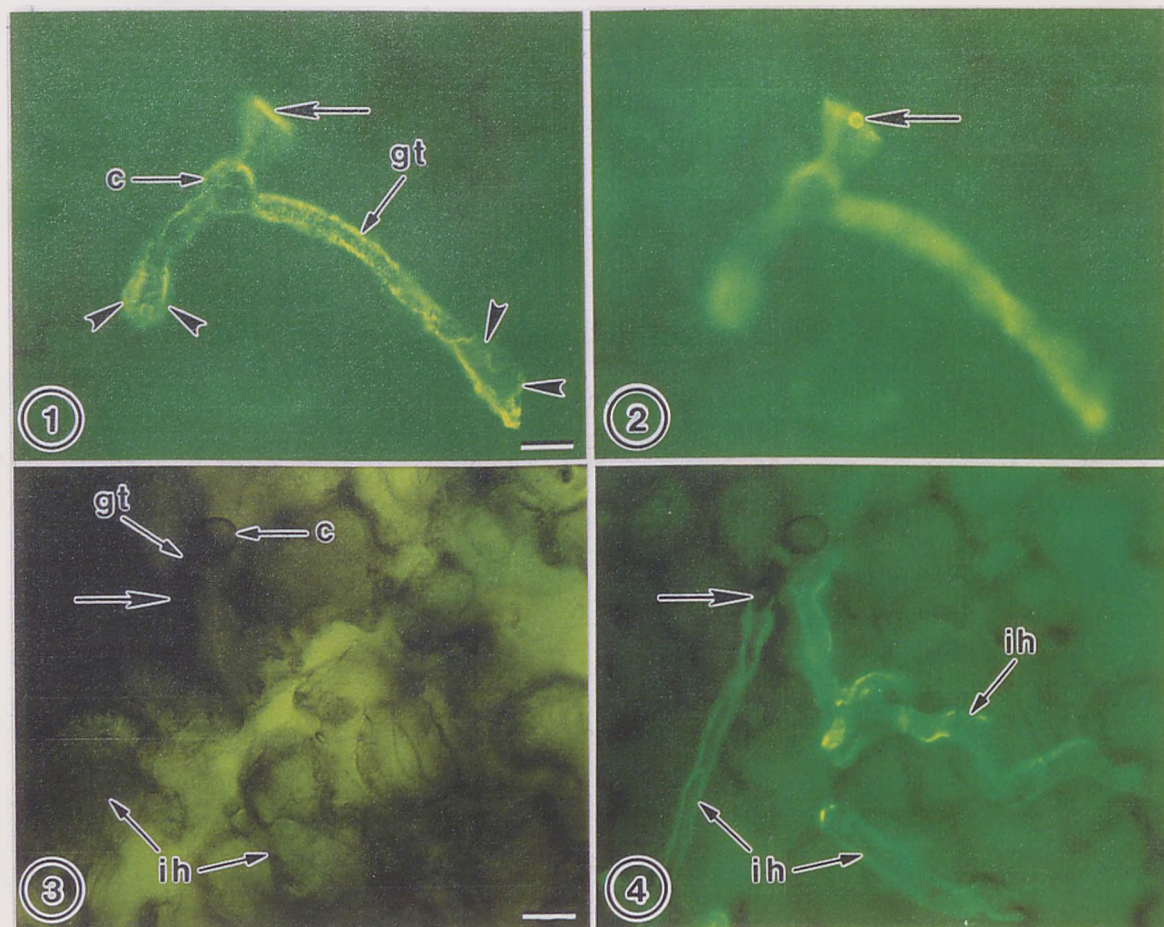




Frontispiece. Figs 1 & 2. Pair of epi-fluorescence micrographs showing *B. fabae*-infected bean leaves (GS/HC for 14 h) immunolabelled with BC-KH4 MAb. Images taken above (Fig. 1) and at the level of the leaf surface (Fig. 2). **Fig. 1.** Extensive fluorescent labelling can be seen at the surface of the conidium (c) and germ tubes (gt) on the leaf substratum. Labelling is also apparent throughout the fibrillar-like sheath (arrowheads) which surrounds the germ tubes. Note the swollen germ tube tip (large arrow) which turns down towards the leaf surface. Bar = 20 μ m. **Fig. 2.** Note the distinct fluorescent ring at the site of penetration. **Fig 3.** DIC image of Fig. 4. Conidium (c) germinated to produce a single long germ tube (gt). Note that the fungus penetrates the leaf surface immediately below the base of the germ tube tip (large arrow). Here, the infection hyphae (ih) are barely distinguishable beneath the leaf surface. Bar = 20 μ m. **Fig. 4.** Epi-fluorescence micrograph showing *B. fabae*-infected bean leaf (GS/HC for 48 h) immunolabelled with BC-FD7-G9 MAb. Fluorescent labelling can be seen at the surface of the infection hyphae (ih) which ramify throughout the host tissue. GS = conidia inoculated in 0.5% (w/v) aqueous glucose solution. [HC = humid chamber].

Immunocytochemical studies of fungal cell surface molecules and the *Vicia faba-Botrytis* interaction

Volume 1: Text

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And so from hour to hour, we ripe and ripe, and then from hour to hour, we rot and rot; and thereby hangs a tale

As You Like It, Act II, Sc. vii

Abstract

Immunofluorescence and immunoelectron microscope studies of fungal cell surface molecules and the *Vicia faba*-*Botrytis*-interaction were conducted using the *Botrytis*-MAbs BC-KH4, BC-AF7 and BC-FD7-G9. The *Botrytis*-MAbs were raised against surface washings of *Botrytis cinerea* and directed against *B. cinerea* and *B. fabae* (Bossi & Dewey, 1992; *Plant Pathology* **41**, 472-482). All three MAbs bound to fibrillar matrices associated with conidia, germ-tubes and hyphae of *B. cinerea* and *B. fabae* grown on agar, glass slides (conidia inoculated in aqueous glucose) and *V. faba* leaves. These matrices provided firm attachment of sporelings to glass and leaf surfaces. Antibody-binding was observed at fungal walls, including septa; however, no labelling was detected inside the fungal cell or at hyphal tips. These surface antigens may be synthesised and/or modified *in muro*. Cryo-fixation and low-temperature freeze-drying of fungal material enhanced the preservation of fibrillar matrices and retention of surface antigens for immunocytochemistry.

Light and electron microscopy of the infection processes of wet and dry conidia inoculated onto glass and *V. faba* leaves showed that in all cases conidia germinated to produce one or more germ tubes. However, the subsequent growth of the developing germ tubes varied according to mode of inoculation applied. In the presence of aqueous glucose, conidia developed long germ tubes. In contrast, dry-inoculated conidia produced short germ tubes only, which penetrated the leaf surface directly. Transmission electron microscopy revealed an amorphous pad of matrix material around short germ tubes on the leaf surface. This pad appeared to consist of fibrillar material in a condensed form. Observation of both wet- and dry-infections revealed that fibrillar material was associated with both penetration and infection hyphae. This fibrillar material was also immunolabelled with the *Botrytis*-MAbs. Fibrils often extended some distance from the penetration and infection hyphae and pervaded the degraded host cell walls. Double-labelling methods using BC-KH4 and specific markers for plant wall components allowed the host-pathogen interface to be investigated further. Interactions between fibrillar matrices and host walls and/or cytoplasm were visualised clearly. These studies provided further support for the wall-modifying action of pectic enzymes. The possible role(s) for *Botrytis* extracellular matrices are discussed.

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LIST OF ABBREVIATIONS

AGP	ArabinoGalactan Proteins
AX	ArabinoXylan
BFA	brefeldin A
CBH1	Cellobiohydrolase
CMC	CarboxyMethylCellulose
ConA	Concanavalin A
CPB	Citrate Phosphate Buffer
CWDE	Cell Wall Degrading Enzyme
DIC	Differential Interference Contrast
DMSO	DiMethyl SulphOxide
ehm	extrahaustorial membrane
ehmat	extrahaustorial matrix
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
GAM	Goat Anti-Mouse
GAMM	Goat Anti-Mouse IgM
GAR	Goat Anti-Rabbit
GAT	Goat Anti-Rat
GGA	Glucose Gelatin Agar
HC	Haustorial Complex
HRGP	Hydroxyproline-Rich GlycoProtein
HRP	HorseRadish Peroxidase
IEM	ImmunoElectron Microscopy
IF	Immunofluorescence

IG	Indirect ImmunoGold
IH	Intracellular Hypha(e)
IPC	IsoPycnic Centrifugation
LSCM	Laser Scanning Confocal Microscopy
LTSEM	Low-Temperature Scanning Electron Microscopy
MAb	Monoclonal Antibody
MEA	Malt Extract Agar
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus Tween-20
PDA	Potato Dextrose Agar
PEG	PolyEthylene Glycol
PG	PolyGalacturonase
PGA	PolyGalacturonic Acid
PGL	Pectate Lyase
PL	Pectin Lyase
PLT	Progressive Low-Temperature
PME	Pectin MethylEsterase
PMG	PolyMethyl Galacturonase
RER	Rough Endoplasmic Reticulum
RG-I	RhamnoGalacturonan-I
RG-II	RhamnoGalacturonan-II
RPMI	Hybridoma Culture Medium
RT	Room Temperature
TEM	Transmission Electron Microscopy
TS	Transverse Section
WGA	Wheat Germ Agglutinin
XG	XyloGlucan

Chapter One

General Introduction

Fungi are responsible for the majority of plant diseases (Aist, 1984). In order for any fungus to establish a plant pathogenic relationship, it has to first enter the plant host. Infection may occur naturally through wounds, decaying or dead plant tissue or via direct penetration of the undamaged host. The intact aerial parts of plants such as leaves, flowers, fruits and young stems are covered by a cuticularised layer, more commonly known as the cuticle (Martin & Juniper, 1970). The plant cuticle is characterised chemically by two types of lipid substances; cutin which constitutes the framework of the cuticle and soluble waxes which are deposited on the surface as epicuticular wax and embedded within the matrix as cuticular wax (Holloway, 1982a). The epicuticular wax, which can be readily extracted with organic solvents, contains many classes of relatively non-polar C₂₀-C₃₂ aliphatic compounds, principally hydrocarbons, wax esters, primary alcohols and fatty acids (Martin & Juniper, 1970; Kolattukudy, 1980). It may be amorphous in form or possess a definite semi-crystalline or crystalline structure and it is often the first component of the plant cuticle encountered by aerial pathogens (Cooper, 1981). Wax crystals may be altered or removed by some plant pathogenic fungi as shown by the clear tracks left beneath germ tubes, hyphae and infection structures following their removal from the leaf surface (Staub, Dahmen & Schwinn, 1974; Sargent & Gay, 1977; Kunoh, Tzuzuki & Ishizaki, 1978). The inability of fungal pathogens to remove or penetrate epicuticular waxes and therefore contact the layers of cuticle beneath undoubtedly contributes to the failure of some pathogens to penetrate non-host plants e.g. *Erysiphe cichoracearum* on barley (Staub, Dahmen & Schwinn, 1974) and *Uromyces phaseoli* on wheat (Wynn, 1976).

The layers of cuticle beneath the epicuticular wax are more commonly referred to as the cuticle *sensu stricto* or cuticle proper (von Mohl, 1847; Holloway, 1982a). Cutin is the main structural component of the plant cuticle; it is an insoluble lipid polyester composed of C₁₆ and C₁₈ substituted aliphatic acids. The main substituent of these monomers is hydroxyl, but aldehyde, ketone, epoxide and unsaturated groups may also be present (Kolattukudy, 1980, 1981; Holloway, 1982b; Kolattukudy & Köller, 1983). In angiosperm cutin, the most

abundant C₁₆ compounds are 9, 16- and 10, 16-dihydroxy palmitic acid, whereas the most common C₁₈ compounds are 9, 10-epoxy-18-hydroxy- and 9, 10, 18-trihydroxystearic acids (Holloway, 1982b; Kolattukudy & Köller, 1983). On most plant organs, cutin consists of a combination of these monomers (Kolattukudy & Köller, 1983). The polymer cutin is considered to be the first physical barrier that the fungus encounters at the plant host surface (Kolattukudy & Köller, 1983; Kolattukudy, 1985).

It is well-known that numerous plant pathogenic fungi can commonly attack cuticularised plant surfaces (Aist, 1984). These pathogenic fungi must therefore possess a means of overcoming the cutin barrier either by the physical force of an infection structure or enzymic digestion of the cuticle. The mechanism of ingress of parasitic fungi through the plant cuticle has been a question of debate for almost a century, as summarised by van den Ende & Linksens (1974). Cutin-hydrolysing enzymes or so-called cutinases have been isolated from a number of plant pathogenic fungi grown on cutin as the sole carbon source e.g. *Fusarium solani* f. sp. *pisi* (Purdy & Kolattukudy, 1975a,b; Coleman, Hiscock & Dewey, 1993), *Colletotrichum gloeosporioides* (Dickman, Patil & Kolattukudy, 1982), *Venturia inaequalis* (Köller & Parker, 1989) and *B. cinerea* (Salinas, Warnaar & Verhoeff, 1986; Salinas, 1992) to name but a few. Antibodies directed against cutinase and chemical inhibitors of this enzyme e.g. di-isopropylfluorophosphate, have been shown to prevent infection of pea epicotyl by the pathogen, *F. solani pisi* (Maiti & Kolattukudy, 1979) and suppress lesion formation on papaya fruits by *C. gloeosporioides* (Dickman, Patil & Kolattukudy, 1982). A cutinase gene-disrupted mutant of *F. solani pisi* has reduced virulence on pea (Rogers, Flaishman & Kolattukudy, 1994). Moreover, the wound pathogen *Mycosphaerella* species was found to be pathogenic on intact papaya fruits following insertion of the *F. solani* cutinase gene (Dickman, Podila & Kolattukudy, 1989). Similarly, transformation of a cutinase-less mutant of *F. solani* with this cutinase gene restored virulence (Kolattukudy *et al.*, 1989). However, despite this evidence, the role of cutinase in pathogenicity is still controversial (Chasan, 1992).

In order to ensure entry into the plant host, the fungus has to initially attach itself firmly to the host surface (Kunoh, Nicholson & Kobayashi, 1991; Nicholson & Epstein, 1991). Traditionally, the attachment of fungi to plant surfaces was considered to be a result of the deposition of fungal spores by impact, lodging or other phenomena of physical entrapment. Furthermore, it was assumed that processes of active fungal attachment did not occur until the formation of germ tubes or infection structures (Nicholson, 1984). It is now widely accepted however, that the phenomenon of attachment is more complex and involves the production of extracellular matrix material by the fungus which in turn, ensures firm

attachment or adhesion of the fungus to the plant surface (Kunoh, Nicholson & Kobayashi, 1991; Nicholson & Epstein, 1991).

This chapter reviews the literature on the role and importance of fungal extracellular matrices in the attachment of spores, germ tubes, hyphae and infection structures to both natural and synthetic substrata. Other putative roles and properties of these matrices in the development of the plant host-pathogen relationship are addressed. Biochemical and ultrastructural evidence on the nature, origin and secretion of fungal extracellular matrices are outlined in brief, as are the possible methods of observation of fungal extracellular matrices by light and electron microscopy. Chocolate spot disease of broad bean, *viz.* the *Vicia faba*-*Botrytis fabae* interaction, is introduced as a model system to be used in this study for investigations of the role of extracellular matrices in plant pathogenesis. In addition, the production and potential uses of monoclonal antibodies directed against fungal cell surface molecules, and in particular *Botrytis* species, as markers for fungal extracellular matrices and disease control agents are described.

1.1 Adhesion of fungal spores to natural and synthetic substrata

Adhesion of fungi to plant host surfaces may occur at a very early stage in the development of the plant host-pathogen relationship. Indeed, there are many examples where ungerminated conidia have been shown to release matrix material immediately upon hydration or upon contact with the substratum (e.g. Paus & Raa, 1973; Young & Kauss, 1984; Hamer *et al.*, 1988; Jones & Epstein, 1989; Beckett, Tatnell & Taylor, 1990; Sela-Buurlage, Epstein, & Rodriguez, 1991; Deising *et al.*, 1992; Doss *et al.*, 1993; Terhune & Hoch, 1993; See Diagram 1.1, [A]). A similar strategy for adherence to plant host surfaces is employed by the zoospores of oomycetes¹ (e.g. *Phytophthora*, *Pythium* species) during encystment (Reichle, 1969; Hemmes & Hohl, 1971; Sing & Bartnicki-Garcia, 1975; Gubler & Hardham, 1988;

¹ Oomycetes (Class: Oomycota) have been commonly included in the fungal kingdom (e.g. Ray, Steeves & Fultz, 1983) as a result of their fungal-like growth habit, aseptate mycelium and their production of biflagellate heterokont zoospores. However, there is considerable evidence that they have close phylogenetic affinities with the chromophyte algae and that they are in fact widely divergent from higher fungi (see Hardham *et al.*, 1994). For example, oomycetes contain cellulose rather than chitin in their cell walls (Bartnicki-Garcia, 1970, 1987) and they possess tubular and not plate-like mitochondrial cristae (Cavalier-Smith, 1981). Nonetheless, oomycetes will often be referred to in this text, not only because they include well-known plant pathogens e.g. leaf blights (e.g. *Phytophthora infestans*: late blight of potato) and downy mildews (*Bremia lactucae*: downy mildew of lettuce), but since *Phytophthora* and *Pythium* species, as mentioned above, are highly adapted for attachment to host plants.

Hardham *et al.*, 1994). In *Phytophthora* species, there are three types of peripheral storage vesicles (large, dorsal and ventral) within the cortical cytoplasm of the unencysted zoospores (Hardham, 1987; Hardham & Gubler, 1990; Gubler & Hardham, 1991; Hardham, Gubler & Duniec, 1991). Within the first two minutes following the induction of encystment, the contents of the dorsal and ventral vesicles are secreted onto the surface of the cysts (Hardham & Gubler, 1990). The timing of secretion of these vesicles has been shown to coincide with the timing of the acquisition of adhesiveness of the existing cells (Gubler, Hardham & Duniec, 1989). Furthermore, it has been suggested that it is the ventral vesicles that contain the adhesive that sticks the zoospores to the root surface because when zoospores swim towards the root of a potential host they orient themselves so that their ventral surface faces the root and they maintain this orientation during encystment (Hardham *et al.*, 1994).

In some cases, the attachment of the ungerminated spore to the plant host surface is a non-specific process (Hickman & Epstein, 1988; Jones & Epstein, 1989; Doss *et al.*, 1993). Water droplets and nutrients (e.g. leaf exudates, pollen grains, insect excrements) on the plant surface may provide a stimulus for the release of extracellular matrices by the fungal spore (Hamer *et al.*, 1988; Jones & Epstein, 1989). Nutrient-competing microbes (e.g. bacteria and yeasts), extracellular host molecules and surface molecules (e.g. leaf waxes; see above) may, on the other hand, restrict the development of fungal infection (Blakeman, 1993). Furthermore, in some plant host-parasite interactions, there is a notable degree of specificity of attachment of the fungal spore to the host surface. For example, evidence suggests that in some fungi (e.g. *Fusarium* species) and in *Phytophthora* and *Pythium* species, the extracellular matrix material that is produced by the pathogen is highly specific for the recognition of, and binding to, a particular host species (Hinch & Clarke, 1980; Hardham & Suzaki, 1986; Longman & Callow, 1987; Hohl & Balsiger, 1988; Boyer & Charest, 1989; Nicholson & Epstein, 1991). Experiments with adhesion-reduced macroconidia of *Nectria haematococca* [anamorph: *F. solani* f. sp. *cucurbitae*] have suggested that spore adhesion is required for a compatible (susceptible) host-pathogen interaction (Jones & Epstein, 1990). Thus, spore adhesion is considered to be a virulence factor for *N. haematococca* (Jones & Epstein, 1990).

It is generally accepted that spore mucilages provide firm adhesion of the spore to the substratum. In the rice blast fungus, *Magnaporthe grisea*, conidia deposited on a synthetic film (Teflon-PFA) for 75 minutes were able to resist water flow rates as high as 200 ml/min (Hamer *et al.*, 1988). In addition, the zoospores of oomycetes become firmly attached to any substratum on which they settle as a result of the release of adhesive material on encystment.

This adhesion process is also strong enough to resist extensive washing (Hinch & Clarke, 1980). It seems likely that adhesion of ungerminated conidia ensures that these propagules are not displaced from the plant surface by wind, rainfall or simply by inoculum droplets rolling off the leaf surface (Young & Kauss, 1984; Hamer *et al.*, 1988). Thus, it follows that adhesion of ungerminated conidia contributes significantly to the development of plant diseases (Mercure, Kunoh & Nicholson, 1994).

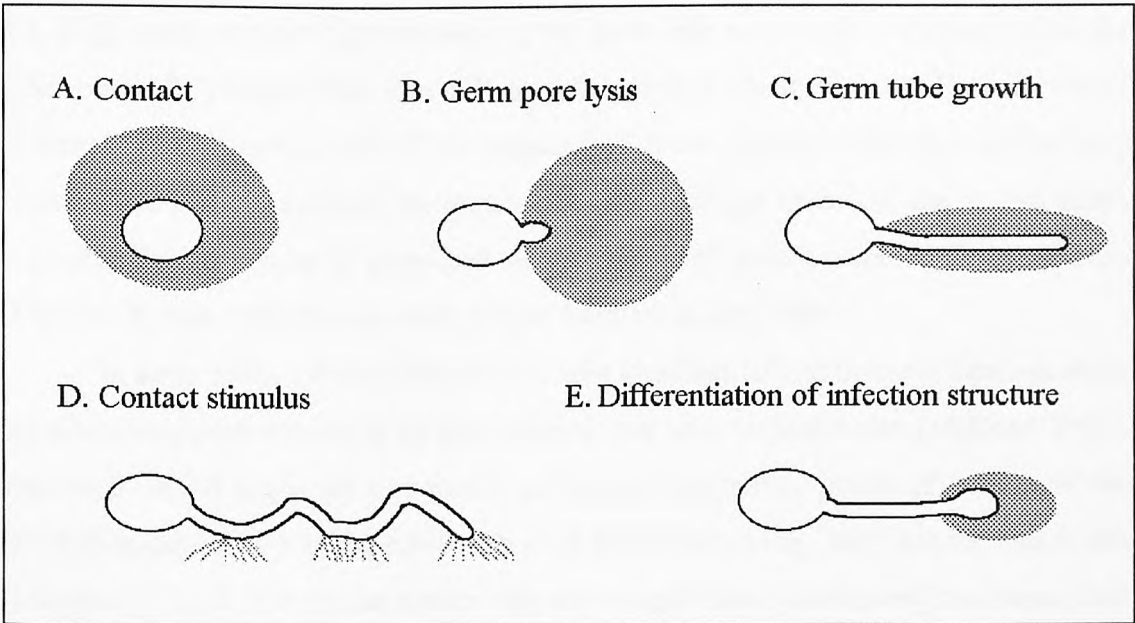


Diagram 1.1 Summary of the pre-penetration processes of fungal spores and the production of extracellular matrices

Ungerminated conidia may produce extracellular matrix material upon contact with the substratum [A]. Fungal spores may release matrix material following germ pore lysis [B] and concurrent with germ tube growth [C]. Additional matrices may also be produced at points of contact of the germ tube with the substratum [D] and during the development of infection structures [E]. Modified from Nicholson (1984).

1.2 Adhesion of fungal germ tubes, hyphae and infection structures

Soon after adhesion of the fungal spore, the conidium may germinate to produce one or more germ tubes (Epton & Richmond, 1980). At the point of germ tube emergence additional matrix polymers, possibly acid mucopolysaccharides, may also be released (Evans, Stempen & Frasca, 1982; Beckett, Tatnell & Taylor, 1990; Clement *et al.*, 1993: See Diagram 1.1, [B]). Extracellular matrices are also known to be liberated concurrent with germ tube growth (Epstein *et al.*, 1985; Epstein *et al.*, 1987; Kunoh, Nicholson & Kobayashi, 1991), and as a consequence, the developing germ tube is often encased in a matrix sheath (Chaubal, Wilmot & Wynn, 1991; Nicholson & Epstein, 1991; Durso, Lehen & Powell, 1993; See Diagram 1.1, [C]) which provides tight adhesion of the germ tube to natural or synthetic substrata (McKeen, 1974; Pringle, 1981; Hau & Rush, 1982; Gold & Mendgen, 1984; Beckett, Tatnell & Taylor, 1990; Clement *et al.*, 1993). Immunological and electron microscope studies have revealed that in *Microbotryum violaceum* (formerly *Ustilago violacea*), the matrix sheath enclosing the germ tube is composed of a network of protein microfibrils or fimbriae (Gardiner & Day, 1985; Svircev *et al.*, 1986a; Gardiner & Day, 1988).

In some cases, adhesive material has been identified only at irregular intervals along the developing germ tube or at the germ tube tip just prior to penetration (McKeen, 1974). Nicholson (1984) suggested that matrix production occurred at points of contact of the developing germ tube with the substratum or at sites of stress (e.g. bends) in the fungal wall (Diagram 1.1, [D]). The contact surface may play a major role in determining the amount and site of secretion of adhesive material by the fungus. Close adherence of germ tubes to substrata may also be essential for the morphological development of the sporeling i.e. the orientation of hyphal growth and for the induction of appressoria (Wynn, 1981; Hoch *et al.*, 1987).

In parasitic fungi, further binding to substrata is often provided by the development of infection structures termed “appressoria” (Emmett & Parbery, 1975), whose induction, formation and action form integral parts of the infection process. Induction of appressorial formation may be achieved by contact stimuli (Emmett & Parbery, 1975; Wynn, 1981; Epstein *et al.*, 1987) and as a result the appressorium is often formed terminally on the germ tube. The most notable morphological requirement of a plant pathogenic fungus to date is the precise height of the stomatal lip of bean which triggers the initiation of appressorium formation in *Uromyces appendiculatus* (Hoch *et al.*, 1987). Extracellular adhesives are often

associated with such organs of attachment (Ward, 1888; Emmett & Parbery, 1975; See also Diagram 1.1, [E]). Thus, it is essential to regard the contact, adhesion and subsequent pre-invasion development of the fungal spore as an on-going unified process which, in turn, is prerequisite to penetration and hence survival of the fungus.

Following maturation, the appressorium may invade the host epidermal cells directly (Salinas & Verhoeff, 1995) or *via* an infection peg through the cuticle (McKeen, 1974; Staub, Dahmen & Schwinn, 1974; Rijkenberg, De Leeuw & Verhoeff, 1980; Mansfield & Richardson, 1981) or they may enter into the leaf tissue *via* natural openings on the leaf surface e.g. stomata (Pring, 1980; Hoch *et al.*, 1987). During this penetrative process and the subsequent invasion of the host tissue, the fungus may continue to secrete a number of extracellular compounds. For example, during the infection of *Vicia faba* L. leaves by the fungus *Uromyces viciae-fabae*, at least two types of extracellular matrices are thought to be produced; one is formed at the contact points between adjacent hyphae and the other at similar points between hyphae and host cells (Beckett & Woods, 1987). Both matrices are considered to be of fungal origin, but only the latter is thought to possess an adhesive role (Beckett & Porter, 1988).

