filled by a number of intermediate species.

Recent papers dealing with phylogenic relationships and species identification in other fungal genera have reported the use of biochemical characters, either exclusively or in conjunction with morphological characters (Bridge,1985; Itavarra, 1988; Mordue, Bridge & Currah, 1989; Bridge, Hawksworth, Kavishe, & Farnell, 1989).

The use of biochemical characters has been on the periphery of *Alternaria* taxonomy for some years. The prominence of *Alternaria* species as producers of biologically active metabolites has meant that biochemical analysis of the anamorph-genus has tended to concentrate on certain types of compound, and strictly taxonomic considerations have been secondary to those of pathology or toxicology. However, Kinoshita *et al.* (1972) discussed the distribution of tenuazonic acid in the anamorph-genus and found that it was produced by a range of species including those in the *Longicatenatae* (*e.g. A.citri*) and the *Noncatenatae* (*e.g. A.crassa*). Cotty & Misaghi (1984) investigated production of zinniol by seven species of *Alternaria*, including *A.brassicae.* With the exception of *A.brassicae* and *A.zinnae* Cotty & Misaghi (1984) found that zinniol was produced by species in the *Noncatenate* but not by *Longicatenatae* species; they proposed that

the ability to produce zinniol might be a indicate a phylogenic divergence between the *Longicatenatae* and the *Noncatenatae* species, and that the apparent inability of *A.brassicae* to produce zinniol might indicate a different phylogenic origin for it from other large-spored species. The strength of this argument is somewhat weakened by the fact that not all isolates of *A.zinnae* produce zinniol, and, as the authors stated, only a few isolates of each species were examined.

Variability in the Alternaria

The preceding discussion has indicated the occurrence of variation in biochemical (Cotty & Misaghi, 1984) and physical (Ellis, 1971; 1976) characters within *Alternaria* species. However, the results of these and other studies allow only limited inferences to be made of the extent of any underlying genetic variability, since genotype by environment interactions have rarely been assessed. Apparently, only a small body of work has been aimed at assessing genetic variability in the *Alternaria* anamorph-genus. Neergaard (1945) examined morphological and physiological characters in two isolates of *A.brassicae* on two substrates, and reported differences which were stable between the substrates. Based on these observations and those of Fjardo & Palo (1934), Neergaard proposed that *A.brassicae* is composed of several morphological races. This proposal carries an obvious implication of genetic variation between the races. The existence of races within *A.brassicae* was partly supported by the variation which Neergaard observed in the pathogenicity of different isolates towards a standard set of test plants, although he did not comment on this variation. More recently, Humpherson-Jones & Hocart (1983) reported that they found no difference in the pathogenicity of a number of isolates of *A.brassicae*, from different sources, to a group of different *Brassica* species. However, using a different host series Saharan and Kadian (1983) found that different isolates of *A.brassicae* could be differentiated on the basis of their pathogenicity; the differential hosts being *B.oleracea* var *botrytis* and *B.o.* var *captitata*.

In similar studies to those conducted on *A.brassicae*, Neergaard (1945) found no stable variation between isolates of *A.brassicicola*. With *A.alternata* Neergaard concluded that variation in spore length between different isolates on the same substrate was often no greater than the variation within an isolate between different media. Unfortunately, there have been no direct attempts to determine the genetic

variability in any *Alternaria* species, or to establish how variability is produced and maintained in apparently sexless populations.

Mutation ultimately provides all variability at individual loci. Precise details of spontaneous mutation rates giving rise to increased virulence in phytopathogenic fungi are scarce. Zimmer, Schafer, & Patterson (1963) reported high mutation rates in *Puccinia graminis* race 202. On one host cultivar mutation to virulence occurred at a rate of one in 200 infections, while on a second cultivar at a rate of one in 6540 infections. Day (1974) and Wolfe & Schwarzbach (1978) have suggested that mutation rates in *Erysiphe graminis* are probably similar to those calculated for *Neurospora* species; *i.e.* between one in 10^6 and one in 10^8 per locus per individual. Watson (1957) detected three spontaneous mutations to virulence in *P.graminis*. Watson's experiments did not allow calculation of actual mutation rates, but the level of replication which was required to isolate single mutant colonies suggests rates lower than one in 10^8.

Induced mutation rates are better documented than spontaneous mutation rates. Day (1974) summarised known examples of induced mutation to virulence, mainly from work on the rusts and smuts. Recorded rates ranged from one in 250 000 to one in 200. In general, even with mutation rates as low as one in 10^8, fungi which produce large numbers of asexual spores have the potential to generate new genotypes in high numbers.

In the absence of a sexual cycle, the Fungi Imperfecti may mimic the conseqences of outbreeding through the formation of heterokaryons, heteroplasmons or by undergoing a parasexual cycle. Heterokaryosis has been known in phytopathogenic fungi for many years (Tinline & MacNeill, 1969; Hastie, 1981) although its significance in nature is still uncertain. Heterokaryosis which results in increased virulence is known to occur in *Thanatephorous cucumeris* (Flentje, Stretton, & McKenzie, 1967). However, in *Alternaria solani* Stall & Alexander (1957) found no correlation between the occurrence of heterokaryosis and pathogenicity. Netzer & Kenneth (1970) produced heterokaryons with equal pathogenicity to their parents by plating together auxotrophic and prototrophic isolates of *A.dauci*. However, conidia taken from the heterokaryons were consistently found to be homokaryotic, providing circumstantial evidence that in this species one or few nuclei are allocated to the initial cell of each conidium. No reports of heterokaryosis

are known in either *A.brassicae* or *A.brassicicola*. Knox-Davis (1979) demonstrated the multinucleate nature of *A.brassicicola* and thus at least the potential exists in this species for the occurrence of heterokaryons. Both Hartmann (1964; 1966) and Louw (1976) have shown that mycelial cells of *A.alternata* (*A.tenuis*) are multinucleate. Louw (1976) also provided evidence about the mechanism of nuclear exchange in *A.alternata*: hyphae which grew parallel for short distances gave rise to side branches which grew together and apparently fused; nuclei were occasionally seen in these connections in fixed material.

Variation in the pathogen population may be generated directly by the formation of novel heterokaryons, as noted above. In addition, heterokaryon formation allows the potential for recombination through a parasexual cycle. Parasexual cycles have been reported in a number of plant pathogenic fungi; *Fusarium oxysporum* (Buxton, 1962), *Verticillium albo-atrum* (Hastie, 1962), *V.dahliae* (Fordyce & Green, 1964).

The generalised model for the parasexual cycle is based on the pattern of events which occurs in *Aspergillus nidulans* (Tinline and McNeill, 1969; Hastie, 1981); there may, however, be considerable variation in the actual events which occur between different genera or species (Caten, 1981). Hastie (1981) provided a list of Ascomycetes and Fungi Imperfecti for which there was evidence of a parasexual cycle; *Alternaria* was not included. Caten (1981), however, suggested that parasexual cycles are probably widespread in the fungi and that lack of published reports in many genera reflects a lack of research rather than a lack of the process.

Variability for certain characters in some fungi is known to have a cytoplasmic rather than nuclear origin. Day (1974) noted that in 50 percent of cases of reported cytoplasmic inheritance in plant pathogenic fungi, virus-like particles (VLP) were implicated. Lau, Reid & Kim (1981) identified two types of VLP in isolates of *A.alternata* which displayed morphological variation. However, the role which these VLP play in determining variability was unknown.

The spread and production of variability by heterokaryosis, parasexual recombination, or heteroplasmosis, relies on the absence of vegetative incompatibility (VI) between isolates. The subject of

VI has been reviewed by Fincham, Day, & Radford (1978). In *Thanatephorous cucumeris* the interactions which result in VI can take place before hyphal contact or after hyphal fusion (Tinline & McNeill, 1969), implicating cytoplasmic or extracellular components. Vegetative incompatibility has not been examined in *Alternaria*. It is therefore not known what part it might play in restricting gene flow in populations of *Alternaria* species.

Variation in characters other than pathogenicity and virulence occurs in many plant pathogenic fungi. Fungicide resistance (or insensitivity) is of concern to field pathologists since its widespread occurrence can directly lead to loss of yield, reduce the options available for disease control and increase the price of crop protection in some cases.

Insensitivity to fungicides may be considered at several levels in relation to taxonomy. It is certain that sensitivity to particular chemical groups is indicative of taxonomic relationships. This is clearly illustrated, for example, in the sensitivity of the powdery mildews, and insensitivity of *Alternaria* and related fungi, to the morpholines. Within broad ecological/taxonomic groups further differentiation may occur on the basis of fungicide sensitivity. For example, *Leptosphaeria* species are sensitive to MBC-generating fungicides, while the *Alternaria* display insensitivity to this group of chemicals at the genus level. Kato, Coe, New, & Dick (1990) examined the relationship between phylogeny and insensitivity to hymexazol and metalaxyl in a group of Oomycete genera and concluded that, broadly, phylogenic relationships were correlated to patterns of insensitivity.

Iprodione and related dicarboximide fungicides have been recomended for the control of *Alternaria brassicae* and *A.brassicicola* in brassicas in the U.K. since these pathogens became a major problem in the 1970's and early 1980's. Control of seed borne infection is particularly effective (Prasanna, 1984) and relatively cheap, and there has been no reported problem of insensitivity in either pathogen to date.

The mode of action of the dicarboximides remains unclear. Early observations on insensitive isolates of *Botrytis cinerea* revealed that insensitivity was linked to an inability to withstand high osmotic pressures (Beever, 1983), although subsequent studies have shown that there is often no correlation

between fungicide insensitivity and osmotic instability (Pommer & Lorenz, 1987). As a result of their work with *B.cinerea* and *Mucor mucedo* Edlich & Lyr (1987) have suggested that the activity of dicarboximides results from inhibition of a flavin enzyme which causes a reduction of electron flow from NADPH to cytochrome C. The pathological effect results from the oxidation of the target enzyme, NADPH, and other essential cellular phospholipids.

Insensitivity to dicarboximides has been reported from laboratory tests on many fungi including at least three *Alternaria* species (Pommer & Lorenz, 1987). In *A.alternata* insensitivity was shown to be stable through *in vitro* sub-culture and to be passed on to single spore isolates from parental strains (McPhee, 1980). With *A.linicola* the problem of insensitivity has arisen relatively recently and has led to seed lots of flax being failed in Northern Ireland because of *Alternaria* contamination after treatment with Iprodione (Mercer *et al.*, 1987). In *A.kikuchiana*, insensitivity was reported as early as 1979 by Kato, Hisade, & Kawase.

The experimental work in this chapter covers a number of relatively diverse subject areas which are related by an underlying consideration of the variation which exists in the *Alternaria*. The first section of work is an examination of the homogeneity of the genus from published descriptions of many of its member species. This study is intended to examine morphological and ecological groupings which occur in the *Alternaria*, and to relate these to knowledge of behaviour in order to provide a preliminary assessment of any association which might exist between host range, conidium morphology and phylogeny.

The second section of work is concerned with examining the physiological similarity between *A.brassicae*, *A.brassicicola*, other Fungi Imperfecti isolated from *Brassica* species and species of *Alternaria* from other substrates.

The final section briefly examines the variation in fungicide insensitivity present in *A.brassicae* and *A.brassicicola* isolates used in the previous experiment in this chapter, treating fungicide insensitivity as a further character, not directly related to host specificity, which might indicate genetic differences between *A.brassicae* and *A.brassicicola*.

This experimental work is intended to provide an complementary assessment to that developed in Chapters 2 and 3 of the relatedness of *A.brassicae* and *A.brassicicola* by providing comparison with other related fungi or other fungi which share their ecological niche. The analysis of the relationships between species in this section of work involved the application of several multivariate statistical techniques and the experimental details precede a brief discussion of these techniques in relation to this type of study.

4.2 MATERIALS AND METHODS

Experiment 4.1: An examination of morphological groups within the *Alternaria*, based on published descriptions.

Collection of the data

Readily available sources were consulted to obtain descriptions of *Alternaria* species (Neergaard, 1945; Ellis, 1971 ; 1976; Simmons, 1967; 1981; 1982a; 1982b; 1986a; 1986b; 1986c). The authors used slightly different methods for describing the various species which meant that an initial comparison of the descriptions was required to obtain a list of characters which could be confidently abstracted from all of the sources. After this initial literature search a list of 14 characters (variates) which had been used by all authors was compiled. The variates were:

1. Is sporulation catenulate on hosts? (1 = yes, 0 = no).
2. Is sporulation catenulate in culture? (1 = yes, 0 = no).
3. Is the species reported from plant hosts? (1 = yes, 0 = no).
4. Is the species reported from animal hosts? (1 = yes, 0 = no).
5. Is the species reported to have a restricted host range? (1 = yes, 0 = no).
6. Do conidia normally have a true beak? (1 = yes, 0 = no).
7. Are conidiophores normally geniculate? (1 = yes, 0 = no).
8. Maximum conidium length (μm).
9. Minimum conidium length (μm).
10. Maximum conidium width (μm).
11. Minimum conidium width (μm).
12. Maximun number of transverse septa.
13. Maximum number of longitudinal septa.
14. Normal number of conidia in a chain (minimum = 1).

The list of variates allowed standard descriptions of 60 species to be produced. The full list of species used and the corresponding data matrix are presented in Appendix 4.1.

Cluster analysis

Several different cluster analyses were performed to test theories relating to morphology and ecology in the *Alternaria*. Hierarchical Cluster analysis was performed to examine homogeneity in the anamorph-genus. The allocation of species to three clusters after Neergaard's (1945) separation of the *Alternaria* into the *Longicatenatae*, *Brevicatenatae*, and *Noncatenatae*, was examined by non-Hierarchical Cluster analysis and Canonical Variates analysis. These techniques were also used to test the theory that conidium morphology and other morphological characters may be correlated with ecological behaviour.

Experiment 4.2: An analysis of variation and similarity in *Alternaria* species and related fungi from brassica seed and other sources.

Isolation of test isolates

Seed samples of *B.napus* from U.K. sources and of various *Brassica* species from Nepal were used to obtain isolates of *Alternaria* spp., anamorphs known to be related to *Alternaria*, and morphologically similar anamorphs. The seed was surface sterilised in 2% sodium hypochlorite for five minutes, shaken to remove excess hypochlorite solution and plated directly on to CMA in 9 cm triple-vented petri dishes. Approximately 15 seeds were placed in each dish and 5 dishes were prepared from each seed sample. The dishes were incubated at 20± 2° C. In the case of the *Alternaria* species only one isolate of each species was retained from each seed lot, unless there was a marked difference in morphology between isolates. Thirty two isolates were made from these seed samples. An additional eight isolates of *Alternaria* species from a number of different sources were also included in the experiment. Isolates from infected brassica seed sources were identified before transfer to petri dishes containing PDA on which all isolates were maintained during this experimental section.

Characters examined for cluster analysis

Actively growing colonies on PDA were scored for two morphological characteristics: (1) Production of pycnidia as opposed to naked conidia; (2) Production of pigment in the substrate. Nuclear distribution was examined by u.v microscopy, by observing hyphae growing on glucose-coated microscope slides. Briefly, standard microscope slides and a 1% solution of glucose in tap water were sterilised by

autoclaving. The slides were immersed in the hot glucose solution in a sterile 100 ml beaker under aseptic conditions and were placed, one per dish, in 9 cm petri dishes containing two sterilised Whatman seed test papers soaked in 4 ml of sterile tap water. After cooling, each slide was inoculated with a 2 mm cube (approximate dimensions) from the colony edge of a PDA-grown culture of one test isolates. The petri dishes containing the slides were sealed with parafilm and kept in sealed polythene bags in constant darkness at 15 - 22°C for 3 to 4 days, after which the hyphae were fixed and stained, as follows, to reveal nuclei. The inoculum plug was carefully removed from the centre of each small colony and sufficient ethanol to cover the colony pipetted gently on to the slide. The hyphae were fixed in ethanol for 30 seconds, rinsed in distilled water, and then a few drops of a 1μg/ml aqueous solution of DAPI were added. The DAPI was replaced after two minutes by a 0.05% v/v aqueous solution of Tinopal which was rinsed off after 30 seconds by immersing the slides in two changes of 100 ml of distilled water. The slides were then mounted with coverslips in a drop of the DAPI solution and nuclei observed with a Leitz Ortholux II microscope system as described in Appendix 2.5. Nuclear numbers in the tip compartment and the three compartments immediately proximal to the tip were noted in 10 hyphae for each isolate. Hyphal width at the third distal compartment was also measured in these hyphae.

Tests for the ability to grow at pH 2 and pH 12, and to hydrolyse Tween 80 were conducted as described by Bridge (1985). For the pH tests the isolates were grown in modified Czapeck's liquid medium containing 0.05 g/l bromocresol purple as a pH indicator. For growth at pH 2, 10.5 g/l citric acid was added and the pH adjusted with 1M HCl. For growth at pH 12, the concentration of KH_2PO_4 was reduced to 0.2 g/l and 3.75 g/l of glycine were added; the medium was adjusted to pH 12 by the addition of 1M NaOH. Sufficient sterilised liquid medium was poured into 5 cm petri dishes to half-fill them and these were then inoculated with one 0.5 cm cube of mycelium from actively growing colonies of the test isolates. Positive results for the pH growth tests were a change in pH and the formation of actively growing colonies in the liquid medium. Three replicates of each isolate were tested in individual dishes. The basal medium for tests for the ability to hyrolyse Tween 80 consisted of 10 g/l mycological peptone, 5 g/l NaCl, 0.1 g/l $CaCl_2.2H_2O$, 0.05 g/l bromocresol purple and 15 g/l Standard Davies agar. The basal medium and a 10% v/v solution of Tween 80 in distilled water were sterilised separately and mixed in proportions of 9:1 before pouring. Tests were conducted in 9 cm petri dishes inoculated with three 0.5 cm cubes of actively growing

mycelium taken from three separate colonies of each isolate. A positive result was a rise in pH and the appearance of white crystals in the medium.

For comparison of metabolites produced by the isolates, colonies were grown in 9 cm petri dishes on PDA for 14 days at 20± 2°C under a 12/12 hour n.u.v./dark cycle. After 14 days growth three 1.5 cm diameter plugs were cut from the colony edge and placed in the bottom of a large boiling tube to which 2 ml of chloroform were added. The tubes were shaken for 30 seconds and allowed to stand for 5 minutes after which the chloroform was collected through Whatman 1PS papers. The chloroform was dried sequentially under nitrogen to allow the residue to be taken up in a final volume of 0.1 ml of chloroform. Thin layer chromatography was conducted as described in Experiment 3.1 and the presence of fluorescent and u.v. quenching bands was recorded for each isolate in the following manner. All bands occurring were scored first on a present (1) or absent (0) basis for all isolates. In cases where bands ocurred at the same R_F in different isolates but fluoresced with different colours an additional score of 1 or 0 was used to indicate the different colour states. The complete data matrix employed in cluster analysis is shown in Appendix 4.2. Cluster analysis within the group of isolates followed the approach used for the literature-based study in Experiment 4.1.

Experiment 4.3: Fungicide insensitive isolates of *Alternaria brassicae* and *A.brassicicola* in relation to variability.

Isolates of *A.brassicae* and *A.brassicicola* obtained in Experiment 4.2 were tested initially for insensitivity to iprodione by assessing colony growth on PDA supplemented 5 μg/ml iprodione (as Rovral 50% WP). Three replicates of each isolate were tested. Plates were incubated at 20± 2° C for 14 days, then assessed for colony growth. Of the three isolates of *A.brassicae* tested one gave rise to a single colony which was able to grow on the supplemented PDA. The insensitive isolate was designated Abi/Bo/UK, and all isolates including Abi/Bo/UK were sub-cultured and maintained for 4 weeks on unsupplemented PDA, before colony growth rates and osmotic sensitivity were examined. *A.brassicicola* isolates Aci/Bo/UK, (iprodione insensitive, obtained from Dr. H.A.S. Epton, University of Manchester) Ac3/Bo/UK, and Ac1/Bn/UK were treated similary to the *A.brassicae* isolates, and used in the colony

growth and tests for osmotic sensitivity.

Colony growth rate on PDA supplemented with different concentrations of iprodione.

In order to examine the degree of insensitivity of the fungicide-resistant isolates a colony growth experiment was conducted using PDA supplemented with a range of concentrations of iprodione (Rovral 50% WP); 0, 20, 50, 200, and 1000,μg/ml active ingredient (a.i.). Petri dishes (9 cm diameter) containing the supplemented agar were inoculated at their centres with 2 mm plugs of single isolates and perpendicular lines were drawn on the lids across the centre of the dishes, all measurements of colony diameter were made along these lines. The plates were incubated at 20± 2° C in 12 hours dark and 8 hours cool white light for 13 days. Colony diameter was measured first after 7 days and then at 2 day intervals. The experiment was arranged as a randomised block design with each of three shelves in an incubator forming blocks.

Assessment of osmotic sensitivity.

To test whether the insensitive isolates of *A.brassicae* and *A.brassicicola* showed increased osmotic sensitivity petri dishes containing PDA supplemented with NaCl at a concentration of 0.7M were inoculated as described above for the fungicide-supplemeted plates. The inoculated plates were examined after 14 days incubation when photomicrographs were also taken of hyphal characteristics on unsupplemeted PDA, and PDA supplemented with iprodione or NaCl.

4.3 STATISTICAL PROCEDURES

Multivariate approaches to cluster analysis

The examination of relationships between individuals in taxonomic studies has been made easier recently by the development of several computer packages intended as aids to numerical taxonomy. The most widely used packages for phylogenetic analysis have been compared (Fink, 1986; Felsenstein, 1990), revealing the variety of approaches which are available for deriving linkages between individuals.

Phylogenetic models attempt to produce the shortest spanning tree between the test subjects based

on the theories that (1) there is a genealogical connection between test subjects, and (2) that evolutionary changes in the traits of a given species are passed to descendent species thus marking the connections between them. One of the most common approaches to the classification of organisms based on nucleotide or peptide sequence data, is the use of parsimony algorithms (Fink, 1986). However, where data are of morphological characters alternative approaches to deriving phylogenies are recomended (Fink, 1986; F.G. Wright, SASS Edinburgh, pers. comm.).

The data used in this experimental section were a mixture of morphological, ecological, and physiological characters, and were therefore better suited to approaches other than parsimony for assessing linkages between the subjects. In this context, the use of multivariate analyses are appropriate for examining the similarities between the various species.

The statistical methods employed fall into two categories: (1) those based on a similarity matrix calculated from the data collected for each species; (2) those based on matrices of sums of squares and products of the data collected for each species. The first category includes the techniques of Hierarchical Cluster Analysis (HCA), including dendrogram derivation, and Principal Coordinates Analysis (PCO). The second category includes the techniques of Principal Components Analysis (PCP), Canonical Variate analysis (CVA) and Non-Hierarchical Cluster Analysis (nHCA). Descriptions of these techniques and the underlying statistical theories have been given by Gower & Digby (1981), Digby & Kempton (1987), Digby *et al.* (1989), and Kempton & Nicoll (1990).

Hierarchical cluster analysis and principal coordinates analysis

The techniques of HCA and PCO are related through their operation on a similarity matrix of the experimental units calculated from the original data. In fact, PCO can be applied to actual data matrices rather than similarity matrices, but in this situation the graphical representation of the units available from PCO does not correspond to the groupings of the units derived from HCA. Similarity matrices are obtained by the application of various coefficients to the recorded data. Similarities based on qualitative data can be derived from the application a number of coefficients, the most commonly used being the simple matching coefficient and Jaccard's coefficient. The derivation of the similarity, S, between two units,

X and Y, based on character, i, is shown for each of these:

Simple matching coefficient: $S_{xy,i} = 1$, where $X_i = Y_i$

$= 0$ otherwise

Jaccard's coefficient: $S_{xy,i} = 1$, where $X_i = Y_i$, if $X_i \neq 0$

$= 0$ otherwise

Since Jaccard's coefficient produces a value of $S = 0$ for matching negative results, it avoids any implication that two individuals might be similar on the basis that they both lack a certain character.

For quantitative variates similarities can again be derived from a number of different coefficients. These share the feature that the similarity between two units is based on the maximum possible difference between them; in most experimental situations this will be the observed range (r_i) for the given variate. Two of the most common methods for calculating similarities are the city-block metric, and the Euclidean distance coefficients. Derivation of S by these methods is as shown:

city-block metric; $S_{xy,i} = 1 - |X_i - Y_i| / r_i$

Euclidean; $S_{xy,i} = 1 - (X_i - Y_i)^2 / r_i^2$

The choice of coefficient is dependent on the data under consideration. The Euclidean method is appropriate for normalised data since it is susceptible to inaccuracies with data which have a large range. In this respect the city-block metric is a more robust method for length or size data. With data sets which consist of a mixture of qualitative and quantitative variates the values of S derived are the means of the similarities based on each variate.