In the early stages of infection of *Phaseolus vulgaris* by the hemibiotroph, *Colletotrichum lindemuthianum*, a thin infection peg develops from the base of a melanised appressorium and penetrates the plant cuticle and cell wall directly (O'Connell, Bailey & Richmond, 1985; Bailey *et al.*, 1992). In a susceptible host, the penetrated epidermal cell remains alive and the tips of the infection pegs swell to form infection vesicles, which in turn, produce one or more primary hyphae. Both the infection vesicles and primary hyphae invaginate the host plasma membrane and are separated from it by an interfacial matrix layer which is thought to be composed of both plant and fungal molecules (Bailey *et al.*, 1992). Similar matrices envelop the intracellular biotrophic hyphae of *C. gloeosporioides* on infection of tangerine (Brown, 1977). However, there is no evidence to suggest that these or similar matrices e.g. the extrahaustorial matrices produced during infection of plant hosts by obligate biotrophic fungi², are involved in adhesion.

² Obligate biotrophic plant pathogenic fungi, such as the powdery mildew fungi, produce specialised infection structures inside host cells (Manners & Gay, 1983). After development of an appressorium on the leaf surface, an infection peg grows directly through the wall of the host cell to form a narrow tube, the haustorial neck. On reaching the host plasma membrane, the infection peg develops further to form a large sac-like structure, the haustorium, which is surrounded by the invaginated host plasma membrane, otherwise known as the extrahaustorial membrane (ehm). The ehm is separated from the haustorial cell wall, plasma membrane and cytoplasm by a gel-like substance called the extrahaustorial matrix (ehmat). In powdery mildew interactions, the precise composition and derivation of the ehmat is unknown (Green *et al.*, 1995).

Evidently, the release of extracellular matrices or mucilages appears to span a broad-range of fungi and fungal structures (Nicholson & Epstein, 1991). It is clear that a greater knowledge of the involvement of extracellular matrices produced by plant pathogenic fungi during the pre-penetration and pre-infection processes of the plant host-pathogen interaction is vital to our understanding of the disease process as a whole.

1.3 Methods of observation of fungal extracellular matrices

Fungal extracellular matrices may be visualised simply by phase contrast and differential interference contrast (DIC) microscopy, especially where matrix material is abundant and viscous. On the other hand, if matrix material is less abundant, observation may necessitate the use of stains e.g. negative staining (Evans, Stempen & Frasca, 1982), histochemical stains and more recently fluorescently-labelled and gold-conjugated lectins (Benhamou & Ouellette, 1986; Bonfante-Fasolo *et al.*, 1987; Hamer *et al.*, 1988; Harder *et al.*, 1989; Chaubal, Wilmot & Wynn, 1991). Labelled lectins have also been employed to follow changes in the surface components of zoospores and cysts of *Phytophthora* and *Pythium* species during encystment by both light and electron microscopy (Bacic, Williams & Clarke, 1985; Hardham, 1985; Hardham & Suzaki, 1986; Gubler, Hardham & Duniec, 1989; Hardham *et al.*, 1994). Water-soluble fungal matrices are often the most difficult to localise by light and electron microscope methods as a result of their inherent solubility in aqueous histochemical stains and chemical fixatives.

Low-temperature scanning electron microscopy (LTSEM) which permits the observation of partially freeze-dried or fully-frozen hydrated material in a condition which closely reflects the natural state (Beckett, Porter & Read, 1982; Read, Porter & Beckett, 1983; Beckett & Read, 1986) is considered to be an invaluable technique for investigations into plant host-pathogen relationships and observation of fungal extracellular matrices (Beckett & Woods, 1987; Beckett & Porter, 1988; Beckett, Tatnell & Taylor, 1990; Read, 1991; Braun & Howard, 1994, Mims, 1994). This technique involves rapid-freezing of biological samples in liquid nitrogen slush and observation of frozen samples at ultra-low temperature (-160°C). Invariably, water-soluble components of fungal extracellular matrices are well-preserved by this method of preparation. The removal of adherent spores by micro-manipulation techniques or simply

by the use of adhesive tape prior to observation by LTSEM, have also confirmed the production of copious amounts of matrix material at the sites of contact of the fungal spores and the substratum (Deising *et al.*, 1992)

Immunocytochemical studies conducted at both the light and electron microscope levels, using monoclonal antibodies (MAbs) directed against fungal pathogens, have proved to be useful for labelling fungal cell surface molecules and in investigations of their possible role(s) in the plant host-pathogen relationship (see also Table 1.1. and references therein). Furthermore, similar immunological techniques using MAbs generated towards components of zoospores of *Phytophthora cinnamomi* (Hardham, Suzaki & Perkin, 1986; see also Table 1.1) and *Pythium aphanidermatum* (Estrada-Garcia *et al.*, 1989; see Table 1.1) have not only greatly increased the knowledge of the ultrastructure of these cells but they have also contributed to a better understanding of the role of cell surface components during encystment (Hardham *et al.*, 1994).

1.4 Nature, origin and secretion of fungal extracellular matrices

Fungal extracellular matrices appear to be composed of two classes of adhesive components: polysaccharides and proteins (mainly glycoproteins) (Nicholson & Epstein, 1991). For example, sporelings of *Bipolaris sorokiniana* have been shown to produce an adhesive, possibly a positively-charged galactosamine polymer, when conidia are inoculated onto glass (Pringle, 1981). On *Colletotrichum graminicola* appressoria, a “hemicellulose” is apparently involved in adhesion (Lapp & Skoropad, 1978). In addition, a protein or glycoprotein appears to adhere rust germlings (Epstein *et al.*, 1987) and zoospores (Sing & Bartnicki-Garcia, 1975; Pring, 1980; Gubler & Hardham, 1988; Gubler, Hardham & Duniec, 1989; Estrada-Garcia, Callow & Green, 1990; Kwon & Epstein, 1993) to substrata.

It is well known that, in filamentous fungi, as in all other eukaryotes, polysaccharides and/or glycoproteins bound for the cell surface or extracellular matrix are synthesised and/or modified in Golgi-like bodies (known here as Golgi equivalents) and subsequently secreted from the fungal cell *via* the exocytosis of small vesicles (Peberdy, 1994). Although it is widely accepted that the production of adhesive materials by zoospores of *Phytophthora* species

Table 1.1 Examples of specific monoclonal antibodies employed previously to label cell surface molecules of fungi and certain oomycetes by immunofluorescence and/or immunoelectron microscopy

MAb	Immunogen	IF [†] microscopy	IEM [‡]	Specificity	Reference
UB20	homogenates of <i>Colletotrichum lindemuthianum</i> sporelings	<i>C. lindemuthianum</i> sporelings grown <i>in vitro</i>	infected bean hypocotyls	surface of conidia, wall and matrix around IH [†]	Pain <i>et al.</i> , 1992
UB22	homogenates of <i>C. lindemuthianum</i> sporelings	<i>C. lindemuthianum</i> sporelings grown <i>in vitro</i>	infected bean hypocotyls	fibrillar sheath and in apical regions of germ tubes, wall and matrix around IH	Pain <i>et al.</i> , 1992
UB25	co-immunisation with IPC [*] preparations of infection structures isolated from bean leaves	<i>C. lindemuthianum</i> sporelings grown <i>in vitro</i> and isolated infection structures	infected bean hypocotyls	cell wall/matrix of IH. Expressed only during biotrophic phase of development	Pain <i>et al.</i> , 1994
UB26	co-immunisation with IPC preparations of infection structures isolated from bean leaves	<i>C. lindemuthianum</i> sporelings grown <i>in vitro</i> and isolated infection structures	—	fibrillar sheath around germ tubes and circular halo of material around appressoria	Pain <i>et al.</i> , 1994; Green <i>et al.</i> , 1995
UB27	co-immunisation with IPC preparations of infection structures isolated from bean leaves	isolated infection structures	<i>C. lindemuthianum</i> sporelings grown <i>in vitro</i>	plasma membrane-wall interface of appressoria, except pore region	Pain <i>et al.</i> , 1994; Green <i>et al.</i> , 1995; Pain, O'Connell & Green, 1995
UB8 UB10	isolated HCs ^{**} from pea leaves infected by <i>Erysiphe pisi</i>	isolated HCs	infected pea leaf material	haustorial plasma membrane within the haustorial complex.	Mackie <i>et al.</i> , 1991, 1993
UB7	isolated HCs from pea leaves infected by <i>E. pisi</i>	isolated HCs	infected pea leaf material	fungus wall and plasma membranes present in both haustoria and mycelia	Mackie <i>et al.</i> , 1991, 1993; Green <i>et al.</i> , 1992
UB11	isolated HCs from pea leaves infected by <i>E. pisi</i>	isolated HCs, mycelia, uninfected or infected pea leaves	isolated HCs	extrahaustorial membrane	Green <i>et al.</i> , 1992; Roberts <i>et al.</i> , 1993
UB40	isolated HCs from pea leaves infected by <i>E. pisi</i>	—	isolated HCs	extrahaustorial matrix of all HCs and host plasma membrane and cell wall	A.J. Mitchell, S.Mc. Cready, J.A. Callow & J.R. Green unpublished data

continued overleaf

Table 1.1 continued

BC-AF7 BC-KH4	surface washings of <i>Botrytis cinerea</i>	<i>B. cinerea</i> sporelings grown <i>in vitro</i>	<i>B. cinerea</i> sporelings grown <i>in vitro</i>	hyphal wall, septa and fibrillar sheath around hyphae	Bossi & Dewey, 1992; Bossi <i>et al.</i> , 1994
9E 11	extracellular material obtained from a mixture of ten <i>B. cinerea</i> isolates	<i>B. cinerea</i> conidia	—	surface of conidia	Salinas, 1992, Salinas & Schots, 1994
4H 10	whole conidia of <i>B cinerea</i>	<i>B. cinerea</i> conidia	—	surface of conidia	Salinas, 1992, Salinas & Schots, 1994
14 E5	cutin esterase	<i>B. cinerea</i> conidia	—	surface of conidia	Salinas, 1992, Salinas & Schots, 1994
PI-01	surface washings of <i>Penicillium islandicum</i>	suspension cultures of <i>P. islandicum</i> and sporulating hyphae grown on cellophane	suspension cultures of <i>P. islandicum</i> and young or sporulating hyphae grown on solid agar	hyphal wall, septa and fibrillar sheath around hyphae. Antigen located in the conidiophore wall including metulae, but absent from the phialides and conidia	Cole, Hawes & Dewey, 1991
HL-EC6	surface washings of <i>Humicola lanuginosa</i>	—	<i>H. lanuginosa</i> grown on solid agar	hyphal wall, septa and fibrillar sheath around hyphae.	Dewey <i>et al.</i> , 1993
AF-BBG1	surface washings of <i>Aspergillus flavus</i>	—	<i>A. flavus</i> and <i>A. niger</i> grown on solid agar	fibrillar sheath surrounding hyphae, septas and regions of hyphal wall	B.G.H. Martin & F. M. Dewey, unpublished results
EB-A2	<i>Aspergillus fumigatus</i> spores or mycelium extracts	germinating conidia of <i>A. fumigatus</i>	—	growth tips of hyphae	Stynen <i>et al.</i> , 1991
4G11	<i>Pyricularia grisea</i> sporelings	<i>Pyricularia grisea</i> sporelings	—	surface of conidia	Xia <i>et al.</i> , 1992
8H1 3E4	<i>Pyricularia grisea</i> sporelings	<i>Pyricularia grisea</i> sporelings	—	germ-tubes	Xia <i>et al.</i> , 1992
6A5 3F12	isolated cell wall fragments of <i>Gliocladium roseum</i>	—	fungal cells and isolated cell walls of <i>G. roseum</i>	separate layers of the cell wall	Breuil <i>et al.</i> , 1992

continued overleaf

Table 1.1 continued

Zp 1, 2	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	—	zoospore periphery	Hardham, <i>et al.</i> , 1986, 1994
ZCp1, 2	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	—	zoospore and cyst periphery	Hardham, <i>et al.</i> , 1986, 1994
Cpa 2	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	dorsal vesicles, cyst coat	Hardham, <i>et al.</i> , 1986, 1991, 1994; Gubler & Hardham, 1988
Cpw 1	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	peripheral cisternae, cyst wall	Hardham, <i>et al.</i> , 1986, 1991, 1994
Vsv 1	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	ventral vesicles, cyst adhesive	Hardham, <i>et al.</i> , 1991, 1994
Lpv 1	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	<i>P. cinnamomi</i> zoospores and cysts	large peripheral vesicles	Gubler & Hardham, 1988; Hardham, <i>et al.</i> , 1991, 1994
PA 1	<i>Pythium</i> <i>aphanidermatum</i> zoospores and cysts	zoospores of <i>Pythium</i> <i>aphanidermatum</i>	—	zoospore surface	Estrada-Garcia <i>et al.</i> , 1989
PA 3 through PA 6	<i>Pythium</i> <i>aphanidermatum</i> zoospores and cysts	cysts and zoospores of <i>Pythium</i> <i>aphanidermatum</i>	—	cyst surface	Estrada-Garcia <i>et al.</i> , 1989
PA 7, 8	<i>Pythium</i> <i>aphanidermatum</i> zoospores and cysts	cysts and zoospores of <i>Pythium</i> <i>aphanidermatum</i>	—	zoospore and cyst surface	Estrada-Garcia <i>et al.</i> , 1989
HB 5, 6	<i>Saprolegnia</i> <i>parasitica</i> zoospore and cysts	<i>S. parasitica</i> zoospore and cysts	—	zoospore and cyst surface	Burr & Beakes, 1994

† Immunofluorescence
‡ Immunogold labelling at the transmission electron microscope level.
/ IH = intracellular hypha(e), i.e. infection vesicles and primary hyphae.
* Isopycnic centrifugation.
** Haustorial complexes.
Hardham *et al.*, 1986 = Hardham, Suzaki & Perkin, 1986
Hardham *et al.*, 1991 = Hardham, Gubler & Duniec, 1991

involves vesicle excretion upon contact with the host surface (Gubler & Hardham, 1988), there is as yet little evidence to suggest that these vesicles are ER- (endoplasmic reticulum) and/or Golgi-derived.

Interestingly, Hamer and co-workers (1988) demonstrated that spore tip mucilage released on germ pore lysis of conidia of *Magnaporthe grisea* was located in the periplasmic space of dormant conidia. They suggested that adhesive material may be stored in older hyphae, in a space between the pellicle or hyphal coating and the cell wall and released when required. It is conceivable that adhesive compounds are released into the fungal wall in a soluble form and they may become less water-soluble when liberated into the extracellular medium (Mims & Richardson, 1989). In short, little is known about the production of extracellular matrices in filamentous fungi and it is often assumed that they are secreted at the hyphal tips (Kunoh, Nicholson & Kobayashi, 1991). Evidently, specific monoclonal antibodies which recognise adhesive compounds could be used to determine the origin and secretion of such material at the intracellular level. Further immunocytochemical studies using brefeldin A, a well-known inhibitor of the secretory pathway in animal, plant and yeast cells (Rothman & Orci, 1992; Satiat-Jeunemaitre & Hawes, 1993; Rambourg *et al.*, 1995) may also provide invaluable information on the putative secretory pathway of such cell surface antigens.

1.5 Properties or functions of fungal extracellular matrices

Fungal extracellular matrices, regardless of their adhesive properties, have been shown to possess additional properties and/or functions which may be essential for the development of the disease process. They may act as an anti-desiccant (Nicholson & Moraes, 1980). They may provide a barrier to diffusion thereby reducing the loss of endogenous nutrients from sporelings and maintaining extracellular pH and ion balances in aqueous environments (Moloshok *et al.*, 1993). They may possess signalling properties which elicit the host defense responses (Darvill & Albersheim, 1984). For example, fungal cell wall oligosaccharides, also known as *oligosaccharins*, have been shown to be elicitors of phytoalexins (Darvill & Albersheim, 1984). Phytoalexins are low molecular weight, anti-microbial compounds which are absent in healthy plants and are produced by host plants in response to infection by a microbial pathogen (Darvill & Albersheim, 1984).

In some cases, extracellular matrices are able to sequester toxic phenols produced by the host in order to counteract infection (Nicholson, Hipskind & Hanau, 1989). In addition, a number of enzymes are also known to be actively secreted with fungal extracellular matrices e.g. glycosidase (Clement, Butt & Beckett, 1993), hydrolase, invertase (Nicholson & Moraes, 1980), polygalacturonases, esterases (McKeen, 1974; Nicholson *et al.*, 1988; Clement, Butt & Beckett, 1993) and cutinase (Kolattukudy, 1985; Köller, 1991). Such enzymes, in particular esterases and cutinase, can degrade the plant cuticle and/or cell walls and may be required by the fungus for the preparation of the infection court (Kunoh *et al.*, 1988; Nicholson *et al.*, 1988; Nicholson & Epstein, 1991). In a recent report, Deising and coworkers (1992) suggested that cutinase may also be required to effect adhesion of the fungus to the substratum.

1.6 The *Vicia faba*-*Botrytis fabae* interaction: a model system for investigating the role of fungal extracellular matrices in plant pathogenesis

The necrotrophic fungi, *Botrytis fabae* [Sard.] and *B. cinerea* Pers.:Fr. [asexual stage of the teleomorph, *Botryotinia fuckeliana* (de Bary) Whetzel] are responsible for important diseases of dicotyledonous plants (Coley-Smith, Verhoeff & Jarvis, 1980). Infection of host plants by *Botrytis* species may arise directly from conidia, mycelium growing on decaying or dead plant tissues or from mycelium growing on extraneous organic material (Verhoeff, 1980). Conidia are a primary inoculum source (Diagram 1.2). They may be dispersed by air currents, water droplets and insects (Harrison, 1988).

Although *B. fabae* has a very limited host range, it is well known as the major cause of chocolate spot disease of broad bean (*Vicia faba* L.) in the field (Ogilvie & Munro, 1947; Leach, 1955; Deverall & Wood, 1961a,b) where the fungus forms dark brown spreading lesions on the leaf surface. *B. cinerea*, on the other hand, is a ubiquitous polyphagous fungus and is often referred to as “grey mould” because of the appearance of the fungus as grey-brown clusters of spores (blastosporangia) on the plant host (Diagram 1.2). It is responsible for the post-harvest spoilage of many fruits [e.g. red raspberry (McNicol, Williamson & Dolan, 1985; Dashwood & Fox, 1988) and cucumber (Elad, 1988)] and vegetables [e.g. cabbage, artichoke, onion, potato, carrot (Jarvis, 1977; Coley-Smith, Verhoeff & Jarvis, 1980)]. *B. cinerea* also causes great economic losses in the

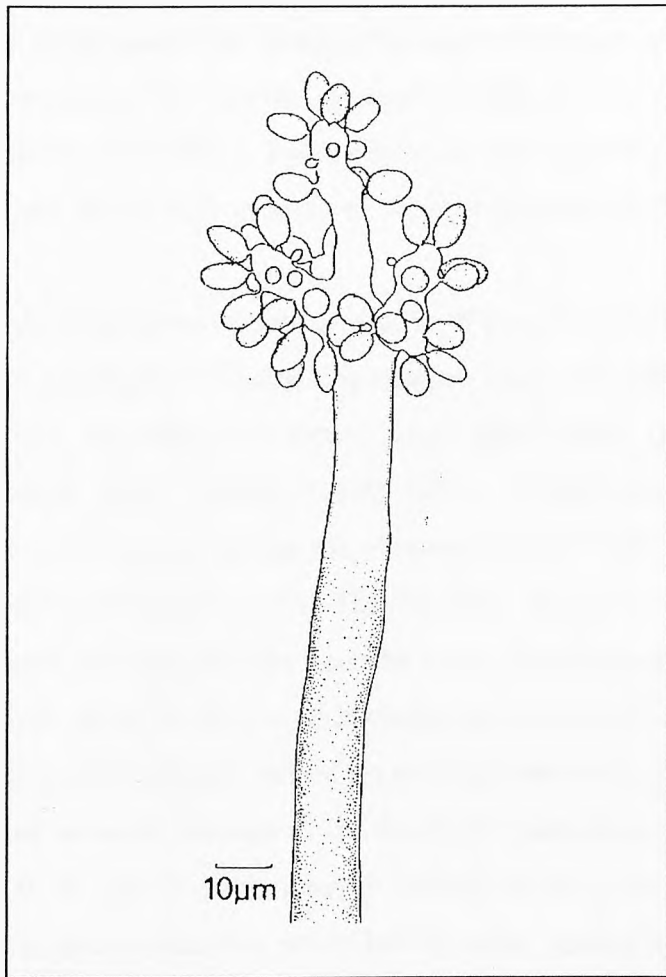


Diagram 1.2 Detail of spore-bearing structure or conidiophore of *Botrytis cinerea*

Numerous elliptical macroconidia (blastospores) are formed by budding from the tips of the thin-walled branches of the conidiophore. (Redrawn from Webster, 1980).

production and export of glasshouse-grown ornamental crops e.g. rose and gerbera flowers (Elad, 1988; Salinas, 1992). In the field, *B. cinerea* may also cause chocolate spot of bean, however only restricted lesions are formed on the leaf surface (Leach, 1955; Deverall & Wood, 1961b; Purkayastha & Deverall, 1965; Mansfield & Deverall, 1974; Harrison, 1988).

There are several reports in the literature which describe in detail the necrotrophic growth of *B. cinerea* and *B. fabae* on various host plants (Rijkenberg, de Leeuw & Verhoeff, 1980; Pie & De Leeuw, 1991; Salinas & Verhoeff, 1995) including *Vicia faba* L. (McKeen

1974; Deverall & Wood, 1961b). The development of chocolate spot disease of Dwarf broad bean is an excellent model system for studying the mode of infection of *Botrytis* species. It allows a close comparison of the infection processes of both *B. cinerea* and *B. fabae*. In addition, the infection of *Vicia faba* L. leaves can be studied simply in the laboratory by the inoculation of excised leaves with droplets of conidial suspensions (Deverall & Wood, 1961b).

Previous light and electron microscope studies of plant-*Botrytis* interactions revealed that the germ tubes and hyphae of *Botrytis* species are often enclosed within a sheath of fibrillar material which may serve as a sort of fungal glue (Ward, 1888 [Diagram 1.3]; Blackman & Welsford, 1916; McKeen, 1974). More recently, studies of *B. cinerea* sporelings grown *in vitro* have shown that the adhesion of conidia and sporelings occurs in two distinct stages (Doss *et al.*, 1993, 1995). The first stage, defined as *immediate adhesion*, occurs upon hydration of freshly-deposited conidia and is characterised by relatively weak adhesive forces which appear to involve hydrophobic interactions (Doss *et al.*, 1993). In contrast, the second stage of adhesion termed *delayed adhesion* involves the secretion of an ensheathing film that remained attached to the substratum even after physical removal of sporelings (Doss *et al.*, 1995). Such research findings support the idea that *Botrytis* extracellular matrices possess adhesive properties. However, further investigations of the possible role(s) of *Botrytis* extracellular matrices in the early stages of development of the *Vicia faba*-*Botrytis* relationship are warranted.

1.7 Production and use of specific monoclonal antibodies to cell surface molecules of *Botrytis* species

To date, the development of immunodiagnostic techniques and the use of antibodies in particular, to detect specific fungi in diseased plants has been limited as a result of the difficulty in raising antisera that are species-specific (Dewey, 1992). Many polyclonal antisera to immunogens (e.g. fungal spores, hyphal fragments, solubilised extracts or concentrates of culture filtrates) cross-react widely with related species, species from unrelated genera and host molecules (Banowetz, Trione & Krygier, 1984). In some cases, where apparently species-specific antisera have been obtained, they were often prepared by cross-adsorption of the antiserum with antigens from other species (Kitagawa *et al.*, 1989). Such antisera, when

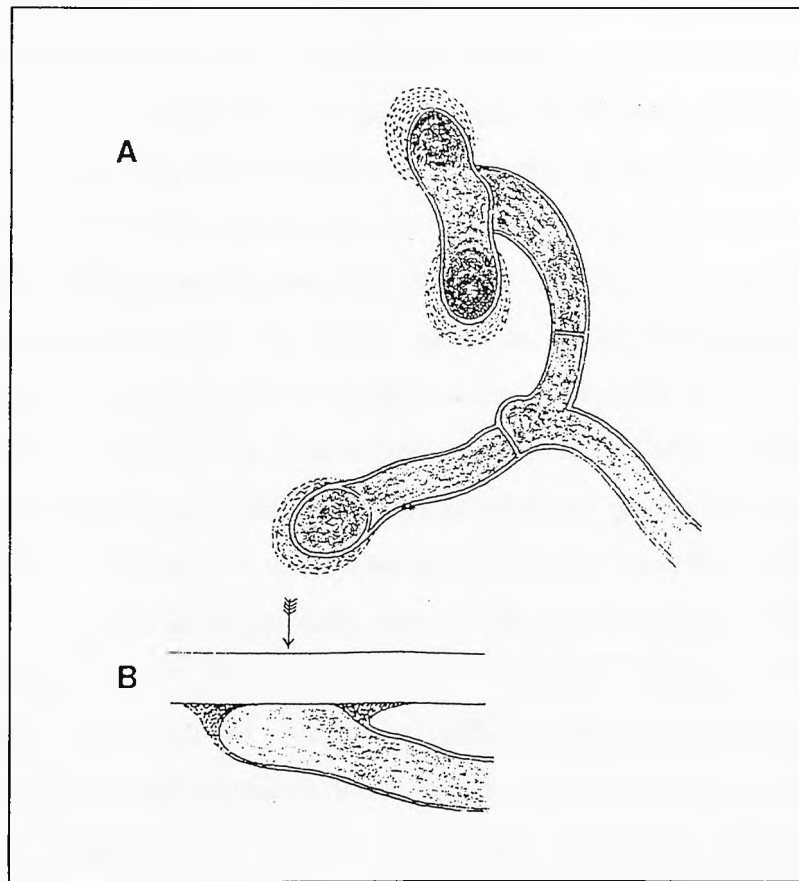


Diagram 1.3 Illustrations showing fungal glue associated with germ tubes of *Botrytis elliptica* grown on glass surfaces

Sporelings of *B. elliptica* were grown in a liquid suspension using the hanging-drop technique. [A] Coverslip viewed from above in direction of arrow in [B]. Germ tubes adhered to glass coverslip by organs of attachment (appressoria) which were surrounded by a fungal glue as shown in profile in [B]. Drawings by Marshall Ward (1888).

tested by immunodiffusion may appear to be specific but when tested by more sensitive methods, such as immunofluorescence or enzyme-linked immunosorbent assays (ELISAs) invariably prove to be non-specific (Dewey, 1992). In many cases, it is the choice of immunogen which limits the specificity of the antisera obtained (Dewey *et al.*, 1991). However, using cell-free surface washings of fungi as the immunogen, Dewey and co-workers were able to raise MAbs that were specific to *Humicola lanuginosa* (Dewey, MacDonald & Phillips, 1989) and *Penicillium islandicum* (Dewey *et al.*, 1990) and *Aspergillus flavus* (F. M. Dewey unpublished data) with relatively little difficulty.

More interestingly, using water-soluble surface washings of *Botrytis cinerea*, Bossi and Dewey (1992) have produced three monoclonal antibodies (MAbs) BC-KH4, BC-AF7 and BC-FD7-G9, that are considered to be genus-specific for *Botrytis* species. They have shown by enzyme-linked immunosorbent assays (ELISAs) that all three MAbs do not cross-react with other fungi normally involved in the post-harvest spoilage of fruits and vegetables. In addition, biochemical analyses involving heat, protease and periodate treatment of the *Botrytis*-antigens confirmed that the MAbs recognise carbohydrate epitopes on a glycoprotein. Further tests by ELISAs showed that one hybridoma cell line, BC-KH4, secreted antibodies that gave the lowest background values with healthy tissue. Therefore, antibodies produced by BC-KH4 were selected and employed to develop the immunoassay for the detection of *B. cinerea* antigens in the expressed juice from infected fruits of strawberries and grapes. Similarly, using MAbs raised against whole conidia, extracellular proteins and a putative cutin-esterase isolated from conidia of *B. cinerea*, Salinas and Schots (1994) developed a monoclonal antibody-based immunofluorescence test for the detection of conidia of *B. cinerea* on cut flowers. Both detection assays provide a means by which early infections of *B. cinerea* can be detected. Furthermore, as a result of the specificity of these antibodies for *Botrytis* species, it is clear that they represent ideal tools for immunocytochemical studies of *Botrytis* extracellular matrices and the role(s) of such matrices in the early stages of development of *Botrytis*-infection processes on a variety of host plants.

1.8 Monoclonal antibodies to Botrytis cell surface molecules as disease control agents

Control of diseases caused by *B. cinerea*, in particular, is difficult because the fungus can attack crops at any stage of growth and it can infect all plant parts (Elad & Evensen, 1995). Many of the strategies currently employed to control grey mould, particularly the use of fungicides, kill or inhibit the growth of the fungus (Elad & Evensen, 1995). However, as the application of fungicides becomes more restricted, as a result of environmental and economical problems, and fungicide resistance in pathogen populations becomes more widespread, the effectiveness of such control measures is questioned. Thus, new methods for disease control are often desired.

A relatively new approach to the control of plant disease involves the use of specific MAbs directed against fungal components. It is well documented that the ability of antibodies to bind to molecules is sufficient to inactivate the function of the antigen. Thus, it is feasible that such antibodies may be employed to inhibit the biological activity of molecules which are essential for pathogenesis (Schots *et al.*, 1992) and in turn, provide effective disease control. In an early study, Shaykh and coworkers (1977) used a polyclonal antiserum raised against an homogeneous preparation of cutinase (isolated from *Fusarium solani* f. sp. *pisi*) to investigate whether or not this enzyme was required for successful penetration of the plant host by *F. solani* f. sp. *pisi*. It was observed that by simply adding the antiserum to the inoculum droplets, infection of pea stems was prevented. It was suggested therefore that cutinase is required for the development of the *Fusarium*-pea interaction. Similarly, Gardiner and his colleagues (1989) found that the simultaneous application of spores of *B. cinerea* and polyclonal antisera, raised against conidia of *B. cinerea* and the fimbrial protein of *Ustilago violacea*, to *Vicia faba* L. leaves delayed lesion formation by inhibiting spore germination. It was observed that F(ab')₂ fragments of IgG molecules were also as effective as the complete IgG molecule. Thus, it was concluded that some constant portion of the antigen-binding region of immunoglobulins inhibited spore germination and hence, lesion formation. Likewise, it is feasible that the *Botrytis*-MAbs produced by Bossi and Dewey (1992) may be possible agents for the control of the widespread diseases caused by *Botrytis* species. It is manifest that any information regarding the adhesion and pre-penetration processes of *Botrytis* species on *Vicia faba* L. leaves is essential knowledge in the quest for fungal molecules that are primarily responsible for pathogenesis. Ultimately, with this knowledge, specific monoclonal antibodies may be raised against such target molecules and thus, provide alternative agents of disease control.

1.9 Aims of study

The aims of this study were four-fold;

- to investigate the idea that the specific monoclonal antibodies BC-KH4, BC-AF7 and BC-FD7, raised against surface washings of *B. cinerea* and directed against *Botrytis* species (Bossi & Dewey, 1992), could be used to label extracellular matrices liberated by *Botrytis* sporelings grown *in vitro* at both the light and electron microscope levels. Preliminary results and the immunocytochemical methods used have been published (Bossi, Cole, Spier & Dewey, 1994).
- to determine the nature, origin and secretion of *Botrytis* cell surface molecules at the intracellular level using immunosorbent assays, and both ultrastructural and inhibitor studies.
- to investigate the role(s) of *Botrytis* cell surface molecules in the early stages of development of the *Vicia faba-Botrytis* interaction by light and electron microscopy and immunocytochemistry. It was hoped that the *Botrytis*-MAbs would prove to be useful markers for location of the fungus in the infected host plant. The *Botrytis*-MAb BC-KH4 was selected for further study since it has been shown previously by enzyme-linked immunosorbent assays that this MAb gave the lowest background values when tested against healthy host tissue (Bossi & Dewey, 1992). Part of this work has been published recently (Cole, Dewey & Hawes, 1996).
- to study the necrotrophic behaviour of the fungus in more detail using affinity labelling studies involving an enzyme-gold complex, cellobiohydrolase conjugated to colloidal gold (Berg *et al.*, 1988; Vian, 1986), and an anti-pectin antibody, JIM 7 (Knox *et al.*, 1990).

Chapter Two

Materials & Methods

2.1 Fungal cultures

Cultures of *Botrytis cinerea* P-6g were provided by R. Pezet (Federal Agricultural Research Station, CH-1260 Nyon, Switzerland) and were obtained originally from a single-spore isolate of a culture derived from infected grapes in Switzerland (Table 2.1). Cultures of *B. fabae* 225851 were obtained from the International Mycological Institute (IMI), Surrey, U.K. (Table 2.1).

Both *Botrytis* species were grown on potato dextrose agar (PDA; Oxoid CM 139, Oxoid Ltd., Basingstoke, Hampshire, England, U.K.) either in Petri dishes (9 cm diameter: Sarstedt Ltd., 68 Boston Road, Beaumont Leys, Leicester, LE4 1AW, U.K.) or on slants (10 ml) for 7-21 days at $17 \pm 2^\circ\text{C}$ prior to sub-culturing. For sub-culturing: (i) Using PDA plates; a circular plug (1 cm diameter) of agar bearing sporulating hyphae was removed with a cork-borer and placed at the centre of a fresh agar plate. (ii) Using agar-slants; a sterile loop was used to stroke the surface of the culture growing on an agar slant in order to collect spores. These spores were then transferred to a fresh agar-slant. The two fungi, *B. cinerea* and *B. fabae* were grown under continuous Osram White fluorescent light and Philips Black light (fluorescent tubes with an emission spectrum of 310-410 nm and maximum at 360 nm) respectively to produce a dense carpet of aerial mycelium and conidia (Plate 2.1, Figs 1 & 2). Other fungi used for screening the *Botrytis* monoclonal antibodies and their respective culture methods are given in Table 2.1. Apothecia of *Botryotinia fuckeliana* were obtained from Dr. Peter Long, Massey University, Palmerston North, New Zealand (see also Plate 3.12, Fig. 1).

Table 2.1 Details of fungal cultures and growth conditions

	Isolate Number	Source	Isolated by	Growth Medium	Temperature (°C)
<i>Botrytis cinerea</i>	P-6g	grapes, Switzerland	RP	PDA	17
<i>B. fabae</i>	225851	IMI	-	PDA	17
<i>Aspergillus flavus</i>	M044	NRI	SP	MEA	30
<i>A. flavus</i>	OD.10.21	NRI	SP	MEA	30
<i>A. niger</i>	-	LHW	FMD	MEA	30
<i>A. parasiticus</i>	3014	NRI	SP	MEA	30
<i>Penicillium expansum</i>	300462	NRI	SP	MEA	30
<i>Heliscus submersus</i>	82609	IMI	-	MEA	15
<i>Tetracladium marchalianum.</i>	-	leaves, R Don	SB	MEA	15

RP: R. Pezet, Federal Agricultural Research Station, CH-1260 Nyon, Switzerland. FMD: F. M. Dewey. SP: S. Phillips. SB: S. Bermingham. IMI: International Mycological Institute, Surrey, U.K. LHW: Little High Wood, University of Durham, U.K. NRI: Natural Resources Institute, Chatham, Kent, U.K. R Don: River Don, South Yorkshire, U.K. PDA: potato dextrose agar (CM 139, Oxoid Ltd., U.K.), MEA: malt extract agar (CM 59, Oxoid Ltd., U.K.).

2.2 Isolation of conidia

Conidial suspensions were obtained by the addition of two ml of a sterile solution of 0.01% (w/v) aqueous Tween-20 (Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, BH17 7BR, U.K.) to sporulating cultures of *Botrytis* species on PDA slants followed by vigorous shaking to dislodge conidia. Conidia were washed with sterile water and pelleted by centrifugation [1 000 g (r_{av} 7.5cm) for 2 min]. This process was repeated twice prior to dilution of conidia of *B. cinerea* and *B. fabae* in 0.5% (w/v) aqueous glucose to give a final concentration of 1×10^5 and 3×10^4 conidia.ml⁻¹ respectively determined using a haemocytometer.

Unwashed dry conidia were harvested from sporulating cultures of *B. cinerea* and *B. fabae* on PDA slants by careful suction into a sterile plastic pipette (Alpha Laboratories Ltd., 40 Parham Drive, Eastleigh, Hampshire, SO5 4NU, U.K.). Using the bulb of the pipette, dry conidia were subsequently blown onto glass slides or leaf surfaces.

2.3 Antibodies

The murine monoclonal antibodies: BC-KH4, BC-AF7, BC-FD7-G9 and AF-BBG1 were obtained from Dr F. M. Dewey (Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RA, U.K.). The rabbit polyclonal antiserum, Av-95 was a kind gift from Dr Martina Celerin (Department of Plant Sciences, The University of Western Ontario, London, ON N6A 5B7, Canada). The β -1,3-glucopyranose and β -1,4-glucopyranose antibodies were purchased from Lynxvale Ltd., 20 Trumpington St., Cambridge, CB2 1QA, U.K., with permission from Professor Northcote (University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge, CB2 1QW, U.K.). JIM 7 murine monoclonal antibody was provided by the John Innes Institute, Colney Lane, Norwich, NR4 7UH, U.K. Further details concerning the aforementioned antibodies are outlined in Table 2.2.

Additional monoclonal antibodies employed in immunocytochemical studies were as follows: JIM 84, 2E7 and MAC 256. JIM 84 was obtained from Dr C. R. Hawes (School of Biological & Molecular Sciences, Oxford Brookes University, Gipsy Lane Campus, Headington, Oxford, OX3 0BP, U.K.). 2E7 and MAC 256 antibodies were provided by Dr R. Napier (Horticulture Research International, Wellesbourne, Warwickshire, U.K.).

2.4 Germination of conidia

2.4.1 *In vitro* studies: Preparation for light microscopy

Dry conidia or aliquots (20 μ l/well) of conidial suspensions were placed on a four- or eight-well Multiwell slide (C.A. Hendley (Essex) Ltd., Oakwood Hill Industrial Estate, Loughton, Essex, IG10 3TZ, England) which was supported on a bent glass rod on moistened Whatman No. 1 filter paper (6 cm: Whatman International Ltd., St Leonards Road, 20/20 Maidstone, Kent, U.K.) in a Petri dish (See Diagram 2.1). The Multiwell slides were sterilised by autoclaving (121°C for 15 mins) before use. Petri dishes were sealed with Parafilm or Nescofilm (Agar Scientific Ltd., 66a Cambridge Road, Stansted,

Table 2.2 Details of antibodies employed in immunocytochemical studies

Antibody	Antigen	Host animal	Antibody type	Specificity	Tested by	Reference
BC-KH4 BC-AF7 BC-FD7-G9	surface washings of <i>Botrytis cinerea</i>	mouse	monoclonal IgM	almost genus-specific [§]	ELISA, IF	Bossi & Dewey 1992; F. M. Dewey unpublished data
AF-BBG1	surface washings of <i>Aspergillus flavus</i>	mouse	monoclonal IgM	specific for <i>Aspergillus</i> species tested [§]	ELISA, IF	F.M. Dewey unpublished data
Av-95	denatured fimbrial subunits from <i>Microbotryum violaceum</i>	rabbit	polyclonal	not tested, recognises 74 kDa glycoprotein subunit	WB	M. Celerin unpublished data
β -1,3-gluco-pyranose	β 1 $\rightarrow$ 3 Glc ₃₋₈	rabbit	polyclonal	recognises β 1 $\rightarrow$ 3 Glc ₃₋₈ and β 1 $\rightarrow$ 4 Glc ₃₋₈ in certain plant species	ELISA, ID, CFA, IG	Northcote, Davey & Lay, 1989
β -1,4-gluco-pyranose	β 1 $\rightarrow$ 4 Glc ₃₋₈	rabbit	polyclonal	recognises β 1 $\rightarrow$ 4 Glc ₃₋₈ and β 1 $\rightarrow$ 3 Glc ₃₋₈ in certain plant species	ELISA, ID, CFA, IG	Northcote, Davey & Lay, 1989
JIM 7	carrot protoplasts	rat	monoclonal IgA	recognises methyl-esterified pectins in plant tissues	ELISA, DB, IIF, IG	Knox <i>et al.</i> , 1990
JIM 84	carrot cell coated vesicles	rat	monoclonal IgM	recognises epitope [§] in Golgi cisternae and plasma membrane in range of plant cell types	WB, IIF, IG	Horsley <i>et al.</i> , 1993
2E7	synthetic peptide corresponding to C terminus of BiP	mouse	monoclonal IgG	recognises HDEL motif	ELISA, WB, IF, IG	Napier <i>et al.</i> , 1992
MAC 256	maize membrane ABP	rat	monoclonal	recognises epitope close to C terminus of ABP including the KDEL motif	ELISA, WB, IF, IG	Napier <i>et al.</i> , 1988; Napier & Venis, 1990; Napier <i>et al.</i> , 1992

[§]All recognise carbohydrate epitopes on a glycoprotein. ELISA: enzyme-linked immunosorbent assay. WB: Western blotting. ID: immunodiffusion assay. CFA: Complement fixation assay. IG: immunogold labelling at the electron microscope level. DB: immunodot-blot analysis. ABP: auxin-binding protein. BiP: luminal binding protein.

Essex, CM24 8DA, U.K.) and conidia were incubated overnight at $17 \pm 2^\circ\text{C}$ as described in Section 2.1. In order to compare the rate of *in vitro* germination of *B. cinerea* and *B. fabae* conidia, samples were observed by light microscopy using a x40 objective lens after approximately 2, 4, 6, 8, 20 and 24 h. Positive germination occurred when the length of the developing germ tube was equal to or greater than the maximum diameter of the spore. Five hundred conidia/sporelings were observed in each case.

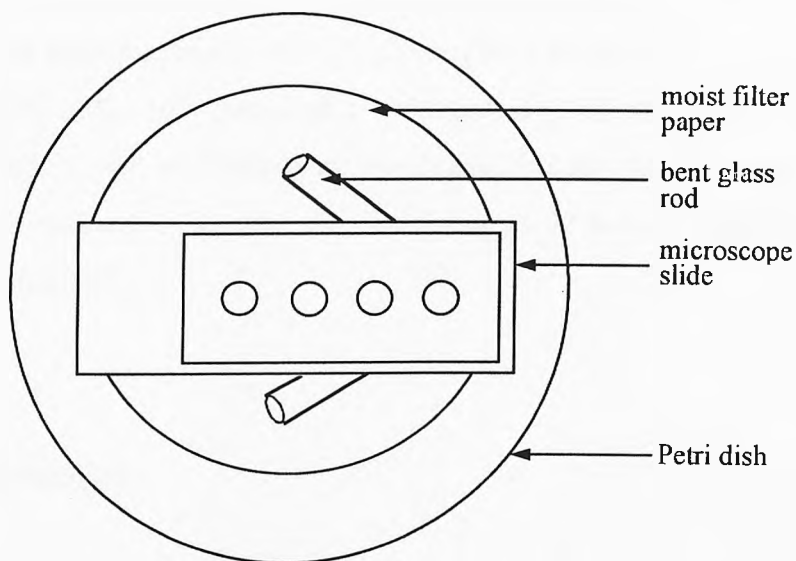
For histochemical staining, unless otherwise stated, aliquots (20-50 μl) of a conidial suspension were placed on glass coverslips (round, 6 mm diameter: A. R. Horwell Ltd., 73 Maygrove Road, London, NW6 2BP, U.K.) which were supported on a glass microscope slide and incubated in a moist chamber as described above. Round glass coverslips were cleaned by sonication in chloroform (Glass-distilled grade: Rathburns Chemicals Ltd., Walkerburn, Scotland, U.K.) for 5 min before use.

2.4.2 *In vitro* studies: Preparation for electron microscopy

Young or leading edge hyphae (including hyphal tips) were obtained by stab-inoculating fresh PDA plates at the centre of the plate with a 10 mm plug of agar taken from a heavily-sporulating culture of *B. cinerea* or *B. fabae* using a cork-borer. Inoculated plates were placed in the incubator for 1 h prior to inversion and further incubation (see Section 2.1). After 3-4 days, squares of agar (3-4 mm^2) supporting leading edge hyphae (including hyphal tips) were cut from the margins of the fungal colony and fixed directly. Alternatively, aliquots (100 μl) of conidial suspensions were applied directly to the surface of PDA in a Petri dish and distributed evenly over the surface of the agar using a glass-spreader. Inoculated plates were then incubated as described above for 1-2 days. Squares of agar (3-4 mm^2) supporting sporelings were subsequently excised and fixed directly.

Suspension cultures of *B. cinerea* were prepared by inoculating 100 ml of PDA broth (Sigma Chemical Co. Ltd., U.K.) in a 250 ml Erlenmeyer conical flask with a single aliquot (100 μl) of a conidial suspension (1×10^4 conidia. ml^{-1}) obtained as above (see Section 2.1). Inoculated flasks were placed in a G25 incubator shaker (New Brunswick Scientific Co. Inc., Watford, Hertfordshire, U.K.) with constant illumination at 25°C for up to 4 days. For conventional transmission electron microscopy (TEM) and

A:



B:

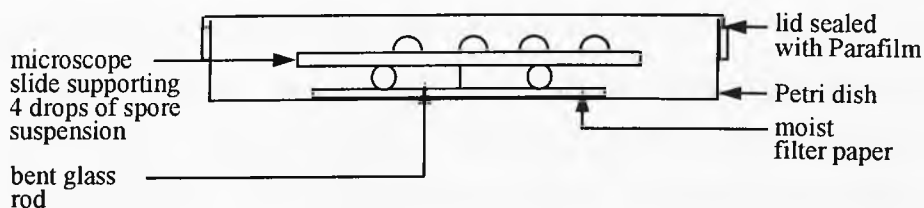


Diagram 2.1 *In vitro* germination of *Botrytis* conidia: Preparation for light microscopy

Diagram showing surface [A] and side [B] views of apparatus required for *in vitro* germination of *Botrytis* conidia for light microscopy. Dry conidia or aliquots of conidial suspensions were placed in the wells on the 4-well microscope slide which was supported on a bent glass rod on moist filter paper in a Petri dish. The lid of the Petri dish was sealed with Parafilm.

immunoelectron microscopy (IEM), aliquots (1 ml) of sporelings were pelleted [1 000 g (r_{av} 7.5 cm) for 5 mins] and resuspended in 4% (w/v) bacto-agar (Sigma Chemical Co. Ltd., U.K.: gelling temperature equal to 35°C) at 4°C. Once set, the agar was cut into small pieces (3 mm³) which were fixed directly.

For low-temperature scanning electron microscopy (LTSEM), freshly-isolated conidia of a 9 day-old culture of *B. cinerea* {washed once in sterile water and resuspended after centrifugation [2 200 g (r_{av} 7.5 cm) for 5 mins] in sterile water to give a final concentration of 5×10^5 conidia. ml⁻¹} were applied (20 µl aliquots) to sterile glass coverslips (round; 6 mm) or Nucleopore membranes (round, 13 mm; Agar Scientific Ltd., U.K.) and incubated in a moist chamber for up to 72 h in a daylight-lit constant temperature room (20°C).

2.4.3 *In vivo* studies

Dwarf broad bean plants (*Vicia faba* L. cv. The Sutton) were grown in Fisons Leavington peat-free Universal Compost mixed 1:50 (w:w) with Dupre Vermiculite under glasshouse conditions. Plants were harvested after 3-4 weeks by which time the second and third bifoliate leaves had fully emerged (Plate 2.2, Figs 1 & 2). The second and third bifoliate leaves were excised and their petioles wrapped immediately in moist tissue. Leaves were washed thoroughly with a jet of distilled water and blotted dry in order to gently remove any traces of extraneous spores or dust and wax (Sutton, Rowell & James, 1984) from the leaf surface without damaging the cuticular membrane. Excised leaves were then placed in a transparent polystyrene storage box (174 x 115 x 60 mm: Fisons Scientific Equipment, Bishop Meadow Road, Loughborough Leicestershire, LE11 0RG, U.K.) with a tight fitting lid and lined with tissue moistened with tap water. Leaf laminae were supported by glass test tubes and two bifoliate leaves were placed in each box. Detached leaves were inoculated on their adaxial surface with unwashed dry conidia or with five µl droplets of conidial suspension. Droplets were placed at three sites on each side of the mid-vein (Diagram 2.2). Storage boxes containing inoculated leaves were sealed with Parafilm and incubated in a growth cabinet set at 20°C with 16 h daylight provided by Compton Warm White fluorescent tubes. After 14-71 h, small

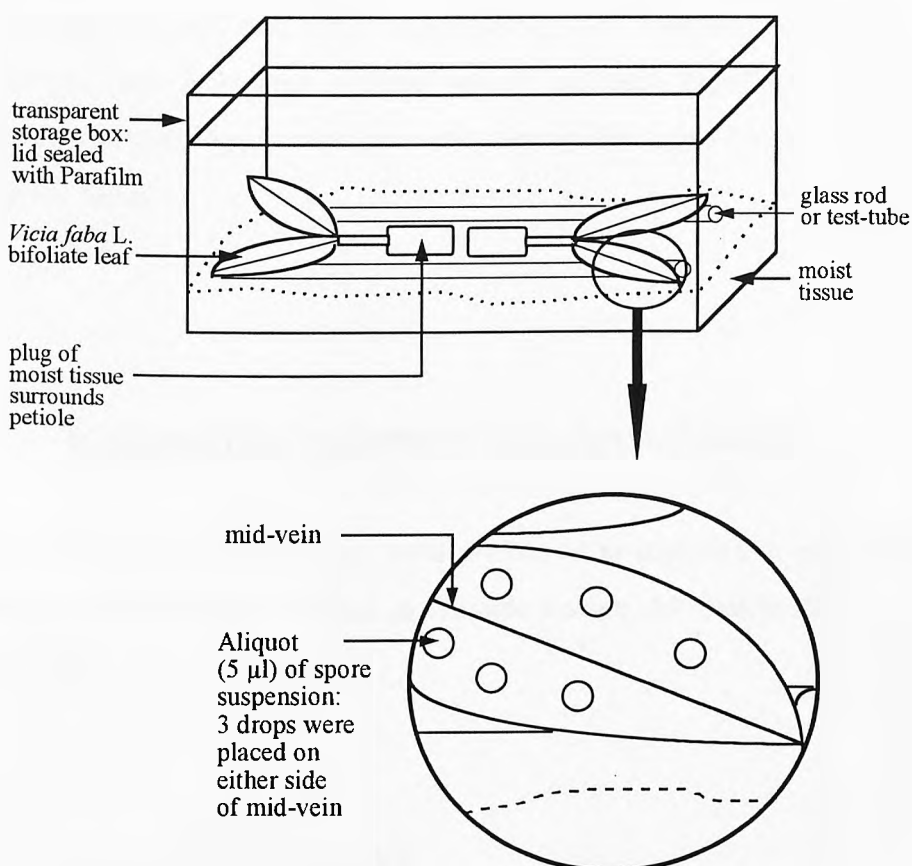


Diagram 2.2 *In vivo* germination of *Botrytis* conidia: Preparation for light and electron microscopy

Vicia faba L. leaves were excised and their petioles were immediately wrapped in moist tissue. Leaves were placed in a transparent storage box lined with moist tissue. Leaf laminae were supported by glass test tubes. Leaves were inoculated on their adaxial surface with aliquots of conidial suspension or dry spores (see Section 2.2). The lid of the storage box was sealed with Parafilm.

circles of leaf tissue beneath inoculum droplets/bearing lesions were excised using a 5 mm diameter cork borer and prepared directly for light and electron microscopy. Circles of leaf material were cut up further into smaller pieces (3 mm³) prior to fixation for transmission electron microscopy and immunoelectron microscopy.

In each experiment, at least three discs per treatment were examined for the presence of attached conidia or sporelings by differential interference contrast (DIC) microscopy at x100 and x400 magnification. Each treatment consisted of at least 12 replicates (two inoculated bifoliate leaves) and each experiment was carried out in triplicate at least. Appropriate controls were applied when necessary and are presented with the results.

2.5 Enzyme-linked immunosorbent assays (ELISAs)

Unless otherwise stated, assays were performed in triplicate in polystyrene micro-titre wells in 12-well strips arranged in reusable frames (Microstrip PS, ICN Biochemicals Ltd., U.K.).

2.5.1 Preparation of antigen

In each case, the antigen was solubilised by the addition of 1-2 ml of phosphate-buffered saline [PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na₂HPO₄, 0.02% (w/v) KH₂PO₄, pH 7.2] to the fungus growing on a solid agar-slant. The agar surface was stroked gently with the edge of a sterile plastic pipette in order to wet the hyphae fully (Dewey, Munday & Brasier, 1989). The surface washings were then removed by suction, placed in an Eppendorf tube and centrifuged at 13 350 g (r_{av} 7.5cm) for 5 mins in order to pellet any cell debris. The supernatant or antigen solution was diluted further in PBS to give a total 20-fold dilution.

2.5.2 Checkerboard ELISAs to test the cross-reactivity of the *Botrytis* MAb, BC-KH4, against *Botrytis* species and a range of other fungi

An indirect plate-trapped antigen ELISA test, as described by Dewey and Brasier (1988) and outlined in Table 2.3, was employed together with a checkerboard titration system for both the antibody and antigen, to test the cross-reactivity of the *Botrytis* murine monoclonal antibodies against a range of other fungi. Cultures of *Botrytis* species were grown and maintained as described previously (see Section 2.1). Cultures of a range of other fungi, as listed in Table 2.1, were obtained from Dr F. M. Dewey. The antigen solutions used for coating wells were obtained from 7-10 day old fungal cultures as described above (see Section 2.5.1). Unless otherwise stated, undiluted hybridoma culture medium (RPMI) plus 5% (w/v) foetal bovine serum (FBS) or PBS plus 0.05% (w/v) Tween-20 were employed as negative controls.