The derived similarity matrix is employed differently by PCO and HCA. With PCO the similarity matrix is viewed as the inverse of a matrix of distances between the units. The analysis attempts to produce a set of coordinates for the experimental units in a hypothetical Euclidean space in which the distances between the units are preserved. Additionally, each dimension explains the maximum possible of the total squared distance between all the units, following the removal of the distance accounted for by all lower

dimensions. The result of such an analysis is similar to the PCP used in Chapter 2 (section 2.3) in that a two or three dimensional scatter plot of the units in the derived principal coordinates will give a reasonable representation of the true multidimensional distance between them.

In the case of HCA the similarities between the units are used in an iterative process to form progressively larger groups, the initial position of all of the units being single-member groups. A number of methods are available which differ in the detail of how the likages are produced. The differences between these methods allow a test of the homogeneity of the *Alternaria* and they are worth further discussion.

In a complete-linkage analysis the selection of groups which are to be joined is made on the basis of combining the groups whose **furthest** members have the maximum possible similarity. This form of agglomerative clustering typically results in compact groups which are joined to each other at relatively low similarities. In contrast, in single-linkage analysis two groups are joined if the similarity between their **nearest** neighbours has the maximum possible similarity. Typically this type of clustering results in the formation of large groups at high similarities whose members show great variation. The differences between the two clustering methods is shown diagramatically in Figure 4.2. A comparison of the linkage groups produced by these clustering methods will provide an analysis of the homogeneity of the group of experimental units to which they are applied.

Non-hierarchical cluster analysis and canonical variates analysis

In contrast to HCA and PCO, nHCA and CVA operate directly on data matrices rather than on similarity matrices. Both techiques are concerned with examination of groupings within a set of experimental units and both require some *a priori* grouping to be enforced by the experimenter. With nHCA, in contrast to HCA, the starting position is normally a number of pre-determined groups of units rather than the individual units. The analysis moves or swaps units between groups to form a pre-determined number of new groups while attempting to optimise a given criterion, such as minimising the within-groups sum of squares. The technique can therefore be used to examine the robustness of existing classification systems or to provide a comparison with HCA clustering.

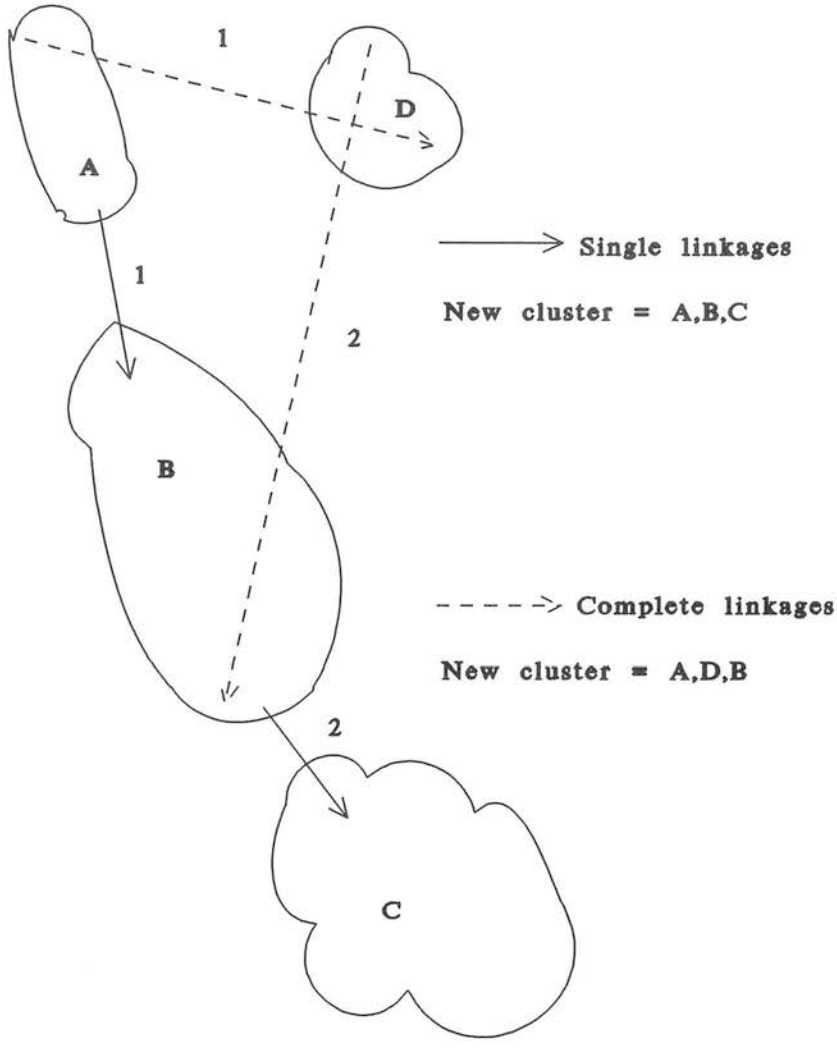


Figure 4.2: A diagramatic representation of hypothetical outcomes of the application of two methods of agglomerative HCA applied to three clusters (after Kempton & McNicol, 1989).

The outcome of nHCA can be displayed graphically through the use of CVA. In this type of analysis the distinctness of groups within a set of units is displayed through the application of a technique which is related to PCP. The analysis generates a new set of variables which are linear combinations of the original variates that can be used to describe the units in a reduced number of dimensions. However, unlike PCP, in CVA the new variables are formed in such a way that the ratio of within-group to between-group sums of squares is minimised. Distinct groups are revealed in a scatter plot of the group centroids in the first two Canonical variates. The distinctness of groups on the scatter plot can be emphasised by marking the boundaries of the 95% confidence limits for group membership. Where two such boundaries do not overlap it may be assumed that the groups are distinct.

The techniques of nHCA and CVA can be used in conjunction to examine the difference between the initial grouping used as input to nHCA and the final groups derived from the clustering process.

Statistical analysis of Experiment 4.3

The colony diameter measurements were analysed by analysis of variance; the analysis was restricted to intra-specific comparisons. The response of the fungi to the increasing iprodione concentration was examined by fitting orthogonal polynomials over the levels of this factor (section 2.2). An initial ANOVA was conducted which included analysis of colony growth over time by fitting orthogonal polynomials over times. This analysis indicated that differences in the growth rate between isolates were more or less constant and that differences between the isolates were summarised by the colony diameters at 13 days; only data for these measurements were considered for further analysis. In order to calculate ED_{50} values (the concentration of iprodione required to reduce colony growth to 50% of the control value), colony diameters at 13 days after inoculation were plotted against Log_{10} of the fungicide concentration.

4.4 RESULTS AND DISCUSSION

Experiment 4.1: An examination of morphological groups within the *Alternaria* based on published species

Results

Homogeneity of the Alternaria

The results of single-linkage cluster analysis (SLCA) and complete-linkage cluster analysis (CLCA) are shown in dendrogram form in Figures 4.3 and 4.4. Under clustering by SCLA all species were found to have formed a single cluster at a similarity (S) of 65%. At S = 80% all but four species, *A.longissima*, *A. mouchacce*, *A.chlamydospora*, and *A.molesta* had formed a single cluster. These four species are distinct either morphologically (e.g. *A.longissima* has exceptionally long conidia), by ecological behaviour (e.g. *A.molesta* has been reported only as a parasite of *Phocena* sp.) or by a combination of relatively unique characteristics. The third condition refers to *A.mouchacce* and *A.chlamydospora*, two of the four soil saprophytes in the group of species examined, which both produce conidia with unusual ratios of length to breadth and of longitudinal to transverse septa. The results of the SLCA were consistent with the hypothesis that quantitative changes in a relatively small number of morphological characters in the *Alternaria* allow morphologically distinct species to be grouped together. However, the SLCA dendrogram indicated that a number of clusters occurred at values of S > 85%. At S = 90% 42 of the 60 species were clustered into seven groups containing two or more species and this separation into a number of distinct morphological groups was highlighted by the results of the CLCA (Figure 4.4).

Cutting the CLCA dendrogram at S = 50% gave rise to three clusters: The first of these contained species which produce large, non-catenulate condia with long beaks. The second and largest cluster contained a variety of morphological forms including both *A.brassicae* and *A.brassicicola*. The third cluster contained species distinct by ecological behaviour and morphology and included all four soil-inhabiting saprophytes and *A.molesta*. At S = 90% the CLCA produced 16 clusters containing two or more species which accounted for 41 of the 60 species.

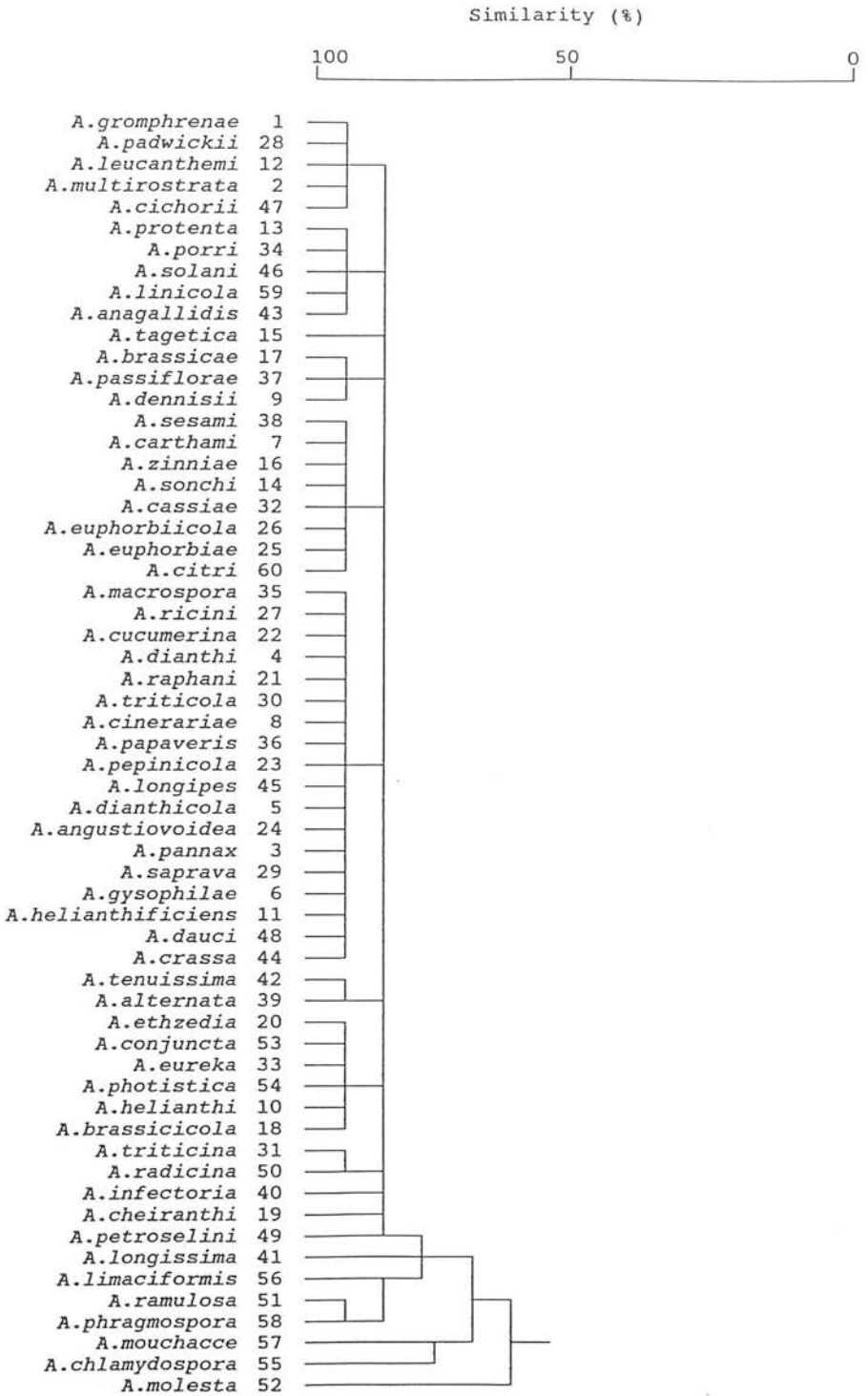


Figure 4.3: A single-linkage dendrogram based on 14 characters for 60 species of *Alternaria* described in published sources.

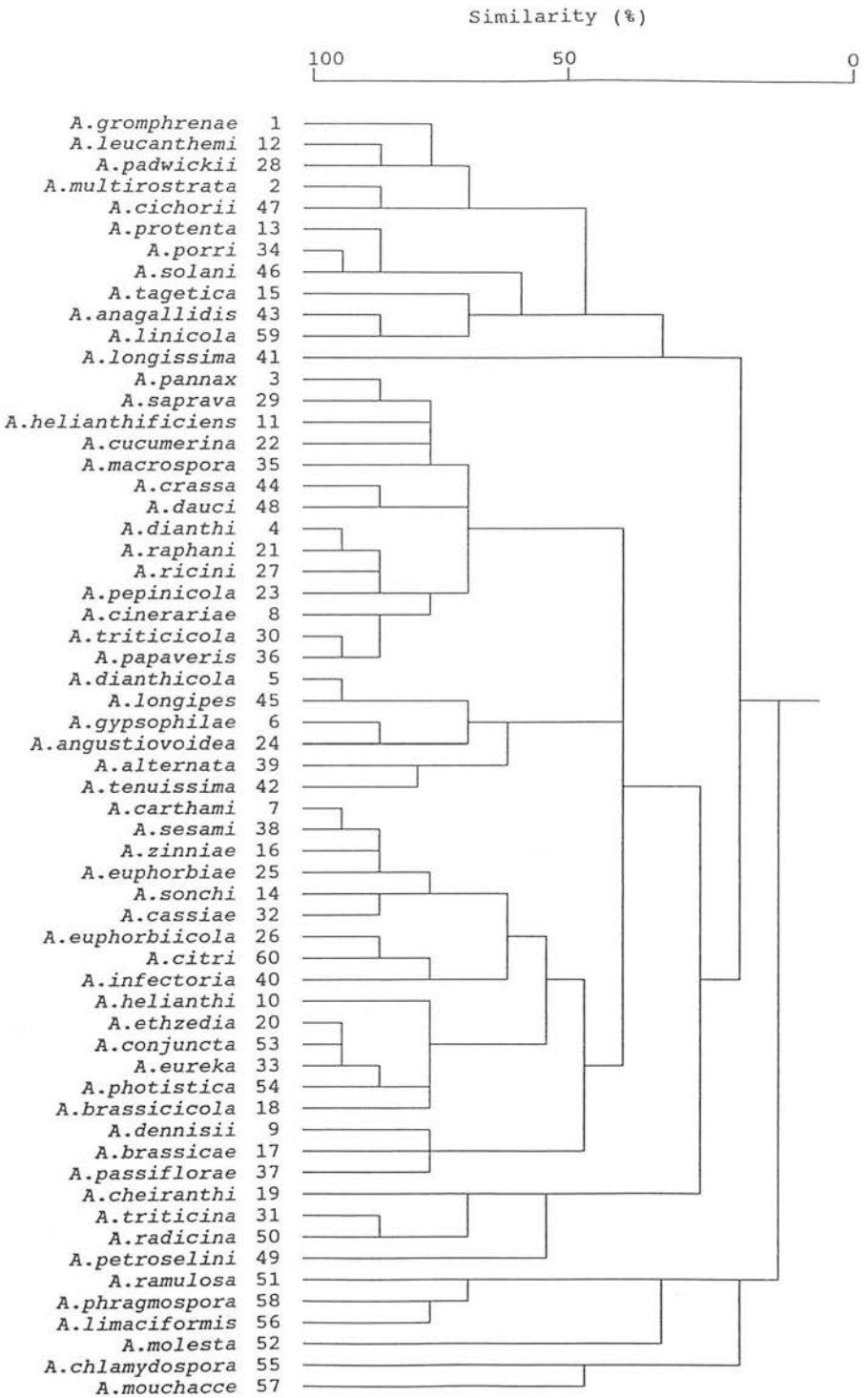


Figure 4.4: A complete-linkage dendrogram based on 14 characters for 60 *Alternaria* species from published sources.

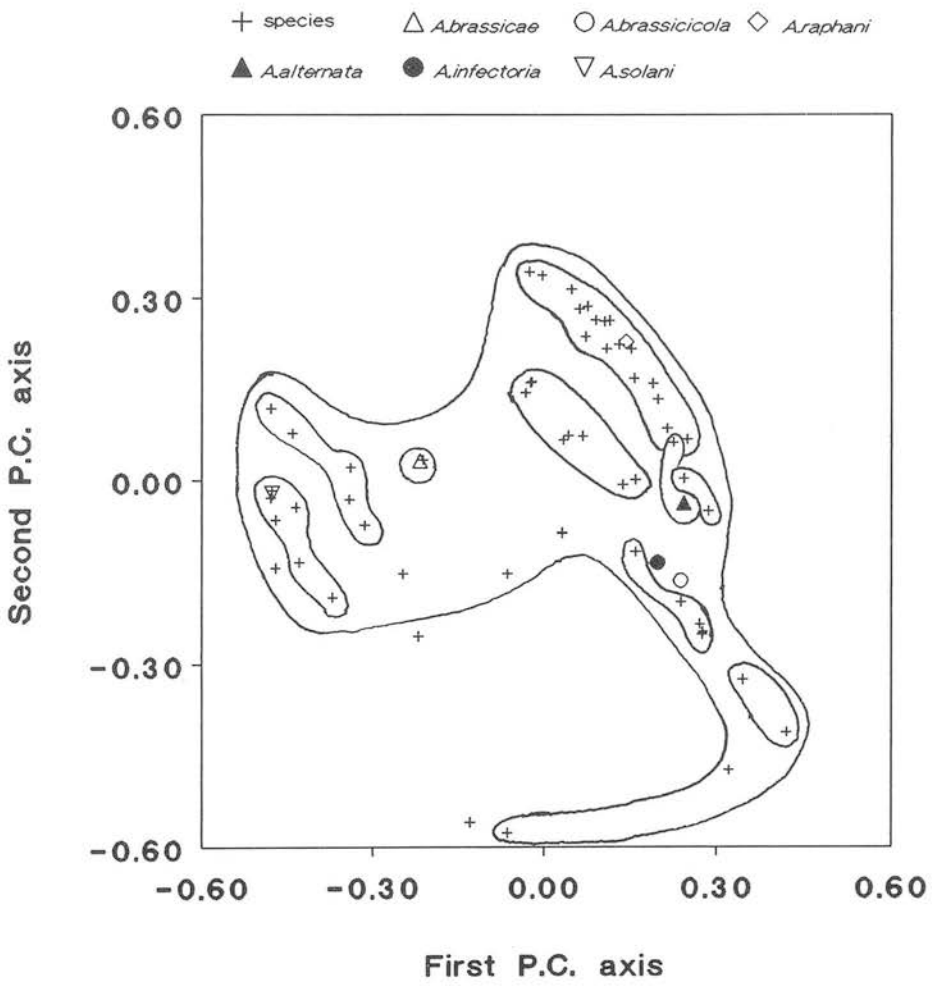


Figure 4.5: Hierarchical clusters from a single-linkage cluster analysis of a group of 60 *Alternaria* species represented in two principal coordinate axes. Clusters formed at 95% similarity (small groups) and 75% similarity (large group and two individuals) are indicated by solid lines.

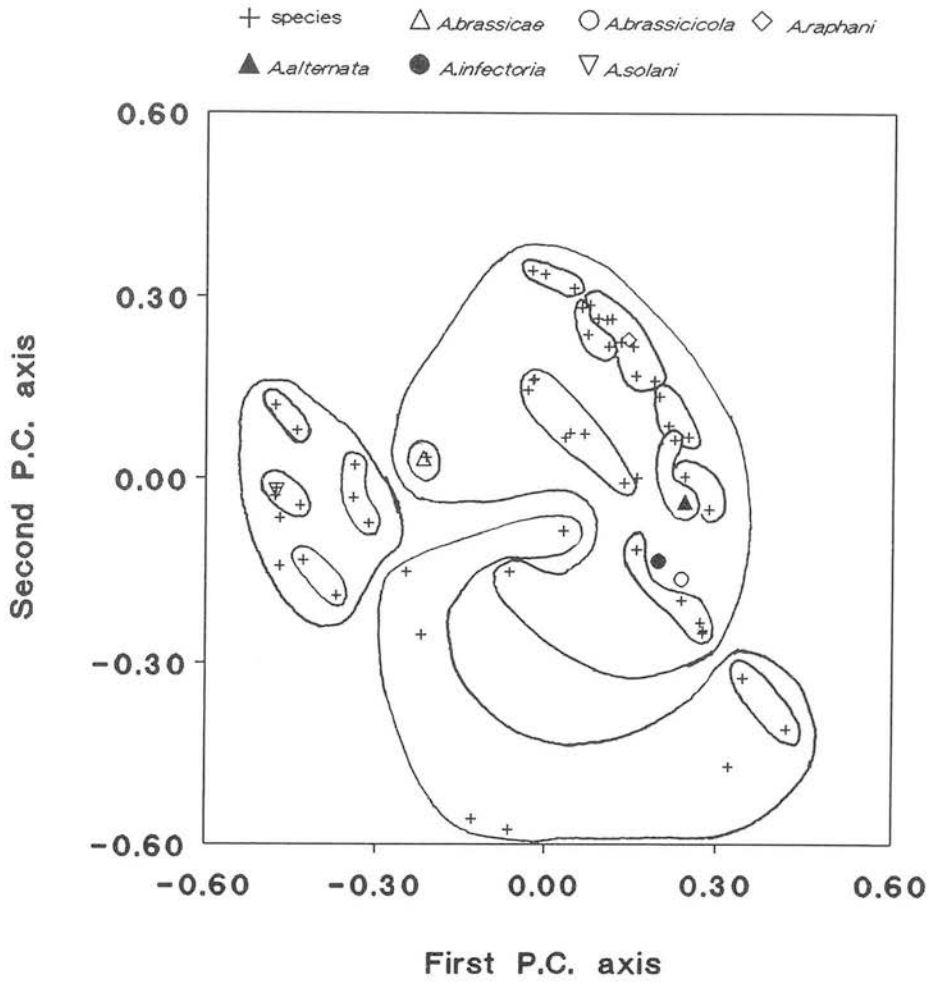


Figure 4.6: Hierarchical clusters from a complete-linkage cluster analysis of a group of 60 *Alternaria* species represented in two principal coordinate axes. Clusters formed at 95% similarity (small groups) and 50% similarity (large groups) are indicated by solid lines.

Table 4.3: Principal Coordinates Analysis of 60 *Alternaria* species based on 14 characters.

	Principal Axis				
Principal axis	1	2	3	4	5
Percentage Squared distance	22	16	13	6	6
Latent roots	3.8	2.8	2.2	1.1	1.1
Trace = 17.5					

The clusters produced by both methods are displayed in the scatter plots in Figures 4.5 and 4.6. The plots show the allocation of species to clusters at S = 95% and the allocation of species when both clustering methods had formed three groups. The difference in the allocation of species and the level of simlarity required to produce three clusters provides a comparison of the difference between the two clustering methods. The PCO analysis produced no negative latent roots, meaning that the derived coordinates for the units represented exactly the Euclidean distances between the species. However, a relatively low proportion of the total variation (50%) was represented by the first three principal coordinates (Table 4.3) and it was found neccessary to include the fourth principal coordinate in order to obtain a visual representation of the distances between the species.

The first principal axis provided a contrast between those species which produce large, long-beaked non-catenulate conidia, and other morphological types. The second principal axis separated the two clusters containing the plant pathogens and plurivorous species from the small cluster containing *A.molesta* and the saprophytic species.