2.5.3 Lectin studies

A plate-trapped antigen ELISA test (Table 2.3) was employed in order to investigate the lectin-binding properties of surface washings of *B. cinerea* and *B. fabae*. Following overnight coating of microtitre wells with the appropriate antigen and thorough rinsing with PBS plus 0.05% (w/v) Tween-20, the wells were treated with peroxidase-labelled lectins directly at a concentration of 1.5 units.ml⁻¹ in PBS (pH 5.4). The peroxidase-conjugated lectins employed were obtained from Sigma Chemical Co. Ltd., U.K., and are described in Table 2.4.

Table 2.3 Protocol for enzyme-linked immunosorbent assays (ELISAs)

Step	Reagents [†] /procedure [‡]	Duration
Antigen-binding	Coat microtitre wells with antigen diluted using a checkerboard titration system	overnight at 4°C
Rinse	Flick out unbound antigen, then wash wells thoroughly with a jet of PBS plus 0.05% (w/v) Tween-20 four times, once with PBS only and briefly with ultra-pure water. Place wells ^{**} in a laminar flow hood to dry.	1 minute per wash
Incubation	Incubate wells with BC-KH4 MAb ^{***} or peroxidase-conjugated lectin diluted using a checkerboard titration system	1 h
Rinse	Four times in PBS plus 0.05% (w/v) Tween-20	
Incubation (step omitted in direct assay)	Incubate wells with secondary antibody [†] peroxidase conjugate diluted 1:1000 in PBS plus 0.05% (w/v) Tween-20	1 h
Rinse	Four times in PBS plus 0.05% (w/v) Tween-20	
Addition of substrate	Add TMB [‡] substrate solution containing 0.01% (w/v) H ₂ O ₂	15-30 mins
Stop reaction	Reaction stopped using 2.5 M H ₂ SO ₄	
	Read absorbance immediately at 450 nm using an ELISA plate reader [§]	

[†] Unless otherwise stated, all steps were carried out at room temperature. During incubation steps, plates were placed in a transparent polyethene bag to prevent evaporation.

[‡] Unless otherwise stated, all test volumes were 50 µl per well and all solutions were prepared in glass or propylene tubes.

^{**} Once dry, wells were either processed directly or sealed in a polyethene bag and stored overnight at 4°C.

^{***} RPMI plus 5% (w/v) FBS was used as a control in lieu of the primary antibody.

[†] Goat anti-mouse polyvalent immunoglobulin (IgM + IgG) horseradish peroxidase conjugate: Sigma Chemical Co. Ltd., U.K. Product No: A-0412.

[‡] TMB: 3,3',5,5'-tetramethylbenzidine (T-2885: Sigma Chemical Co. Ltd., U.K.) prepared as a stock solution [100 mg TMB in 10 ml of undiluted dimethyl sulphoxide (DMSO)] and stored at 4°C. Small aliquots of stock solution were freshly diluted 100-fold into acetate/citrate buffer pH 5.4 containing 0.01% (w/v) H₂O₂ before each assay.

[§] blanked against clean and empty wells.

Table 2.4 List of lectins employed for identification of components of surface washings of *B. cinerea*

Lectins	Product code [†] and Lot number	Origin	Haptens
Wheat Germ Agglutinin (WGA)	L-3892 10H-4012-1	<i>Triticum vulgaris</i>	<i>N</i> -acetyl chitotriose
Concanavalin A (Con-A)	L-6397 54H8255	<i>Canavalia ensiformis</i> (Jack bean)	α -D-mannose, α -D-glucose
<i>Glycine max</i>	L-2650 81H4046	Soybean	<i>N</i> -acetyl galactosamine
<i>Arachis hypogaea</i> (PNA)	L-7759 83H4072	Peanut	D-galactose

[†] obtained from Sigma Chemical Co. Ltd., U.K.

2.5.4 Checkerboard ELISAs to determine the reactant concentrations of the BC-KH4 MAb and the corresponding antigen in surface washings of *B. cinerea* and *B. fabae*

An indirect plate-trapped antigen test (Table 2.3) was employed, using checkerboard titration systems, in order to determine the reactant concentrations of the BC-KH4 MAb and the corresponding antigens of *B. cinerea* and *B. fabae*. The antigen solutions used for coating wells were obtained from 7-10 day old fungal cultures as described above (see Section 2.5.1). Unless otherwise stated, undiluted RPMI plus 5% (w/v) FBS or PBS plus 0.05% (w/v) Tween-20 were employed as negative controls.

2.6 Light microscopy

2.6.1 Calcofluor staining

Sporelings of *B. cinerea* grown in the presence of aqueous glucose on glass coverslips (see Section 2.4.1) were stained with 0.01-0.02% (w/v) aqueous Calcofluor White M2R (Sigma Chemical Co. Ltd., U.K.) for 5 mins and mounted on a microscope slide with a coverslip prior to observation by epi-fluorescence microscopy using a G365 excitation filter, LP 420 barrier filter and an FT395 dichroic mirror.

2.6.2 Staining for mucin

Sporelings of *B. cinerea* grown as described for Calcofluor staining (Section 2.6.1) were stained for mucin using a method modified from Grimley (1964). A drop of toluidine blue and malachite green mixture, freshly-prepared by mixing equal volumes of 4% (w/v) aqueous toluidine blue and 4% (w/v) aqueous malachite green, was added to sporelings on coverslips supported on a glass microscope slide. The slide was warmed to 60°C for approximately 30 seconds, until the edges of the stain turned a copper colour and then flooded with 1% (w/v) aqueous basic fuchsin for 1 minute at room temperature. After staining, the coverslip was rinsed thoroughly with distilled water, placed on a clean microscope slide in a drop of distilled water, sealed with a square (20 x 20 mm) coverslip and observed by bright field microscopy.

2.6.3 Preparation of whole-mounts

Circles (5 mm diameter) of excised leaf material were fixed directly in 96% (v/v) ethanol: 96% (v/v) acetic acid (mixed 1:1, v:v) for 24 h, cleared in 75% (v/v) aqueous lactic acid (BDH, Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, U.K.) for 48 h, stained with 0.06% (w/v) aniline blue (Polysciences Inc.,

Warrington, PA, U.S.A.) in lactophenol (BDH, Merck Ltd., U.K.) for 1-2 hours essentially as described by Pie & De Leeuw (1991). Samples were mounted in 50% (v/v) aqueous glycerol (BDH, Merck Ltd., U.K.) on glass microscope slides prior to observation by DIC microscopy.

2.6.4 Colloidal gold staining

Sporelings attached to Multiwell slides (Section 2.4.1) were stained *in situ* with Protogold [a colloidal suspension of gold particles (17-20 nm diameter) stabilised with Tween-20; British BioCell International, Cardiff Business Technology Centre, Senghenydd, Cardiff, CF2 4AY, U.K.] (20 µl/well) and placed in a moist chamber for one hour at room temperature with gentle agitation on a rocking table or overnight at 4° C without agitation, as described by Jones, Bailey & O'Connell (1995). Multiwell slides were soaked in 0.3% (w/v) Tween-20 detergent for 2 h, rinsed thoroughly by submergence in 500 ml of ultra-pure water (4 times, 15 mins each) and placed in an oven at 60°C prior to inoculation. Sporelings grown in the presence of aqueous glucose were rinsed three times by immersion in ultra-pure water (500 ml) for 5 minutes prior to staining. Thorough rinsing prior to staining was important since gold colloids are precipitated by dissolved salts and proteins (Lucocq, 1993). In contrast, dry-inoculated conidia were stained directly.

Following colloidal gold labelling sporelings were rinsed thoroughly with ultra-pure water (3 times, 5 mins). Sporelings were then either mounted directly and observed by bright field microscopy or they were stained further using a silver enhancement kit (British BioCell International, U.K.) according to the manufacturer's instructions. Equal volumes of initiator and enhancer were mixed immediately before use and drops (20 µl) were applied to each well. Enhancement was monitored by bright field microscopy at low intensity illumination. At room temperature, optimum staining was obtained after 3-5 mins. The enhancement process was terminated by rinsing slides thoroughly by immersion in ultra-pure water (3 times, 5 min). Slides were mounted as described in Section 2.6.3, and observed by bright field or epi-polarisation microscopy.

2.6.5 Squash preparations of apothecia of *Botryotinia fuckeliana*

Small pieces (4 mm³) of the apothecial cup of the ascomycete *Botryotinia fuckeliana*, were placed in a drop of water or stain [0.06% (w/v) aniline blue in lactophenol] on a clean microscope slide and squashed under a coverslip by gently tapping the surface of the coverslip with the handle of a mounted needle. Asci were teased out further if necessary using a mounted needle. Slides were observed by bright field or DIC microscopy.

2.6.6 Semi-thin resin-embedded sections of infected bean leaves

Semi-thin sections (400 nm) of LR White resin-embedded (see Section 2.10.2) infected *Vicia faba* leaves were obtained as described in Section 2.11 and collected onto a clean microscope slide. Sections were dried by placing the microscope slide on a hot plate and stained with 1% (w/v) toluidine blue (Raymond A. Lamb, 12, The Viaduct, Ealing Road, Alderton, Middlesex, U.K.) in 1% (w/v) sodium borate (BDH, Merck Ltd., Merck House, Poole, Dorset, BH15 1TU, U.K.) solution for 2-3 min on the hot plate or until the edges of the stain turned a copper-colour. Sections were then rinsed with ultra-pure water and mounted with a coverslip prior to observation by DIC microscopy.

2.7 Immunofluorescence microscopy (IF)

Fungal sporelings and discs (5 mm diameter) of infected leaf material (14-21 h post-inoculation) were prepared for immunofluorescence microscopy as shown in Table 2.5. For control treatments, sporelings and infected leaf discs were incubated with hybridoma culture medium (RPMI) plus 5% (w/v) FBS or the murine monoclonal antibody, AF-BBG1 (see Table 2.2) in lieu of the primary antibody. In the case of sporelings attached to Multiwell slides, 20 µl aliquots of treatment solutions were used throughout. Excess liquid was removed from each well using a pipette prior to each treatment step. In

contrast, leaf discs were submerged in 400 µl aliquots of solutions throughout. Prior to observation by epi-fluorescence microscopy, immunolabelled sporelings and leaf discs were mounted on glass slides in Citifluor glycerol solution (Agar Scientific Ltd., U.K.) with a coverslip and sealed with nail varnish.

All samples prepared for light microscopy were observed with a Zeiss Axioplan microscope using bright field, DIC, epi-polarisation or epifluorescence optics as appropriate. For epi-polarisation microscopy, an oil-immersion anti-reflection objective lens was used. For epi-fluorescence microscopy, FITC fluorescence was observed using a 450-490 excitation filter, 515-565 barrier filter and an FT510 dichroic mirror. Photomicrographs were recorded on Fujichrome colour negative film at 100 ASA or Ilford HP5/Pan F film at 400/50 ASA respectively and developed in Ilford Microphen (Ilford Ltd., Maberley, Cheshire, U.K.).

2.8 Laser Scanning Confocal Microscopy (LSCM)

Immunolabelled sporelings attached to Multiwell slides were observed using a Zeiss LSM 410 laser scanning microscope at Carl Zeiss (Oberkochen) Ltd., Welwyn Garden City, Hertfordshire, U.K. Immunolabelled leaf discs were observed using a BioRad MRC 600 laser scanning confocal microscope with the kind permission of Dr Mark Fricker (Department of Plant Sciences, University of Oxford, U.K.). Photomicrographs were recorded on Polaroid film.

2.9 Low-temperature scanning electron microscopy (LTSEM)

After 24-71 h, infected leaves were removed from the incubation chamber and immediately prepared for LTSEM. Leaves inoculated with aqueous conidial suspensions were washed thoroughly with a jet of sterile-filtered water and blotted dry in order to remove residual glucose. Leaves inoculated with dry conidia were not washed. Squares (approx. 5 x 5 mm) of infected leaf tissue and glass coverslips or Nucleopore membranes

Table 2.5 Indirect immunofluorescence labelling of fungal sporelings and infected *Vicia faba* L. leaves for light microscopy

Step	Reagents/procedure [†]	Duration
1. Fixation (optional step)	Submerge fungus or 4 mm discs of infected leaf material in 3% (w/v) PFA* in PBS plus 0.025-0.05% (w/v) Tween-20	30 mins to 1 h for fungal sporelings or 3 hours for infected leaves
2. Rinse	Four times in PBS only to remove all traces of fixative	10 mins each
3. Blocking	Submerge fungus or infected leaves in blocking buffer**	1 h
4. Rinse	Three times in PBST to remove excess BSA	10 mins each
5. Incubation	Incubate fungus or infected leaves in full-strength primary antibody, hybridoma culture medium or PBST only	1-2 h at RT
6. Rinse	Four times in PBST only	10 mins each
7. Incubation	Incubate fungus or infected leaves in secondary antibody*** diluted in blocking buffer	1 h in the dark
8. Rinse	Four times in PBST only	10 mins each

[†] All steps were carried out at room temperature (RT) unless otherwise stated.

* PFA = paraformaldehyde (Sigma Chemical Co. Ltd., U.K.)

** Blocking buffer consisted of 1% (w/v) bovine serum albumin (BSA:essentially fatty acid-free, Sigma Chemical Co. Ltd., U.K.) in phosphate-buffered saline [PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na₂HPO₄, 0.02% (w/v) KH₂ PO₄, 0.02% (w/v) NaN₃, pH 6.9] plus 0.1% (w/v) Tween-20 (PBST).

*** Goat anti-mouse FITC-conjugate (IgG, whole molecule): Sigma Chemical Co. Ltd., U.K., Product code F-9006. Diluted 1:30 or 1:40 (v:v) in blocking buffer.

bearing sporelings (see Section 2.4.2) were mounted directly onto copper specimen stubs using methylcellulose, plunged rapidly into liquid nitrogen slush and transferred to the work chamber of an EMscope SP2000 Sputter-Cryo Preparation Unit for orientation and fracturing (Beckett & Porter, 1988). Samples were etched at -65°C, sputter-coated with gold and observed at approximately -160°C on a temperature-controlled stage in a Philips 501B scanning electron microscope at 9 kV. Photomicrographs were recorded on FP4 (220) film.

2.10 Preparation of fungal and leaf material for ultramicrotomy and transmission electron microscopy (TEM)

2.10.1 Conventional fixation and preparation of fungal conidia, sporelings and hyphae and non-infected and infected *Vicia faba* L. leaves

Conidia, sporelings and hyphae (including hyphal tips) grown on agar or in suspension culture (see Section 2.4.2) and small pieces (3-4 mm³) of infected and non-infected leaves (see Section 2.4.3) were prepared for TEM using a conventional preparation method involving post-fixation in osmium tetroxide (OsO₄) and embedding in Spurr resin (Table 2.6). Apothecia of *Botryotinia fuckeliana* were also prepared for TEM essentially as described in Table 2.6. Apothecia were cut up into small pieces (4 mm³) in primary fixative and placed under vacuum (26 Hg) at ambient temperature (24-27°C) for 1 h prior to further fixation (2 h) in the absence of a slight vacuum.

Table 2.6 Conventional procedure for the preparation of fungal conidia, sporelings and hyphae and infected *Vicia faba* L. leaves for transmission electron microscopy

Step	Reagents/procedure ^f	Duration
1. Primary fixation	Submerge fungus or leaf material in 1% (w/v) PFA [*] and 1% (v/v) GA ^{**} in sucrose- and mineral-supplemented cacodylate buffer [†]	30 mins to 1 h for fungal conidia, sporelings and hyphae or 3 hours for leaf material ^{***}
2. Rinse	Four times in cacodylate buffer only	10 mins each
3. Secondary fixation	Submerge fungus or leaf material in 1% (w/v) aqueous OsO ₄ ^{****}	1 h at RT
4. Rinse	Four times in ultra-pure water	10 mins each
5. In-block staining	Used 0.5% (w/v) aqueous uranyl acetate	overnight at 4°C
6. Rinse	Four times in ultra-pure water	10 mins each
7. Chemical dehydration	Dehydrate fungus and leaf material samples completely using a graded water/ethanol series: 10, 20, 30, 50, 70, 90, 100% (v/v) and pure ethanol	10 mins per step
8. Infiltration with epoxy resin	Infiltrate fungus and leaf material with Spurr [§] resin gradually using resin diluted in 100% ethanol: 25% (v/v) resin 50% (v/v) resin 75% (v/v) resin 100% resin 100% resin 100% resin 100% resin 100% resin [†] Place specimens in fresh resin into flat silicone rubber moulds	 1 h 1 h 1 h 1 h Overnight 8 h Overnight 8 h
9. Polymerisation	Place specimens in oven	10 h at 70°C

^f Unless otherwise stated, all steps were carried out at room temperature.

^{*} PFA = paraformaldehyde.

^{**} GA = glutaraldehyde solution (25%, v/v), EM Grade: Agar Scientific Ltd., U.K..

^{***} Initial 10 mins under vacuum (26 Hg).

^{****} OsO₄: Osmium tetroxide (Agar Scientific Ltd., U.K.).

[†] Primary fixatives were diluted in 0.1 M sodium cacodylate buffer (pH 7.2; Agar Scientific Ltd., U.K.) containing 2% (w/v) sucrose (BDH, Merck Ltd., U.K.), 0.025% (w/v) CaCl₂ and 0.04% (w/v) Tween-20.

[†] Additional resin changes and increased infiltration times were included where necessary

[§] Spurr (1969). Purchased from Agar Scientific Ltd., U.K.

2.10.2 Low-temperature embedding of fungal conidia, sporelings and hyphae and infected and non-infected *Vicia faba* L. leaves for immunogold labelling

Conidia, sporelings and hyphae (including hyphal tips) grown on agar or in liquid media (see Section 2.4.2) and small pieces (3-4 mm²) of infected and non-infected leaves (see Section 2.4.3) were prepared for immunocytochemistry using a progressive low-temperature (PLT) method essentially as described by VandenBosch (1991) and outlined in Table 2.7. Apothecia of *Botryotinia fuckeliana* were also prepared for TEM essentially as described in Table 2.7. As described above (Section 2.10.1), apothecia were fixed for 3 h (the first hour under vacuum).

2.10.3 Molecular Distillation Drying

Small blocks of agar (3-4 mm²) supporting the leading edge of hyphal growth of a 3 day-old culture of *B. cinerea* grown on PDA were excised and frozen by rapid immersion in liquid propane pre-cooled to -180°C using liquid nitrogen. Following cryo-fixation, samples were stored in liquid nitrogen until required or processed directly by freeze-drying in a Life Cell molecular distillation drying apparatus (Plate 2.3, Fig. 1). Samples to be freeze-dried were loaded into the sample chamber (Plate 2.4, Fig. 1) under liquid nitrogen, which was then transferred to the liquid nitrogen dewar. Once the dewar was fully connected to the drying column, rough pumping of the sample chamber and drying column was carried out using the mechanical vane pump and liquid nitrogen slush (-210 °C) was formed. At this stage, a drying schedule, pre-programmed into the Barber Colman 570 temperature controller (Plate 2.3, Fig. 1), was initiated in order to heat the nitrogen slush and convert it to the vapour phase which was subsequently removed from the sample chamber by the roughing pump. At this point (-150°C), the roughing valve was closed and the vane pump was switched off. The samples were then exposed to an ultra-high vacuum (10⁻⁶ mbar) which was generated by the turbo-molecular pump.

Table 2.7 Progressive low-temperature embedding of fungal and plant material for immunocytochemistry

Step	Reagents/procedure	Duration
1. Fixation	Submerge fungus or leaf material in 1% (w/v) PFA and 1% GA (v/v) in 0.1M PIPES ^f buffer plus 0.1% (w/v) Tween-20	1-3 h at RT
2. Rinse	Four times in PIPES buffer only to remove all traces of fixative	10 mins each
3. Dehydration at progressively lower temperatures	In a graded water:ethanol (v:v) series: 10% ethanol 30% ethanol 50% ethanol 70% ethanol 90% ethanol 100% ethanol pure* ethanol	30 mins per step RT 0°C (on crushed ice) -20°C -20°C -20°C -20°C -20°C
4. Infiltration with acrylic resin	Infiltrate fungus and leaf material with LR White** resin containing catalyst [†] , using resin diluted in ethanol (v/v): 50% resin 67.7% resin 75% resin 100% resin 100% resin 100% resin 100% resin 100% resin 100% resin 100% resin [‡] Place specimens in fresh resin in gelatin capsules; fill to exclude air	All steps at -20°C 1 h 1 h 1 h Overnight 8 h Overnight 8 h Overnight 24 h 24 h
5. Polymerisation	Polymerise using U.V. light [§]	24 h at -20°C, followed by 24 h at 0°C

^f PIPES = Piperazine-N, N'-bis-(2-ethanesulfonic acid); Sigma Chemical Co. Ltd., Poole, Dorset, U.K., pH 7.2.

Absolute ethanol: A.R. Quality, Hayman Ltd., 70 Eastways, Industrial park, Witham, Essex, CM8 3YE, England, U.K.

** LR White: London Resin White (Agar Scientific Ltd., U.K.)

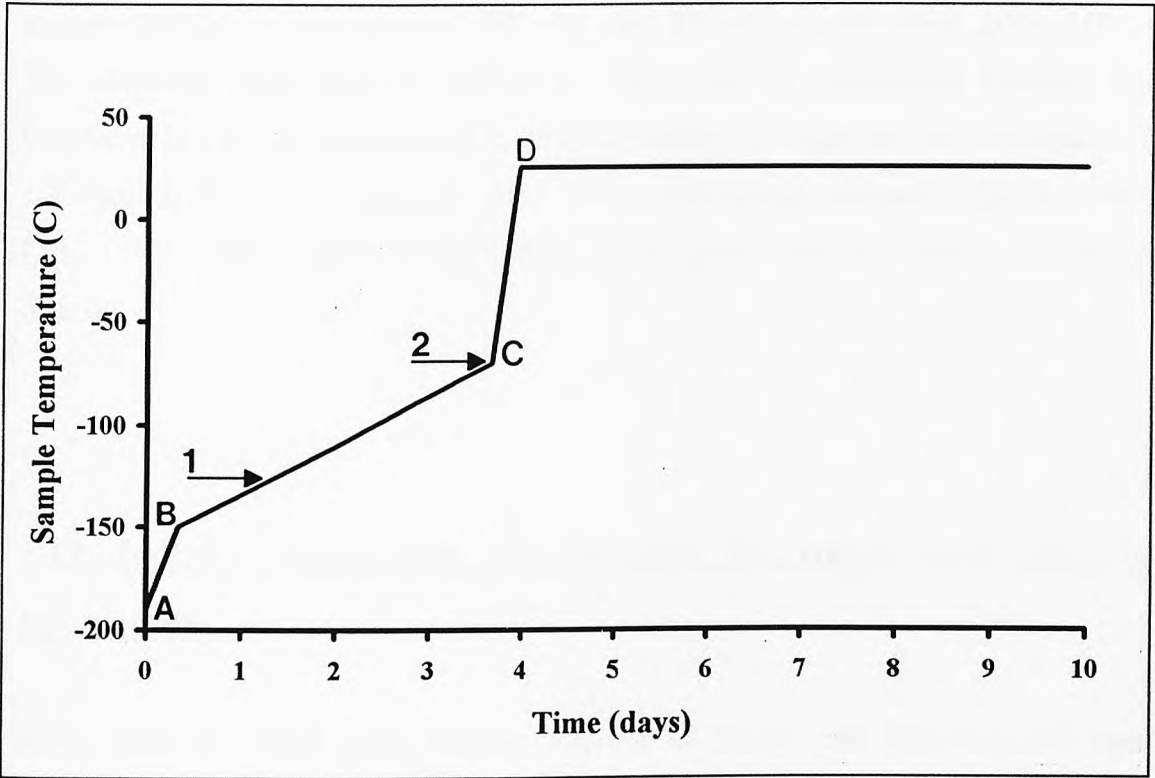
[†] Plus 0.5% (w/v) benzoin methyl ether (Sigma Chemical Co. Ltd., U.K.)

[‡] Additional resin changes and increased infiltration times were included where necessary

[§] The light source should be approximately 20 cm from the capsules

Samples were dried under vacuum for 4 days from -196°C to 6°C. During the critical drying phase (-140°C to -70°C) sample temperatures were increased continuously at a rate of 1°C per hour (Graph 2.1).

At the completion of the drying cycle, paraformaldehyde vapour was introduced into the column through the gas inlet valve and resin through the specialised resin inlet valve under low vacuum pressure (10^{-1} mbar) [Plate 2.4, Fig. 2]. Following resin introduction, samples were removed from the drier, infiltrated several times with fresh resin at 6°C and polymerised using U.V. light (24 or 48 h at 6°C).



Graph 2.1 Freeze-drying schedule

A: Equilibration temperature of the system (-192°C). B: Starting-point of the gradual warming period (-150°C). Points 1 and 2 indicate the beginning and end of the molecular distillation drying process. C: The stage at which the samples were considered to be dry (-70°C). Temperatures were then raised rapidly to D (25°C) where paraformaldehyde vapour was introduced and resin infiltration began.

2.11 Ultramicrotomy

Both semi-thin (400 nm) and ultra-thin (70-90 nm) sections of resin-embedded fungal and leaf material were cut using a Reichert Ultracut E microtome. In all cases, semi-thin sections were cut first and observed by light microscopy essentially as described in Section 2.6.6 in order to obtain the correct orientation of the resin block prior to further sectioning. This procedure was especially important when ultra-thin sections of hyphal tips and penetration sites were required.

Ultra-thin sections of Spurr- and LR White-embedded fungal and leaf material were collected onto copper grids (200 hexagonal mesh: Agar Scientific Ltd., U.K.) prior to post-staining for conventional TEM and onto Formvar-coated nickel grids (100 or 200 hexagonal mesh: Agar Scientific Ltd., U.K.) prior to immunogold labelling. For enzyme-gold staining, sections of LR-White embedded of fungal and leaf material were collected on Formvar-coated gold grids (100 or 200 hexagonal mesh: Agar Scientific Ltd., U.K.). Grids bearing sections were either stored prior to staining or stained directly.

2.12 Indirect immunogold (IG) labelling for transmission electron microscopy

Formvar-coated nickel grids bearing sections of fungal and leaf material were immunostained with the primary antibodies (see Table 2.2) using an indirect immunogold labelling technique as outlined in Table 2.8. Secondary antibody-gold conjugates (EM grade) were obtained from British BioCell International, U.K., and all were raised in goats against antibodies of the species in which the primary antibody was raised. Goat anti-mouse IgM (GAMM) gold conjugates were employed for detection of the murine monoclonal antibodies, BC-KH4, BC-AF7, BC-FD7-G9 and BBG1; goat anti-mouse IgG (GAM) gold conjugates for the detection of 2E7; goat anti-rabbit (GAR) gold conjugates for the location of Av-95, β -(1 $\rightarrow$ 3)-glucan and β -(1 $\rightarrow$ 4)-glucan antisera and finally, goat anti-rat (GAT) gold conjugates for the detection of MAC 256 and JIM 7 MAb. In single immunogold labelling protocols either 5 or 10 nm colloidal gold

Table 2.8 Indirect immunogold labelling of ultra-thin sections for transmission electron microscopy

Step	Reagents [§] /procedure ^f	Duration
1. Blocking	Float grid with sections on a drop [*] of blocking buffer ^{**}	10-30 mins
2. Rinse	Pass grid through a series of three drops of PBST only. Remove excess buffer from grid with filter paper ^{***}	3 mins per drop
3. Incubation [†]	Submerge grid with sections uppermost in a drop of primary antibody at full-strength or diluted in blocking buffer or PBST only	1 h at RT or overnight at 4°C
4. Rinse	Pass grid through a series of four drops of PBST as above	3 mins per drop
5. Incubation [†]	Incubate grid in a drop of secondary antibody-gold conjugate [‡] diluted 1:20 (v:v) in blocking buffer	1 h at RT
6. Rinse	Four times in PBST as above. The grids were post-stained for immediate examination or stored for later staining and observation	
7. Staining	Float grid on a drop of 2% aq. uranyl acetate. Followed by Reynold's lead citrate ^{****}	8 mins in the dark 10 mins

[§] Where possible, all solutions were filtered through a 0.2 µm Schleicher & Schuell membrane filter (pyrogen-free, Anderman & Co. Ltd., 145 London Road, Kingston-upon-Thames, Surrey, KT2 6NH, U.K.) before use.

^f All steps were carried out at RT unless otherwise stated.

^{*} Grids were floated on 20 µl aliquots of solutions on Parafilm, unless otherwise stated.

^{**} Blocking buffer contained 1% (w/v) bovine serum albumin (BSA:essentially fatty acid-free, Sigma Chemical Co. Ltd., U.K.) in phosphate-buffered saline [PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na₂HPO₄, 0.02% (w/v) KH₂PO₄, 0.02% (w/v) NaN₃, pH 6.9] plus 0.1% (w/v) Tween-20 (PBST).

^{***} Whatman No. 50.

^{****} Reynolds (1963)

[†] Grids were agitated by vibration created by a small air-pump.

[‡] containing 5, 10 or 15 nm-colloidal gold particles, as appropriate.

conjugates were employed. When 5 nm gold conjugates were used, silver enhancement was often carried out prior to post-staining. The silver enhancement procedure was essentially as described previously for the intensification of colloidal-gold labelled sporelings for light microscopy (see Section 2.6.4) using the silver enhancement kit obtained from British BioCell, U.K., according to the manufacturer's instructions. Equal volumes of initiator and enhancer were mixed immediately before use and droplets (20 μ l) were placed on small strips of dental modelling wax (Agar Scientific Ltd., U.K.). Immunolabelled sections on grids were floated on these droplets for 30-60 sec, rinsed thoroughly in ultra-pure water and blotted dry with Whatman No. 50 filter paper prior to post-staining. If no silver-enhancement was carried out, then lead citrate staining was occasionally omitted in order to reduce the contrast of these specimens. In each case, specificity of gold labelling was determined by treating sections in the same way as outlined in Table 2.8, but following replacement of the primary antibody with PBST, blocking buffer or hybridoma culture medium/pre-immune serum. All immunolabelling experiments were repeated several times.

Double-immunolabelling protocols were essentially as described in Table 2.8. A small volume (50-100 μ l) of undiluted murine monoclonal antibody, BC-KH4 was mixed with an equal volume of the rabbit polyclonal antiserum to $\beta(1\rightarrow3)$ glucopyranose which had been previously diluted 1:400 in blocking buffer (see Table 2.8). Grids bearing sections were incubated in droplets of the primary antibody mixture as described in step 3, Table 2.8. The corresponding secondary antibodies, GAMM 5 nm- and GAR 15 nm-gold conjugates were diluted 1:10 in blocking buffer and then mixed together in equal volumes to give a final dilution of 1:20 in each case. Grids bearing sections were incubated in droplets of the secondary antibody mixture as described in step 5, Table 2.8. Secondary antibody-gold conjugates bearing colloidal gold particles of disparate sizes were employed in order to allow distinction between the primary antibodies. Control treatments consisted of the replacement of the BC-KH4 MAb with hybridoma culture medium and the $\beta(1\rightarrow3)$ glucan antiserum with blocking buffer.

2.13 Preparation of cellobiohydrolase-gold complex (CBH1-Au)

The enzyme, cellobiohydrolase (CBH1, EC 3.2.1.91) isolated from *Trichoderma reesei* and purified as described by Schulein (1988) was kindly provided by Dr. B. Vian (Laboratoire de Phytopathologie, INRA-INA P-G, 16 rue Claude Bernard, 75231 Paris Cedex 05). The complex, CBH1-colloidal gold, was prepared partially according to Chanzy and co-workers (1984) and Berg and coworkers (1988), as described by Bonfante-Fasolo and colleagues (1990).

2.13.1 Production of 15 nm gold particles

The 15 nm gold particles were prepared by the reduction of tetrachloroauric acid (HAuCl_4) as described by Roland & Vian (1991): All glassware was pre-rinsed with concentrated nitric acid and washed thoroughly with ultra-pure water before use. Using ultra-pure water, 100 ml of aqueous 0.01% (w/v) HAuCl_4 (BDH, Merck Ltd., U.K.) was prepared in a 100 ml Erlenmeyer conical flask and heated to boiling. As soon as the solution had started to boil, 4 ml of 1% sodium citrate (Frens, 1973) was added rapidly and the mixture was allowed to continue to boil gently. The colour of the mixture turned violet-purple and after 4-5 mins stabilised at a reddish-orange colour (like young wine turning to old mature wine). At this stage, the colloidal gold-complex was ready to use.

2.13.2 Adjustment of pH of the gold colloid

The pH of the mono-disperse colloidal gold suspension (average particle diameter, 15 nm), prepared as described in Section 2.13.1, was adjusted to the isoelectric point of the enzyme cellobiohydrolase (pH 4.55) by the drop-wise addition of 0.1 M acetic acid. During adjustment of pH, the electrode was protected by the addition of 100 μl of 1% (w/v) aqueous polyethylene glycol (PEG, 20 000 Da: Sigma Chemical Co. Ltd., U.K.) to a small volume (5 ml) of the gold colloid to be measured in order to avoid plugging the pores of the electrode with gold particles.

2.13.3 Estimation of the minimal amount of enzyme necessary for the full stabilisation of the gold colloid

A stock solution of enzyme (E: 2 mg.ml⁻¹) was prepared in 0.05 M acetate buffer (pH 4.6) and diluted successively in acetate buffer in ten small tubes to produce a series of double dilutions. Each tube contained 100 µl of enzyme solution E, or E/2 or E/4 or E/8...and so on to E/512. An aliquot of gold colloid (500 µl) was placed in each tube, the tube was shaken and after 5 min, 100 µl of 1% (w/v) NaCl was also added. At this stage, the stabilisation of the gold colloid was checked visually by observation of the solutions and the changes in colour from blue (flocculated gold) to red (stabilised gold). The stabilisation of the gold colloid was also estimated photometrically using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer. The lowest amount of enzyme that was required to stabilise the gold colloid i.e. to produce a red solution, was selected and this concentration of enzyme was used for the preparation of the CBH1-gold complex. In this case, 0.15 mg of CBH1 was necessary to protect 10 ml of the gold colloid, which was equivalent to 75 µl of the enzyme stock solution (E).

2.13.4. Preparation of the enzyme-gold complex

In order to produce a stable gold complex, a small aliquot of enzyme (in this case, 75 µl of solution E) determined previously (see Section 2.13.3) was added to 10 ml of a freshly-prepared gold suspension at adjusted pH (4.5). The mixture was stirred and allowed to stand for 5 minutes before adding a small volume (500 µl) of 1% (w/v) aqueous PEG 20 000 in order to saturate any uncoated gold sites. The suspension was then centrifuged at 60 000 g for 1 h at 4°C using a Sorvall RC 28S ultracentrifuge. After centrifugation, three phase suspensions were recognised (see Diagram 2.3). The red mobile pool was removed carefully with a long-form glass pipette, resuspended in 5 ml of citrate-phosphate buffer (CPB; pH 4.8) containing 0.02% (w/v) PEG 20 000 [100 µl of 1% (w/v) PEG 20 000; CPB-PEG] and re-centrifuged as before. Finally, the mobile pellet was removed carefully and resuspended in a small amount (200-500 µl) of CPB-

PEG. Once prepared, the complex was used within 2 weeks and when not in use, it was stored in the fridge at 4°C. The enzyme-gold complex was also re-centrifuged at 60 000 g for 1 h at 4°C as described above before each use.

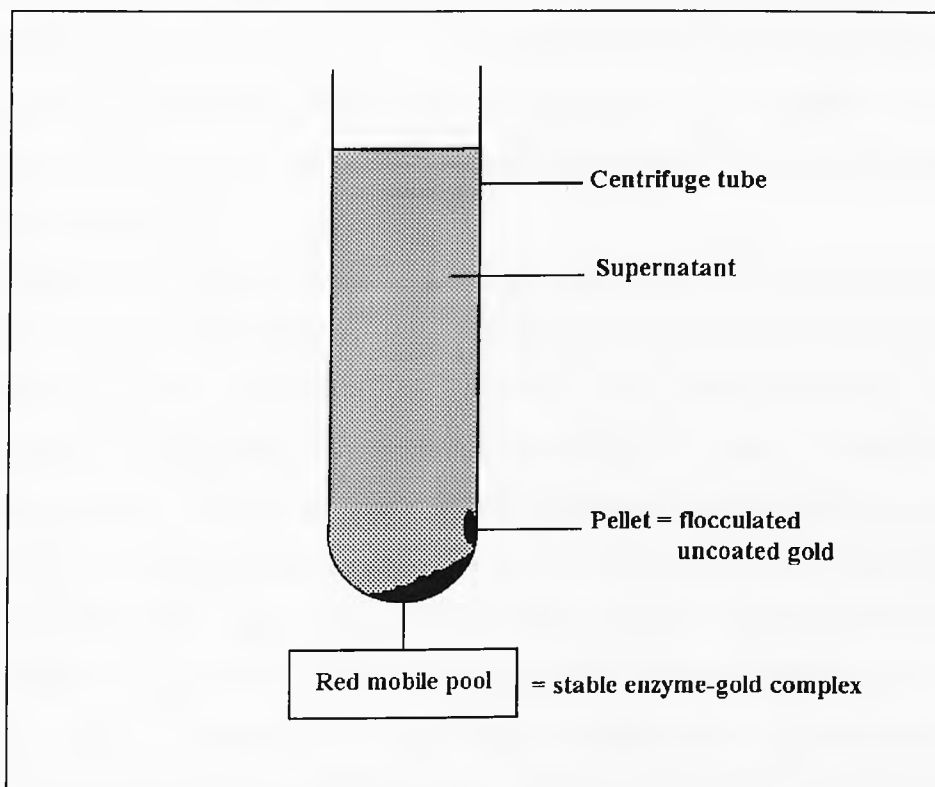


Diagram 2.3 Preparation of cellobiohydrolase gold-complex

At the end of centrifugation, three phases were recognised: a clear supernatant that contained free, i.e. non-complexed enzyme, a dark pellet that corresponded to flocculated, uncoated gold particles and a red mobile pool located at the bottom of the centrifuge tube that represented the usable enzyme-gold complex.

2.13.5 A one-step procedure for direct labelling of ultra-thin sections for transmission electron microscopy: Using the cellobiohydrolase-gold complex

Ultra-thin sections of PLT-dehydrated and LR White-embedded samples (see Section 2.10.2) were collected onto Formvar-coated gold grids (100 hexagonal mesh: Agar Scientific Ltd., U.K.). Grids were floated on drops (20 μ l) of solutions on Parafilm and maintained in a moist chamber throughout to prevent the sections drying out. Great care was also taken so that, in each case, the sections were always in contact with the surface of the incubation fluid. All solutions were filtered through a 0.2 μ m Schleicher & Schuell membrane filter (Anderman & Co. Ltd., U.K) before use and all steps were carried out at room temperature.

Grids were incubated with 0.05 M citrate-phosphate buffer (pH 4.8) plus 0.02% (w/v) PEG 20 000 [CPB-PEG] or CPB-PEG plus 0.1% (w/v) Tween-20 and 1% (w/v) BSA [CPB-PEG-TB] for 10 minutes. Grids were then transferred onto a drop of enzyme-gold complex diluted 1:50 (v:v) in CPB-PEG for 60 mins, washed thoroughly with CPB (3 times, 5 min each), followed by brief rinsing in distilled water and allowed to air-dry. In each case, excess liquid was removed from grids using Whatman No. 50 filter paper. Sections on grids were counter-stained with uranyl acetate and lead citrate or uranyl acetate only prior to observation by transmission electron microscopy.

For control treatments, the enzyme-gold complex was pre-incubated with 1 mg.ml⁻¹ carboxymethylcellulose (CMC: Sigma Chemical Co. Ltd., U.K.) in CPB-PEG buffer for 1 h at room temperature before use.

2.13.6 Double labelling studies for transmission electron microscopy: Using the cellobiohydrolase-gold complex

Double labelling studies, using the cellobiohydrolase-colloidal gold (CBH1-Au) complex and the monoclonal antibodies, BC-KH4 or JIM 7 were essentially as described in Table 2.8, although 0.05 M citrate-phosphate buffer (pH 4.8) plus 0.02% (w/v) PEG 20 000 [CPB-PEG] was employed instead of PBS buffer. In addition, CPB-PEG plus 0.1% (w/v) Tween-20 was used to rinse grids throughout and CPB-PEG plus 0.1% (w/v)

Tween-20 and 1% (w/v) BSA [CPB-PEG-TB] was employed as the blocking buffer. A small volume of the enzyme-gold complex diluted 1:20 (v:v) in CPB-PEG was added to an equal volume of undiluted BC-KH4 or JIM 7 MAb. Grids were incubated in a drop of this mixture as described in step 3, Table 2.8. The grids were then processed further as described in steps 4-6 using CPB-PEG plus 0.1% (w/v) Tween-20 to wash grids and CPB-PEG-TB as the blocking buffer throughout. In step 5, the appropriate secondary antibody 5 nm-gold conjugate (GAMM or GAT) was employed in order to distinguish between the location of the primary antibody and the pre-labelled enzyme-gold complex. For control treatments, the enzyme-gold complex was pre-incubated with 1 mg.ml⁻¹ carboxymethyl cellulose in CPB-PEG buffer for 1 h at room temperature before use. In addition, the BC-KH4 MAb was replaced with hybridoma culture medium and the JIM 7 MAb with CPB-PEG.

2.14 Post-embedment labelling of ultra-thin sections for transmission electron microscopy: Using Concanavalin A (Con-A)

Ultra-thin sections of glutaraldehyde-OsO₄-fixed sporelings of *B. cinerea* (see Section 2.10.1) were labelled with Concanavalin A (Con-A: C-7275, Sigma Chemical Co. Ltd., U.K.) using an indirect, single-sided, post-embedment labelling method as reported by Bourett & Howard, 1994. Sections were incubated in 0.01 M PBS buffer, pH 7.3 containing 0.1 mM CaCl₂, 0.1 mM MnCl₂, 0.5% (w/v) Tween-20 and 0.5% (w/v) BSA for 15 mins, washed thoroughly in PBS only and then transferred onto drops of uncomplexed Con-A (30 µg.ml⁻¹ of PBS) for 60 mins in a moist chamber. After thorough rinsing of sections on grids using a "jet" of PBS, grids were floated on drops of buffer for 5 mins, "jet"-washed again and blotted dry by holding the grids edgewise on Whatman No. 50 filter paper. Great care was taken to avoid drying the grids completely. Con-A binding sites were subsequently detected using a horseradish peroxidase-gold (10 nm) conjugate (HRP-gold: P-0304, Sigma Chemical Co. Ltd., U.K.) diluted 1:20 in PBS containing 0.5% (w/v) Tween-20 and 0.5% (w/v) BSA for 60 mins. Grids were then washed thoroughly with PBS as above, followed by brief rinsing in sterile-filtered ultra-

pure water (three times, one min) and counter-stained with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds, 1963).

For control treatments, the lectin was either omitted or pre-adsorbed using 200 mM α -methyl-D-mannoside (Sigma Chemical Co. Ltd., U.K.) diluted in PBS for 45 mins. Unless otherwise stated, grids were floated on drops (20 μ l) of solutions throughout and all solutions were filter-sterilised using a 0.2 μ m membrane filter (Anderman & Co. Ltd., U.K.) before use. All steps were carried out at room temperature.

2.15 Post-staining and transmission electron microscopy (TEM)

Unless otherwise stated, Spurr-embedded sections were post-stained with lead citrate only and LR White-embedded sections were post-stained with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds, 1963) prior to observation by TEM. In all cases, sections were observed with a JEOL 1200EXII transmission electron microscope at 100 kV.

2.16 Mode of action studies of the secretory inhibitors, brefeldin A and pyrimethanil on *B. cinerea*

2.16.1 Chemicals

A stock solution of brefeldin A (BFA: Sigma Chemical Co. Ltd., U.K.) was prepared by dissolving 5 mg of BFA in 500 μ l of absolute ethanol. When not in use, the BFA stock solution was stored at -20 °C. Pyrimethanil was supplied by Dr. R. Milling (Agrevo U.K. Ltd., Saffron Walden, Essex, CB10 1XL, England, U.K.) as a 40% (w/w) suspension concentrate formulation. For each experiment, a stock solution of pyrimethanil was

freshly prepared by dissolving 5 mg of the concentrate in 500 μl of sterile dimethyl sulphoxide (DMSO).

2.16.2 Effect of BFA and pyrimethanil on conidial germination

Equal volumes of a spore suspension of *B. cinerea* [9.65×10^5 conidia. ml^{-1} of 1% (w/v) aqueous glucose] prepared as described in Section 2.2, and inhibitor solution (0, 0.2, 2, 20 and 200 μg inhibitor. ml^{-1} of ultra-pure water) were mixed together and aliquoted (20 μl) onto wells of a 4-well Multiwell slide (one slide per treatment). In the absence of the inhibitor, conidia were incubated in 0.5% (w/v) aqueous glucose only (Control A) or 0.5% (w/v) aqueous glucose containing 10 $\mu\text{l}.\text{ml}^{-1}$ absolute ethanol (Control B). Slides bearing conidia were incubated in a moist chamber as described in Section 2.4.1. After 24 h, slides were flooded with sterile water, mounted with a coverslip and observed by phase contrast microscopy. The percentage germination of conidia was determined in each case. Positive germination was recorded when the germ tube length was equal to or greater than the length of the conidium. A total of 500 conidia or ten fields of view using a x40 objective lens were counted in each case.

2.16.3 Effect of BFA and pyrimethanil on radial growth of colony

The effect of BFA and pyrimethanil on radial growth of *B. cinerea* was investigated using Petri dishes (9 cm) containing agar media (15 ml) supplemented with the secretory inhibitor. Media consisted of PDA or glucose-gelatin agar [GGA: 20 $\text{g}.\text{l}^{-1}$ agar (Technical Grade: Oxoid Ltd. U.K.) containing 3.0 mM MgSO_4 (monohydrate: BDH, Merck Ltd., U.K.), 13 mM KH_2PO_4 (BDH, Merck Ltd., U.K.), 4 $\text{g}.\text{l}^{-1}$ D-glucose (BDH, Merck Ltd., U.K.) and 4 $\text{g}.\text{l}^{-1}$ gelatin (BDH, Merck Ltd., U.K.)]. Plates containing varying concentrations of inhibitors (e.g. 0, 0.1, 1, 10, and 100 $\mu\text{g}.\text{ml}^{-1}$) were prepared by adding the compound to the liquid form of the medium, just prior to pouring. Control treatments consisted of PDA only (Control A) and PDA or GGA supplemented with either 100 $\mu\text{l}.\text{ml}^{-1}$ absolute ethanol or 10 $\mu\text{l}.\text{ml}^{-1}$ DMSO (Control B). Once set, the agar

plates were inoculated at the centre with a single plug of agar bearing sporulating hyphae, placed in polyethene bags and unless otherwise stated, incubated for 3-4 days at 20°C in the light (see Section 2.1). The radial growth of the colony was determined in each case by measuring the distance between the leading edge of the expanding colony and the edge of the inoculum block. A minimum of three measurements (in cm) were taken from each agar plate.

2.16.4 Effect of BFA on conidial adhesion

Conidia isolated from agar-slants (see Section 2.2) were washed thoroughly in sterile water by centrifugation (3 times at 1 000 g for 5 min) to remove all traces of detergent and conidial adhesive matrix. Conidia were finally re-suspended in 1% (w/v) aqueous glucose and mixed with an equal volume of BFA (50 µg.ml⁻¹) to give a final concentration of 0.81×10^5 conidia.ml⁻¹ of 0.5% (w/v) aqueous glucose containing 25 µg.ml⁻¹ BFA. This conidial suspension was inoculated onto Multiwell slides and incubated for 24 h at 17°C in a moist chamber (see Section 2.4.1). After 24 h, each slide bearing conidia and sporelings was placed inside a 50 ml volume plastic centrifuge tube (Bibby Sterilin Ltd., Stone, Staffordshire, ST15 0SA, U.K.) that had been modified by cutting 5 mm diameter holes in the centre of the bottom of the tube and the lid. Conidia and sporelings were then dislodged from slides using a modification of the method described by Doss and co-workers (1993). The centrifuge tube with slide inserted was placed in a one litre beaker containing one litre of ultra-pure water (RT) whilst holding a clean finger over the hole in the lid. Once the tube was fully submerged (5 mm from the bottom of the beaker), the finger was removed to allow water to fill the centrifuge tube. The finger was then replaced and the tube was removed from the water. Finally, the finger was removed to allow the water to drain. This washing cycle was repeated eight times. Fresh ultra-pure water was replaced in the beaker after each slide and slides were kept in a moist chamber prior to counting in order to prevent the slides from drying out. The percentage adhesion was calculated from the number of conidia that remained adhered after washing relative to the number of conidia that had been applied to the surface. For each treatment, 500 conidia or ten fields of view observed using a x40

objective lens were counted by phase contrast microscopy and a minimum of four replications were included in each treatment.

2.16.5 Ultrastructural studies of the mode of action of BFA and pyrimethanil

Somatic hyphae were obtained from cultures (3-4 days old) of *B. cinerea* grown on PDA or GGA supplemented with varying concentrations of BFA or pyrimethanil (see Section 2.16.3) by cutting out small agar blocks (approx. 3 mm³) supporting the leading edge hyphae. Agar blocks were then fixed and processed directly for conventional transmission electron microscopy and immunocytochemistry at the TEM level as described in Table 2.6 and 2.7 respectively.

For growth in liquid culture, conidia were harvested using 0.1% (w/v) Tween-20 as described previously (see Section 2.2) and inoculated into 0.1 x strength PDA broth (Sigma Chemical Co. Ltd., U.K.) to give a final concentration of 1.85×10^4 conidia.ml⁻¹. Cultures were agitated (shaker incubator; 120 rpm) for 2-4 days at 22°C. Sporelings were harvested by centrifugation [1 000 g (r_{av} 7.5cm) for 5 mins] and resuspended in 5 ml of 0.1 x PDA broth containing 10 µg.ml⁻¹ BFA in a small Petri dish (6 cm diameter, 5 mm deep; Bibby Sterilin Ltd., U.K.). Control treatments contained 10 µl.ml⁻¹ absolute ethanol. Petri dishes containing sporelings were then placed in the shaker incubator (120 rpm, 17-22°C) for 2 h. After treatment, 2 x one ml aliquots of each suspension were removed, placed in an Eppendorf tube and washed by centrifugation (three times, as above) in sterile 0.1 x PDA broth in the presence or absence of the inhibitor as appropriate. Sporelings from each treatment were fixed for conventional TEM and IEM as described above.

Chapter Three

Visualisation of fungal extracellular matrices

3.1 Introduction

Extracellular matrices are often associated with conidia, germ tubes, hyphae and infection structures of plant pathogenic fungi (Kunoh, Nicholson & Kobayashi, 1991; see also Chapter One; Sections 1.1 and 1.2). Such matrices are composed of polysaccharides and proteins (mainly glycoproteins). Therefore, traditionally, general histochemical stains for polysaccharides or negative stains are employed for the detection of fungal extracellular matrices by light microscopy. For example, a mixture of toluidine blue-malachite green-basic fuchsin; a blend of metachromatic dyes to stain for mucin (Beckett, Tatnell & Taylor, 1990) or the negative stains, nigrosine (Beckett, Tatnell & Taylor, 1990) and India ink (Evans, Stempen & Stewart, 1981; Evans, Stempen & Frasca, 1982). Such histochemical staining methods *sensu stricto* are based on the chemical functions of polysaccharides and do not distinguish between different classes of polysaccharides.

Lectins, on the other hand, are a heterogeneous group of proteins or glycoproteins of non-immune origin, which bind to specific carbohydrate residues (Lis & Sharon, 1973; Goldstein & Hayes, 1978). They are isolated from a wide variety of biological sources, although the most well-known lectins are of plant-origin e.g. Concanavalin-A (Con-A) from the seeds of *Canavalia ensiformis* and wheat germ agglutinin (WGA) from wheat germ, *Triticum vulgare*. There are now over 60 lectins, available commercially, which recognise a wide range of carbohydrate moieties and which can be labelled directly or indirectly with a fluorophore or colloidal gold for observation by microscopy. Although the specificity of lectins is limited (Vian, 1986; Roth, 1987), they have been used extensively at both the light and electron microscope levels to label components of fungal cell walls and extracellular matrices and the surface of zoospores (e.g. Keen & Legrand, 1980; Bacic, Williams & Clarke, 1985; Chamberland *et al.*, 1985; Hardham 1985; Benhamou & Ouellette, 1986; Bonfante-Fasolo *et al.*, 1987; Hamer *et al.*, 1988; O'Connell & Ride, 1990; O'Connell, 1991; O'Connell, Nash & Bailey, 1992; Durso, Lehnert & Powell, 1993).