The poor representation of the real distance between some of the species in two dimensions is indicated by the distortions in the outlines of the clusters in Figures 4.5 and 4.6. The 3D scatter diagrams in Figure 4.7, which show plots of the species in the first, second, and fourth principal axes from different orientations, give a better representation of the distances between the species. *A.longissima* is seen to be distinct in the fourth principal axis, and the cluster comprising *A.molesta* and the four saprophytic species

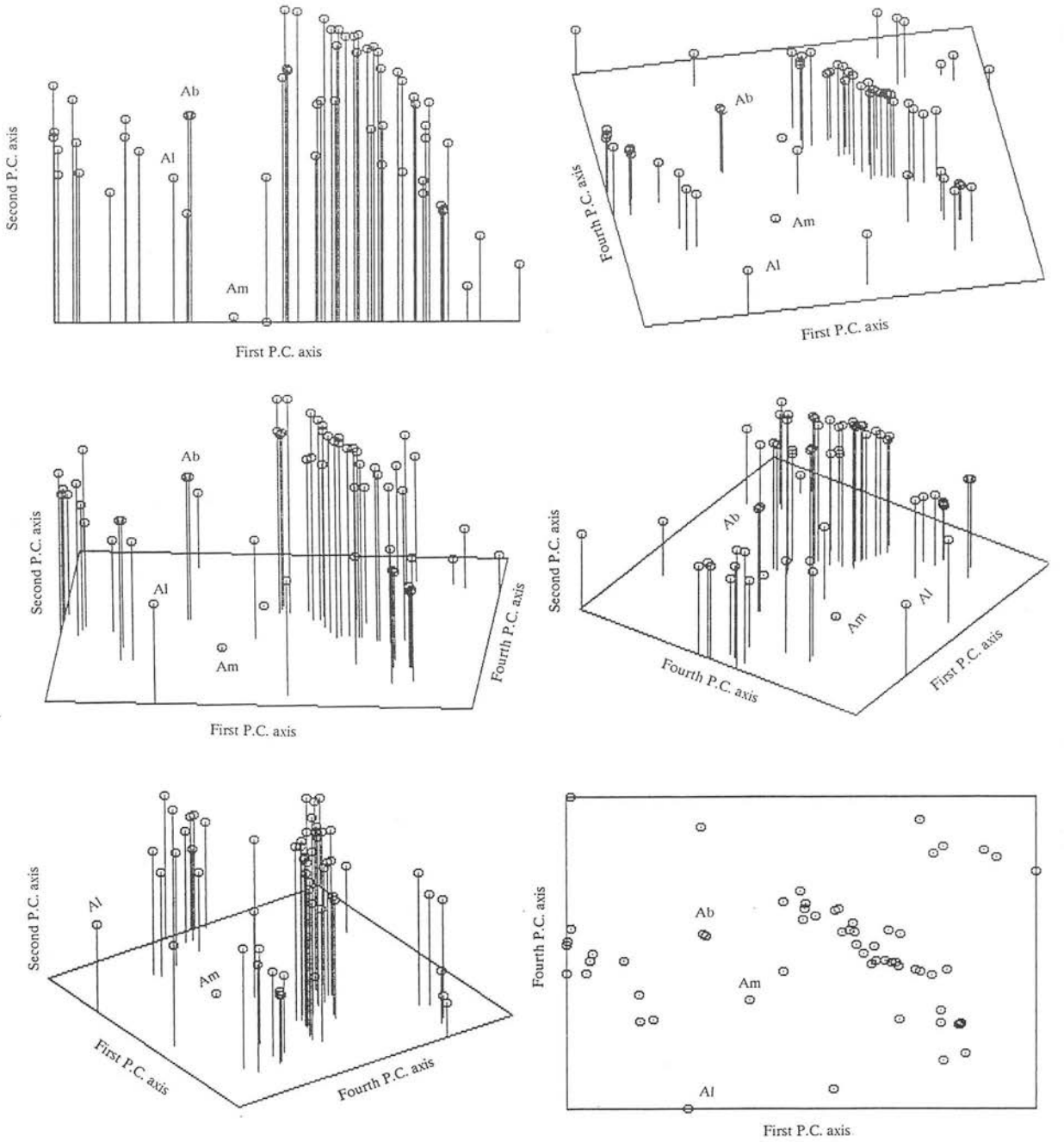


Figure 4.7: Six representations the relative distance between 60 *Alternaria* species in three principal coordinate axes.

can be seen to occupy a position of low altitude on the second principal axis, below the two other major clusters. In keeping with Neergaard's (1945) suggestion, *A.brassicae* was found to be intermediate between the small-spored species and the large-spored non-catenulate species.

Derivation of three Sub-Groups (after Neergaard (1945) by nHCA

Non-hierarchical clustering of the 60 species by the minimum within-groups sums of squares criterion into three clusters produced two entirely distinct groups which overlapped with a third, smaller, intermediate group. The inter-group distances and the allocation of indvidual species to the three groups is shown in Figure 4.8. The contribution of each of the original data variables to the separation of the groups in the CVA is shown in Table 4.4.

Table 4.4: Latent roots and latent vectors from a canonical variates analysis of 3 groups of *Alternaria* species, derived by non-Heirarchical Cluster analysis based on 13 ecological and morphological characters.

CVA axis	**1**	**2**
Latent Root	9.4	0.3
Percentage variation	97	3
Latent Vectors		
Max. spore length	0.022	-0.011
Min. spore length	0.014	0.012
Max. spore width	0.017	0.039
Min. spore width	0.007	-0.042
Max. transverse septa	0.062	0.088
Max. longitudinal septa	-0.026	-0.057
Number of spores per chain	-0.076	-0.210
Catenulate *in vivo*	-0.138	1.617
Catenulate *in vitro*	0.479	-1.193
Plant pathogen	-0.631	1.283
Animal pathogen	0.299	-0.714
Spores with beaks	0.438	1.281
Conidiophores geniculate	1.024	0.043

The separation of species into groups 1 and 2 used a combination of variates describing the presence of a beak, the catenulation characteristics of the spores, and whether the conidiophores are

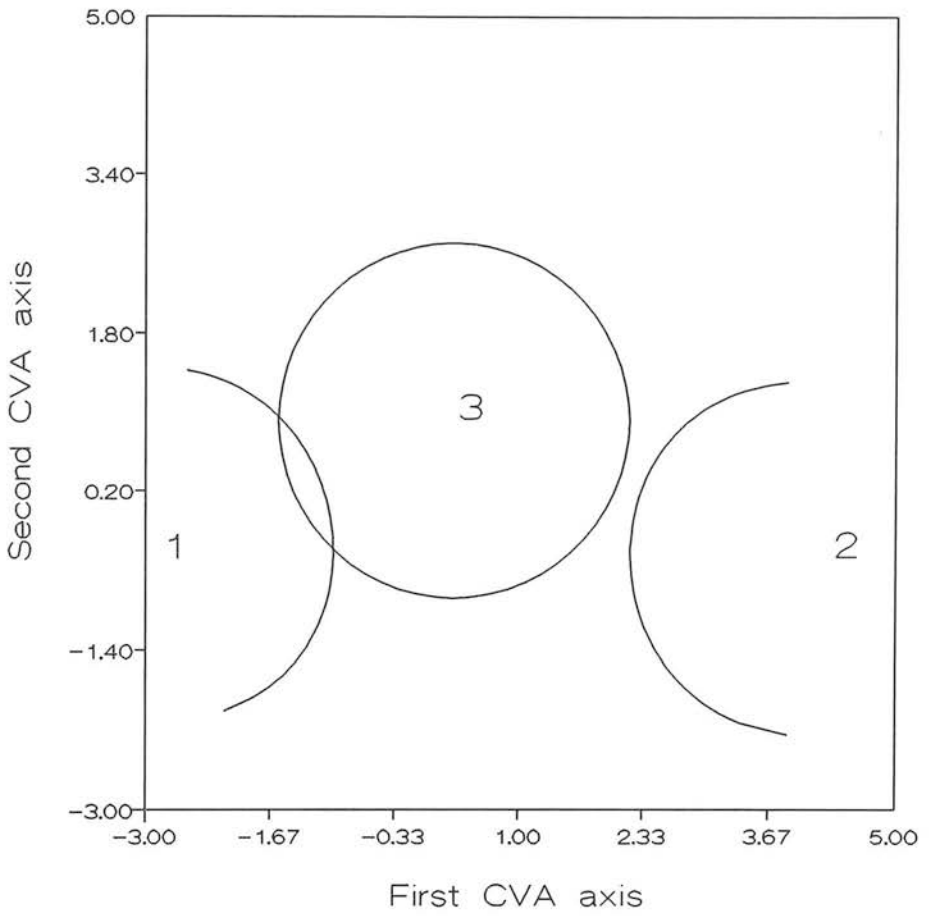


Figure 4.8: A scatter plot of the distances between three groups of *Alternaria* species from based on group centroid positions from a canonical variates analysis. Group 95% confidence limits are shown as circles.

geniculate. Thus, group 1 contained primarily small-spored species which produce chains of spores which may not have true beaks, while group 2 contained primarily species with large, individual spores with prominant beaks. The third group was a relatively heterogeneous collection of species with intermediate conidium size. However, there were certain characters which several of the species shared, apart from an intermediate conidium size. For example, the group contained species which produce conidia with many transverse but few longitudinal septa (e.g. *A.helianthi*).

Morphological variation and ecological groups

The results of the assessment of the morphological distinctness of *Alternaria* species associated with particular host plant families or ecological niches are shown in Table 4.5 and Figure 4.9.

Table 4.5: Latent roots and latent vectors from a canonical variates analysis of 23 groups of *Alternaria* species, based on 13 ecological and morphological characters.

CVA axis	1	2	3	4	5
Latent Root	8.0	2.3	1.8	1.5	0.9
Percentage variation	45	13	10	9	5
Latent Vectors					
Max. spore length	-0.004	-0.003	0.001	0.005	-0.006
Min. spore length	0.006	-0.004	0.006	0.010	0.014
Max. spore width	0.009	0.055	0.134	0.028	0.051
Min. spore width	0.075	-0.093	-0.226	0.036	0.058
Max. transverse septa	0.002	0.043	-0.053	-0.032	0.122
Max. longitudinal septa	-0.089	-0.003	-0.116	0.046	-0.036
Number of spores per chain	0.017	0.024	0.084	0.168	0.136
Catenulate *in vivo*	-0.536	-0.115	-0.984	-3.821	-1.109
Catenulate *in vitro*	1.472	-0.203	0.138	3.746	1.713
Plant pathogen	6.641	0.688	-1.060	-0.432	-1.644
Animal pathogen	-1.842	5.060	-2.958	-0.364	2.663
Spores with beaks	0.580	1.310	1.517	-1.421	1.364
Conidiophores geniculate	-0.130	2.06	0.252	0.478	-1.009

Two groups, *A.molesta* as a single-species group, and the group of strictly saprophytic species were distinguished from the other species by ecological behaviour. Approximately 45% of the variation was accounted for in the first CVA axis which was derived mainly from the pathogenicity characteristics

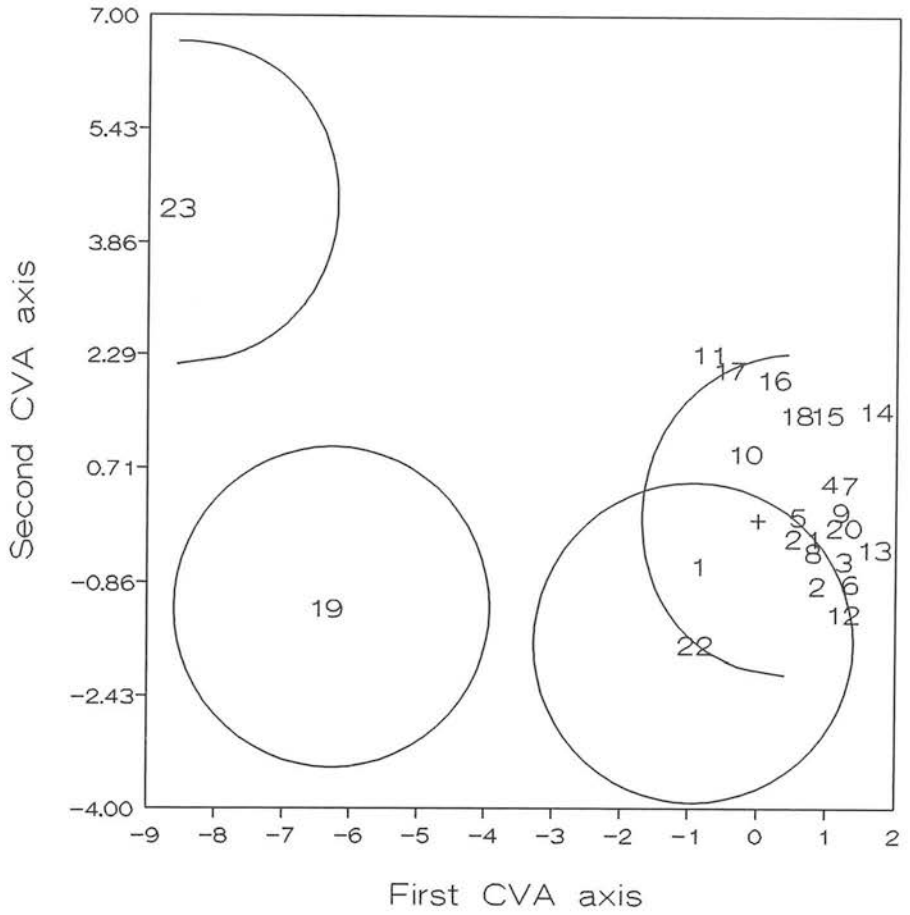


Figure 4.9: A representation of the distance between 23 host-family groups of *Alternaria* species based on group centroid scores in two canonical variate axes following CVA of morphological and ecological characters. Group 95% confidence limits are shown as circles.

of the groups, as indicated the large latent vectors for these characters (Table 4.5). The second CVA axis differentiated primarily between *A.molesta* and all other species. Among the plant pathogenic and plurivorous species there was little evidence of association of specific morphological types with particular host families or ecological behaviour. Host plant families which were found to be distinct were represented by single *Alternaria* species which were distinguishable on the basis of only a few characters. For example, the Amaranthaceae and the Passifloraceae were separated in the fourth CVA axis by the difference in the catenulation characteristics of *A.gomphrenae* and *A.passiflorae*.

Since the results of this analysis indicated that morphological groups were not correlated with host species or ecological behaviour, the 60 species were reallocated to 23 groups on the basis of a minimum within-groups sums of squares criterion by non-hierarchical cluster analysis (nHCA). The criterion value decreased to 3% of its initial value, following this reallocation of, indicating the morphological diversity among *Alternaria* species from indvidual plant hosts. The relative distance between the 23 new groups in a canonical variate space is shown diagramatically in Figure 4.10.

Few species with common hosts remained clustered together after reallocation by nHCA. With species from the Crucifereae for example, all five were allocated to different clusters. *A.brassicae* was placed in a cluster with *A.zinniae* and *A.porri* (cluster 17 in Figure 4.10). *A.brassicicola* was clustered with *A.linicola* (cluster 22). *A.raphani* was gouped with *A.dianthicola* and *A.tagetes* in cluster 7. *A.cheiranthi* was separated as a single member cluster (cluster 3). *A.ethzedia* was clustered with *A.tenuissima* and *A.triticina* in cluster 6.

Discussion

Morphological groups and homogeneity in the Alternaria.

The results of this survey of published *Alternaria* species suggested that Neergaard's (1945) division of the *Alternaria* into three groups provides a usable starting point for examining variation within the anamorph-genus. Hierarchichal clustering indicated that the majority of species are morphologically similar, but there is at least one sub-group which is distinct in having large, long-beaked conidia.

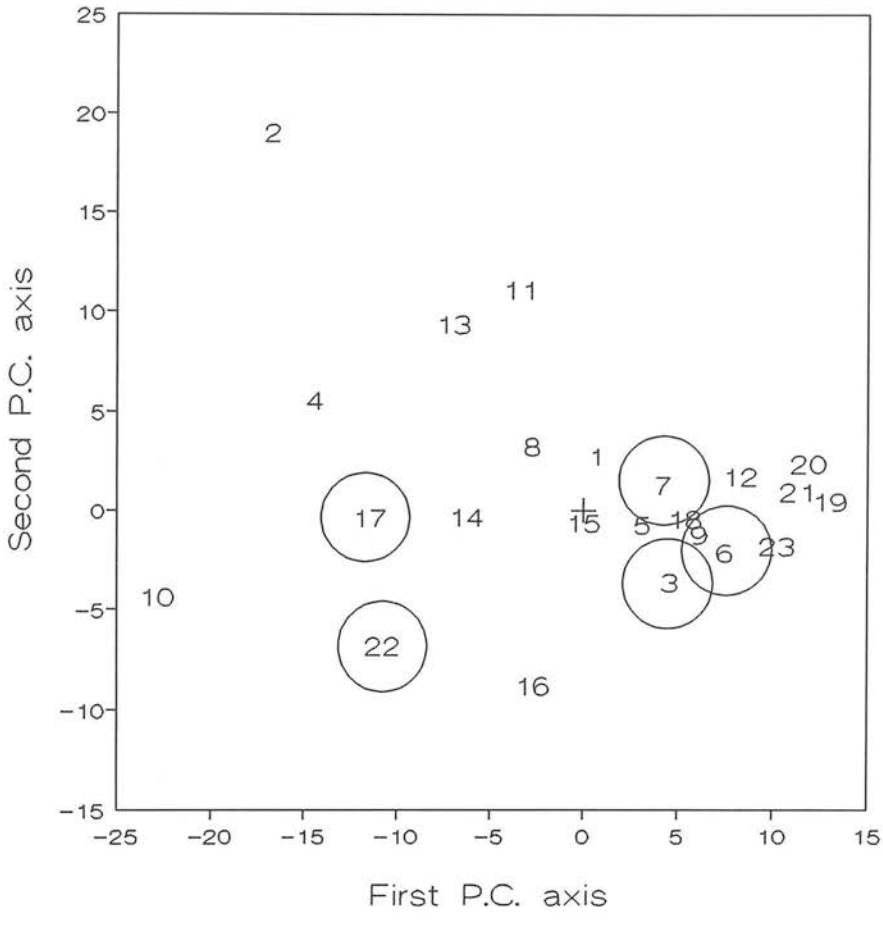


Figure 4.10: A diagramatic representaion of the relative distance between 23 clusters of *Alternaria* species in a canonical variate space following reallocation of species from host family groups by nHCA. Circles represent cluster 95% confidence limits.

The cluster analyses successfully separated morphologically distinct species and clustered together similar species. The morphological similarity between many *Alternaria* species is easily recognised and in this respect formal numerical methods provide only a relative numerical scale for placing species on obvious morphological trends. However, a secondary result of the clustering methods has been the production of various graphical representations of the relative similarity between species. The phylogenic value of these models is limited by the fact that they are based only on morphological and ecological data. However, they could now be tested against other models derived from biochemical and molecular biological data to determine if morphological similarity is associated with phylogenic similarity.

Neergaard's *Longicatenatae* and *Noncatenatae* may represent phylogenically distinct groups within the *Alternaria*. The *Noncatenatae* are distinguishable from many of the species which make up the bulk of the *Alternaria*, by the overall size of the spores, by the length of beaks, and by the abruptness of the transition from the conidium body to the beak. However, even within the large-spore sub-group there is some general morphological variation, with *A.longissima* being distinct because the transition from conidium body to beak is rather gradual and also because the maximum length achieved by conidia is far greater than in any other species.

To date only a few anamorphs, all of which are morphologically similar to *A.alternata*, have been connected to *Lewia* sexual stages; teleomorphs for the full range of morphological types within the *Alternaria* have not been noted and distinct conidium types may be found to be associated with different sexual genera. The teleomorphs of the *Noncatenatae*, for example, may belong to genera other than *Lewia*. Some of the large-spored *Alternaria* species have a broad morphological similarity to *Nimbya* species. *Nimbya* species typically have large, long-beaked, solitary conidia which are morphologically a half-way-house between the large *Alternaria* species and *Drechslera* (Simmons, 1989). The ascospores of the *Macrospora* teleomorphs of *Nimbya* appear more similar to those of *Pyrenophora* than those of *Lewia* (Wehmeyer, 1961; Simmons, 1986c; 1989). The similarity between large-spored *Alternaria* species and *Nimbya* species may suggest a position for teleomorphs of the large-spored species in this group of the Pleosporaceae rather than in *Lewia*, which more closely resembles *Pleospora* Rab.

Comparison of pathogenic behaviour and morphology

None of the plant families or ecological niches examined was found to have a morphologically unique population of *Alternaria* pathogens. However, the strictly saprophytic species produce rounded condia which lack beaks and which might therefore be easily spread in soil water. Wehmeyer (1961) found correlations between ascospore morphology, host range, and geographical distribution in the teleomorphs of the Pleosporaceae. The results of the present study indicate that there is no parallel correlation between conidium morphology and host range. In addition, it is not known whether morphologically distinct species with identical host ranges (such as *A.brassicae* and *A.brassicicola*) are more closely related than morphologically similar species with very different host ranges (such as *A.solani* and *A.porri*).

Neergaard's (1945) description of many *Alternaria* phytopathogens indicates that the early classifications considered morphologically similar species occurring on different plant species as *formae speciales* (ff. sp.) of the most well known species. Neergaard noted that 9 ff.sp. had been suggested for *A.brassicae* on hosts outside the Cruciferae, but he pointed out that none of them could actually be considered to pathotypes of *A.brassicae*. The pathotypes, or *formae speciales*, suggested for *A.alternata* represent an extreme case of the situation in which morphologically similar species may have different host ranges. Simmons (1990) has suggested that in some cases the allocation of formerly recognised species to the status of pathotypes of *A.alternata* may not be accurate, as noted in section 4.1. Simmons noted that anamorphs of the *A.alternata* group occur relatively freqently on citrus and tobacco, but in each case they are distinguishable from the previously recognised pathogens, *i.e. A.citri* and *A.longipes*. One possible conclusion from these apparently conflicting views is that there are host-specific *Alternaria* species on both tobacco and citrus for which specificity may not be determined by the production of HSTs (*i.e. A.citri* and *A.longipes*), and also that there are *A.alternata*-like anamorphs which produce HSTs, these being the ff.sp. of Nishimura & Kohmoto (1983a ; b). In the present study the analyses of groupings within the genus suggested that although the *A.alternata*, *A.citri*, and *A.longipes* lie within the *Longicatenatae*, they are not especially closely related within that group. For example in the nHCA derived from the initial grouping of all 60 species, *A.alternata* and *A.citri* were placed in a large cluster (group 23, Figure 4.10) while *A.longipes*

was placed in a non-overlapping cluster (group 15, Figure 4.10). In the initial HCA itself the relative similarity between between *A.citri* and *A.alternata* and *A.longipes* was 60%, while the similarity between *A.alternata* and *A.longipes* was 76%. The nearest neighbours of *A.alternata*, *A.citri* and *A.longipes* were respectively *A.tenuissima* (86%), *A.euphorbiicola* (93%), and *A.gypsophilae* (95%). Reference to Simmons (1981; 1982a; 1990) suggests that the this result accurately reflects the differences in morphology between these species.

The detection of double toxin-producing isolates suggests that the use of HST production in the *Alternaria*, may not provide a suitable basis for establishing ff.sp.. In other fungal genera it is common to consider morphologically identical fungi which have distinct and non-overlapping host ranges as ff.sp. (section 4.1). It would be difficult to maintain a traditional classification of the toxin producing isolates as ff.sp. if such double toxin producers are able to parasitise both susceptible hosts. In species outside this group of toxin producers the production of specific metabolites may have limited value in the taxonomy of the *Alternaria* in the near future. This is due partly to the paucity of knowledge on the range of metabolites produced by most species, but also because, for practical purposes, morphological characters and knowledge of ecological behaviour are likely to be more accessible.

Questions have been raised in this study in relation to the taxonomic position of the holomorphs which have *Alternaria* anamorphs within the Pleosporaceae, the relationship between conidium morphology, host range, and phyolgeny, and the possible use of physiological and biochemical characters to examine these questions. Experiment 4.2 addresses these questions in an examination of the similarity between a number of fungal isolates from brassica seed and other sources, with particular reference to *A.brassicae* and *A.brassicicola*.

Experiment 4.2: An analysis of variation and similarity among *Alternaria* species isolated from brassica seed and other sources.

Results

Species isolated.

The sources of the various seed lots and infected plant tissue, and the isolates derived from them which were included in the study are shown in Table 4.6. The predominant species isolated from the Nepalese seed was *A.alternata*. In addition *A.brassicicola*, *A.cheiranthi*, *A.tenuissima*, *Alternaria* spp., *Leptosphaeria maculans* (*Phoma lingam*), *Pleospora* sp. (*Stemphylium botryosum*), and *Ulocladium* sp. also occurred.

Of the three unidentified *Alternaria* species isolated from the Nepalese seed, two produced a small number of conidia in the colonies formed immediately around the infected seed. On subsequent transfer to PDA sporulation ceased and attempts to stimulate sporulation by growing the fungi on CMA, V8 juice agar, water agar, and filter papers all failed. In addition, these isolates also failed to sporulate when the method of Senior *et al.* (1987) was tested. The morphology of the spores initially produced by each isolate and their subsequent colony growth habit in culture suggested that they were probably within the *A.alternata-A.tenuissima* complex of species. The third unidentified *Alternaria* species produced conidia on PDA plates after 28 days in culture when colonies had ceased expansion and desiccation had begun. Colonies on PDA were white and cottony, with the aerial mycelium approximately 2 mm in height. Conidia were produced singly, in sparse bunches on geniculate conidiophores, near the colony edge. The conidia (Plate 4.1a) were of the *A.helianthi* type and were large (150 - 250 μm in length) and tapered toward the terminal end. The basal and terminal ends were rounded, and there was no beak; conidium colour was mid-brown. A sample of this isolate was sent to IMI, Kew for identification but failed to sporulate, and the identification was not completed.

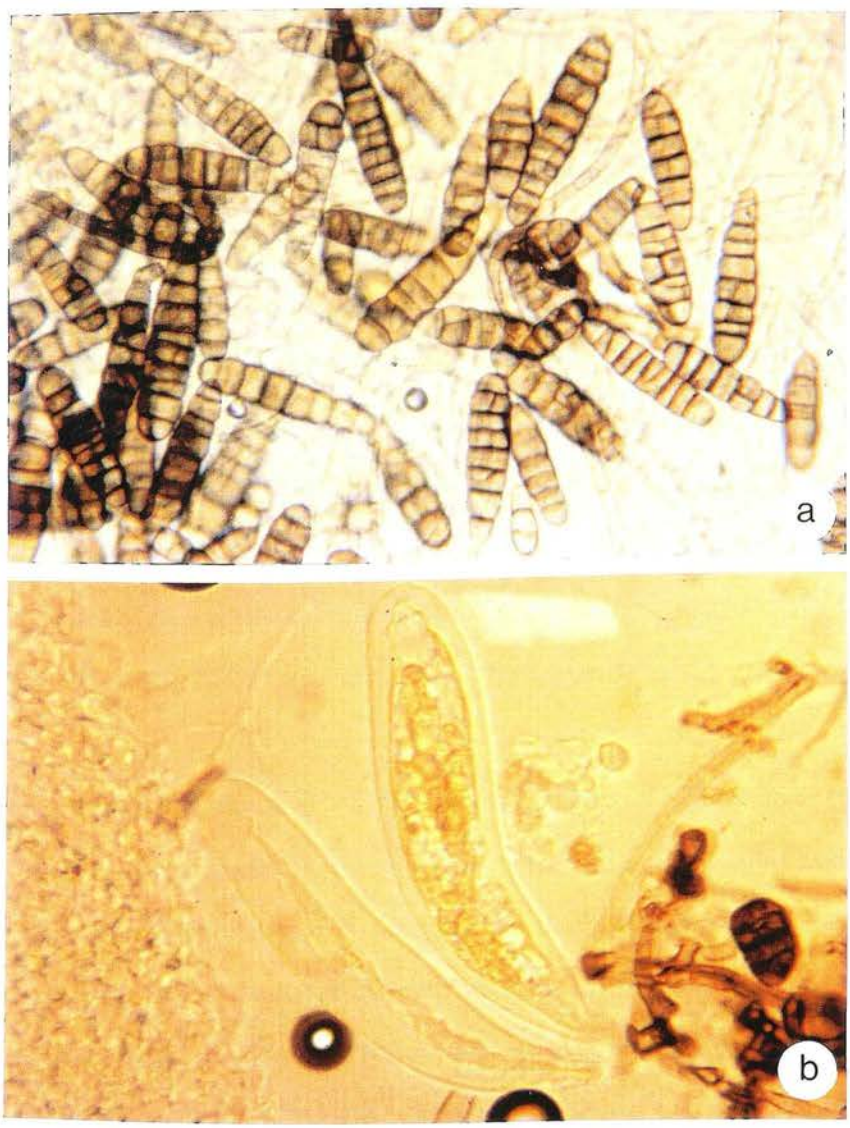
Table 4.6: Sources of *Alternaria* species and related fungi, or morphologically similar fungi examined.

Country of Origin	Plant source	Species isolated	Isolate designation
Israel*	*Solanum tuberosum*	*A.solani*	As1/88/I, As2/88/I
Nepal	*Brassica oleracea*	*A.alternata*	Aa1/7/N
		Alternaria sp.	Asp3/7/N
		L.maculans	Lm1/7/N
		Ulocladium sp.	Usp/6/N
U.K.	*B.napus*	*A.alternata*	Aa1/108/UK, Aa2/108/UK, Aa1/113/UK, Aa1/148/UK Aa2/148/UK, Aa3/148/UK
		A.brassicicola	Ac1/Bn/UK, Ac2/Bn/UK
		A.tenuissima	At2/Bn/UK
		Bipolaris sp	Bsp/87/UK
		L.maculans	Lm1/Bn/UK
		Ulocladium sp	Usp/87/UK
	B.oleracea	*A.alternata*	Aa1/1/UK, Aa2/1/UK, Aa1/4/UK
		A.brassicae	Ab1/Bo/UK, Ab2/Bo/UK, Abi/Bo/UK, Ab4/Bo/UK
		A.brassicicola	Ac3/Bo/UK, Aci/Bo/UK Ac5/3/UK
		A.cheiranthi	Ach/3/UK
		A.tenuissima	At1/4/N
		Alternaria sp	Asp1/1/UK
		Alternaria sp	Asp2/1/UK
		S.botryosum	Sb1/2/UK
		S.botryosum	Sb1/8/UK, Sb2/8/UK
	Cheiranthus cheiri	*A.cheiranthi*	Ach/I/UKÒ
	Raphanus sativus	*A.raphani*	Ar1/I/UKÒ
	Triticum aestivum	*A.alternata*	Aa1/Ta/UK, Aa3/Ta/UK
		A.infectoria	Ai1/Ta/UK

* Isolates provided by Helen Stewart, S.C.R.I., Invergowrie

Ò Isolates obtained from IMI culture collection, Kew

Plate 4.1: Conidia (a) and immature asci (b) produced by fungi isolated from brassica seed samples.


a
b

In the case of *L.maculans* only the *Phoma* state occurred in culture during the course of these studies. In contrast, although the *Pleospora* isolates were initially identified on isolation as their *Stemphylium* anamorphs, after approximately 21 days culture on PDA all three isolates produced ascocarps. Although ascocarp production (Plate 4.1b) was subsequently maintained by these isolates through subculturing, mature asci were never observed.

Physiological, cytological, and biochemical data

Only the *L.maculans* isolates produced pycnidia, all other isolates produced naked conidia or were sterile in culture, while only the *A.solani* isolates were chromogenic. Only 10 of the 40 isolates were able to grow at pH 2, with five of these 10 being *A.alternata* (Aa1/113/UK, Aa3/148/UK, Aa3/Ta/UK, Aa2/1/UK, Aa/4/UK). The other isolates which grew at pH 2 were, *A.brassicae* (Ab4/Bo/UK), *Bipolaris* sp. (Bsp/87/UK), *A.infectoria* (Ai1/Ta/UK), *L.maculans*, (Lm1/7/N), and *A.solani* (As1/88/I, As2/88/I). Only four isolates were unable to grow at pH 12, these were *A.alternata* (Aa1/7/N), *A.infectoria* (Ai1/Ta/UK), *L.maculans* (Lm1/7/N), *A.raphani* (Ar1/I/UK). Six of the 40 isolates were unable to hydolyse Tween 80; half of these were *A.brassicicola* isolates (Ac2/Bn/UK, Ac3/Bo/UK, Aci/Bo/UK), the others being *A.raphani* (Ar1/I/UK), *Alternaria* sp. (Asp3/7/N), and *Ulocladium* sp (Usp/87/UK).

Examining the results of the observations of nuclear distribution and numbers in hyphae, clear differences were found between species although there was also considerable variation within species. Generally, the isolates formed two sub-groups with respect to nuclear distribution, with a third small group composed of two species. Hyphal compartments of the large-spored *Alternaria* species (*A.brassicae*, *A.solani*, and *Alternaria* sp3), *L.maculans*, and *S.botryosum* were generally mutlinucleate, particularly with respect to the hyphal tip compartment. The multinucleate condition of the tip compartment arises apparently from a combination of active nuclear division and a lack of septum formation in the actively growing region of the hyphae. In compartments proximal to the tip, nuclear number in these species was found to be typically two to four, although uninucleate compartments were common with *A.brassicae* and compartments with more than four nuclei occurred in all of these species. Hyphal width was variable in this group with species such as *L.maculans* and *A.brassicae* having hyphae typically 4 - 5 m wide, while those of *S.botryosum* were 3 - 4 m in width.

In the second group of species the hyphal tip compartment was found to contain one to four nuclei and subsequent compartments to be uninucleate or to contain two to three nuclei. Hyphal width in this group of species was predominantly 3 m, except in the case of the *Ulocladium* isolates in which the hyphae were typically 2 - 3 m wide. In *Ulocladium* the width of the hyphae also resulted in the nuclei being thinner and longer than those of *Alternaria* species, in which non-dividing nuclei were generally spherical or elliptical. The intermediate group comprised *A.raphani* and *A.cheiranthi*. In these species hyphal width was typically 3 - 4 m, hyphal tip compartments were multinucleate, but proximal compartments generally contained one to two nuclei. Features of hyphal morphology and nuclear distribution are illustrated in Plates 4.2 and 4.3.

Examination of chloroform extracts from mycelium by TLC showed that all isolates produced a green fluorescing band between R_F 0.65 and 0.70 (Appendix 4.2). This band had a slightly lower R_F than ergosterol, but may be a related compound or hyphal wall fraction. Extracts from *A.alternata* isolates generally contained the greatest number of metabolites while extacts from *A.brassicae* and *A.brassicicola*, in common with many of the other fungi, contained few metabolites. A total of 21 differences were found between the isolates with respect to R_Fs of fluorescing and u.v.-quenching bands, and colours of the fluorescing bands.

Cluster Analysis

Hierarchical cluster analysis by complete linkage: At a similarity of 95% (S = 95%) 17 of the 40 isolates were allocated to 6 clusters as shown below:

Cluster 1: Aa3/Ta/UK, Aa1/4/UK

Cluster 2: Aa1/113/UK, Ach/I/UK

Cluster 3: Sb/2/UK, Sb2/8/UK

Cluster 4: Ab1/Bo/UK, Ab2/Bo/UK

Cluster 5: Abi/Bo/UK, Asp2/1/UK, Ac1/Bn/UK, Asp1/1/UK, Lm/Bn/UK

Cluster 6: Ac3/Bo/UK, Aci/Bo/UK, Usp/87/UK, Asp3/7/N

Plate 4.2: Illustrations of nuclear distribution in fungi isolated from brassica seed lots.

a. A hyphal tip of *A.brassicae* (Ab4/Bo/UK) with several dividing nuclei visible. Stained with DAPI and Tinopal.

b. Distal compartments of the hypha illustrated in plate **a.** showing spherical non-dividing nuclei unequally distributed in the compartments. Stained with DAPI and Tinopal.

c. A hyphal tip of *A.brassiciciola* (Ac1/Bo/UK) showing uninucleate compartments. Nuclei and septa are visible. Stained with DAPI only.

d. A hyphal tip from *A.raphani* (Ar1/IMI/UK) showing multinucleate character of the tip compartment. Stained with DAPI and Tinopal.

e. Hyphal tip and distal compartments in *A.alternata* (Aa1/7/N) showing the regular distribution of nuclei. Stained with DAPI and Tinopal.

f. Hyphal tip in *A.solani* (As1/88/I) showung several nuclei. Nuclear division appears to be occurring in the most distal brightly stained nucleus

Bar = approximately 5 μm in all cases.

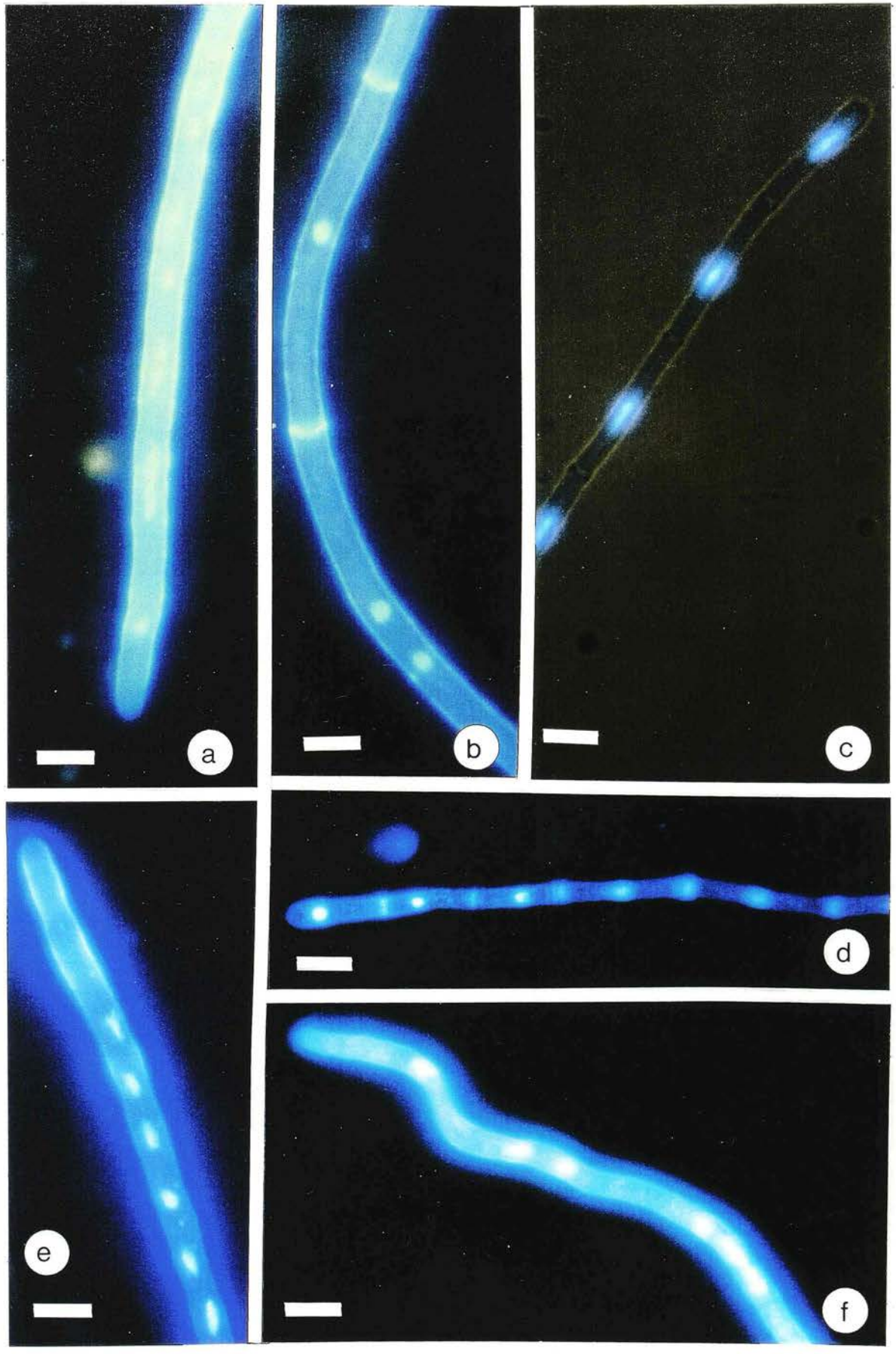

a
b
c
d
e
f

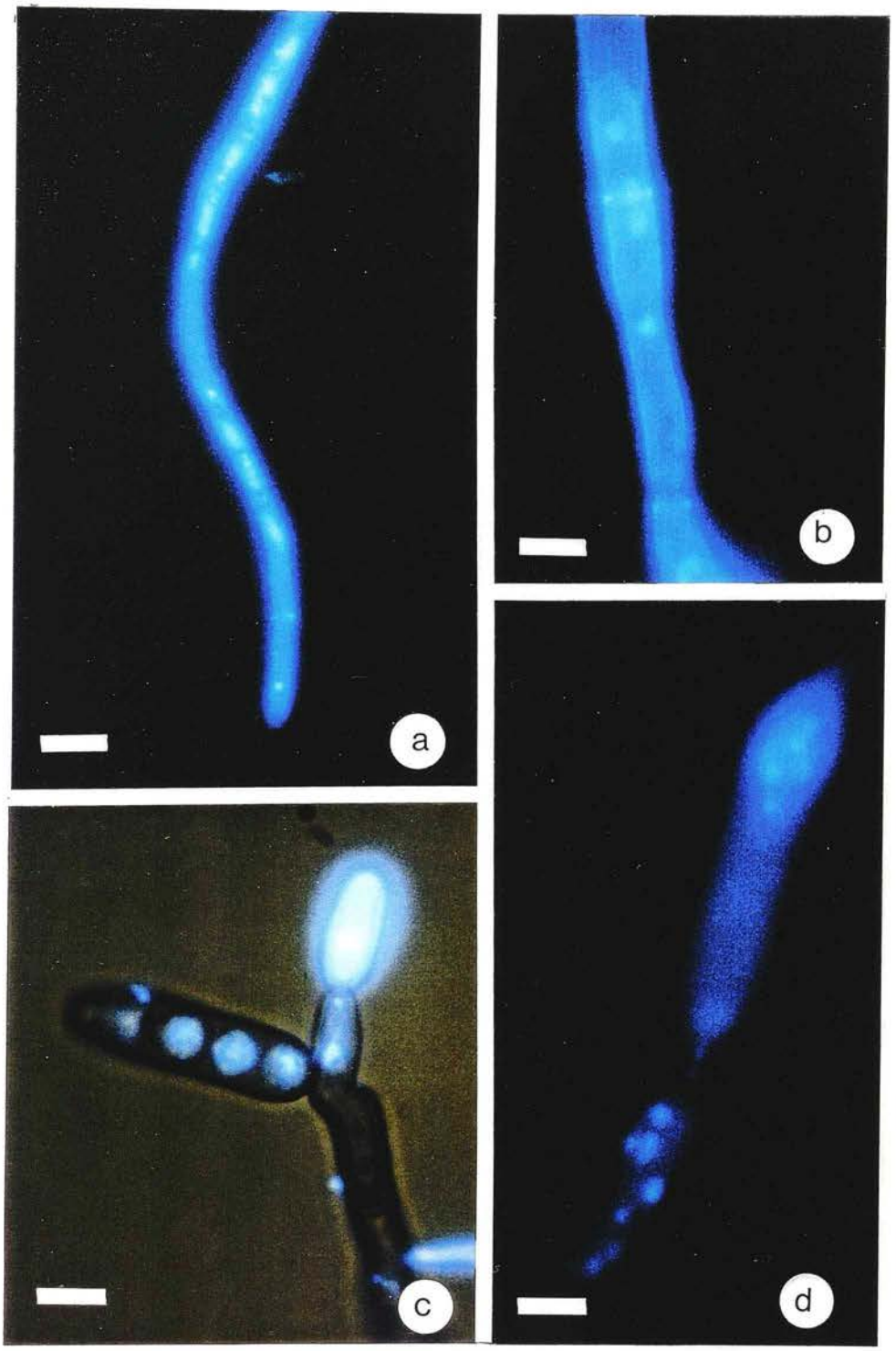
Plate 4.3: Illustrations of nuclear distribution in fungi isolated from brassica seed lots.

a. Non-specific stining in a hyphal tip fom *A.brassicicola* (Ac2/Bo/UK) in an unfixed preparation. Stained with DAPI. Bar = 5 *u*m

b. Apparent nuclear migration through a septumin *A.brassicae* (Abi/Bo/UK). Lodging of nucleai in septa may arise as an artifact of fixation. Bar = 5 *u*m.

c. Conidiogenesis in *Bipolaris* sp. (Bsp/87/UK). The nuclei are visible in the pigmented conidium and conidiophore, but not in the brightly fluorescing juvenile, single-celled conidium. Stained with DAPI and Tinopal. Bar = 4 *u*m.

d. Multinucleate conidiophore tip (lower left) and sigle-celled juvenile conidium (upper right) of *A.brassicae* (Abi/Bo/UK). The cytoplasmic connection between the conidium and conidiophore is faintly visible. Bar = 5 *u*m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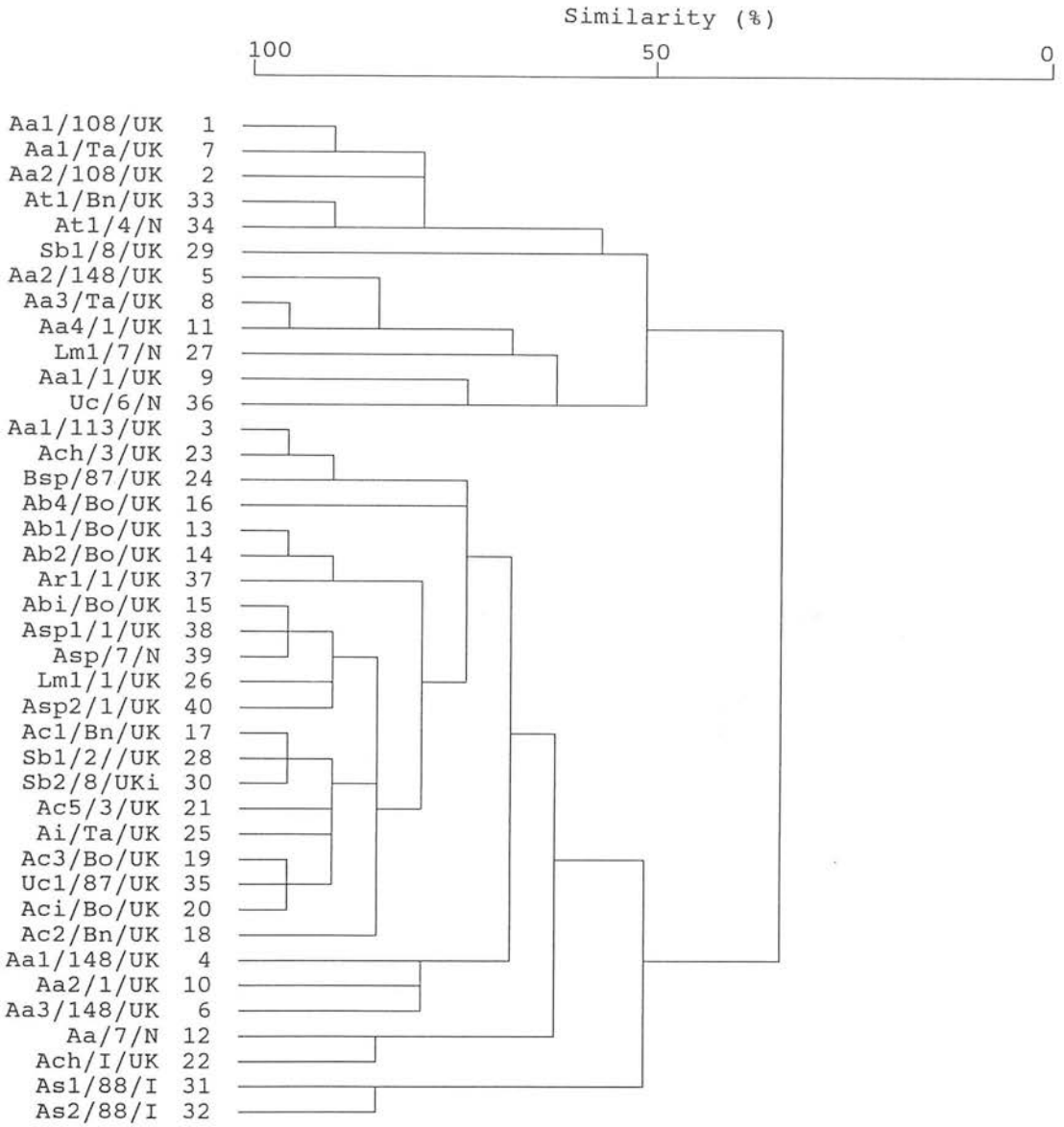


Figure 4.11: A complete-linkage dendrogram for 40 isolates of *Alternaria* and related fungi based on physiological and biochemical characteristics.

At S = 85% all *A.brassicae* and *A.brassicicola* isolates, with the exception of Ab4/Bo/UK, were clustered together into a single group which also contained the isolates listed in clusters 5 and 6 above and in addition Ar1/I/UK, and Bsp/87/UK; Ab4/Bo/UK was added to this cluster at S = 75%.

All 40 isolates formed a single cluster at S = 40%. At S = 45% however there were two distinct clusters, one containing 12 isolates, the other containing 28 isolates. The smaller group consisted mainly of *A.alternata* isolates, but also included *A.tenuissima*, one *Stemphylium* isolate (Sb/8/UK) and one *L.maculans* isolate (Lm/7/N). This cluster was characterised by isolates which produced a large number of metabolites in extracts, but which tended to have low numbers of nuclei in hyphal compartments. The larger group of 28 isolates was characterised by the presence of few metabolites in extracts but by multinucleate hyphal compartments. The clusters formed are shown in dendrogram form in Figure 4.11 and as a scatter plot, following Principal Coordinates (PCO) analysis in Figure 4.12.

The PCO analysis did not provide an exact representation of the distance between isolates. However, the first five latent roots accounted for 65% of the variation (Table 4.7). There were a small number of negative roots which implied that the distance between the isolates was slightly overestimated in the first few roots. This notwithstanding, the main clusters in the data, as detected by HCLA, are visible in the scatter plots in Figure 4.12.

The positions of the *A.brassicae* and *A.brassicicola* isolates on the PCO scatter plot suggested that these species were likely to be near neighbours of the unidentified *Alternaria* species, *A.cheiranthi*, *A.raphani*, *Bipolaris* sp., *L.maculans*, and *S.botryosum*. This grouping was assessed further by Canonical Variates analysis (CVA) and non Hierarchical Cluster analysis.