More recently, naked *viz.* non-conjugated colloidal gold has been employed to label the extracellular matrices of plant pathogenic fungi, including *B. cinerea* (Harder *et al.*, 1989; Jones, Bailey & O'Connell, 1995; Mercure, Kunoh & Nicholson, 1995). Colloidal gold is often used as a total protein stain to detect proteins on nitro-cellulose or polyvinylidene difluoride membranes, after dot-blotting or electrophoretic blotting from gels (Rohringer, 1989). The colloidal gold particles are negatively charged. They bind strongly to proteins and other macromolecules through hydrophobic and electrostatic forces and through interactions with thiol groups (Lucocq, 1993). This affinity labelling technique is simple, rapid to perform and accumulations of gold particles are visualised as a pink to red coloration by bright field microscopy (De Mey *et al.*, 1986). Individual gold particles are less than 20 nm in diameter, and hence too small to be resolved by bright field microscopy. The colloidal gold staining can be intensified by means of silver enhancement with a physical developer. During enhancement, colloidal gold catalyses the reduction of silver ions to metallic silver which in turn is deposited in concentric rings around the gold particles. Thus, as these particles enlarge, the colour observed by bright field microscopy changes from pink-blue through yellow and brown to grey-black due to the reflection of light by the gold/silver particles of different sizes (De Mey *et al.*, 1986). Further enhancement of these intensified particles can also be obtained by the use of epi-polarisation microscopy (De Waele, 1989). The sensitivity of this technique (Jones, Bailey & O'Connell, 1995) has been compared to that achieved using fluorochrome-conjugated lectins (O'Connell, Nash & Bailey, 1992).

Previous studies have shown that, in most instances, conidia of *B. cinerea* and *B. fabae* require nutrients and/or conditions of high relative humidity in order to germinate (Snow, 1949; Harrison, 1984b; Harrison, 1988). Under such conditions, conidia germinated readily on a variety of artificial substrata, including glass, to produce one or more germ tubes. In this study, conidia were inoculated dry or in an aqueous suspension supplemented with glucose onto glass surfaces. Conidial germination was monitored in each case and the production of extracellular matrix material was investigated by bright field, differential interference contrast (DIC) and/or epi-polarisation microscopy using a commercially-available non-conjugated colloidal gold sol (particle size: 17-20 nm) in combination with silver enhancement. In addition, the fluorescent brightener Calcofluor White M2R, which binds to β -glucans in fungal cell walls (Maeda & Ishida, 1967; Gull & Trinci, 1974; Butt *et al.*, 1989) and labels the extracellular matrices associated with unfixed germ tubes of the common maize rust fungus *Puccinia sorghi* Schw (Chaubal, Wilmot & Wynn, 1991), was also employed to visualise sporelings grown *in vitro* by epi-fluorescence microscopy.

Further enhancement of the resolution of extracellular matrices can be achieved by electron microscopy. Indeed, transmission (Gull & Trinci, 1971; Mims & Richardson, 1989) and low-temperature scanning electron microscope studies (Read, Porter & Beckett, 1983; Beckett, Tatnell & Taylor, 1990; Mims, 1991; Read, 1991; Braun & Howard, 1994; see also Chapter One, Section 1.3) have shown that when germ tubes and hyphae of plant pathogenic fungi are grown on artificial membranes, they are often surrounded by an extensive fibrillar sheath which provides firm attachment to the substratum. In the present study, low-temperature scanning electron microscopy (LTSEM) of sporelings grown *in vitro* was carried out in order to preserve the extra-mural substances *in situ*.

Increased sensitivity of labelling of extracellular matrices, compared to that afforded by lectins, can be obtained using specific enzymes or antibodies. Enzymes can be labelled directly with colloidal gold and, in turn used directly as specific probes for the corresponding enzyme-substrate at both the light (following controlled silver intensification of gold particles) and electron microscope levels (Vian, Brillouet & Satiat-Jeunemaitre, 1983; Bendayan, 1984; Roland & Vian, 1991). For example, Chamberland and co-workers (1985) reported that a chitinase-gold complex was more suitable for the ultrastructural localisation of chitin in the walls of *Fusarium oxysporum* f. sp. *radicis-lycopersici* than a chitin-specific gold-conjugated lectin.

Since the advent of hybridoma technology which permits the production of homogeneous MAbs secreted by a single cell line and directed against a unique epitope of the immunising antigen (Köhler & Milstein, 1975), many antibodies have been raised which are specific for cell wall components and surface molecules of fungi and zoospores (Mendgen *et al.*, 1988; Wycoff & Ayers, 1990; see Chapter One, Table 1.1 and references therein). Such MAbs may be produced in unlimited amounts. They may be isolate-, race-, species- or genus-specific and as a result, they represent a diverse set of molecular probes for investigations of components of the fungal cell wall and extracellular matrices. In addition, such MAbs have considerable potential as tools for immunocytochemical studies, for instance they may be applied to immunofluorescence, immunoenzymatic-detection assays and immunoelectron microscopy.

Indirect immunofluorescence (IF) microscopy is a well-established method for the visualisation of the spatial and temporal distribution of specific antigens. In brief, the principle of the immunofluorescence labelling protocol is simple: Specific antibodies are employed as the affinity probes for the *in situ* localisation of antigens. Fluorophores conjugated to a

secondary antibody attach via the secondary antibody to the primary antibody and thus, serve as markers for the antigen.

The application of laser scanning confocal microscopy (LSCM) in the epifluorescence mode for the detection of fluorescent markers, has several advantages over conventional fluorescence microscopy. Confocal fluorescence microscopy improves the spatial resolution of the fluorescent label with the removal of out-of-focus fluorescent blur, it allows for precise optical serial-sectioning of biological specimens and visualisation of these sections by three-dimensional reconstruction and digital image processing (Brakenhoff *et al.*, 1985; Robert-Nicoud *et al.*, 1989; Wijnaendts-van-Resandt *et al.*, 1989; Pawley, 1990).

Post-embedding indirect immunogold labelling follows essentially the same principle as immunofluorescence, except that in this case, the primary antibody binds to the antigens exposed at the cut surface of ultra-thin sections of resin-embedded tissue. In addition, colloidal gold is used instead of the fluorochrome, and it is conjugated directly to the secondary antibody or to Protein-A for observation by transmission electron microscopy (VandenBosch, 1991). Early immunogold studies investigating the nature and binding properties of fungal cell wall components and extracellular matrices were carried out using samples fixed in glutaraldehyde, post-fixed with osmium tetroxide and embedded in epoxy resins (Bendayan & Zollinger, 1983). Although, in some instances, such conventional preparation methods yielded satisfactory results following immunogold labelling, particularly where the antigen was abundant, the substitution of milder fixation conditions and acrylic resins e.g. LR White, which are hydrophilic in nature, are now considered to be necessary precautions to take in order to increase the sensitivity of the labelling technique. In general, low-temperature dehydration and embedding using acrylic resins are thought to reduce antigen denaturation and thus, to increase antigen preservation (Roth *et al.*, 1981). In addition, osmium tetroxide post-fixation is often omitted from preparation protocols for immunocytochemistry because it may mask the antigenic sites and therefore cause considerable reduction in antibody recognition. Furthermore, the use of osmium tetroxide also precludes U.V. polymerisation of acrylic resins (VandenBosch, 1991). As a result, samples prepared for immunocytochemistry using mild aldehyde-fixation and progressive low-temperature embedding in acrylic resin often exhibit low contrast when observed by transmission electron microscopy.

In this study, specific monoclonal antibodies raised against surface washings of the fungus, *Botrytis cinerea* and directed against a number of *Botrytis* species tested [e.g. *B. cinerea*, *B. fabae* (Bossi & Dewey, 1992), *B. allii* and *B. squamosa* (F. M. Dewey, unpublished observations)] were employed in order to determine the sites of antibody-binding in *Botrytis* sporelings grown *in vitro* by immunofluorescence and immunoelectron microscopy. Both conventional and confocal immunofluorescence microscope methods were carried out in order to compare the spatial distribution of the fluorescent label in each case. Post-embedding immunogold labelling was performed on sections of *Botrytis* conidia and sporelings prepared for immunocytochemistry using both conventional and progressive low-temperature dehydration and embedding protocols. In addition, the occurrence and role(s) of matrix molecules throughout the sexual stage of *B. cinerea* (fruiting body: *Botryotinia fuckeliana*) was investigated by immunoelectron microscopy. Non-specific binding of MAbs to components of tissue and embedding resin was reduced by using a buffer containing an inert protein (Van Noorden & Polak, 1993) for diluting the antisera and pre-rinsing the sections.

3.2 Results

3.2.1 Germination of conidia of *B. cinerea* and *B. fabae* on glass surfaces

DIC microscopy revealed that when conidia of *B. cinerea* and *B. fabae* were inoculated onto glass surfaces and incubated in a moist chamber for 23 h, they germinated to produce one (Plate 3.1, Figs 1-4) or more germ tubes. In the presence of aqueous glucose, the germ tubes were long i.e., the length of the germ tube was greater than the maximum diameter of the spore (Plate 3.1, Figs 1 & 2). In contrast, unwashed dry conidia produced only short germ tubes which remained firmly attached to the substratum even after thorough washing (Plate 3.1, Figs 3 & 4). In all cases, little or no extracellular matrix was detected at the surface of the conidia, germ tubes or substratum.

3.2.2 Visualisation of extracellular matrices of *Botrytis* species grown *in vitro* by light microscopy using histochemical stains

Epi-fluorescence microscopy of *B. cinerea* sporelings grown *in vitro*, in the presence of aqueous glucose, and stained with the fluorescent brightener Calcofluor White M2R showed an extensive network of branched septate hyphae (Plate 3.2, Fig. 1). The walls of the conidia and hyphae were stained intensely. In addition, the septal walls and hyphal tips were highly fluorescent (Plate 3.2, Fig. 1). However, no extracellular matrix material was observed at the surface of the substratum, conidia or hyphae.

In contrast, bright field microscopy of similar sporelings stained for mucin using the histochemical stains toluidine blue, malachite green and basic fuchsin showed that the hyphae were surrounded by a thin coating of mucilaginous material. (Plate 3.2, Fig. 2 arrows). In addition, a more extensive pad of mucin was observed around the swollen hyphal tips (Plate 3.2, Fig. 2 arrowheads). Using the affinity stain, colloidal gold followed by silver enhancement, the germ tubes of *B. cinerea* sporelings were also found to be enclosed by a sheath of positively-stained fibrillar material (Plate 3.2, Fig. 3).

Epi-polarisation microscopy of dry-inoculated spores of *B. fabae* grown on glass surfaces and labelled with colloidal gold followed by silver-enhancement, revealed that, in many cases, the sporelings were surrounded by a pool of heavily-stained matrix material (Plate 3.2, Fig. 4 arrowheads). This matrix pool remained firmly attached to the glass surface even after thorough washing and extended as far as 65 μm across the substratum. No fibrillar material was observed along the length of the germ tubes. No such matrix pool was observed around conidia or germ tubes of *B. cinerea* by DIC or epi-polarisation microscopy following the inoculation and germination of dry conidia onto glass surfaces and subsequent labelling with colloidal gold and silver enhancement (data not shown).

3.2.3 Visualisation of extracellular matrices of *B. cinerea* sporelings by low-temperature scanning electron microscopy (LTSEM)

When *B. cinerea* sporelings were grown on Nucleopore membranes or glass coverslips in the presence of exogenous nutrients and observed by LTSEM, in most cases, little or no extracellular matrix material was detected at the surface of the conidia or germ tubes (Plate 3.3, Figs 1 & 2). However, by low-angle viewing (Clement *et al.*, 1994; Clement, 1995) which allowed the sporeling to be viewed within the cryo-chamber of the microscope at an angle of 10° to the horizontal, abundant fibrillar-like matrix material could be seen associated with the conidium, germ tubes and the substratum (Plate 3.3, Fig. 3 arrows).

3.2.4 Indirect immunofluorescence (IF) microscopy of *Botrytis* sporelings using the *Botrytis* monoclonal antibodies (MAbs)

Indirect immunofluorescence microscopy of non-fixed, glucose-supplemented *B. cinerea* and *B. fabae* sporelings, using the *Botrytis* murine monoclonal antibodies: BC-KH4, BC-AF7 or BC-FD7-G9 (data not shown) revealed that, in all cases, patches of fluorescence were located along the length of the germ tubes (*B. cinerea*: Plate 3.4, Figs 1 & 2; *B. fabae*: Plate 3.4, Fig. 4). In a few cases, this punctate fluorescence appeared to aggregate at the hyphal tip (*B. cinerea*: Plate 3.4, Fig. 1 arrows; *B. fabae*: Plate 3.4, Fig. 4 arrows). In *B. cinerea* sporelings, fluorescence was observed over the entire surface of the conidia (Plate 3.4, Figs 1 & 2). However, in the case of *B. fabae*, conidia were often non-fluorescent (Plate 3.4, Fig. 4).

In contrast, intense fluorescence was observed over the surface of the conidia and germ tubes of glucose-supplemented and paraformaldehyde-fixed sporelings of both *Botrytis* species (*B. cinerea*: Plate 3.5, Figs 2 & 6; *B. fabae*: Plate 3.5, Fig. 4) after immunolabelling with each of the *Botrytis*-MAbs. In addition, the developing germ tube was often encased within a sheath of fluorescent matrix material (*B. cinerea*: Plate 3.5, Figs 2 & 6; *B. fabae*: Plate 3.5, Fig. 4). Furthermore, an extensive pad of fluorescently-labelled fibrillar material was often observed at the tip of the germ tube (*B. cinerea*: Plate 3.5, Figs 2 & 6 arrowheads; *B. fabae*: Plate 3.5, Fig. 4 arrowheads). It was also

interesting to note that, in many cases, the germ tubes were slightly swollen at their tips (Plate 3.5: Figs 3-6). In control treatments, i.e. when *Botrytis*-specific MAbs were replaced with hybridoma culture medium only, little or no fluorescence was detected at the surface of non-fixed and fixed sporelings of both *Botrytis* species (for example, *B. cinerea*: Plate 3.5, Fig. 7 & 8).

The fibrillar nature of the ensheathing film of matrix material surrounding the germ tubes of paraformaldehyde-fixed and BC-KH4-labelled sporelings of *B. cinerea* was visualised more clearly using a laser scanning confocal microscope (LSCM) in the epifluorescence mode (Plate 3.6, Figs 1 & 2).

In comparison, when dry-inoculated conidia of *Botrytis* species grown *in vitro* were fixed, immunolabelled with the MAb BC-KH4, BC-AF7 or BC-FD7-G9 and observed by conventional fluorescence microscopy, intense fluorescence was detected at the surface of the conidia (Plate 3.7, Figs 1, 2, 4-6). However, little or no fluorescence was observed at the surface of the short germ tubes (Plate 3.7, Figs 1, 2 & 6). Moreover, *B. cinerea* sporelings appeared to be surrounded by a pool of fluorescent matrix material which remained firmly attached to the glass slide even after thorough washing (Plate 3.7, Figs 1 & 2, arrowheads). In contrast, only traces of fluorescent matrix material were associated with dry-inoculated conidia of *B. fabae* attached to glass slides (Plate 3.7, Figs 4 & 5). In control treatments, no fluorescence was observed at the surface of *Botrytis* sporelings or the glass surface (Plate 3.7, Fig. 8).

In all cases, the pattern of labelling of conidia, germ tubes and fibrillar sheath was similar using all three *Botrytis*-MAbs. However, the intensity of fluorescent labelling of sporelings, observed following indirect immunolabelling using the MAbs, often varied according to the antibody titre.

3.2.5 TEM and indirect immunogold (IG) labelling of extracellular matrices of *Botrytis* species grown *in vitro* using the *Botrytis*-MAbs

TEM of ultra-thin sections of *Botrytis* hyphae and sporelings, post-fixed with osmium tetroxide (OsO₄) and embedded in Spurr resin, showed excellent ultrastructural preservation of nuclei, membranes and membrane-bounded organelles (Plate 3.8, Figs 1 & 2). The fungal cell wall was clearly discernible and it was surrounded by a broad

sheath of lightly-stained fibrillar matrix material (Plate 3.8, Figs 1-4). Immunoelectron microscopy of ultra-thin sections of OsO₄-fixed and Spurr-embedded *B. cinerea* sporelings, using the MAb BC-KH4, revealed traces of gold-labelling throughout the extracellular matrix material (Plate 3.8, Fig 3), however no antibody-binding sites were detected at the fungal cell wall or inside the fungal cell. In control sections, where the primary antibody was omitted, traces of gold labelling were also observed in the extracellular matrix material (Plate 3.8, Fig. 4).

When ultra-thin sections of *Botrytis* sporelings, prepared using mild aldehyde fixation and progressive low-temperature (PLT) dehydration and embedding in LR White (acrylic) resin, were observed by TEM, the fungal ultrastructure compared reasonably well with that observed following conventional methods of preparation (cf. Plate 3.8, Fig. 2 with Plate 3.9, Fig. 1 and Plate 3.10, Fig. 1). The overall contrast of LR White-embedded sections was low and reduced membrane preservation was observed. Nonetheless, following immunogold-labelling of similar sections using the *Botrytis*-MAbs, BC-KH4, BC-AF7 or BC-FD7-G9, numerous antibody-binding sites were localised throughout the fibrillar matrix (*B. cinerea*: Plate 3.9, Figs 1-4 and *B. fabae*: Plate 3.10, Figs 1-3). In addition, gold-labelling was observed in distinct regions throughout the translucent cell wall (Plate 3.9, Figs 1-4 and Plate 3.10, Fig. 1) and across the septum (Plate 3.9, Fig. 3 and Plate 3.10, Fig. 3). However, no labelling of the cytoplasm was observed. In control sections, i.e. in the absence of the primary antibody, no antibody-binding was located throughout the septa, translucent germ tube walls or fibrillar matrix material (Plate 3.9, Figs 5 & 6 and Plate 3.10, Fig. 4).

Indirect immunogold labelling of ultra-thin sections of germinating conidia of *B. cinerea* with the BC-AF7 MAb, revealed that numerous antibody-binding sites were located throughout the extensive fibrillar matrix material surrounding the conidium following germination (Plate 3.11, Fig. 1). Such matrix material also formed a sheath around the emerging germ tube (not shown here). Gold labelling was also observed throughout the outermost layer of the mesospore wall (Plate 3.11 Fig. 1).

3.2.6. Localisation of extracellular surface molecules in the ascomycete, *Botryotinia fuckeliana* (perfect stage of *Botrytis cinerea*) by immunoelectron microscopy (IEM) using the *Botrytis*-MAbs

Apothecia of the ascomycete *Botryotinia fuckeliana* (the perfect stage of *Botrytis cinerea*) were cupulate, stalked and red-brown in colour (Plate 3.12, Fig. 1). The cup of the apothecia varied in shape from infundibuliform to discoid. DIC microscopy of unstained and aniline blue-stained squash preparations of the apothecial cup revealed that this tissue was comprised of a layer of cylindrical asci and sterile hairs or paraphyses (Plate 3.13, Figs 1-3). This palisade layer or hymenium was supported by the hypothecium, a tightly-arranged mass of hyphae (Plate 3.13, Fig. 2). On closer examination, it was observed that the asci were clavate and inoperculate (Plate 3.13, Fig. 2). In all cases, each ascus contained eight ascospores which were in turn, ellipsoidal in shape and appeared smooth in texture (Plate 3.13: Fig. 3). The paraphyses were approximately the same length as the asci.

Observation of ultra-thin sections of the apothecial cup prepared for IEM using mild aldehyde fixation and PLT dehydration and embedding in LR White resin, revealed that the ultrastructure of the apothecial tissue compared reasonably well with that observed in conventionally-prepared samples (Plate 3.14, cf. Figs 1 & 2). The hymenium (containing asci and paraphyses) and hypothecium could be readily distinguished (not shown here). Immunogold labelling of PLT-dehydrated and LR White-embedded sections using the *Botrytis*-MAbs revealed that antibody-binding occurred in regions of the translucent ascospore wall and throughout the fibrillar-like matrix material surrounding each ascospore in the ascus (Plate 3.14, Figs 2-4). The walls of the paraphyses were rarely labelled except across the septum (Plate 3.14, Fig. 5). In addition, in most cases, the ascus wall remained unlabelled. In one case, using the BC-AF7 MAb, the ascus wall and in particular, the thickened tip of the ascus were heavily-labelled (data not shown here). However, further immunolabelling experiments are required in order to confirm these latter observations. In control sections, i.e. in the absence of the primary antibody, no gold labelling was located at the walls of the ascospores, asci, paraphyses or throughout the hyphae comprising the hypothecium (Plate 3.14, Fig. 6).

3.3 Discussion

3.3.1 Germination of *Botrytis* conidia on artificial substrata may depend upon the energy source of the fungal spore

Light and electron microscope studies of the germination of conidia of *B. cinerea* and *B. fabae* revealed that in all cases, conidia germinated on artificial substrata to produce one or more germ tubes. However, the subsequent growth of the developing germ tubes varied considerably according to the mode of inoculation. It is conceivable that the observed differences in germ tube growth following germination of dry and wet (i.e. in the presence of aqueous glucose) conidia on glass surfaces, may be a direct result of the energy source of the fungal spore.

In this study, it is clear that unwashed dry conidia of *B. cinerea* and *B. fabae* require only moist conditions for successful germination on glass. In both cases, dry-inoculated conidia germinated to produce one or more short germ tubes. Such a phenomenon has not been described previously for *Botrytis* conidia grown *in vitro*, although previous researchers have reported that dry conidia of *B. cinerea* infect various host plants held at conditions of high humidity (Rijkenberg, De Leeuw & Verhoeff, 1980; Salinas *et al.*, 1989; Pie & De Leeuw, 1991; Fourie & Holz, 1994; Williamson *et al.*, 1995; See also results of *in vivo* studies presented in Chapter Five). In view of the above results, it is presumed that (i) free moisture activated the germination processes of dry conidia of *Botrytis* species on glass surfaces and that (ii) these conidia contained sufficient reserves to supply the energy as well as the building blocks for the *de novo* synthesis of protein, RNA, polysaccharides and lipids which in turn, are required for the synthesis and assembly of cell walls, membranes and organelles. Even so, it is evident that the conidial reserves of unwashed dry conidia were extremely limited to produce short germ tubes only. In contrast, the fact that conidia germinated in the presence of aqueous glucose to produce long germ tubes may be explained by the suggestion that the conidia were provided with an additional carbon source which increased germ tube growth.

3.3.2. Evidence for the production of extracellular matrices by conidia and germ tubes and hyphae of *Botrytis* sporelings grown *in vitro* by light and electron microscope techniques

Although no extracellular matrices were detected on *Botrytis* sporelings grown *in vitro* by DIC or epi-fluorescence microscopy using the fluorescent brightener Calcofluor White M2R, matrix material ensheathing germ tubes and hyphae was visualised clearly by bright field microscopy using a histochemical stain for mucin. Abundant matrix material was also observed around the swollen tips of hyphae. Such appressorial-like swellings have been reported previously for *Botrytis* species (see Chapter Five, Table 5.1 and references therein).

Low-temperature scanning electron microscopy (LTSEM) and in particular, the use of a low-angle tilted holder (Clement *et al.*, 1994; Clement, 1995) also revealed that copious amounts of matrix material were released by the fungus at points of close adherence of the sporelings to the glass surface. Such matrix material was not readily detected by LTSEM using conventional holders. In addition, LTSEM allowed the preservation of water-soluble surface molecules that may have been lost during the aqueous staining and washing procedures.

3.3.2.1 Evidence for the release of proteinaceous material by *Botrytis* sporelings grown *in vitro* using colloidal gold/silver staining

The use of non-conjugated colloidal gold particles followed by silver enhancement by a physical developer and bright field microscopy revealed a distinct fibrillar sheath surrounding the long germ tubes of *B. cinerea* sporelings grown on glass surfaces and in the presence of aqueous glucose. Jones and co-workers (1995) first described the use of non-conjugated colloidal gold particles as a sensitive staining method for visualising extracellular matrices of plant pathogenic fungi, including *B. cinerea*. They observed that colloidal gold staining of sporelings of *Colletotrichum gloeosporioides* was prevented at high pH (12) and was unaffected by the presence of the non-ionic detergent, Tween-20. As a result, they suggested that colloidal gold binds to macromolecules through ionic rather than hydrophobic forces. Jones and co-workers (1995) also observed that colloidal gold staining of germ tubes of *C. gloeosporioides* was abolished by pre-treatment with proteolytic enzymes, but was unaffected

by treatment with glutaraldehyde or paraformaldehyde. Thus, they assumed that colloidal gold binds to protein or protein-linked molecules, however, they were unable to explain why reaction with aldehydes did not block the binding process. Furthermore, since colloidal gold staining of extracellular matrices of *C. gloeosporioides* was prevented by deamination with nitrous acid, acetylation with acetic anhydride and reaction with 1-fluoro-2,4-dinitrobenzene, they concluded that the colloidal gold particles bound to cell matrices via ionic interactions with the free amino groups of basic proteins (Jones, Bailey & O'Connell, 1995). In the present study, since the negatively-charged colloidal gold particles labelled the fibrillar sheath of *B. cinerea* sporelings, it follows therefore that this matrix sheath contained positively-charged molecules, which are probably protein in nature. However, further analyses of the components of the matrix sheath is required.

Similarly, in view of the properties of colloidal gold, the results of colloidal gold/silver staining of dry-inoculated conidia of *B. fabae*, germinated on glass, indicated that these sporelings were surrounded by a thin pool of proteinaceous matrix material (see Diagram 3.1). Circular films of basic protein around germinating conidia of *C. gloeosporioides* and *Erysiphe graminis* and around appressoria of *Magnaporthe grisea* have been observed previously by staining with colloidal gold (Jones, Bailey & O'Connell, 1995). In an earlier study, using a fluorochrome-conjugated lectin, O'Connell and co-workers revealed haloes of *N*-acetylglucosamine around conidia of *C. gloeosporioides* isolated from avocado (O'Connell, Nash & Bailey, 1992). Similar haloes were also observed around conidia of *E. graminis* by scanning electron microscopy and after staining with Coomassie Brilliant Blue (Kunoh *et al.*, 1988; Nicholson *et al.*, 1993). In both cases, the haloes were considered to result from the contact-stimulated release of a protein exudate (Kunoh *et al.*, 1988; Nicholson *et al.*, 1993). More recently, Mercure and colleagues employed colloidal gold/silver staining to observe the release of adhesive material from ungerminated conidia of *Colletotrichum graminicola* onto hydrophobic surfaces (Mercure, Kunoh & Nicholson, 1995). They reported that colloidal/gold silver staining of adhered conidia revealed patterns of staining which in turn, indicated that proteinaceous materials were released from the tip of the conidium and at the site of contact of the conidium with the substratum (the contact interface). Furthermore, using various fluorescein-labelled lectins at the light microscope level and SEM, it was concluded that the material at the conidium-substratum contact interface contained glycoprotein (Mercure, Kunoh & Nicholson, 1995).