Table 4.7: PCO analysis of 40 isolates of *Alternaria* and related fungi based on 31 physiological and biochemical variates.

Principal axis	**1**	**2**	**3**	**4**	**5**
Percentage squared distance	32.9	10.9	7.6	7.2	6.2
Latent roots	3.1	1.0	0.7	0.7	0.6
Trace = 9.33					

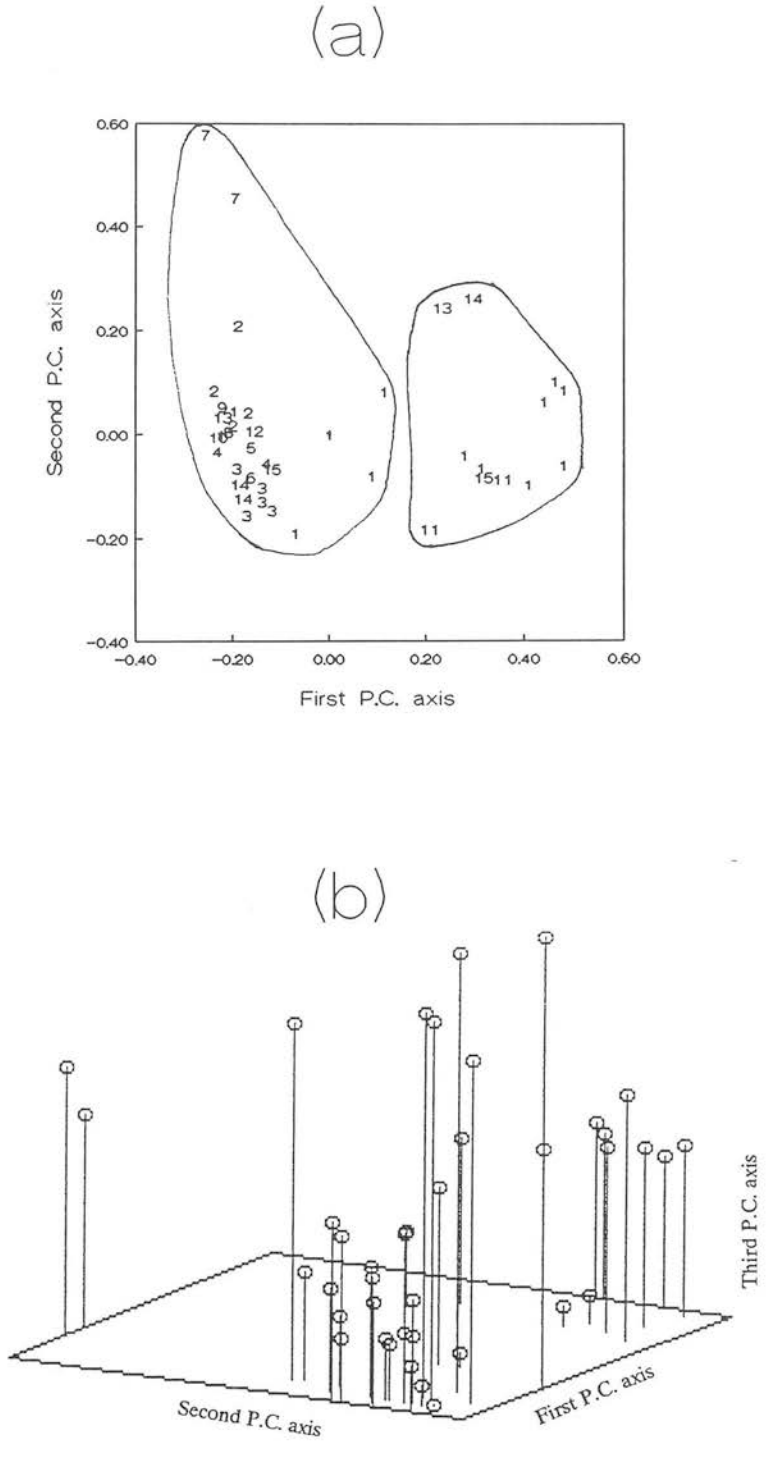


Figure 4.12: Representations of the distance between 40 isolates of *Alternaria* and related fungi in (a) two, and (b) three principal coordinates. In (a) numbers refer to the designation of groups of isolates shown in Table 4.9 columm 1 (page 213), while the solid lines show clusters S = 45%.

Canonical variates analysis and non hierarchical cluster analysis

Three Canonical Variates analyses were conducted. The first used the physiological and cytological data only, the second the biochemical data only, and the third, used the whole data set. The separation of the species in the first two Canonical variates for each of the three analyses is shown in Figure 4.13. In considering the physiological and cytological data only, the group of species was found to form two distinct clusters, with a further four species more or less distinct both from the main clusters and from each other (Figure 4.13a). *A.brassicicola* was found to be clustered with *A.alternata*, *A.infectoria*, *S.botryosum*, and *A.tenuissima*. *A.cheiranthi* was closely related to this cluster, and showed the greatest similarity to *S.botryosum*, but being distinct from the other species. *A.brassicae* was located in the second large cluster with *L.maculans*, *A.solani*, *Alternaria* sp1, and *Alternaria* sp3. *A.raphani* was located close to this group, and showed greatest similarity with *A.solani* and *L.maculans*. *Ulocladium* and *Alternaria* sp2, were found to be distinct from the other isolates and from each other.

When the biochemical data alone were examined, there was less distinction between the species than was revealed by the physiological and cytological data (Figure 4.13b). *A.alternata*, *A.cheiranti*, and *Bipolaris* formed a distinct cluster. *A.solani* and *A.tenuissima* formed distinct individual clusters, while none of the other species was clearly differentiated. *A.brassicicola* lay in a compact cluster of species with the three unidentified *Alternaria* isolates, *A.infectoria*, and *L.maculans*. *A.brassicae* and *A.raphani* formed a small partially distinct cluster, while *Ulocladium* and *S.botryosum* formed a cluster between the group containing *A.brassicicola* and the group containing *A.alternata*.

The CVA of the combined data set is shown as scatter plot in Figure 4.13c, and a summary of the analysis is given in Table 4.8. *A.brassicae* was found to be closely related to the unidentified *Alternaria* species represented by isolates Asp3/7/N and Asp1/1/UK, and to *L.maculans*, while *A.brassicicola* was distinct from *A.brassicae*, but occurred in a tight cluster of species comprising *A.brassicicola*, *Bipolaris* sp., and *S.botryosum*. *A.brassicicola* was found to be more closely related to *A.alternata* than *A.brassicae* was.

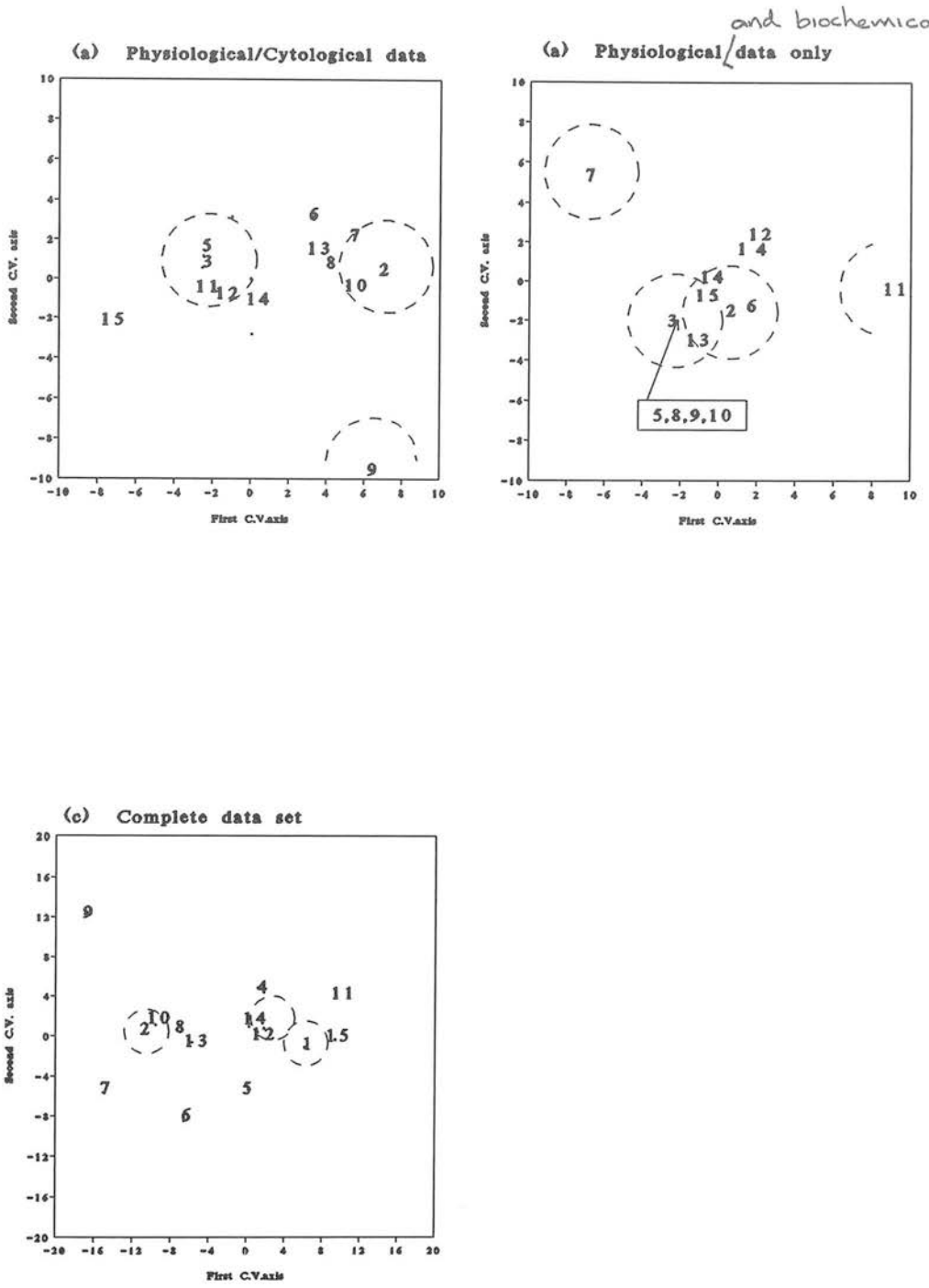


Figure 4.13: Representations of inter-group distances in two canonical variate axes for 15 groups of fungal isolates derived from a set of 15 species using (a) physiological and cytological data, (b) biochemical data, (c) a combined data set. Species 95% confidence limits are shown by circles.

Table 4.8: A summary of a Canonical Variates analysis based on biochemical, and physiological and cytological data of 40 isolates of *Alternaria* and related fungi which had been allocated to 15 morphological species.

CVA axis	1	2	3
Latent root	91.4	16.7	10.6
Percentage variation	67.4	12.3	7.8
Latent vectors			
Growth at pH 2	2.2	-2.4	-2.2
Growth At pH 12	1.9	6.2	-3.6
Hydrolysis of Tween 80	-1.8	2.7	-2.4
Chromogenic	0.0	0.0	0.0
Pycnidial	0.0	0.0	0.0
Nuclei in hyphal tip	-1.5	1.0	-0.2
Nuclei in 1st distal compartment	2.7	-5.4	-3.1
Nuclei in 2nd distal compartment	-0.9	4.4	3.5
Nuclei in 3rd distal compartment	-2.2	2.3	2.4
Hyphal width	-3.1	-3.8	-3.5
Metabolite profile variate 1 *	0.2	0.7	-0.7
2	-0.3	0.7	-1.0
3	-3.8	-3.6	4.3
4	0.4	-0.4	1.5
5	7.1	2.6	-2.9
6	-0.8	-0.2	1.4
7	-4.6	-4.4	2.9
8	-0.5	0.9	-1.2
9	-0.3	-2.7	-1.0
10	2.3	-0.1	-0.9

* In order to utilise the metabolite band data from the 21 scores used in the HCA and PCO the scores for the bands were combined into groups of three and a new score was allocated for the sequence of each triplit of bands.

The unrelated species, *Ulocladium*, was also found to cluster quite closely to *A.alternata*. Four species were found to be relatively distinct; these were *A.infectoria*, *A.raphani*, *A.solani*, and Asp2/1/UK.

In order to assess whether the 15 designated species represented a natural grouping of the 40 isolates into 15 species, the initial morphological classification of isolates (clustering method 1) was used as the starting point for a non-Hierarchical Cluster Analysis (nHCA) utilising a minimum within-group sum of squares criterion (clustering method 2). The nHCA was also conducted using the 29 groups formed by hierarchical cluster analysis at 95% similarity (clustering method 3). The allocation of isolates to the initial

15 species and their allocation to the 15 groups formed by the nHCA from both starting positions are shown in Table 4.9.

The separation of the 15 groups of isolates under each of the clustering methods is shown in Figure 4.14. The main result of concern to the present study from these clustering procedures was the difference in the behaviour of the *A.brassicae* and *A.brassiciciola* isolates under the clustering procedures. Irrespective of the starting classification the *A.brassicicola* isolates tended to remain clustered together while the *A.brassicae* isolates were separated under both clustering procedures. Reffering to Table 4.9 it can be seen that four of the five *A.brassicicola* isolates (Ac1/Bn/UK, Ac3/Bn/UK, Ac5/3/UK/, Aci/Bo/UK) were in cluster 8 following nHCA method 2, with the remaining isolate (Ac2/Bo/UK) being in cluster 3. Similarly under nHCA method 3, four of the five *A.brassicicola* isolates (Ac1/Bn/UK, Ac2/Bn/UK, Ac3/Bo/UK, Aci/Bo/UK) remained in a single cluster, with the remaining isolate (Ac5/3/UK) in cluster 1. In contrast, irrespective of the starting classification, all of the *A.brassicae* isolates occurred in different clusters, except when grouped simply by morphology (method 1).

Discussion

The present study found that *A.brassicae* and *A.brassicicola* differ with respect to morphological, physiological, and biochemical characteristics. Ellis (1971) reported hyphal widths for the two species as 1.5 - 7.5 m and 4 - 8 m respectively. In the present study hyphae of *A.brassicicola* were found to be slightly narrower than those of *A.brassicae*, but the ranges recorded were lower than those reported by Ellis. The difference between the results of the present study and Ellis's may be accounted for by two factors. First, a limited number of isolates was examined in the present study. Second, Ellis examined hyphae from a number of substrates, while all observations made in the present study were of hyphae grown in only one set of standardised conditions. This not withstanding, the isolates examined in this experiment appear to be representative of each species.

A.brassicae and *A.brassicicola* differed with respect to numbers of nuclei in their hyphal compartments. Knox-Davies (1979) examined nuclear distribution in *A.brassicicola*, while Campbell (1969)

Table 4.9: The allocation of 40 isolates to 15 groups on the basis of, (1) morphological characters; (2) nHCA utilising biochemical and physiological characters starting from the grouping at (1); (3) nHCA, as in (2), but starting from an allocation of the 40 isolates to 29 groups on the basis of heirarchical cluster analysis.

Group number	Clustering method (1)		(2)		(3)
1	Aa/7/N, Aa1/108/UK, Aa2/108/UK, Aa1/113/UK Aa1/148/UK, Aa2/148/UK Aa3/148/UK, Aa1/1/UK Aa2/1/UK, Aa4/1/UK Aa1/Ta/UK, Aa3/Ta/UK	1	Aa2/1/UK, Aa/7/N	1	Aa1/148/UK, Aa3/148/UK Ac5/3/UK, Ach/3/UK Uc1/87/UK
		2	Ab4/Bo/UK		
		3	Aa1/108/UK, Aa/148/UK Ac2/Bn/UK, Ai/Ta/UK Uc1/87/UK	2	Aa1/108/UK, Aa1/Ta/UK
				3	Aa2/108/UK, At1/148/UK
2	Ab1/Bo/UK, Ab2/Bo/UK, Abi/Bo/UK, Ab4/Bo/UK	4	Ach/I/UK	4	Uc1/6/N
		5	Aa1/113/UK, As1/88/I	5	Ab1/Bo/UK, Lm1/7/N Lm1/7/N, Ar1/I/UK Asp1/1/UK
3	Ac1/Bn/UK, Ac2/Bn/UK, Ac3/Bo/UK, Aci/Bo/UK, Ac5/3/UK	6	Aa2/148/UK, Aa1/Ta/UK Aa3/Ta/UK, Aa4/1/UK		
				6	Sb/8/UK
4	Ach/3/UK, Ach/I/UK	7	Lm1/7/N		
				7	Aa2/148/UK
5	Ai/Ta/UK	8	Aa1/1/UK, Ac1/Bn/UK Ac3/Bo/UK, Ac5/3/UK	8	Aa1/1/UK, Aa1/7/N
6	Ar1/I/UK		Aci/Bo/UK, Bsp/87/UK Sb1/2/UK, Sb/8/UK	9	Abi/Bo/UK, As2/88/I
7	As1/88/I, As2/88/I		At1/4/N	10	Aa1/1/UK, At1/4/N
8	Asp1/1/UK	9	Abi/Bo/UK, As2/88/I		
9	Asp2/1/UK	10	Aa2/108/UK, Aa3/148/UK At1/148/UK	11	Aa2/1/UK, Aa1/7/N
10	Asp3/7/N	11	Uc1/6/N	12	Ab2/Bo/UK, Bsp1/87/UK Asp3/7/N, Asp2/1/UK
11	At1/4/N, At1/Bn/UK	12	Sb1/8/UK		
				13	Aa1/113/UK, Ach1/I/UK As1/88/UK,
12	Bsp/87/UK	13	Ab1/Bo/UK, Lm1/Bn/UK Ar1/I/UK, Asp1/1/UK		
13	Lm1/7/N, Lm1/Bn/UK			14	Ac1/Bn/UK, Ac2/Bn/UK Ac3/Bo/UK, Aci/Bo/UK Ai1/Ta/UK, Sb1/2/UK Sb2/8/UK
14	Sb1/2/UK, Sb1/8/UK, Sb2/8/UK	14	Asp2/1/UK, Asp3/7/N Ab2/Bo/UK		
15	Uc1/6/N, Uc1/87/UK	15	Ach/3/UK	15	Ab4/Bo/UK

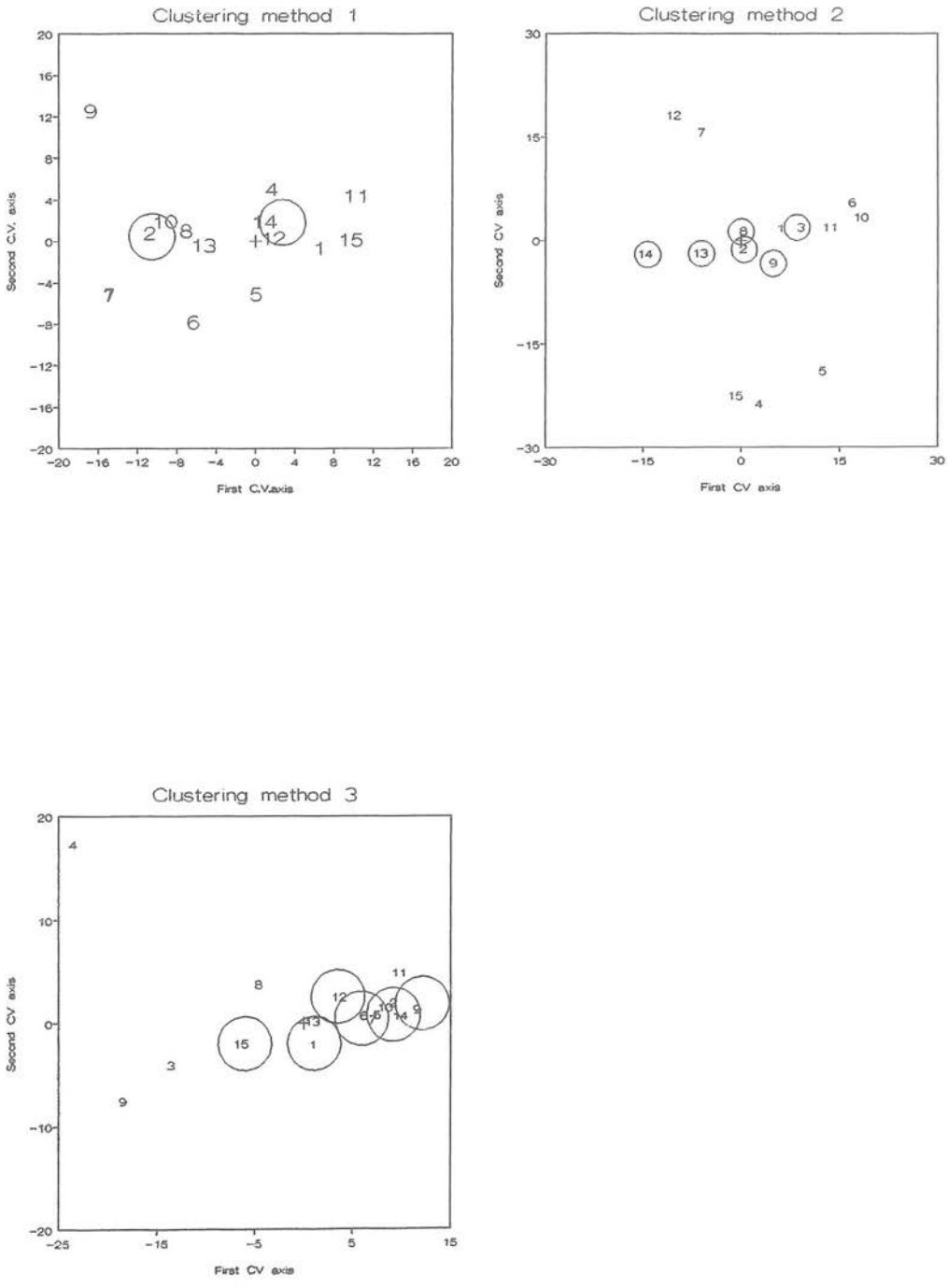


Figure 4.14: Representations of inter-group distances in two Canonical Variate axes for 15 groups of fungal isolates derived from a set of 40 isolates by different clustering methods. Group membership is given in Table 4.10. Group 95% confidence limits are shown by circles.

made a detailed study of conidium formation in this species which included examination of nuclear distribution in the conidiophore. Knox-Davies, found that *A.brassicicola* was multinucleate, with hyphal compartments containing up to 33 nuclei and tip compartments containing a maximum of 27 nuclei. In the present study, the maximum number of nuclei recorded for *A.brassicicola* in hyphal tips was 10, while intercalary compartments were never observed with more than six nuclei. The majority of cells examined contained one to three nuclei, which is fewer than the number commonly found by Knox-Davies (four to nine), but similar to that recorded for *A.alternata* in this study and by *Hartmann* (1966), and for *A.solani* (this study; Stall, 1958). Nuclear numbers in cells of *A.brassicae* have apparently not been examined before. The results of the present study suggest that individual compartments generally contain two to six nuclei, while hyphal tips contain five to 10 nuclei.

Knox-Davies (1979) noted that the multinucleate condition of *Alternaria* species provides them with the means to maintain a heterokaryotic condition. This would allow species to maintain genetic diversity since nuclei carrying deleterious alleles can be supported in heterokaryotic isolates. However, *A.brassicicola* shows little intraspecific variation while *A.brassicae*, *A.alternata*, and *A.solani* all show considerable variation with respect to cultural characteristics. This difference betweeen species may suggest that the mechanisms which determine the level of genetic diversity in each species are different. Knox-Davies suggested that control over diversity in *A.brassicicola* is partly determined by the control over nuclear migration into juvenile conidia. Thus, if only one or a few conidia are responsible for the nuclear content of each conidium genetic diversity present in the mycelium can be reduced at conidium formation. Furthermore, if certain genotypes confer a selective advantage on nuclei for inclusion in conidia, diversity will be further reduced in the nuclear population of the conidium as compared with that in the mycelium. It was also suggested by Knox-Davies that in specialised pathogens such as *A.brassicicola* selection pressure is likely to favour reproductive capacity over maintenance of genetic variability, so that conidia which carry genes for sporulation as opposed to vegetative growth will tend to be selected for. This theory requires further investigation, but it is likely that a range of factors are responsible for the production of conidia by *Alternaria* species. One question which is immediately apparent is why *A.brassicicola* and *A.brassicae* should differ so much in their capacity for maintaining sporulation in culture. The results of Chapters 2 and 3 of the present study and those of other workers (Neergaard, 1945; Changsri & Weber, 1963; Prasanna,

1984) suggest that *A.brassicae* is a more specialised a pathogen as *A.brassicicola*, and presumably faces similar selection pressures in its environment. It may be that species such as *A.brassicae* show a reduced capacity for sporulation because their specialisation as pathogens results in a general lack of "fitness" for growth in axenic culture.

The Hierarchical Cluster analysis suggested that *A.brassicae* and *A.brassicicola* were relatively similar in comparison with the least similar species examined. In studies which used primarily enzymatic markers rather than secondary metabolites Mugnai *et al.*, (1989) reported a comparable level of homogeneity in a group of 32 *Beauveria* isolates and two isolates of *Tolypocladium*, and stated that the low similarities found in the group of isolates resulted partly from the variability within the group and partly because Jaccard's coefficient, rather than a simple matching coefficient, was used to construct the similarity matrix. Both of these factors could also account for the low level of similarity recorded between isolates in the present study.

In Canonical Variates analysis the species were differentiated on the basis of differences in a range of secondary metabolites, and their ability to hydrolyse Tween 80, in addition to differences in nuclear numbers in hyphal tip compartments. In considering differences between the species in relation to metabolites produced, the results of the present experiment confirm the findings of the experiments in Chapter 3, in that there appear to be metabolites which are representative of individual species, and that species can be partly differentiated on this basis. However, the clear distinction between *A.brassicae* and *A.brassicicola* noted on the basis of physiological and cytological characters was not supported by the examination of secondary metabolites. Bridge *et al.* (1989) found that an overall similarity in the secondary metabolite profiles of isolates of *Sarocladium*, in conjunction with other characters, allowed them to conclude that two previously recognised species were probably a single species. Secondary metabolites have also been useful in the separation of species of *Penicillium* (Bridge, Hawksworth, Kozakiewicz, Onions & Paterson, 1986). However, secondary metabolites profiles must be used with caution in taxononmic studies since the metabolites produced by any given isolate may vary considerably with incubation period (Experiment 3.1) prior to analysis, and also over longer periods if isolates are maintained in axenic culture.

Alternatives to the use of secondary metabolites in taxonomic studies include, enzyme and soluble protein markers (Bridge *et al.*, 1989; Mordue *et al.*, 1990), pyrolysis-mass spectrometry (Berkeley, Goodacre, Heleyer, & Kelley, 1991), or RAPD DNA (or AFLP) mapping (Williams, Kubelik, Livak, Rafalski, & Tingey, 1991). Of these techniques, analysis of protein markers, either individual isozymes or total protein content, is susceptible to some variation due to culture conditions, in a similar manner to secondary metabolites, but is probably more reproducible and is used more widely. Pyrolysis-MS involves the controlled combustion of samples of the organism in an inert atmosphere under carefully controlled conditions, followed by spectrophotometric analysis of the volatile products released. It has been shown that organisms have characteristic combustion spectra and multivariate spectrum data can be subjected to CVA to reveal clusters of isolates. Analysis of DNA polymorphisms has, until recently, relied on the time consuming process of RFLP map construction. However, it has been shown by independent groups of workers that reproducible DNA polymorphism fingerprints can be produced by the amplification of arbitary DNA sequences using random primers in the polymerase chain reaction (PCR) technique (Welsh & McClelland, 1991; Williams *et al.*, 1991). Furthermore, primers with only eight bases can give reproducible results (Caetano-Anolles, Bassam & Gresshoff, 1991). Direct analysis of the differences in the DNA of *Alternaria* species would provide the most attractive means of relating morphological and ecological variation to phylogenic origin. Recent studies of *A.linicola* isolates have shown that the protocol described by Caetano-Anolles *et al.* (1991) gives suitable amplification for use in this type of study (Evans, McRoberts, & Finch unpbl).

In relating the results of this experiment to those of Experiment 4.1 (the study of the published descriptions of *Alternaria* species) the present experiment provided some evidence that spore morphology may give an indication of taxonomic relationships within the *Alternaria*, and additionally it indicated the relationships of *A.brassicae* and *A.brassicicola* with other genera in the Pleosporaceae. The morphological study presented in Experiment 4.1 suggested that *A.brassicicola* was clustered with other small-spored, chain-forming species, including *A.alternata*, while *A.brassicae* was identified as morphologically distinct from both of the main classes within the anamorph-genus, but was more closely associated with the *Noncatenatae*. In this experiment *A.brassicicola* was again found to be closely related to the chain-forming

or small-spored species, including the *Stemphylium* isolates, while *A.brassicae* was found to be most closely related to two unidentified *Alternaria* isolates and *L.maculans* (Figure 4.13c).

Considering the possibile identity of the teleomorphs for *A.brassicae* and *A.brassicicola*, the present experiment suggests that teleomorphs for *A.brassicae* and *A.brassicicola* may lie in different genera. The teleomorph genus, *Lewia*, is a close segregate of *Pleospora* Rab., which produces only *Stemphylium* anamorphs (Simmons, 1986c). In the present experiment, *A.brassicicola* was found to be similar to *A.alternata* and *Stemphylium*, suggesting that the teleomorph of *A.brassicicola* may occur in this section of the Pleosporaceae. Additional evidence of the close relationship between *A.alternata* and *Stemphylium* was provided by Heiny & Gilchrist (1991) who found that *A.alternata* f.sp. *lycopersici* and *S.botryosum* produce an identical peptide phytotoxin. These independant results suggest that the methedology used in this experiment identified true similarities and differences among the fungi examined. This being the case, the apparent relatedness of *A.brassicae* to *L.maculans* rather than to *Stemphylium* suggests a separation of *A.brassicae* and *A.brassicicola* at the generic level in the Pleosporaceae. In addition Wehmeyer (1961), considered that *Pleospora* could have arisen from ancestral *Leptosphaeria* species, a theory which tentatively implies that *A.brassicae* may be a morre ancient form species than *A.brassicicola*

It is tempting to speculate further on this theory since it is relatively simple to imagine that the type of conidium formation in *A.brassicae* could give rise to a chain-forming habit if there was suitable selection pressure for an increase in reproductive rate. Indeed, in *A.longipes* Simmons (1981) noted that in rapidly sporulating cultures new spores are produced so rapidly that the transition from conidium body to *pseudorostrum* becomes shortened and difficult to identify. However, although *A.brassicae* may be more closely related to *Leptosphaeria* than *A.brassicicola*, *Leptosphaeria* species do not produce naked conidia and a degree of divergence between *A.brassicae* and modern *Leptosphaeria* species has obviously occurred. Elucidation of the actual phylogenic relationships within the group would require treatment of a large number of isolates of *A.brassicae*, *A.brassicicola*, *Leptosphaeria*, and *Pleospora* to a comprehensive array of physiological and biochemical tests, and preferably some examination of DNA polymorphism. It would be

instructive also to include in this type of experiment a range of *Alternaria* species covering the range of morphological forms. A study based more on molecular genetics would perhaps provide definitive answers to the likely trends in phylogeny which have been suggested by this experiment.

Experiment 4.3: Fungicide insensitive isolates of *Alternaria brassicae* and *A.brassicicola* in relation to variability.

Results

With *A.brassicae* and *A.brassicicola*, the ANOVA of data for colony growth indicated that both of the main effects (isolate and fungicide concentration) and the interaction between them were significant. Examining the results for *A.brassicae*, some difference was found between the isolates in their sensitivity to iprodione, but the general shape of the response curve of colony diameter against iprodione concentration was the same in most cases. It was found that the insensitive isolate, Abi/Bo/UK, produced larger colonies than Ab1/Bo/UK at all concentrations of iprodione, except in the control treatment where Ab1/Bo/UK produced larger colonies. Isolate Ab2/Bo/UK was intermediate in its response between the other two isolates and showed a more erratic response to increasing fungicide concentration. Thus, although no growth was noted for Ab2/Bo/UK at iprodione concentrations of 50μg/ml and 1000μg/ml, at 200μg/ml mean colony diameter at 13 days after inoculation was 13 mm. The most sensitive *A.brassicae* isolate, Ab1/Bo/UK produced small colonies after 13 days growth at an iprodione concentration of 1000μg/ml, but not at lower concentrations. The growth of Ab1/Bo/UK and Ab2/Bo/UK at the higher fungicide concentrations meant that a polynomial function of iprodione concentration fitted rather poorly to colony diameter for the *A.brassicae* isolates. The interaction between isolates and fungicide concentration with respect to colony diameter is shown in Figure 4.15.

The interaction between isolates, fungicide concentration, and sampling time for the *A.brassicicola* isolates is also shown in Figure 4.15. The *A.brassicicola* isolates displayed a wider range of ED_{50} values than the *A.brassicae* isolates and were found to have a higher growth rate in the control plates than the *A.brassicae* isolates. Isolate Aci/Bo/UK showed a high level of insensitivity to iprodione, while

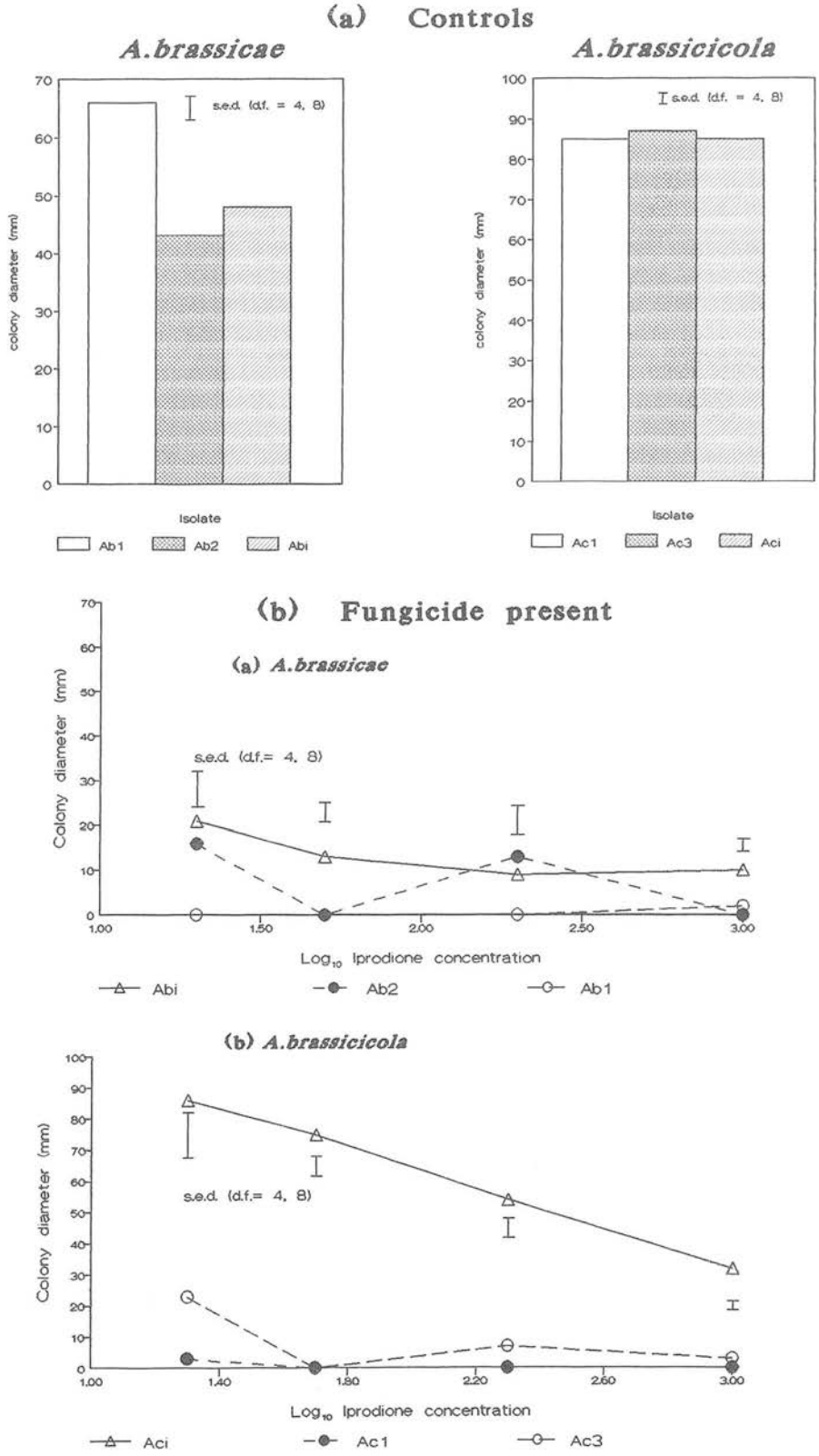


Figure 4.15: The effect of increasing concentration of iprodione on colony diameter of isolates of (a) *A.brassicae* and (b) *A.brassicicola* on supplemented PDA. Measurements made at 13 days after inoculation.

Aci/Bn/UK was the most sensitive, and Ac3/Bo/UK showed an erratic response to increasing iprodione concentration, producing colonies at 20, 200 ,and 1000 but not 50 μg/ml.

Estimated ED_{50} values for all six *Alternaria* isolates are shown in Table 4.10, which also records the results of tests on the response of each isolate to high osmotic pressure in the PDA/NaCl growth medium, and observations on hyphal growth habit. Photomicrographs of hyphae, and photographs of colonies are shown in plate 4.4.

Table 4.10: Characteristics of hyphal growth for six *Alternaria* isolates in relation to their sensitivity to iprodione and osmotic sensitivity.

Isolate	ED_{50} μg/ ml	Osmotic sensitivity	Hyphal growth and colony characteristics
(*A.brassicae*)			
Ab1/Bo/UK	5	LOW	normal, colonies even
Ab2/Bo/UK	12	LOW	normal, colonies slightly uneven
Abi/Bo/UK	15	LOW	coiled with profuse branching, colonies uneven and feathered
(*A.brassicicola*)			
Aci/Bn/UK	5	LOW	normal, colonies even
Ac3/Bo/UK	8	LOW	normal, colonies even
Aci/Bo/UK	501	LOW	normal, colonies even

For both *A.brassicae* and *A.brassicicola*, the most sensitive isolates had ED_{50} values of 5 g/ml, with other isolates in a range from 8 - 501 μg/ml iprodione. The ED_{50} value for Aci/Bo/UK (501 g/ml) was, therefore, greater than those for all other isolates tested by a factor of 33-100.

While the *A.brassicicola* isolates produced no abnormal growth characters in any growth medium, in *A.brassicae* isolates Ab2/Bo/UK, and Abi/Bo/UK produced uneven colonies edges, which were more pronounced in the case of Abi/Bo/UK, while additionally, individual hyphae of Abi/Bo/UK, but not Ab2/Bo/UK, were found to be unable to maintain linear growth resulting in pronounced coiling; the hyphae also branched profusely at colony margins giving colony edges a feathered appearance. These growth abnormalities appeared in all media.

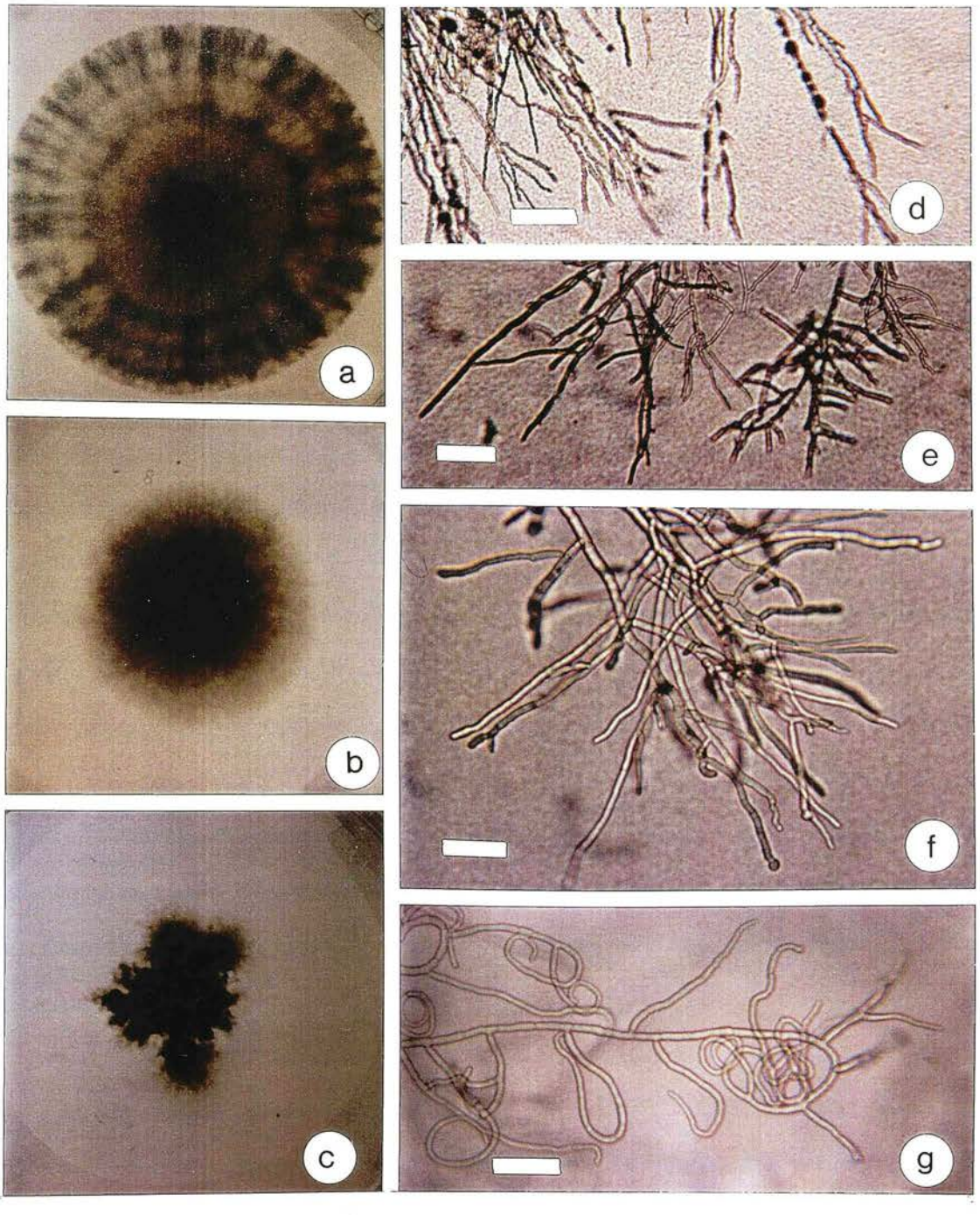
Plate 4.4: Colony and hyphal growth characteristics of *A.brassicae* isolates with different sensitivities to iprodione.

a. - c. Colony morphology of *A.brassicae* isolates on unsupplemented PDA after selection for resistance to iprodione. **a.** Ab1/Bo/UK (ED_{50} = 5 μg/ml), showing typical, even colony edges. **b.** Ab2/Bo/UK (ED_{50} = 8.5 μg/ml) showing smaller colony size, increase in pigmentation, and slightly uneven colony edges. **c.** Abi/Bo/UK (ED_{50} = 12.5 μg/ml) showing small colony size, deep pigmentation and uneven, feathered colony margins.

d. Hyphae at the colony margin of Ab1/Bo/UK showing normal hyphal growth habit in agar culture. Bar = 40 μm.

e. and f. Hyphae at the colony margin of Abi/Bo/UK showing exagerated branching. Bar = 30 μm.

g. Hyphae at the colony margin of Abi/Bo/UK showing coiling and lack of linear growth. Bar = 40 μm.


a
b
c
d
e
f
g

Discussion

Although only a few isolates of each species were examined, there was an indication that *A.brassicae* and *A.brassicicola* may differ in their potential for insensitivity to iprodione, and perhaps in the genetic basis of insensitivity. Thus, the pattern of insensitivity displayed by *A.brassicicola* was suggestive of a single-gene effect, with sensitivity present either at a very high level (Aci/Bo/UK) or absent (Ac1/Bo/UK, Ac3/Bo/UK), while in *A.brassicae* variation in insensitivity was more quantitative in nature, which may suggest a polygenic basis. The need for an examination of a larger number of isolates to test this theory further is emphasised.

The difference in the characteristics of the fungicide insensitive isolates of *A.brassicae* and *A.brassicicola* suggested that the mechanism of insensitivity may differ between the two species. In the case of *A.brassicae* greater insensitivity was correlated with a lower colony growth rate, morphological abnormality, and disruption to linear hyphal growth suggesting that insensitivity is associated with a disruption in normal cell growth. These observations are consistent with a disfunction in membrane transport, suggesting that insensitivity might be related to reduced uptake of the fungicide in this species. However, Edlich & Lyr (1987) noted that it is often difficult to relate the site mode of action of a fungicide to the symptoms expressed in the fungus, and similar abnormal hyphal growth characteristics in the less sensitive strains of *A.brassicae* may have arisen from a primary cause which is not directly involved in membrane function or cell growth. Although insensitivity to dicarboximides has been related to alterations in membrane characteristics leading to increased osmotic insensitivity in some species (Pommer & Lorenz, 1987), none of the isolates of *A.brassicae* tested showed osmotic sensitivity.

An interesting ecological question which arises from the present study is the failure of either *A.brassicae* or *A.brassicicola* to generate fungicide-insensitive populations in the field when the physiological, and presumably genetic, potential to produce insensitive strains exists in both species. Dekker (1984) pointed out that production of insensitive isolates under intense selection pressure in the laboratory does not automatically mean that a species will generate insensitivity in the field at a level which is economically important. With *A.brassicae* and *A.brassicicola* fungicides are used predominantly to control seed-borne inoculum with the result that the target population of the fungicide is relatively small

and not actively growing at the point when the fungicide is applied. It may be the case that treatment of the fungal population while it is in an inactive state may prevent it from generating insensitive and fit strains, which might otherwise arise in the field. Some support for this hypothesis is provided by the a similar findings in the case of *Ascochyta pisi* (M.Zziwa, pers. comm.) in relation to resistance to the aromatic hydrocarbon fungicides. Cross resistance between the dicarboximides and the aromatic hydrocarbon fungicides has been reported, and they may have similar modes of action (Edlich & Lyr, 1987). However, problems of insensitivity to mercurial seed treatments in the barley leaf stripe pathogen *Pyrenophora graminea* and the oat leaf spot pathogen, *P.avenae* suggest that it is possible for insensitivity to arise in fungi even when the target population is predominantly seed-borne, although it should be noted that an ability to become insensitive to mercury is in itself a rather exceptional characteristic.

4.5 GENERAL DISCUSSION

Many of the workers who have studied the *Alternaria* have commented on and examined the variability in this group of fungi. Reviews of the literature conducted in this study, in conjunction with experimental observations of variation in features of pathogenicity, biochemistry, and physiology, have indicated the diversity which exists within the anamorph-genus.

This chapter has attempted to examine variability in the *Alternaria* in relation to morphology and ecological behaviour in order determine the relations of *A.brassicae* and *A.brassicicola* with the rest of anamorph-genus. *A.brassicae* was found, as suggested by Neergaard (1945), to be morphologically distinct from both the *Longicatenatae* and *Noncatenatae*, although its is morphologically more closely related to the latter. On a broad morphological and ecological basis the nearest neighbour of *A.brassicae* was *A.passiflorae* (97% similarity), *A.brassicicola* was found to be most similar to *A.eureka* (94% similarity), while the similarity between *A.brassicae* and *A.brassicicola* was found to be 75%. The relatively low similarity between *A.brassicae* and *A.brassicicola* suggested by the morphological examination in Experiment 4.1 was supported in the second experiment which indicated that in a heterogeneous collection of fungi, *A.brassicae* and *A.brassicicola* were more similar to species from other genera within the Pleosporaceae than they were to each other. It might be implied from this separation that the two species represent lines of conidium development in different genera of the Pleosporaceae. Further investigation of the phylogeny of *A.brassicae* and *A.brassicicola* would provide information both on the degree of genetic diversity within the *Alternaria* and also provide valuable information on the Pleosporaceae, one of the most taxonomically complex groups in the Ascomycetes.

As a result of the various observations made on *Alternaria* species during this study several graphical representations, or ordinations, of the relative similarity between species have been produced. Thus, in Chapter 2 a number of pathogenicity characters were used to assess behavioural or ecological similarity between six species (section 2.5), while these six species have also been examined in this chapter either on mainly morphological grounds (Experiment 4.1), or on the basis of secondary metabolism and cytology (Experiment 4.2). Within all of these assessments certain groupings of the fungi were indicated, suggesting

that there may be a correlation between morphology, pathogenic behaviour, and physiology/biochemistry in these fungi. For example, *A.brassicae* and *A.solani* were found to display a predilection for stomatal penetration, are morphologically similar, and have relatively high numbers of nuclei in hyphal compartments.

Considering any individual ordination, the relationships between the points representing the species are unaffected if the whole ordination is rotated, reflected or isotropically scaled. By employing these procedures it is possible to assess whether two or more ordinations of the same set of units display similar relationships between the units, by attempting to map all of the ordinations to each other. The iterative process for performing this is known as Generalised Procrustes analysis (GPA) (Krzanowski, 1990).

A GPA was performed on the six species examined in Chapter 2 to assess the similarities between the ordinations produced in section 2.5, and Experiments 4.1 and 4.2. The influence of the additional species examined in Experiments 4.1 and 4.2 on the calculated relative distances between species was removed by abstracting the data for the six species in question and re-analysing them. The data used for GPA were the coordinates of the species in the first five principal coordinate axes, derived from PCOs of similarity matrices constructed from the original data sets.