It is well-known that adhesive material is often released by a fungal spore upon immediate contact with the substratum and prior to germination (Gold & Mendgen, 1984;

Tronchin, Poulain & Vernes, 1984; Douglas, 1985; McCourtie & Douglas, 1985; Kunoh *et al.*, 1988; Mercure, Kunoh & Nicholson, 1994: See also Chapter One, Section 1.1 and references therein). However, in the case of dry-inoculated conidia of *B. fabae*, it seems likely that the pool of matrix material associated with the sporelings on the glass surface (see Diagram 3.1) may have been released by the ungerminated conidium following germ pore lysis and concurrent with germ tube growth. However, the possibility that the release of such material occurred before the conidia contacted the substratum or upon contact of the conidia with the substratum and prior to germ tube emergence cannot be ruled out. Regardless of this fact, it is suggested that such matrix material may ensure adhesion of the conidium and/or sporeling to the substratum. In corroboration with the results of previous researchers (Jones, Bailey & O'Connell, 1995), it is clear that the colloidal gold staining method represents a sensitive technique for the visualisation of *Botrytis* extracellular matrices. Such a simple technique could be applied to studies investigating the timing of the release of proteinaceous extracellular matrices by fungal spores.

3.3.2.2 *Botrytis*-MAbs are useful markers for the location of specific wall components and extracellular matrices of *Botrytis* sporelings at the fluorescence microscope level

Further details of the content and nature of *Botrytis* extracellular matrices were provided by indirect IF microscopy using the *Botrytis*-MAbs antibodies BC-KH4, BC-AF7 and BC-FD7-G9 (Bossi & Dewey, 1992). Since, all three MAbs were raised against water-soluble surface washings of *B. cinerea*, the effects of aqueous paraformaldehyde fixation and no fixation of sporelings prior to immunofluorescence labelling were investigated. Immunofluorescence microscopy of glucose-supplemented sporelings demonstrated clearly that in the presence or absence of an aldehyde fixation step, the cell wall components and/or extracellular surface molecules of both fungi were antigenic. In addition, all three *Botrytis*-MAbs showed similar binding to germ tubes, hyphae and conidia of both *B. cinerea* and *B. fabae*. Hence, it can be assumed that they recognise similar antigenic sites. However, the pattern of immunolabelling of the MAbs varied according to the presence or absence of a chemical fixation step prior to immunolabelling. In all cases, increased preservation of surface antigens was observed following chemical fixation. This is perhaps not surprising in view of the soluble nature of the surface antigens. Nevertheless, the observation that surface antigens were located on unfixed sporelings although extensive washing steps were applied prior to immunolabelling also

indicated that some of the *Botrytis*-antigens must be relatively insoluble in aqueous media. Such *Botrytis*-antigens may be cross-linked to the fungal cell wall or components of the extracellular matrix material.

In the absence of chemical fixation, it is suggested that the aggregation of surface molecules may have occurred as a result of multiple cross-linking of the MAbs with the surface molecules during the immunolabelling procedure. This idea is supported by the fact that, following fixation and immunofluorescence microscopy of *Botrytis* sporelings, no such cross-linking was observed. Instead, the antigenic sites were located over the entire surface of the conidia and germ tubes. Extensive antibody-labelling was also located throughout the extracellular matrix material enveloping the germ tubes which in turn, appeared fibrillar in structure. The fibrillar layer surrounding germ tubes of *B. cinerea* sporelings was more distinct when observed by laser scanning confocal microscopy in the epifluorescence mode.

In the light of the above results and previous studies which have confirmed the existence of a layer or sheath of fibrillar material surrounding germ tubes and hyphae of many fungi, including *Botrytis* species (e.g. Evans, Stempen & Frasca, 1982; Gardiner & Day, 1988; Beckett, Tatnell & Taylor, 1990; Van Dyke & Mims, 1991; Bossi & Dewey, 1992; Pain *et al.*, 1992; Pain *et al.*, 1994; Jones, Bailey & O'Connell, 1995), it is postulated that prior to immunofluorescence of *Botrytis* sporelings, a paraformaldehyde fixation step is required in order to preserve the extracellular matrices *in situ*. In addition, the fact that the sporelings were rarely dislodged during the immunostaining process suggested that the fibrillar sheath provided tight adhesion of the sporeling to the substratum.

In a previous study, it was shown from heat, protease and periodate treatment of the surface antigen that all three MAbs, BC-KH4, BC-AF7 and BC-FD7-G9 recognised carbohydrate epitopes on glycoproteins (Bossi and Dewey, 1992). It follows therefore that the cell wall components and the fibrillar sheath of both *Botrytis* species may be in part composed of glycoproteins. In oomycetes and many parasitic fungi, the extracellular surface molecules which have been implicated in adhesion of the spore to a substratum appear to be glycoproteins (see Chapter One, Section 1.4 and references therein). It is manifest that immunofluorescence microscopy of *Botrytis* sporelings using the *Botrytis*-MAbs provides an excellent means for the detection of a specific glycoprotein component of the extracellular matrices associated with the sporelings grown *in vitro*. Additional studies are required in order to characterise this glycoprotein component further (see Chapter Four).

Preliminary IF studies of dry-inoculated conidia of *B. cinerea*, using the *Botrytis* MAbs, indicated that following germination the sporelings were surrounded by a pool of

glycoprotein material which extended across the glass surface. However, no such matrix pool was observed around *B. fabae* sporelings or on the substratum following similar treatment. It is pertinent at this stage to recall the results of colloidal gold/silver staining of dry-inoculated conidia of *B. cinerea* and *B. fabae* grown *in vitro*. Collectively, the results of colloidal gold/silver staining and immunofluorescence microscopy of dry-inoculated conidia of *Botrytis* species grown *in vitro* are summarised in Diagram 3.1. Such potential differences in the chemical composition of the matrices produced by germinating conidia of both *B. cinerea* and *B. fabae* require further investigation.

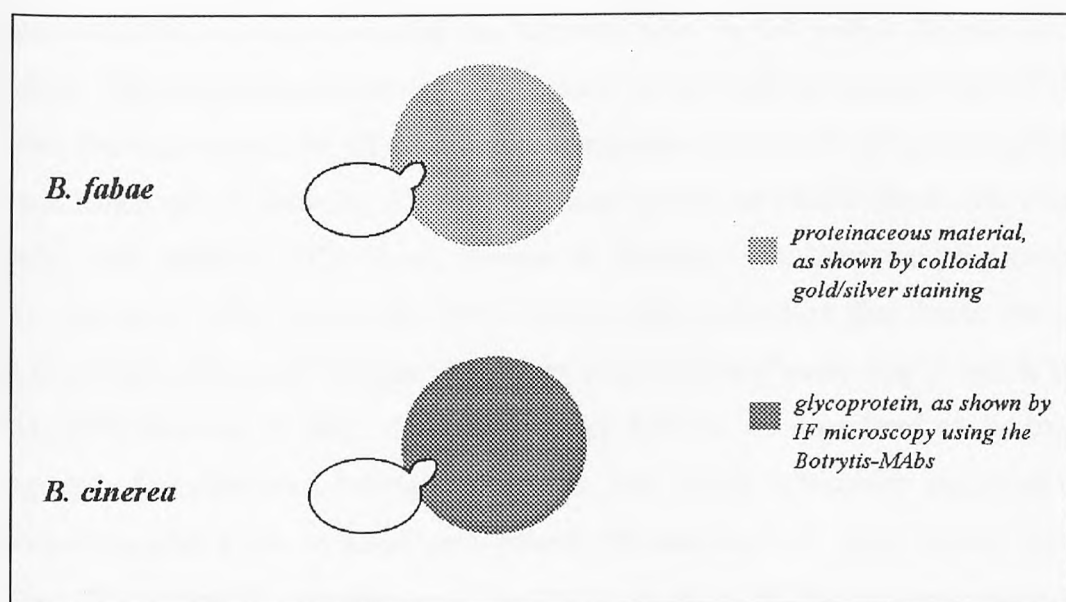


Diagram 3.1 Diagram to illustrate the possible nature of the extracellular matrices associated with dry-inoculated conidia of *Botrytis* species following germination on glass surfaces

Dry-inoculated conidia of *Botrytis* species germinated on glass surfaces to produce one or more short germ tubes. A pool of matrix material was released by each conidium on germ pore lysis and concurrent with germ tube emergence. Epi-polarisation microscopy using colloidal gold/silver staining and IF microscopy using the *Botrytis*-MABs revealed that the content of this matrix pool varied according to the fungus employed. In the case of *B. fabae*, the pool of matrix material was heavily-labelled with colloidal gold indicating the presence of proteinaceous material. In contrast, the matrix material associated with dry-inoculated conidia of *B. cinerea* showed no labelling with colloidal gold. IF microscopy using the *Botrytis*-MABs showed that positive labelling was observed throughout the matrix pool associated with dry-inoculated conidia of *B. cinerea*. No immunolabelling of the matrix pool associated with germinated conidia of *B. fabae* was observed. Positive labelling with the *Botrytis*-MABs indicated the presence of a glycoprotein component. In all cases, the pool of matrix material released by the germinating conidium spread over the surface of the substratum, was firmly attached to the glass surface and probably facilitated adhesion of the sporeling to the substratum.

It was interesting to note that glycoprotein matrix material was also located at the surface of dry-inoculated conidia of both *Botrytis* species, as shown by *Botrytis*-MAB labelling. However, no such material was observed at the surface of the short germ tubes. It is possible that matrix material may have been present at the surface of the short germ tubes but that it may not have been detected by this microscope technique.

3.3.2.3. Further evidence for a fibrillar extracellular sheath around germ tubes and hyphae of *Botrytis* sporelings grown on agar by TEM

TEM studies demonstrated that the walls of germ tubes and hyphae were covered by an extensive fibrillar network of material that extended from the cell wall to the surrounding medium. This observation also confirmed the results of both light microscope and LTSEM studies. Previous research by TEM has also demonstrated that the germ tubes and hyphae of several fungal species, including *B. cinerea*, possess an external fibrillar sheath (Hawker & Hendy, 1963; McKeen, 1974; Evans, Stempen & Stewart, 1981; Benhamou & Ouellette, 1986; Toti *et al.*, 1992; Viret *et al.*, 1994). Previous EM studies have also shown that long fine fibril structures termed “fimbriae” are present at the surface of many fungi (Poon & Day, 1974, 1975; Gardiner & Day, 1985, 1988). Such fimbriae are considered to be largely composed of proteinaceous material (Gardiner & Day, 1985). It has been suggested that fimbriae may play a role in fungal pathogenesis (Benhamou *et al.*, 1986; Svircev *et al.*, 1986a). The superficial resemblance of the fibrillar matrices of *Botrytis* germ tubes and hyphae to fimbriae of other fungi will be examined further in Chapter Four. Undoubtedly, the *Botrytis*-antigens are also major components of the fibrillar sheath. However, it cannot as yet be determined whether they become merely trapped within the mucilaginous material. Regardless of this, it seems likely that they may play an important role in the adhesion of the conidia, germ tubes and hyphae to a substratum.

3.3.2.4 *Botrytis*-MAbs are useful markers for the location of specific components of *Botrytis* cell walls and extracellular matrices at the ultrastructural level

Immunoelectron microscopy (IEM) of sporelings grown *in vitro*, using the *Botrytis*-MAbs, indicated an increased antigenicity of fungal cell walls and extracellular matrices in samples

prepared by mild aldehyde fixation, progressive low-temperature (PLT) dehydration and embedding in the acrylic resin, LR White, compared to that of samples prepared for TEM by conventional methods. Although the fungal cell wall and fibrillar matrix were less clearly distinguished in samples embedded in LR White resin compared to epoxy resin, the spatial distribution of the surface antigens throughout the translucent fungal cell wall and extracellular matrices could be clearly mapped. The results of IEM were also in agreement with those obtained by IF studies. Hence, in each case the *Botrytis*-specific cell surface molecules were located at the germ tube/hyphal walls and in the external fibrillar sheath. By IEM, antigenic sites were also located throughout the septa.

In previous work, a similar distribution of soluble surface molecules was observed in the fungus, *Aspergillus flavus* following immunogold labelling using the MAb, AF-01, specific for *A. flavus* and raised against surface washings of the fungus (L. Cole, C. Hawes, R. Priestley, D. Hughes & M. Dewey, unpublished data). In addition, using the species-specific MAb, PI-01, directed against surface wall components of the fungus, *Penicillium islandicum*, the PI-01-antigen was also located in hyphal walls and throughout the septa (Cole, Hawes & Dewey, 1991). In earlier studies (MacDonald, Dunstan & Dewey, 1989; Dewey *et al.*, 1990) the PI01-antigen was characterised as a glycoprotein (*Mr* approx. 92 000). In addition, it was speculated that the PI01-antigen was an exoenzyme, possibly an autolysin, required for active growth (Cole, Hawes & Dewey, 1991).

The germination of conidia of *Botrytis* species has been studied previously by TEM (Hawker & Hendy, 1963; Buckley, Sjaholm & Sommer, 1966; Gull & Trinci, 1971; Richmond & Pring, 1971). Characteristically, the wall of a *Botrytis* conidium is composed of an outer electron-opaque layer and an inner-translucent layer (Gull & Trinci, 1971; present study). Prior to germination, the conidium swells and the synthesis of new wall material occurs (Hawker & Hendy, 1963; Gull & Trinci, 1971; Smith *et al.*, 1976). Germination of the fungal spore is recognised by the appearance of one or more germ tubes. Soon after its formation, the germ tube is covered by a mucilaginous sheath (Gull & Trinci, 1971). It is prominent that the matrix material associated with the *B. cinerea* conidium in this study was comparable in nature, structure and form to that produced by developing germ tubes and hyphae. In all cases, the fibrillar sheaths around the conidia were also immunoreactive with the *Botrytis*-MAbs. It is also interesting to note that similar antigenic sites were located throughout the translucent layer of both the conidial and germ tube walls. Surprisingly, there have been few comparative studies on the chemical composition of spore and germ tube walls. Perhaps, the *Botrytis*-MAbs described here and the production of other probes specific

for components of fungal cell walls and extracellular matrices may initiate a new interest in this area of research.

3.3.3 *Botrytis*-MAbs label fibrillar-like matrix material around the ascospores of *Botryotinia fuckeliana* (the perfect stage of *B. cinerea*) at the ultrastructural level

Fibrillar-like matrices were also found to be associated with the surface of immature ascospores in asci arranged in the hymenial layer of the apothecia of *Botryotinia fuckeliana* (the perfect stage of *B. cinerea*). Such similarities in the form and content of these matrices and the conidial matrices described previously may be attributed to similar functions. The properties and functions of conidial matrices have been outlined earlier (See Chapter One, Section 1.5). In brief, they may function as a protectant, an anti-desiccant and/or as a means of attachment to the substratum. Likewise, the extracellular matrices associated with ascospores may be required for effective dispersal of these propagules from the ascus. In addition, they may be necessary for attachment of the ascospore to a substratum and they may also provide the spore with a source of nutrients. For example, the ascospores of a marine ascomycetous parasite (*Trichomarix invadens*) of the tanner crab *Chionoecetes bairdi*, have been shown to possess two long filamentous appendages which develop in the ascus and which are thought to be involved in attachment (Porter, 1982). In some newly released spores of *T. invadens*, the remnants of a membranous covering was also visible. This material was considered to be simply a residuum of membranes involved in the development of the ascospores within the ascus and not additional wall layers or modifications. However, the function of such a membranous covering was not discussed.

In conclusion, it is clear that the *Botrytis*-MAbs are suitable tools for further studies of extracellular matrices associated with the conidia, germ tubes and hyphae of *B. cinerea* and *B. fabae* and ascospores of *Botryotinia fuckeliana*. As a consequence, they were employed in immunocytochemical studies in order to determine the role of such surface molecules in the early stages of development of *Vicia faba*-*Botrytis* interactions.

Chapter Four

Nature, origin and secretion of specific components of *Botrytis* cell walls and extracellular matrices

4.1 Introduction

Apart from indirect evidence which suggests that the *Botrytis*-MAbs, BC-KH4, BC-AF7 and BC-FD7-G9, recognise carbohydrate epitopes on a glycoprotein (Bossi & Dewey, 1992), there are at present no available data on the precise nature of the antigenic determinants recognised by these MAbs. This chapter describes the use of a combination of techniques, including two immunosorbent procedures, immunoelectron microscopy (IEM) and enzyme-linked immunosorbent assays (ELISAs), designed to further examine the nature, origin and/or secretion of specific cell surface molecules in *Botrytis* sporelings grown *in vitro*. In the first instance, it is necessary to have a knowledge of the composition of fungal cell walls and the pathways by which polysaccharides and proteins/glycoproteins arrive at the cell surface, in filamentous fungi in general, if the nature, origin, secretion and possibly role(s) of these specific cell surface antigens are to be understood.

4.1.1 Composition of walls of ascomycetous hyphae

The two polymers, chitin [a homo-polymer of (1→4)- β -linked *N*-acetylglucosamine residues] and (1→3)- β -(1→6)- β -glucan are the main components of the walls of ascomycetous hyphae (Wessels & Sietsma, 1981). It is now generally accepted that both polymers are extruded¹ from the cytoplasm at the apices of growing hyphae and modified within the wall domain

¹The plasma membrane-bound chitin synthase and β -(1→3)-glucan synthase add homopolymers of β -(1→4)-linked *N*-acetylglucosamine and β -(1→3)-glucose residues respectively to the growing wall at the apex. Other wall components, including proteins and glycoproteins probably enter the wall by fusion of secretory vesicles with the plasma membrane at the site of wall growth (Wessels, 1993).

(Wessels, 1993). Among such modifications are the formation of covalent cross-links between the chitin and glucan polymers and hydrogen bonds between the homologous polymer chains. Thus, in simple terms, the hyphal wall is essentially a network of chitin microfibrils cross-linked to a glucan matrix or gel (Wessels, 1993). In this present study, IEM studies were carried out, using antisera directed against oligosaccharides containing β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucose (Northcote, Davey & Lay, 1989) in order to compare and contrast the distribution of β -glucan polymers in the walls of young hyphae of *Botrytis* species with that observed previously for the cell surface molecules recognised by the *Botrytis*-MAbs (See Chapter Three, Sections 3.2.5 and 3.3.2.4).

There is considerable evidence to suggest that proteins and/or glycoproteins are also present in fungal cell walls (Reiss *et al.*, 1992; Wessels, 1993). Although the amounts of proteins are small compared to polysaccharides, these proteins, nevertheless, are thought to constitute a considerable portion of the proteins synthesised by the fungus as a whole (Wessels, 1993). The majority of proteins secreted by filamentous fungi are considered to be glycosylated (Peberdy, 1994). Thus, it is tempting to assume that the glycoprotein portion of *Botrytis* extracellular matrices (See Chapter Three) may be released from the fungal cell via the protein secretory pathway i.e. via a vesicle-mediated pathway (see below and Diagram 4.1). Both enzymes and structural proteins are secreted by filamentous fungi (Peberdy, 1994). Such enzymes may be required for assimilatory purposes, they may be necessary for development of the infection process or they may be involved in recognition processes (Wessels, 1993; Peberdy, 1994). These enzymes are released from the surface of the plasma membrane at the hyphal tip region into the periplasmic space, where they may be secreted across the cell wall into the external medium or alternatively, they may be incorporated into the cell wall and slowly released into the medium (Wessels, 1993; Peberdy, 1994). More recently, by cloning genes which were abundantly expressed during the formation of aerial hyphae and fruiting bodies, it has been discovered that a distinct class of proteins, named hydrophobins, are produced by the fungal mycelium only when it has reached a certain stage of maturity (Wessels, 1993). These hydrophobins are secreted by submerged hyphae as monomers into the surrounding medium and conversely self-assemble as insoluble complexes in the walls of emergent hyphae (Wessels, 1993). It is thought that they play a crucial role in morphogenesis and pathogenesis (Wessels, 1993). The possible role(s) of the *Botrytis*-antigens will be discussed further in relation to their location at the cell surface and at the intracellular level in *Botrytis* hyphae.

4.1.2 Protein secretion occurs via a vesicle-mediated pathway at the growing apices of filamentous fungi

Early cytological studies revealed that the growing hyphal apices were filled with vesicles of varying sizes to the exclusion of all other organelles (McClure, Park & Robinson, 1968; Girbardt, 1969; Grove & Bracker, 1970; Grove, Bracker & Morré, 1970). Some vesicles also appeared to be in the process of fusing with the plasma membrane (Girbardt, 1969; Grove & Bracker, 1970). This apical organisation could be identified with the Spitzenkörper, first observed by Brunswick in 1924 and often visible in hyphal apices as a phase-dark region by light microscopy (Girbardt, 1957, 1969). In view of this circumstantial evidence, it was frequently assumed that secretion of proteins by filamentous fungi was probably restricted to the tips of growing hyphae. However, there is other evidence for the occurrence of protein excretion occurring mainly at the apex (Chung & Trevithick, 1970; Pugh & Cawson, 1977; Sprey, 1988; Podila *et al.*, 1995). Current research also suggests that protein secretion in filamentous fungi is intimately associated with the process of growth at the hyphal tip (Wösten *et al.*, 1991; Wessels, 1993; Peberdy, 1994). In this study, the possibility that the antigens recognised by the specific *Botrytis*-MAbs were delivered to the cell surface at the tips of *Botrytis* hyphae via exocytotic vesicles was investigated by IEM methods. Conventional TEM studies were carried out in order to observe the ultrastructure of the growing apices of *Botrytis* hyphae in more detail.

Unfortunately, our understanding of the cellular mechanisms involved in protein secretion is still minimal and for the most part, it is necessary to extrapolate from other eukaryotic organisms. A hypothetical secretory pathway in the hypha of a filamentous fungus has been described recently (Peberdy, 1994) and is illustrated in Diagram 4.1. In yeast, mammalian and plant cells, proteins destined for secretion are synthesised on ribosomes associated with the membranes of the endoplasmic reticulum (ER), the so-called rough ER (RER). However, the secretory process is generally considered to be initiated once the nascent extracellular proteins have been sequestered into the lumen of the RER (Peberdy, 1994). This event is determined by information embodied in the signal sequence, a *N*-terminal domain attached to the protein molecule. It is well-known that the retention of soluble resident-ER proteins in mammalian, plant and yeast systems is mediated by a short, four amino acid sorting signal, identified as KDEL or HDEL (Munro & Pelham, 1987; Pelham, 1989; Herman *et al.*, 1990; Denecke, De Rycke & Botterman, 1992; Napier *et al.*, 1992).

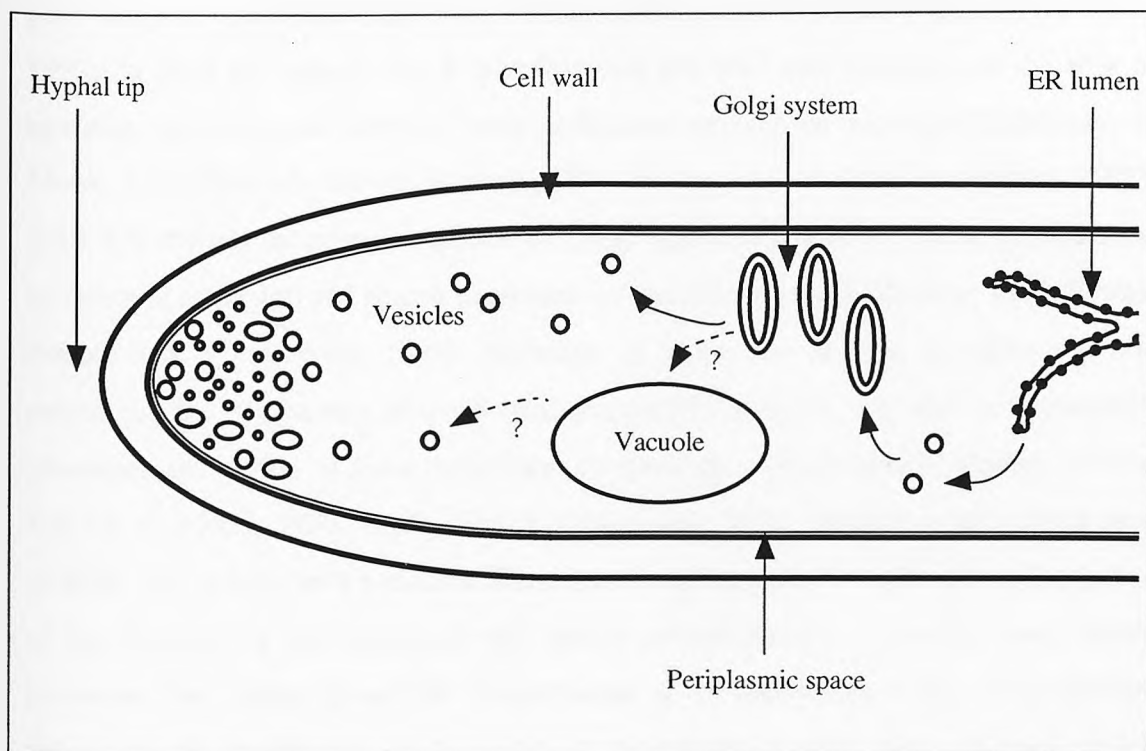


Diagram 4.1 A hypothetical secretory pathway in filamentous fungi

Proteins to be secreted are first transferred to the lumen of the ER where post-translational processes take place. Vesicles carry the molecules to the Golgi system, or its equivalent, where processing continues. Vesicles are then transported vectorially to the apex where they fuse with the plasma membrane, releasing their contents into the periplasmic space. There is speculation concerning the possible involvement of vacuoles in the secretory pathway. Redrawn from Peberdy (1994).

Before proteins are secreted, they must undergo several post-translational modifications. For example, proteolytic cleavage of the protein molecule to remove the signal sequence and glycosylation (Peberdy, 1994). The complexity of the biochemical pathway(s) involved in glycosylation has still to be unravelled in filamentous fungi. However, several clues to the glycosylation process have been obtained from research on yeast cells, where mutants defective in the process have been used extensively (Peberdy, 1994). Such modifications begin in the ER and continue as the proteins pass through the Golgi apparatus.

Most fungal cells do not exhibit the stacked Golgi cisternae characteristic of other eukaryotic cells (Grove & Bracker, 1970; Howard, 1981; Sewall, Roberson & Pommerville, 1989; Bourett & Howard, 1994). However, by careful fixation, oriented sectioning and observation by TEM, it has been shown that filamentous fungi do possess membranous cisternae which are thought to be homologous with the Golgi apparatus observed in animal,

plant and yeast cells (Howard, 1981; Bourett & Howard, 1994; Satiat-Jeunemaitre *et al.*, 1996). In plant and animal cells, it is well-known that the Golgi cisternae are the sites of synthesis, processing and sorting of many surface and extracellular materials (Mollenhauer & Morré, 1994; Driouich, Faye & Staehelin, 1993; Hawes, Faye & Satiat-Jeunemaitre, 1995). Thus, it is strongly tempting to implicate the Golgi equivalents of fungi with the synthesis and secretion of some wall and plasma membrane components (Grove & Bracker, 1970; Sewall, Roberson & Pommerville, 1989). Moreover, it is all too easy to speculate that the polysaccharidic components of the *Botrytis* extracellular matrices may also be synthesised, processed and sorted in these subcellular compartments. Histochemical studies (Grove, Bracker & Morré, 1970; Heath, Gay & Greenwood, 1971; Dargent *et al.*, 1982) have revealed that in oomycetes and some filamentous fungi, the apical vesicles, the maturing face of the dictyosomes and the apical wall contain polysaccharides. It has also been shown previously that certain intracellular compartments of the filamentous fungus, *Magnaporthe grisea*, can be conveniently labelled using the plant lectin, concanavalin-A (Con-A) which recognises α -D-glucosyl and α -D-mannosyl residues (Bourett, Piccollelli & Howard, 1993; Bourett & Howard, 1994). Furthermore, these authors concluded that using a double-sided and indirect postembedding labelling procedure, Con-A can be used to successfully probe the endomembrane system of this fungus (Bourett, Piccollelli & Howard, 1993; Bourett & Howard, 1994). Nevertheless, specific probes for the various components of the secretory pathway in filamentous fungi are hitherto lacking.