Figure 4.16 shows the three ordinations of the six *Alternaria* species in their first two principal coordinate axes prior to GPA, while Figure 4.17 shows the consensus configuration for the six species and the configurations for each of the data sets following the analysis. Table 4.11 shows a summary of the GPA, indicating the main sources of variance within and between the three configurations.

The GPA provided a consensus ordination (Figure 4.17) of the three sets of data which supported the view that similar separations of the six *Alternaria* species occurred in the individual analyses. Thus, the consensus points for *A.brassicae* and *A.solani* were separated from the other species in the first principal axis, while the points for *A.alternata*, *A.infectoria* and *A.brassicicola* were less clearly

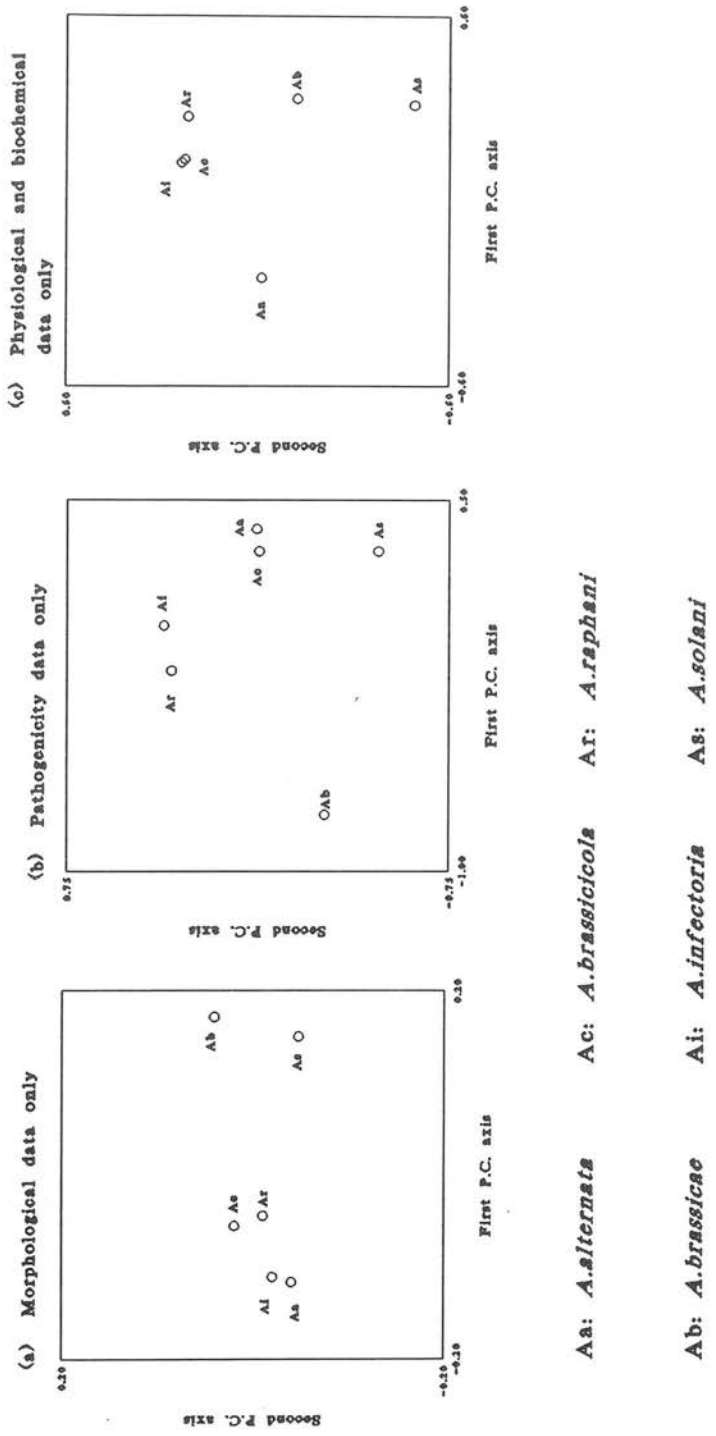


Figure 4.16: Ordinations derived from (a) morphological, (b) pathogenicity, and (c) physiological and biochemical data for six *Alternaria* species.

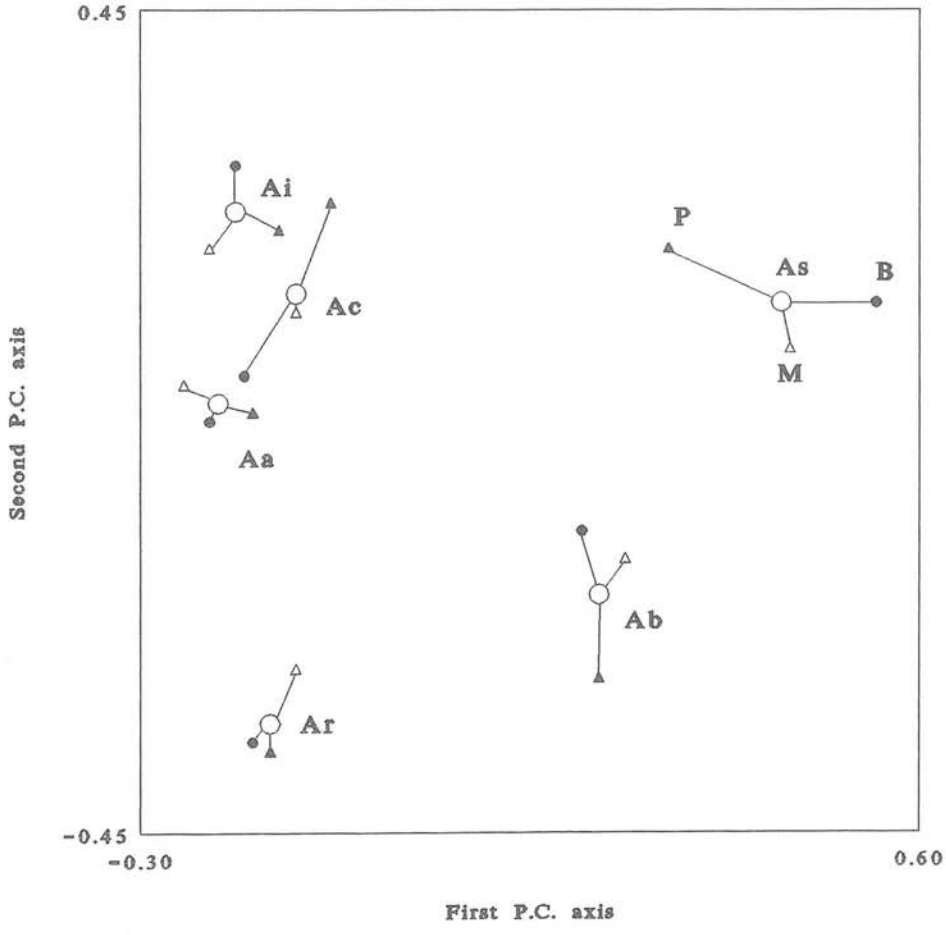


Figure 4.17: A plot of the consensus configuration and configurations derived from morphological (M), pathogenicity (P), and physiological (B) data for six *Alternaria* species following Generalised Procrustes analysis of the original ordinations for the species.

Table 4.11: Summary of a Generalised Procrustes analysis of three ordinations of six *Alternaria* species. The ordinations were based on pathogenicity characteristics, morphological characters, and biochemical/physiological characters of the species.

Analysis of variaton				% Residual sums of squares		
	Principal axis			Within		Within
Configuration	1	2	3	configurations		species
Consensus	*38*	*24*	*18*			
Morphological	40	15	14	20	*A.brassicae*	16
Pathogenicity	23	36	24	43	*A.brassicicola*	25
Physiological	50	24	13	37	*A.raphani*	8
				Total 100	*A.solani*	21
					A.alternata	14
					A.infectoria	16
					Total	**100**

distinguished. The residual variance (Table 4.11) was more or less equally split between species, implying all of the species were similarly placed in the original ordinations. In examining the range in goodness-of-fit displayed across the six species, *A.brassicicola* accounted for 25% of the residual sum of squares, while *A.raphani* accounted for only 8%. The large residual associated with *A.brassicicola* is indicated by the relatively long connections between the consensus point and the individual configurations for this species (Figure 4.16), although these distances give only approximations of the true residuals. Considering the distribution of the residual variance over the configurations, the largest residual was associated with the ordination from the pathogenicity data, while the smallest was associated with the morphological data. This result suggests that although there is a good fit between morphology, pathogenicity characteristics and physiology, of the three types of character, pathogenic behaviour correlates less well to the other two types than they do to each other. If the species examined here are representative of the morphological types to which they belong, the results of this study indicate that the major morphological divisions in the *Alternaria* also represent physiological and to some extent behavioural differences.

A randomisation test was conducted to examine the residual variance of the GPA, in order to assess evidence for a correlation between the original ordinations. The test assessed the significance of the size of the residual variance of the GPA, and thus the correlation between the original ordinations. The procedure for the test was as follows: the morphological ordination of the species was taken to be fixed. The coordinates for each species from both of the other ordinations were randomly allocated any species

(including the species to which they belonged). Following randomisation, a GPA was conducted and the value of residual variance was saved. The steps just outlined were repeated 500 times to obtain a sample of 500 residuals; these showed an approximately normal distribution and indictated the range of possible residuals under conditions where there was random association between morphology, pathogenic behaviour, and biochemistry/physiology. The value of the residual obtained from the original analysis fell in the lower 5% tail of this distribution, suggesting that the original ordinations were more similar to each other than would be expected if there was no association between morphology, pathogenic behaviour, and biochemistry/physiology.

Examining the different morphological types in the *Alternaria* in relation to ecological behaviour further, there is perhaps some support for the theory put forward above. Thus, the *Noncatenatae* are predominantly species with a restricted host range such as *A.solani*, *A.porri*, *A.linicola*, and *A.dauci*, while the *Longicatenatae* are a mixture of ecological types, but include a number of species such as *A.alternata*, *A.infectoria*, *A.conjuncta*, and *A.eureka* which occur as saprophytes or opportunistic pathogens on a wide range of plants. This proposed separation of species is too much of a generalisation if all of the species in the two morphological groups are included however. For example, *A.brassicicola* (*Longicatenatae*) has a restricted host range, while *A.longissima* (*Noncatenatae*) is a plurivorous species. This notwithstanding, careful study of the *Alternaria*, relating morphology to ecological behaviour, may well reveal a core of species in each group which display the correlations outlined above.

Knox-Davies (1979) drew attention to the distinction which between the *Longicatenatae* and the *Noncatenatae*. Species in the former generally maintain their capacity for sporulation in culture, while in the latter this capacity is often lost. Knox-Davies's proposals realting to selection in the nuclear population of heterokaryotic fungi were outlined in the discussion of Experiment 4.2, above. Observations on *A.alternata* (*A.tenuis*) (Hartmann, 1966) and *A.brassicicola* (Campbell, 1970; Knox-Davies, 1979) suggested that low numbers of nuclei are involved in conidiogenesis. These cytological observations are supported by the results of Netzer and Kenneth (1970) who found that conidia isolated from heterokaryotic mycelium of *A.dauci* were predominantly monokaryotic. Netzer and Kenneth's findings suggest that either a single nucleus gave rise to all nuclei in the mature conidia or, if more than one nucleus was transported into the

immature conidum, all of the nuclei were of the same genotype.

The current study was not intended as a detailed cytological study of *A.brassicae* and *A.brassicicola*. However, conidiophores and juvenile conidia, in their initial single-celled state, of *A.brassicae* were found to be multinucleate. The cytoplasmic connection between the the conidiophore and the spore was usually clearly visible (Plate 4.3), although nuclear migration between conidiophores and conidia was never observed. Campbell (1970) and Knox-Davies (1979) found that the cytoplasm in the terminal cell of conidiophores in *A.brassicicola* was degenerate. This condition would restrict the movement of nuclei from the conidiophore after the initial phase of development, thus reducing conidium variability and maintaining the selective advantage of the nuclei which are initially placed in the conidium.

If it is the case that species in the *Longicatenatae* sacrifice variability in favour of reproduction, what is the evolutionaly "strategy" of the members *Noncatenatae* which are typically as specialised pathogens as the species in the *Longicatenatae* ? *A.brassicae* has been found to be a more variable pathogen than *A.brassicicola* (Neergaard, 1945). Whether the increased variability results partly from weaker control over nuclear inclusion in conidia remains to be determined.

The difference in variability and reproductive rate in *A.brassicae* and *A.brassicicola* may also be related to the ecological strategies of the two species discussed in section 2.5. Thus, with *A.brassicicola* the life-cycle includes both the capacity for rapid multiplication and colonisation of host tissue and prolonged periods of dormancy in host seed. With a capacity to remain in association with seed for several years there is a further reduction in the need for *A.brassicicola* to retain variability, since the infected seed will provide a new generation of plants to which the pathogen has a good chance of being well adapted. Thus, adoption of an r-strategy is compatible with a capacity to remain in a non-reproducing state for prolonged periods. In the case of *A.brassicae* a lower capacity for colonising seed would appear to exist in conjunction with the pathogen's greater variability. The variability may allow the *A.brassicae* to exist in an active state in the environment, with the cost of this greater variability being a reduced reproductive capacity, which is in turn partly off-set by the greater energy reserve in each spore.

The results of this chapter have highlighted the variation within and between *A.brassicae* and *A.brassicicola* in relation to characters not directly related to pathogenicity. Furthermore, when a large number of characteristics are considered together it appears that; (1) the two species can be differentiated predictably, by means other than conidium morphology, and (2) the relationships between these species and other members of the *Alternaria* remain constant over different types of characteristic. However, although these studies, by employing a conceptual approach to examining the relationships between the pathogens, have made a contribution to an already developing ecological identity for this group of fungi, it is clear that there are large gaps in our knowledge particularly with respect to understanding genetic and physiological diversity. In comparing our knowledge of the biology *Alternaria* with that of the ecologically and phylogenically related *Drechslera/Bipolaris* group of species, it appears we would know far more about the *Alternaria* if their sexual stages were known.

5. CONCLUDING REMARKS

Specific details of the methodology and results of this study have been considered in the general discussions of each chapter. The purpose of this concluding discussion is to provide a broad perspective of the *Alternaria*, with particular reference to the biology of *Brassica/Alternaria*, host/pathogen systems, in light of the findings of this work.

The *Alternaria* share with other genera of conidial fungi, such as *Stemphylium*, *Bipolaris*, and *Drechslera*, a capacity for saprophytic growth and a general potential for to disease in higher plants. Dickinson (1981) reviewed the behaviour of *A.alternata* in association with cereal plants and suggested that even this unspecialised and opportunistic pathogen represented an intermediate group between the biotrophs and the necrotrophs. A conclusion from the observations of the behaviour of six *Alternaria* species in the present study is that the capacity to form infection structures occurs generally in the *Alternaria* and, furthermore, that all species, irrespective of their host specificity, display the same general pattern of leaf surface behaviour. All of the species examined attempted to penetrate a range of plants, with the outcome of the interaction being determined predominantly by events which occurred after or during attempted penetration, not by the behaviour of the fungi at the plant surface. A further conclusion which arises from these observations is that, as a group, the *Alternaria* are generally insensitive to variation in leaf surface morphology with respect to the formation of appressoria. Some variation in behaviour is apparent within the group however, sensitivity ranging from species such as *A.brassicicola* and *A.alternata* which are relatively insensitive to surface features, to *A.brassicae* which shows a degree of sensitivity which may be related to its apparent capacity for stomatal penetration.

The reactions of host and non-host plants to attempted penetration shared several features, although differences were also apparent. In all cases a variety of cell reactions was noted, implying that local variations in the physiological status of the pathogen or challenged plant, even within a single leaf, can alter the phenotype of the host - pathogen interaction. Considering the general trends which were discernable from these observations, non-host reactions suggested a hypersensitive response, in which single challenged cells reacted to attempted penetration. This type of response has been correlated with the accumulation of phytoalexins in many host/pathogen interactions. In host reactions the pattern of cell

death and reaction were suggestive of an incomplete hypersensitive response (Tommiyama, 1982).

The production of phytotoxins by *Alternaria* species has been implicated as a factor in disease development in many cases (Templeton, 1972; Nishimura *et al.*, 1987). Although a traditional role for these compounds, whether host-specific or non-specific toxins, would be the induction of cell damage to allow nectrotrophic feeding by the fungi, more recent studies have indicated that the role of host specific toxins may be to suppress resistance reactions, without necessarily inducing host cell death (Nishimura *et al.*, 1987; section 2.5). These observations, the restricted host range of many *Alternaria* species such as *A.brassicae* and *A.brassicicola*, and the similarity in the symptoms induced by these species suggest that pathogenicity in is determined by a factor (perhaps a host specific toxin) which specifically inhibits the early defence reactions of host plants. This factor need not be the same in both *A.brassicae* and *A.brassicicola* despite the similarity in the symptoms which arise from infection by these pathogens. Symptoms produced by infection result from the reaction of the host tissue to the presence of pathogens. It is likely that pathogens which have broadly similar modes of pathogenicity will induce the formation of similar symptoms since it appears that tissue reactions by plants operate *via* a limited number of mechanisms (Heath, 1985).

A role for non-specific toxins in disease development is less easy to define, although the results of many studies have shown that the *Alternaria* produce non-specific phytotoxins and in some cases they have been isolated from infected tissue. In the present study both *A.brassicae* and *A.brassicicola* were found to produce unidentified non-specific phytotoxins, while studies by Buchwaldt & Green (1992) have shown that destruxin B , which had been reported as a host specific phytotoxin from *A.brassicae* (Bains & Tewari, 1987), has non-specific toxicity.

A role in pathogenicity for either host specific or non-specific toxins is suggested by their production by the fungi and their isolation from diseased plants. However, lesion development by *A.brassicae* and *A.brassicicola* is determinate and often results in the formation of green islands in senescing leaves. This behaviour has been correlated to the production if cytokinins by the fungi, although it has recently been shown that the eremophilane phytotoxins produced by *Drechslera gigantea* can also induce

green islands (Bunkers & Strobel, 1991). Observations of compatible interactions in the present study showed that although the cells adjacent to the point of penetration become necrotic, those in contact with advancing hyphae were living.

It is clear that several interpretations of the points outlined above are possible. Two possible models which account for these observations are presented below. It is stressed, however, that further interpretations are possible.

A. *Continued specific suppression model.*

1. Successful establishment is dependent on the suppression of early defence reations by a specific supressor (putatively a host specific toxin).

2. Initial establishment of hyphae adjacent to the point leads to intercellular growth with continued specific supression of resistance reactions but followed by host cell necrosis.

3. A physiological balance between the advancing hyphae and reacting plant cells is reached after a period of colonisation, perhaps as a result of the diversion of fungal metabolism from radial extension to sporulation.

4. Intercellular hyphae continue to feed from host cells inducing stress and chlorosis. The infected lesion acts as a nutrient sink in the leaf. Nutrient availability is prolonged by cytokinin production by the pathogen which maintains host cells at the edge of the lesion in a viable condition.

B. *Specific and general toxin model.*

1. Successful establishment is dependent on the suppression of early defence reations by a specific supressor (putatively a host specific toxin).

2. The initial accumulation of hyphae at the infection site provides the pathogen with sufficient inoculum potential for the production of general phytotoxins at sufficient concentrations to debilitate host cells in advance of colonisation and overtake plant defence reactions.

3. A physiological balance between the advancing hyphae and reacting plant cells is reached after a period of colonisation, perhaps as a result of the diversion of fungal metabolism from radial extension to sporulation.

4. Intercellular hyphae feed from degenerating, chlorotic host cells. The infected lesion acts as a nutrient sink in the leaf. Nutrient availability is prolonged by cytokinins production by the pathogen which maintains host cells at the edge of the lesion in a viable condition.

In both models the initial events during contact between the host and pathogen, and pathogen development immediately after penetration are crucial to the outcome of the interaction. It is obvious that there is a need for more detailed examinations of these interactions and of the physiology of lesion development to allow clarification of the role of specific and/or general toxins, the status of host cells during the interaction, and the involvement of cytokinins in green island formation. Bunkers & Strobel (1991) have recently shown that phytotoxins produced by *Drechslera gigantea* can induce the formation of green islands. The possibility that pathogen metabolites can have more than one mode of action may explain the complex series of symptoms which can result from *Alternaria* infection in crucifers.

Irrespective of the details of the host/pathogen interaction, an essential feature of the *Alternaria* would seem to be their ability to exist as saprophytes while maintaining a potential for pathogenicity (Nishimura & Kohmoto, 1983a). These characteristics are shared with the *Alternaria* by a group of closely related species, as noted above. Previous studies of the ecology and pathogenicity of these species (Neergaard, 1945; Scheffer, 1989) have highlighted the similarities between them which are supported by the findings of the present study. It is arguable that this group of toxigenic, conidial, leaf-spotting, hemibiotrophic, parasites / saprophytes, which all have teleomorphs in the *Pleosporales*, are as distinctive a group of plant pathogens as the *Uredinales* or *Erysiphales*, all be it a group which requires further description; indeed Subramanian (1983) dealt with the group in this context in his appraisal of Hyphomycetes. Further investigations of their ecology and phylogeny will hopefully lead to clarification of this identity and to a greater understanding of their pathogenicity.

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Appendices.

Appendix 2.1 Genotypes of leaf type mutants of *B.oleracea* var *gemmifera* used in experiment 2.1

The three lines used in the study comprised a family designated 90/83 which has two mutant genes each controling a single leaf characteristic. The mutant alleles are:

Go^c; dominant leaf glossiness (low waxiness)

An; dominant anthocyanin spot at the leaf tip

The genotypes of the lines used in the study, with respect to waxiness only, were:

Glossy; $Go^c/+$

Intermediate; $Go^c/+$

Waxy; $+/+$

The intermediate leaf type segregated unexpectedly in the cross, and probably resulted from differences at other loci which modulated the expression of the Go^c allele (T. Hodgkin, pers. comm.).

Appendix 2.2: Illumination conditions used for plant growth in Experiments 2.1 - 2.3.

Experiment 2.1: Plants were raised on open benches and natural daylight was supplemented with overhead high pressure mercury vapour lamps which provided approximately 15,000 lux at bench level.

Experiment 2.2: Plants were raised in a Burkard isolation propagator and natural daylight was supplemented by overhead strip lights, which provided approximately 10,000 lux at bench level.

Experiment 2.3: Plants were raised in a Burkard isolation propagator without supplement to natural daylight.

Appendix 2.3: General conditions for the growth and maintenance of *Alternaria* isolates.

All cultures were maintained, in a heated incubator at 20 ± 2° C, with a 16h dark/ 8h n.u.v. diurnal illumination cycle in 9 cm plastic petri dishes, and unless otherwise stated on Oxoid corn meal agar (CMA). Sub-cultures were made at 3 - 4 week intervals, and degenerate isolates were revived either by sub-culture on PDA or V8 Juice agar under the growth conditions outlined above.

APPENDIX 2.4: Tissue preparation protocols for Fluorescence (FM) Microspcopy (LTSEM).

Experiment 2.1:

DAPI:
1. Fix in 95% v/v aqueous ethanol for one minute and rinse in sterile distilled water.
2. Store in sterile distilled water at 4° C until required.
3. Mount and stain in a 1 g/ ml aqueous solution of DAPI (Sigma) at approximately pH 7.

ANS:
1. Fix and store in cold 200% w/v aqueous chloral hydrate.
2. Rinse twice in distilled water.
3. Mount and stain in 0.05% w/v solution of ANS (Sigma) in Sodium/ Phosphate buffer at pH 5.

TINOPAL:
1. Fix and store in 200% w/v aqueous chloral hydrate.
2. Rinse twice in distilled water.
3. Stain for 1 minute in 3% v/v Tinopal (UVITEX BHPT, Ciba Geigy Chemicals) in 20% v/v aqueous glycerol.
4. Rinse over night in distilled water.
5. Mount in 20% v/v aqueous glycerol.

Experiment 2.2 and 2.3:

ANILINE BLUE/ TINOPAL:

1. Fix and clear tissue in hot 200% w/v aqueous chloral hydrate.
2. Rinse twice in sterile distilled water.
3. Stain for 24 hours at 20° C in 0.05% w/v water-soluable Aniline blue in pH 12 Phosphate buffer.
4. Stain in 0.5% v/v aqeous solution of Tinopal for 30 seconds.
5. Rinse twice in distilled water.
6. Mount in Aniline blue stain solution.

Appendix 2.5: Tissue preparation protocol for Low Temperature Scanning Electron Microscopy:

1. Secure tissue on copper stub with Tissue-Tek ^{R} carboxymethylcellulose adhesive.
2. Rapid freezing in Nitrogen slush at -140° C under Argon.
3. Transfer to microscope stage for preliminarly observation.
4. If required, etch on microscope stage at -70° C to remove ice.
5. Sputter-coat with gold under Argon at -140° C.
6. Transfer to microscope stage for observation at -140° C.

Appendix 2.6a: A summary of the analyses of variance for experiment 2.1.

		Probability (F statistic)) of calculated variance ratio VARIATES										
Source of Variation	**d.f.**	**Germ***	**GTN**	**BASE**	**MID**	**TERM**	**BRNCH**	**AP:GT**	**TPEN**	**%PnECWJ**	**%PnCPW**	**%PnSTO**
Replicates	2											
Pathogen	2	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.288	<0.001
Leaf type	2	0.080	0.091	0.446	0.100	0.165	0.284	0.020	0.100	0.119	0.645	0.006
Glossy v. Waxy (GvW)	1	0.163	0.050	0.345	0.270	0.125	0.499	0.376	0.205	0.753	0.681	0.002
Int'med. v. mean (IvM)	1	0.075	0.323	0.397	0.064	0.260	0.152	0.008	0.081	0.042	0.402	0.436
Time	3	<0.001	0.427	0.199	0.058	0.453	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lin.	1	<0.001	0.110	0.571	0.010	0.118	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Quad.	1	0.359	0.668	0.041	0.400	0.784	0.641	0.011	0.050	0.006	0.956	0.666
Deviations	1	0.086	0.924	0.753	0.812	0.803	0.980	0.480	0.751	0.547	0.527	0.427
Pathogen*Leaf type	4	0.344	0.017	0.757	0.920	0.451	0.539	0.088	0.481	0.530	0.523	0.005
Pathogen*GvW	2	0.201	0.044	0.491	0.986	0.614	0.404	0.758	0.854	0.415	0.861	<0.001
Pathogen*IvM	2	0.529	0.043	0.801	0.642	0.261	0.525	0.024	0.210	0.496	0.238	0.572
Pathogen*Time	6	0.071	0.526	0.959	0.960	0.249	<0.001	0.007	<0.001	<0.001	0.425	<0.001
Pathogen*Lin.	2	0.025	0.257	0.918	0.964	0.161	<0.001	0.002	<0.001	<0.001	0.149	<0.001
Pathogen*Quad.	2	0.498	0.927	0.687	0.653	0.124	0.832	0.105	0.278	0.255	0.352	0.948
Pathogen*Deviations	2	0.214	0.332	0.760	0.769	0.993	0.070	0.592	0.320	0.330	0.986	0.422
Leaf type*Time	6	0.426	0.643	0.663	0.718	0.166	0.254	0.428	0.933	0.813	0.546	0.170
GvW*Lin.	1	0.656	0.526	0.077	0.331	0.925	0.129	0.694	0.543	0.477	0.258	0.015
IvO*Lin.	1	0.132	0.658	0.526	0.695	0.742	0.542	0.803	0.568	0.331	0.862	0.348
GvW*Quad.	1	0.766	0.647	0.520	0.205	0.005	0.568	0.221	0.459	0.640	0.096	0.301
IvM*Quad.	1	0.902	0.502	0.916	0.870	0.652	0.044	0.044	0.516	0.428	0.996	0.822
GvW*Deviations	1	0.100	0.183	0.917	0.369	0.532	0.926	0.852	0.729	0.736	0.834	0.407
IvM*Deviations	1	0.425	0.285	0.977	0.785	0.503	0.409	0.821	0.925	0.486	0.382	0.506
Pathogen*Leaf type*Time	12	0.294	0.487	0.951	0.389	0.707	0.770	0.482	0.807	0.992	0.381	0.303
Pathogen*GvW*Lin.	2	0.252	0.340	0.869	0.294	0.719	0.329	0.644	0.690	0.901	0.481	0.041
Pathogen*IvM*Lin.	2	0.095	0.687	0.984	0.586	0.572	0.503	0.512	0.768	0.959	0.676	0.618
Pathogen*GvW*Quad.	2	0.414	0.977	0.371	0.318	0.805	0.842	0.102	0.243	0.525	0.094	0.189
Pathogen*IvM*Quad	2	0.231	0.056	0.283	0.081	0.428	0.394	0.185	0.806	0.860	0.833	0.966
Pathogen*GvW*Deviations	2	0.602	0.988	0.976	0.542	0.119	0.856	0.570	0.336	0.736	0.116	0.469
Pathogen*IvM*Deaviations	2	0.590	0.287	0.982	0.735	0.778	0.400	0.951	0.693	0.699	0.580	0.439
RESIDUAL	57											
Total	94											

Appendix 2.6a (contd.): A summary of the analyses of variance for experiment 2.1.

Description of variates: Germ, Percentage of conidia which germinated; **GTN**, mean number of germ-tubes per germinated conidium; **BASE**, percentage of germinated conidia which produced germ-tubes from basal cells; **MID**, percentage of germinated conidia which produced germ-tubes from mid-body cells; **TERM**, percentage of germinated conidia which produced germ-tubes from terminal cells; **BRNCH**, percentage of germ-tubes with at least one branch; **AP:GT**, the ratio of appressoria to germ-tubes, **TPEN**, the ratio of penetration events to germinated conidia (as a percentage); **%PnECWJ**, percentage of penetrations which occurred at epidermal cell wall junctions; **%PnCPW**, percentage of penetrations which occurred over epidermal cell periclinal walls; **%PnSTO**, percentage of penentrations which occurred via stomata.

Appendix 2.6b: A summary of the correspondance analysis of selection of infection sites by fungi in experiment 2.1

CPA axis	CPA Roots	Percentage of Variance
1	0.39	77
2	0.11	23

Appendix 2.6c: A summary of the analyses of variance for experiment 2.2.

		Probability (F statistic)) of calculated variance ratio VARIATES											
		Germ*	GTN	GTL	APGT	%NPGT	TPEN	%PnECJ	%PnCPW	%PnSTO	%PnLOC	%PnSCR	%PnADJ
Source of Variation	d.f.												
Replicates	2												
Pathogen	2	0.005	<0.001	<0.001	<0.001	<0.001	<0.001	0.446	0.031	<0.001	<0.001	<0.001	0.224
Host	7	0.445	0.423	0.089	0.565	0.388	0.670	0.186	0.343	0.164	<0.001	0.323	<0.001
Pathogen*Host	14	0.265	0.250	0.402	0.756	0.694	0.574	0.796	0.284	0.004	0.009	0.397	0.012
RESIDUAL	48												
Total	71												

* **Description of variates: Germ**, Germination (per cent); **GTN**, mean number of germ-tubes; **GTL**, mean germ-tube length; **APGT**, ratio of appressoria to germ-tubes (%); **%NPGT**, percentage of germ-tubes not associated with penetration; **TPEN**, ratio of penetrations to germinated conidia (%); **%PnECJ**, percentage of penetrations at cell epidermal junctions; **%PnCPW**, percentage of penetrations through periclinal walls; **%PnSTO**, percentage of penetrations *via* stomata; **%PnLOC**, percentage of penetrations associated with localised callose deposition; **%PnCCR**, percentage of penentrations associated with single cell reactions; **%PnACR**, percentage of penetrations associated with adjacent cell reactions.

Appendix 2.6d: A summary of a Principal Components Analysis of host cell reactions in experiment 2.2.

PCP axis	Latent Root	Per cent Interaction Sum of squares
1	2077	67
2	1040	33
3	0	0

Appendix 2.6e: A summary of the analyses of variance for experiment 2.3.

		Probability (F statistic)) of calculated variance ratio										
		VARIATES										
		Germ	GTN	GTL	APGT	%NPGT	TPEN	%PnECJ	%PnCPW	%PnSTO	%PnLOC	%PnADJ	%NcPn
Source of Variation	**d.f.**												
Replicates	2												
Pathogen	5	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.495	0.878	0.003	0.001	<0.001	0.005
Host	3	0.012	0.089	0.041	<0.001	<0.001	0.003	0.398	0.390	0.354	<0.001	<0.001	0.065
Wheat v. Dicots (WvD)	1	0.201	0.107	0.035	0.010	<0.001	0.008	0.094	0.559	0.274	<0.001	<0.001	0.021
OSR v. tomato (OvT)	1	0.089	0.054	0.100	0.832	0.683	0.327	0.995	0.175	0.155	0.001	<0.001	0.165
poppy v. OSR+tomato (PvO+T)	1	0.010	0.611	0.265	<0.001	0.105	0.010	0.719	0.363	0.861	0.012	<0.001	0.864
Time	1	0.102	0.566	<0.001	<0.001	<0.001	<0.001	0.302	0.142	0.373	0.157	0.504	0.017
Pathogen*Host	15	0.011	0.251	0.623	<0.001	<0.001	<0.001	0.510	0.427	0.008	<0.001	<0.001	0.215
Pathogen*(WvD)	5	0.009	0.227	0.991	0.736	0.006	0.270	0.265	0.242	0.002	0.107	<0.001	0.098
Pathogen*(OvT)	5	0.780	0.936	0.872	0.004	<0.001	<0.001	0.283	0.338	0.065	<0.001	<0.001	0.093
Pathogen*(PvO+T)	5	0.018	0.075	0.076	<0.001	0.233	0.045	0.927	0.716	0.804	0.875	0.311	0.999
Pathogen*Time	5	<0.001	0.077	0.008	0.001	0.056	<0.001	0.086	0.840	0.129	0.326	0.838	0.166
Host*Time	3	0.800	0.568	0.125	0.655	0.043	0.090	0.570	0.187	0.206	0.111	0.466	0.047
(WvD)*Time	1	0.549	0.303	0.073	0.312	0.011	0.027	0.571	0.082	0.055	0.018	0.725	0.006
(OvT)*Time	1	0.966	0.364	0.229	0.897	0.693	0.662	0.279	0.184	0.373	0.750	0.763	0.824
(PvO+T)*Time	1	0.425	0.724	0.288	0.451	0.207	0.206	0.475	0.875	0.805	0.596	0.128	0.590
Pathogen*Host*Time	15	0.207	0.224	0.575	0.003	0.017	0.124	0.520	0.200	0.133	0.395	0.148	0.699
Pathogen*(WvD)*Time	5	0.736	0.411	0.677	0.065	0.008	0.345	0.058	0.064	0.007	0.226	0.980	0.092
Pathogen*(OvT)*Time	5	0.350	0.428	0.657	0.721	0.182	0.703	0.838	0.328	0.638	0.394	0.019	0.996
Pathogen*(PvO+T)*Time	5	0.053	0.108	0.236	<0.001	0.233	0.023	0.969	0.659	0.899	0.592	0.277	0.916
RESIDUAL	85												
Total	134												

*** Description of variates is given on the following page**

Appendix 2.6e (contd.)

Description of variates: Germ, Germination (per cent); **GTN**, mean number of germ-tubes; **GTL**, mean germ-tube length; **APGT**, ratio of appressoria to germ-tubes (%); **%NPGT**, percentage of germ-tubes not associated with penetration; **TPEN**, ratio of penetrations to germinated conidia (%); **%PnECJ**, percentage of penetrations at cell epidermal junctions; **%PnCPW**, percentage of penetrations through periclinal walls; **%PnSTO**, percentage of penetrations *via* stomata; **%PnLOC**, percentage of penetrations associated with localised callose deposition; **%PnCCR**, percentage of penentrations associated with single cell reactions; **%PnACR**, percentage of penetrations associated with adjacent cell reactions; %NcPn, percentage of

Appendix 2.6f: A summary of a Principal Components Analysis of plant cell reactions in experiment 2.3.

PCP axis	Latent Root Sum of squares	Per cent Interaction
1	2077	67
2	1040	33
3	0	0

Appendix 2.6g: A summary of the Correspondence Analysis of the distribution of penetration events for the plant * pathogen interaction in Experiment 2.3

CPA Axis	CPA Roots	Percentage of the variance
1	0.26	54
2	0.14	30
3	0.05	12
4	0.01	3
5	<0.01	2

Appendix 3.1: Composition of liquid culture medium (LCM) used for the production of *Alternaria* metabolites in chapter 3.

All masses given are per litre:

Sucrose	10.0g
Carboxymethylcellulose	10.0g
KH_2PO_4	5.0g
KNO_3	5.0g
$Mg_2SO_4.12H_2O$	0.5g
Penicillin	0.1g
Streptomycin	0.12g

Appendix 3.2: Sample preparation protocol for Freeze fracture low temperature scanning electron microscopy (Experiment 3.1).

1. Sample of tissue cut to the correct size
2. Sample mounted in Tissue-Tek^R on a copper stubb
3. Sample immersed in nitrogen slush under vacuum until frozen
4. Sample fractured under vacuum and gold coated under argon.
5. Sample transfered under vacuum to low temperature stage for observation.

Appendix 3.3: Analysis of variance of the leaf disk bioassay reported in experiment 3.1.

Source of variance	d.f.	F
Replication	2	
Isolate (I)	8	<0.001
Plant (P)	1	<0.001
Culture period (Cp)	2	0.002
I * P	8	<0.001
I * Cp	16	0.643
P * Cp	2	0.002
I * P * Cp	16	0.680
Residual	108	
Total	161	

Appendix 3.4: Analysis of variance of the leaf disk bioassay reported in experiment 3.3.

Source of variance	d.f	F
Replication	2	
Isolate	9	<.001
A.brassice v. *A.brassicicola*	1	<.001
Fungi v. control	1	<.001
Deviations	7	0.014
Residual	18	
Total	29	

Appendix 4.1: Data matrix of morphological and ecological characteristics used in Experiment 4.1 for cluster analysis in the *Alternaria*.

VARIATE*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A.gromphrenae	0	0	1	0	1	1	0	140	70	15	11	11	8	1
A.multirostrata	0	0	1	0	1	1	0	400	100	32	20	14	7	1
A.pannax	1	1	1	0	1	1	0	170	150	20	12	11	1	4
A.dianthi	1	1	1	0	1	1	0	120	30	26	15	8	8	4
A.dianthicola	1	1	1	0	1	1	0	130	55	17	14	14	2	5
A.gypsophilae	1	1	1	0	1	1	0	118	22	7	3	11	1	6
A.carthami	1	1	1	0	1	1	1	270	85	26	15	11	8	2
A.cinerariae	1	1	1	0	1	1	0	140	50	40	15	10	8	4
A.denisii	0	1	1	0	1	1	1	70	20	11	7	12	2	4
A.heianthi	1	1	1	0	1	0	1	145	45	30	10	12	5	2
A.helianthificiens	1	1	1	0	1	1	0	275	250	18	10	8	7	2
A.leucanthemi	0	0	1	0	1	1	0	130	25	26	10	15	1	1
A.protenta	0	0	1	0	1	1	1	210	165	15	12	13	10	1
A.sonchi	1	1	1	0	1	1	1	130	60	19	18	7	6	2
A.tagetica	0	0	1	0	1	1	1	310	100	35	30	10	1	2
A.zinniae	1	1	1	0	1	1	1	290	110	28	19	9	5	2
A.brassicae	0	1	1	0	1	1	1	350	75	18	9	19	8	3
A.brassicicola	1	1	1	0	1	0	1	130	18	20	8	11	6	20
A.cheiranthi	0	1	1	0	1	0	0	100	20	30	14	7	11	3
A.ethzedia	1	1	1	0	1	0	1	45	35	14	10	7	1	6
A.raphani	1	1	1	0	1	1	0	130	50	25	10	9	10	3
A.cucumerina	1	1	1	0	1	1	0	220	130	24	15	9	11	2
A.pepinicola	1	1	1	0	1	1	0	75	35	17	11	7	7	3
A.angustiovoidea	1	1	1	0	1	1	0	40	25	8	10	8	3	12
A.euphorbiae	1	1	1	0	1	1	1	220	90	28	11	9	1	4
A.euphorbiicola	1	1	1	0	1	1	1	90	53	20	10	6	3	5
A.ricini	1	1	1	0	1	1	0	170	70	27	13	10	7	2
A.padwickii	0	0	1	0	1	1	0	170	95	20	11	5	0	1
A.saprava	1	1	1	0	1	1	0	300	150	15	8	10	4	3
A.triticicola	1	1	1	0	1	1	0	170	50	35	12	10	8	3
A.tricina	1	1	1	0	1	0	0	90	20	30	9	7	5	4
A.cassiae	1	1	1	0	1	1	1	200	65	20	19	10	1	2
A.eureka	1	1	1	0	1	0	1	30	15	13	12	6	4	6
A.porri	0	0	1	0	1	1	1	300	100	20	15	12	8	1
A.macrospora	1	1	1	0	1	1	0	180	90	22	15	9	9	2
A.papaveris	1	1	1	0	1	1	0	80	50	35	18	7	8	3
A.passiflorae	0	1	1	0	1	1	1	250	100	29	14	12	5	5
A.sesami	1	1	1	0	1	1	1	260	90	33	14	11	7	2
A.alternata	1	1	1	1	0	1	0	63	20	18	9	8	6	7
A.infectoria	1	1	1	0	0	1	1	70	20	27	9	7	4	10
A.longissima	0	0	1	0	0	1	1	500	100	30	20	40	3	1
A.tenuissima	1	1	1	0	0	1	0	95	22	21	11	7	10	4
A.anagallidis	0	0	1	0	1	1	1	210	16	27	7	8	2	1
A.crassa	1	1	1	0	1	1	0	440	120	40	15	11	8	1
A.longipes	1	1	1	0	1	1	0	110	35	16	10	14	4	8
A.solani	0	0	1	0	1	1	1	300	150	19	15	11	8	1
A.cichorii	0	0	1	0	1	1	0	400	100	20	15	11	5	1
A.dauci	1	1	1	0	1	1	0	450	110	25	16	11	11	2
A.petoselini	0	0	1	0	1	0	0	105	35	34	18	11	9	1
A.radicina	1	1	1	0	1	0	0	57	27	32	13	14	7	3
A.ramulosa	1	1	0	0	0	0	0	60	30	25	15	5	3	3
A.molesta	0	0	0	1	1	0	1	38	15	12	11	6	3	1
A.conjuncta	1	1	1	0	1	0	1	45	30	12	10	7	1	5
A.photistica	1	1	1	0	1	0	1	50	45	20	17	8	1	6

A.chlamydospora	0	0	0	0	0	1	0	60	20	40	16	5	15	1
A.limaciformis	1	1	0	0	0	0	1	45	30	18	9	7	1	3
A.mouchacce	0	0	0	0	0	0	0	60	10	25	5	7	3	1
A.phragmospora	1	1	0	0	0	0	0	50	20	13	6	7	0	8
A.linicola	0	0	1	0	1	1	1	292	39	28	7	16	4	1
A.citri	1	1	1	0	1	1	1	63	20	18	9	6	10	7

*** List of variates: 1.** Is sporulation catenulate on hosts? (1 = yes, 0 = no). **2.** Is sporulation catenulate in culture? (1 = yes, 0 = no). **3.** Is the species reported from plant hosts? (1 = yes, 0 = no). **4.** Is the species reported from animal hosts? (1 = yes, 0 = no). **5.** Is the species reported to have a restricted host range? (1 = yes, 0 = no). **6.** Do conidia normally have a true beak? (1 = yes, 0 = no). **7.** Are conidiophores normally geniculate? (1 = yes, 0 = no). **8.** Maximum conidium length (m). **9.** Minimum conidium length (m). **10.** Maximum conidium width (m). **11.** Minimum conidium width (m). **12.** Maximun number of transverse septa. **13.** Maximum number of longitudinal septa. **14.** Normal number of conidia in a chain (minimum = 1).

Appendix 4.2: The data matrix used for cluster analysis in Experiment 4.2

Variate	1	2	3	4	5	6	7	8	9	10	12----------32
Aa/7/N	0	1	1	0	0	2.2	1.6	1.4	1.3	3	1 0 0 0 0 1 1 1 0 1 1 0 0 0 0 0 0 0 0 0
Aa1/108/UK	0	1	1	0	0	2.2	1.3	1.2	1.1	3	1 0 0 0 0 1 1 1 0 1 1 0 0 0 0 0 0 0 0 0
Aa2/108/UK	1	1	1	0	0	4.8	2.3	1.8	2.2	3	1 0 1 1 0 1 0 1 0 1 1 0 0 0 0 0 0 1 0 0
Aa1/113/UK	0	1	1	0	0	1.1	1.5	1.3	1.3	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1
Aa1/148/UK	0	1	1	0	0	0.7	1.0	1.0	1.0	3	1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0
Aa2/148/UK	1	1	1	0	0	1.2	1.3	1.5	1.1	3	1 0 1 1 0 0 1 1 0 1 0 1 0 1 0 0 0 0 0 0
Aa3/108/UK	0	1	1	0	0	1.8	1.2	1.2	1.3	3	1 0 0 1 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0
Aa1/1/UK	1	1	1	0	0	2.9	1.9	1.9	1.4	3	1 0 0 1 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0
Aa2/1/UK	0	1	1	0	0	3.7	1.1	1.0	1.1	3	1 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0
Aa4/1/UK	1	1	1	0	0	3.0	2.1	1.7	1.7	3	1 0 1 1 0 1 1 1 0 1 0 1 0 0 0 0 0 0 0 0
Aa1/Ta/UK	1	1	1	0	0	2.0	1.4	1.2	1.4	3	1 0 1 1 0 1 1 1 0 1 1 0 0 0 0 0 0 0 0 0
Aa3/Ta/UK	0	0	1	0	0	2.0	1.4	1.2	1.4	3	1 0 1 1 0 1 1 1 0 1 0 1 0 0 0 0 0 0 0 0
Ab1/Bo/UK	0	1	1	0	0	7.3	2.3	2.5	2.4	5	1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ab2/Bo/UK	0	1	1	0	0	8.8	3.5	2.9	3.0	4	1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Abi/Bo/UK	0	1	1	0	0	5.8	3.7	3.5	4.1	5	1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ab4/Bo/UK	1	1	1	0	0	10.0	6.3	6.5	4.1	5	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ac1/Bn/UK	0	1	1	0	0	3.3	1.7	1.6	1.3	3	1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ac2/Bn/UK	0	1	0	0	0	4.7	2.0	1.7	1.5	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ac3/Bo/UK	0	1	0	0	0	4.4	1.1	1.5	1.3	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0
Aci/Bo/UK	0	1	0	0	0	3.6	1.5	1.3	1.3	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ac5/3/UK	0	1	1	0	0	1.9	1.1	1.3	1.2	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ach/3/UK	0	1	1	0	0	6.2	2.6	2.3	2.1	3	1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Ach/I/UK	0	1	1	0	0	8.2	2.8	2.4	2.1	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
Ai1/I/UK	0	0	1	0	0	2.0	1.4	1.3	1.8	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0
Ar1/I/UK	0	0	0	0	0	6.4	1.9	1.9	1.7	4	1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
As1/88/I	1	1	1	1	0	5.5	2.0	2.1	2.0	4	1 0 0 0 0 0 0 0 1 0 0 0 0 1 1 1 0 0 0 1
As2/88/I	1	1	1	1	0	7.8	3.7	3.4	3.6	5	1 0 0 0 0 0 0 0 1 0 0 0 0 1 1 1 0 0 0 0
Asp1/1/UK	0	1	1	0	0	6.7	2.4	2.0	2.2	4	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Asp2/1/UK	0	1	1	0	0	10.4	2.3	3.0	3.6	4	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Asp3/7/N	0	1	0	0	0	9.4	2.3	2.1	2.1	4	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
At1/148/UK	0	1	1	0	0	2.1	1.2	1.4	1.3	3	1 0 1 1 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
At1/4/N	0	1	1	0	0	4.2	1.9	1.6	1.5	3	1 0 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
Bsp/87/UK	1	1	1	0	0	4.6	1.2	1.2	1.2	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1
Lm1/Bn/UK	0	1	1	0	1	6.2	2.6	2.3	2.1	4	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Lm1/7/N	1	0	1	0	1	7.1	2.3	2.3	2.7	4	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Sb1/2/UK	0	1	1	0	0	4.1	1.7	1.4	1.8	3	1 0 0 1 0 0 1 1 0 1 0 1 0 0 0 0 0 0 0 0
Sb1/8/UK	0	1	1	0	0	6.0	2.3	2.1	2.0	3	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0
Sb2/8/UK	0	1	1	0	0	4.0	1.7	1.3	1.3	3	1 0 0 1 0 0 1 1 1 1 1 0 1 1 0 0 0 0 0 0
Uc1/87/UK	0	1	0	0	0	0.7	1.2	1.2	1.0	2	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Uc1/6/N	0	1	1	0	0	0.9	1.2	1.1	1.0	2	1 1 0 1 1 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0

List of variates: 1. Ability to grow at pH 2. **2.** Ability to grow at pH 12. **3.** Ability to hydrolyse Tween 80. **4.** Chromogenic activity present. **5.** Production of pycnidia rather than conidia. **6.** No. of nuclei in hyphal tip. **7.-9.** Nos. of nuclei in first, second, and third hyphal compartments proximal to the tip. **10.** Hyphal width at the third compartment proximal to the tip. **12.-32.** Presence / Absence scores for secondary metabolites.