In contrast, considerable advances have been made in the understanding of the plant endomembrane system, membrane flow between the ER and the Golgi apparatus and within the Golgi apparatus itself as a result of the availability of specific marker antibodies for identification of certain subcellular compartments. For example, JIM84, 2E7 [anti-HDEL] and MAC 256 [anti-KDEL] which have been employed successfully to locate a Golgi-membrane glycoprotein and to recognise the HDEL- and KDEL-carboxyterminal retention sequence of the resident ER proteins respectively in both plant and animal cells (Napier *et al.*, 1992; Horsley *et al.*, 1993). In this study, IEM studies were carried out in order to test the usability of all of three plant-MAbs, as specific markers for certain intracellular compartments of the endomembrane system of *B. cinerea*. It was also hoped that close examination of the secretory pathway in *Botrytis* hyphae would yield further information on the mode of secretion of *Botrytis* cell surface molecules.

4.1.3 The use of secretory inhibitors in studies of the secretory pathway in animal, plant and yeast cells

A common approach to further studies of the secretory pathway in eukaryotic cells, is the application of drugs or inhibitors in order to disrupt the transport process. For example, the drug, brefeldin A (BFA), has been used extensively as an inhibitor of the secretory pathway in animal, plant and yeast cells (Klausner, Donaldson & Lippincott-Schwartz, 1992; Rothman & Orci, 1992; Satiat-Jeunemaitre & Hawes, 1992a,b, 1993; Shah & Klausner, 1993; Vogel *et al.*, 1993; Rambourg *et al.*, 1995). BFA is a dihydroxy α - β unsaturated lactone (see Appendix A), it was first isolated from a strain of *Penicillium decumbens* by Singleton and coworkers in 1958 and termed decumbin. To date, very limited research has been carried out on the effect(s) of BFA on the secretory pathway of filamentous fungi (Bourett & Howard, 1995; Satiat-Jeunemaitre *et al.*, 1996). Nevertheless, previous studies have established that this drug², when applied at a concentration within the range of 10-50 $\mu\text{g}.\text{ml}^{-1}$, can affect both fungal growth and differentiation (Betina, Janstová & Spíslaková, 1975; Baráthova, Betina & Ulický, 1977; San-Blas & Yegres, 1978). Pyrimethanil, on the other hand, is a new anilino-pyrimidine fungicide (“Scala”®, “Mythos”®; see Appendix A) for the control of *Botrytis* species in vines, fruits, vegetables and ornamentals and *Venturia* species in apples and pears (Milling & Richardson, 1995). It has also been shown to inhibit the secretion of cell wall-degrading enzymes by *B. cinerea* (Milling, Richardson & Daniels, 1993; Milling & Richardson, 1995). In the present study, the effects of BFA and pyrimethanil on conidial germination and radial growth of *B. cinerea* were investigated and compared using growth media amended with the inhibitor. Preliminary tests on the effects of BFA on adhesion of *B. cinerea* sporelings to glass surfaces were also carried out. Furthermore, the ultrastructural effects of BFA on the secretory pathway and the mode of secretion of the *Botrytis* cell surface molecules in young hyphae/sporelings of *B. cinerea* were studied using conventional TEM and IEM methods respectively. Con-A was employed, in this study, as a probe to label the endomembrane system in *Botrytis* sporelings prepared for TEM using conventional methods (Bourett, Piccollelli & Howard, 1993; Bourett & Howard, 1994). It was hoped that Con-A would also aid in the elucidation of the effects of BFA on the secretory pathway in *Botrytis* sporelings.

² BFA has been identified and named by other groups as cyanein (Betina, Janstová & Spíslaková, 1975; Baráthova, Betina & Ulický, 1977).

4.1.4 Techniques for improving the preservation and retention of soluble antigens for immunoelectron microscopy

Previous TEM and SEM studies have shown that when germ tubes and hyphae of plant pathogenic fungi, including *Botrytis* species were grown on artificial substrata, they were often surrounded by an extensive fibrillar sheath which provided firm attachment to the substratum (See Chapter Three: Section 3.1, p. 57 and references therein; see also results of Chapter Three, Sections 3.2.3 and 3.2.5). However, in some fungi with well-attached propagules, sporelings or infection structures, this fibrillar layer has been reported to be limited or largely electron-translucent (Wheeler & Gantz, 1979; Evans, Stempen & Frasca, 1982; Hoch & Staples, 1983). In such cases, it has been suggested that considerable losses of water-soluble matrix components occurred as a result of the conventional electron microscope preparation protocols employed (Harder *et al.*, 1989). Such protocols involved aqueous fixation, organic solvent dehydration and resin infiltration processes. Thus, alternative methods are required for the increased preservation and hence, observation of water-soluble matrices at the ultrastructural level.

Today, advances in cryo-technology have favoured the application of rapid-freezing methods in combination with low-temperature scanning electron microscopy (See Chapter One, Section 1.3 and references therein) or cryo-dehydration methods, such as freeze-substitution (Howard & Aist, 1979; Hoch & Howard, 1980; Hoch & Staples, 1983; Hamer *et al.*, 1988) for the observation of water-soluble fungal cell surface components. The two main methods of cryo-dehydration of rapidly-frozen biological samples are freeze-substitution and low-temperature freeze-drying. Freeze-substitution depends upon the replacement of the frozen aqueous phase of a specimen with an organic solvent by discrete molecular events (Hoch, 1991). Although this technique has resulted in excellent morphological preservation of fungal specimens, it is contradictory to one of the principle reasons for rapid-freezing the specimen in the first place; that is, the elimination of solvent contact in order to increase the retention of soluble antigens. Thus, low-temperature freeze-drying is considered to be the most suitable method for removing water from biological specimens with minimal disturbance of other cell constituents. Molecular distillation drying is an example of such a process. Here, rapidly-frozen biological material is freeze-dried in a controlled manner at ultra-low temperatures (from $<-150^{\circ}\text{C}$ to -80°C) and ultra-high (10^{-6} mbar) vacuums (Linner *et al.*, 1986a,b; Livesey *et al.*, 1989). Once samples have been dried, they may be fixed using

paraformaldehyde and/or osmium vapour. Vapour fixation has been shown to enhance ultrastructural preservation and to add contrast to biological specimens without causing significant biochemical blocking of antigenic sites (Linner *et al.*, 1986b). Molecular distillation drying is particularly useful for immunocytochemical studies where the antigens are water-soluble, sensitive to chemical fixation, solvent extraction and/or heat treatment. Moreover, the only solvent applied in this process is the resin. Furthermore, resin extraction of soluble molecules can also be reduced by infiltration of samples with acrylic resins at low-temperature and ultra-high vacuums. Thus, by avoiding chemical pre-fixation and solvent contact during resin embedding, molecular distillation drying provides the basis for combining ultrastructural preservation with optimum material for immunocytochemistry (Linner *et al.*, 1986a,b). Using the *Botrytis*-specific MAbs, immunogold labelling of ultra-thin sections of *Botrytis* sporelings and hyphae, prepared by cryo-fixation and molecular distillation drying, was carried out in order to compare antigen retention and ultrastructural preservation in each case. In addition, the results of these studies were compared further with that observed using conventional and progressive low-temperature dehydration and embedding protocols (See Chapter Three).

As a result of early observations (Poon & Day, 1974, 1975; Day & Poon, 1975) which suggested that the surface fibrils of *Microbotryum violaceum* (Pers.) Fuckel (formerly *Ustilago violacea*) were similar in structure to the long proteinaceous surface hairs of bacteria termed fimbriae (Duguid *et al.*, 1955) or pili (Brinton, 1959), extensive research has been carried out in one laboratory in order to investigate the composition and occurrence of fimbriae throughout the fungal kingdom (Gardiner & Day, 1988). Such studies have shown that fimbriae may be observed in fungi from all the major groups. Nevertheless, it has been reported that fungal fimbriae are not readily detected in ultra-thin sections of fungal cells, even in cells of *M. violaceum*, processed by conventional preparative methods and observed by TEM, because they appear electron-transparent (Gardiner & Day, 1988). In the present study, samples of *Botrytis* hyphae grown *in vitro* were prepared for IEM studies by rapid-freezing and low-temperature freeze-drying in order to (i) increase the preservation of the fibrillar matrix sheath around the hyphae and (ii) using an antiserum directed against fimbriae of *M. violaceum* to investigate further the superficial resemblance of the fibrillar matrices associated with the germ tubes and hyphae of *Botrytis* species to fungal fimbriae.

4.1.5 Characterisation of *Botrytis* cell surface molecules using monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs)

Almost two decades have passed since Clark & Adams (1977) first adapted the medical immunodiagnostic procedure, enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of plant viruses. Today, ELISAs are routinely employed as a practical serodiagnostic technique for the detection and identification of plant pathogenic viruses. However, relatively few antibody-based immunoassays have been developed for the detection of fungi (Dewey *et al.*, 1991; Dewey, 1992; Dewey *et al.*, 1993; Schots, Dewey & Oliver, 1994; Dewey & Thornton, 1995). This is partly due to the fact that antisera raised to fungi cross-react widely with related species, species from unrelated genera and host molecules (see Chapter One, Section 1.7). This chapter describes the use of an indirect plate-trapped antigen ELISA method involving the *Botrytis*-specific MAb, BC-KH4, for the characterisation of specific cell surface antigens in surface washings of *B. cinerea* and *B. fabae* (Bossi & Dewey, 1992; see Diagram 4.2). In brief, the immunoassay involves the immobilisation of the fungal antigen (Ag) by its attachment or binding to polystyrene microtitre wells (Voller *et al.*, 1974; Lehtonen & Viljanen, 1980; Dewey & Brasier, 1988; Dewey & Thornton, 1995). Excess antigen is then removed from the wells by thorough washing and the hybridoma supernatant (Ab) is subsequently added. Unbound antibody is removed and the retained antigen-antibody complex is detected using an enzyme-labelled secondary antibody (Ab-E) which specifically recognises murine monoclonal antibodies. Finally, the retained enzyme is assayed by the addition of a suitable substrate. The use of substrates that give coloured hydrosylates permits easy visual qualitative assessment of the presence or absence of the antigen-antibody complex (Clark, 1981). Furthermore, since the retention of enzyme is proportional to the amount of antigen-antibody binding, a quantitative measurement of the antigen-antibody complexes can be obtained by colorimetry. Using this immunoassay, it has previously been shown that all three hybridoma supernatants, BC-KH4, BC-AF7 and BC-FD7-G9 did not recognise fungi normally involved in the post-harvest spoilage of fruits and vegetables (Bossi & Dewey, 1992). In this study, the specificity of the MAb, BC-KH4 towards *Botrytis* species was investigated further using a range of fungi not previously tested. In addition, the nature of the antigenic determinants or epitopes of both *B. cinerea* and *B. fabae* were characterised further by a simple direct ELISA method involving commercially-available peroxidase-conjugated

lectins. In most cases, a “checkerboard” titration system was employed. in order to determine the most suitable combinations of reactant concentrations (Clark & Adams, 1977).

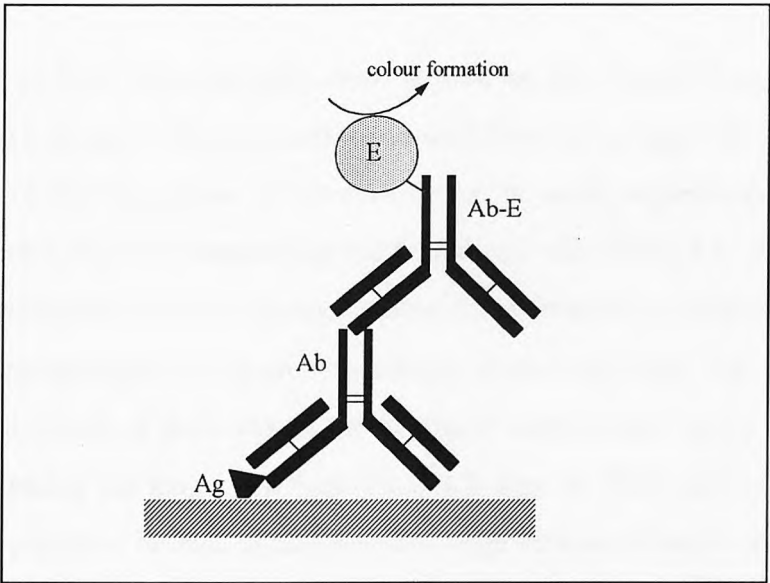


Diagram 4.2 Indirect plate-trapped antigen enzyme-linked immunosorbent assay

4.2 Results

4.2.1 Immunolocalisation of β -glucan polymers in the walls of young hyphae of *Botrytis* species

The antiserum to β -(1 $\rightarrow$ 3) oligosaccharides showed an abundance of polymers with β -(1 $\rightarrow$ 3) glucose linkages in the translucent cell wall (Plate 4.1, Figs 1 & 2) and septa of young hyphae of *B. fabae* grown *in vitro*. However, no such polymers were detected in the fibrillar matrix material surrounding the fungal cell wall (Plate 4.1, Figs 1 & 2). In contrast, the antiserum to β -(1 $\rightarrow$ 4) oligosaccharides indicated the presence of relatively few polymers containing β -(1 $\rightarrow$ 4) glucose linkages in the translucent cell wall (Plate 4.1, Fig. 3). Instead, traces of β -(1 $\rightarrow$ 4) glucan polymers were located in the fibrillar matrix material surrounding the fungal cell wall (Plate 4.1, Fig. 3). Both antisera located their corresponding polymers in small vacuoles and/or large vesicles throughout the cytoplasm (Plate 4.1, Figs. 1 & 3). In addition, traces of gold labelling were observed free in the cytoplasm following immunolabelling with either of the polyclonal antisera (Plate 4.2, Figs 1-3). As described previously (see Plate 3.9, Figs 1-4 and Plate 3.10, Fig. 1), the MAb BC-KH4 recognised antigenic sites throughout the fibrillar matrix material and in distinct regions of the translucent cell wall (Plate 4.1, Fig. 4). Control treatments, i.e. where the primary antibody was omitted, revealed little non-specific binding of the secondary antibody to the fungal cell wall, septa or fibrillar matrix material (Plate 4.1, Figs 5 & 6).

4.2.2 Immunolocalisation of *Botrytis*-MAb binding sites at the growing apices of *Botrytis* hyphae

Ultrastructural observations of the tips of young hyphae of *B. fabae* prepared using conventional TEM preparative methods, e.g. glutaraldehyde/osmium tetroxide fixation and embedding in Spurr epoxy resin revealed the presence of numerous membrane-bounded vesicles at the extreme tips of the growing hyphae to the exclusion of all other

organelles (Plate 4.2, Figs 1 & 2). Two distinct populations of vesicles, apical vesicles and micro-vesicles were visible at the hyphal apex (Plate 4.2, Figs 1 & 2).

Similar highly vesiculated areas were located at hyphal tips in *Botrytis* hyphae prepared for immunocytochemistry using mild aldehyde fixation, PLT-dehydration and embedding in LR White resin (Plate 4.2, Figs 3 & 4). However, because of the overall low contrast and reduced membrane preservation associated with LR White-embedded material (see Chapter Three, Section 3.2.5), individual vesicles were often difficult to discern. Thus, a clear distinction between two vesicle populations, according to their size, was not readily apparent. Moreover, using the MAb BC-FD7-G9 very few antibody-binding sites were observed at the cell wall or associated with the numerous vesicles located at the hyphal tips (Plate 4.2, Fig. 4). Similar results were obtained when sections of hyphal tips were incubated with PBST only, in lieu of the primary antibody (not shown here).

4.2.3 Ultrastructure of the endomembrane system in young hyphae of *Botrytis* species prepared by conventional TEM methods

All components of the endomembrane system, e.g. smooth and rough endoplasmic reticulum (ER), Golgi equivalents and associated small vesicles were clearly apparent throughout the cytoplasm of growing hyphae of *B. cinerea* (Plate 4.3, Figs 1-4). Golgi elements were particularly abundant in a region just behind the hyphal tips (not shown here). In addition, they were often located in close proximity to the cell wall (not shown here). These Golgi equivalents were composed of individual or a few loosely associated smooth cisternae, they were surrounded by vesicles and they often showed connections to the rough ER (RER) (Plate 4.3, Figs 1, 3 & 4).

4.2.4 The use of specific plant antibodies (JIM 84, E27 and MAC 256) to label the components of the endomembrane system of young hyphae of *B. cinerea*

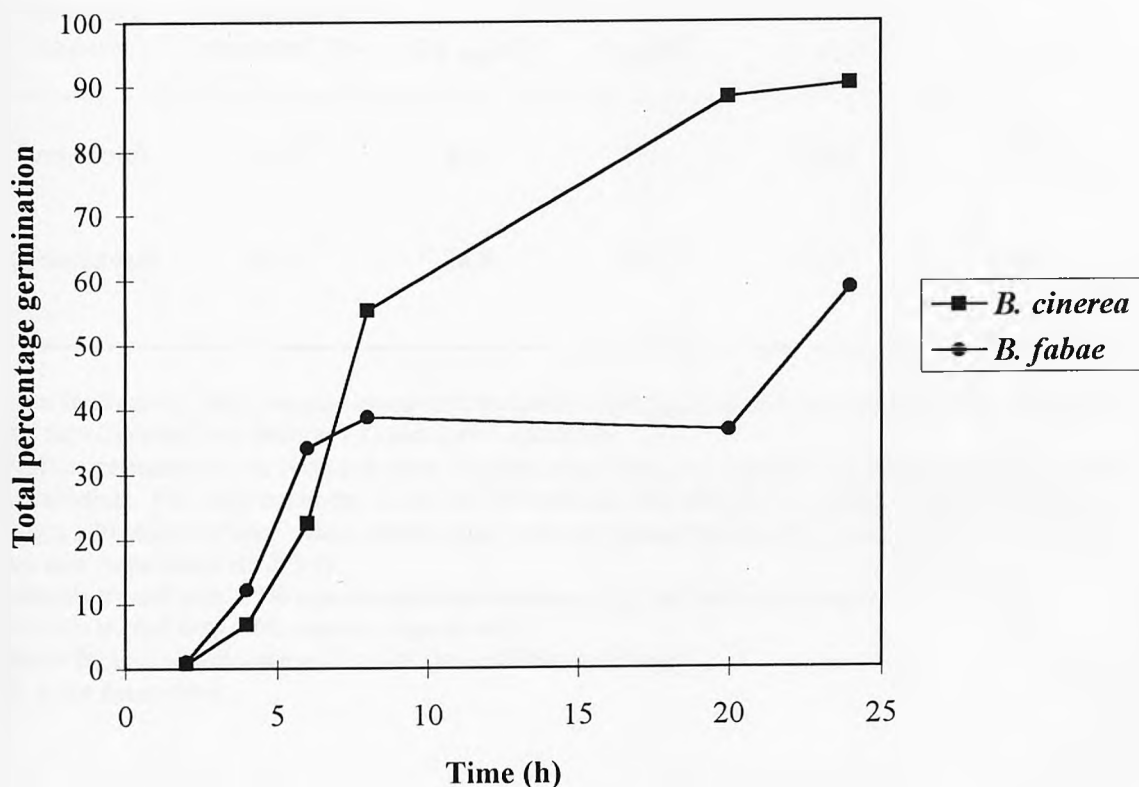
TEM observations of ultrathin sections of PLT-dehydrated and LR White-embedded somatic hyphae of *B. cinerea* grown on agar revealed that although certain organelles e.g. nuclei, mitochondria and vacuoles, were well-preserved, the elements of the endomembrane system were more difficult to distinguish (results not shown). The rough ER was just visible as parallel arrays of tubules or flattened sacs which were coated with ribosomes on their cytosolic surface. In contrast, Golgi complexes were not conspicuous.

IEM studies using the MAbs, JIM 84, 2E7 (anti-HDEL) and MAC 256 (anti-KDEL) revealed that in each case, no specific labelling of endomembrane compartments was detected (data not shown). Instead, traces of antibody-binding were located throughout the cytoplasm and at the hyphal tips. In control treatments, i.e. the absence of the primary antibody, very few antibody-binding sites were located throughout the cytoplasm.

4.2.5 Effect of the secretory inhibitors, brefeldin A (BFA) and pyrimethanil on conidial germination, germ tube growth and adhesion of *B. cinerea*

Time-course studies showed that after 24 h, the percentage germination of *B. cinerea* and *B. fabae* conidia grown *in vitro* was 90.2% and 58.7% respectively (see Graph 4.1; see also Appendix B). On the basis of this criterion alone, *B. cinerea* was selected as a more suitable “model” fungus compared to *B. fabae*, for studies of the effects of brefeldin (BFA) and pyrimethanil on conidial germination.

Preliminary experiments showed that BFA and pyrimethanil, when applied at concentrations of 1 or 10 $\mu\text{g}.\text{ml}^{-1}$ and 0.1, 1 or 10 $\mu\text{g}.\text{ml}^{-1}$ respectively, had no significant effect on the germination of *B. cinerea* conidia grown *in vitro* (Table 4.1). However, at a



Graph 4.1 Time-course study of the percentage germination of *Botrytis* conidia grown *in vitro*

Conidia of *B. cinerea* and *B. fabae* were resuspended in 0.5% aqueous glucose to give a final concentration of 2.56×10^5 and 4.25×10^4 conidia.ml⁻¹ respectively. Conidia (20 μ l aliquots) were inoculated onto Multiwell glass slides and incubated at 17°C as described in Chapter Two, Section 2.1. At the time intervals shown, 500 conidia/sporelings were observed by light microscopy using a x40 objective lens. Positive germination was recorded when the germ tube length was equal to or greater than the length of the spore.

Table 4.1 Effects of brefeldin A and pyrimethanil on the germination of *B. cinerea* conidia grown *in vitro*

Secretory inhibitor ^f	Germination ^g in control (%)	Spore germination (% control)			
		0.1 µg.ml ⁻¹	1 µg.ml ⁻¹	10 µg.ml ⁻¹	100 µg.ml ⁻¹
brefeldin A	84.8 [†]	n.d.	93.4	100.8	80.5*
pyrimethanil	65.1 [‡]	88.6	90.3	103.7	n.d.

^fAfter inoculation, plates supplemented with brefeldin A and pyrimethanil were incubated at 17°C in the light (see Chapter Two, Section 2.1) and dark respectively.
^gPositive germination was recorded when the germ tube length was equal to or greater than the length of the conidium. For each treatment, a total of 500 conidia were observed by phase contrast microscopy using a x40 objective lens. Values shown equal mean percentage germination with respect to *n* fields of view (see Appendices B2 & B3).
[†]Controls treated with 0.5% aqueous glucose containing 10µl.ml⁻¹ absolute ethanol.
[‡]Controls treated with 0.5% aqueous glucose only.
Asterix denotes significance at *P* < 0.05 (Mann-Whitney U test).
n.d. = not determined.

higher concentration (100 µg.ml⁻¹), BFA significantly reduced conidial germination by 19.5% (Table 4.1.) and had a deleterious effect on germ tube growth (data not shown). Analyses of control treatments showed that absolute ethanol (10 µl.ml⁻¹) had no effect on conidial germination (see Appendix B).

Preliminary experiments investigating the effect of the drug BFA on the adhesion of *B. cinerea* sporelings to glass surfaces revealed that when glucose-supplemented conidia were incubated on glass slides in the presence or absence of BFA (25 µg.ml⁻¹), the drug had no effect on conidial or germ tube adhesion after a 24 h time period. In both BFA and control treatments, sporelings were dislodged using a modification of the method described by Doss and co-workers (1993) [See also Chapter Two, Section 2.16.4].

4.2.6 Effects of the secretory inhibitors, brefeldin A (BFA) and pyrimethanil on radial growth of *B. cinerea*

In agar plate tests, BFA significantly inhibited the radial growth of *B. cinerea* on PDA by 43.5% and 65% compared to that of the controls, when the drug was applied at a concentration of 10 and 100 $\mu\text{g.ml}^{-1}$ respectively (Plate 4.4 & Table 4.2). Lower concentrations of brefeldin A tested (0.1 and 1 $\mu\text{g.ml}^{-1}$) had no effect on the radial growth of *B. cinerea* on PDA. Similar effects of BFA were observed when the fungus was grown on GGA supplemented with the drug (Table 4.2). However, in this case, the effects of BFA on radial growth appeared less pronounced. For example, at BFA concentrations of 10 and 100 $\mu\text{g.ml}^{-1}$, the radial growth of *B. cinerea* on GGA was only reduced by 12 and 38% compared to that of the controls.

Furthermore, on both PDA and GGA, the radial growth of the fungus was significantly inhibited by all concentrations of pyrimethanil tested (Plate 4.4 & Table 4.2). However, in this case, the sensitivity of *B. cinerea* to pyrimethanil was enhanced when the fungus was grown on GGA supplemented with the secretory inhibitor, instead of PDA (Table 4.2). This sensitivity to pyrimethanil was particularly apparent even at a concentration as low as 0.1 $\mu\text{g.ml}^{-1}$, where the radial growth of the fungus on PDA and GGA was inhibited by 10 and 36% respectively to that of the controls.

Analyses of control treatments where the fungus was grown on PDA only or PDA containing absolute ethanol (100 $\mu\text{l.ml}^{-1}$) or DMSO (10 $\mu\text{l.ml}^{-1}$) showed that both solvents significantly affected the radial growth of the fungus (see Table 4.2). For example on PDA containing solvent (Control B), the radial growth of the fungus was reduced by 14% in the presence of absolute ethanol (100 $\mu\text{l.ml}^{-1}$) and increased by 8% in the presence of DMSO (10 $\mu\text{l.ml}^{-1}$).

Table 4.2 Effects of the secretory inhibitors, brefeldin A and pyrimethanil, on the radial growth of *B. cinerea*

Agar medium	Secretory inhibitor	Mean [§] radial growth of control (in cm)		Radial growth (% of control)			
		Control A [†]	Control B [‡]	0.1 µg.ml ⁻¹	1 µg.ml ⁻¹	10 µg.ml ⁻¹	100 µg.ml ⁻¹
PDA	brefeldin A	3.13	2.68*	106.4	101.0	56.5*	35.0*
	pyrimethanil	1.68	1.82*	90.0*	9.5*	5.5*	n.d.
GGA	brefeldin A	2.23	1.66*	99.1	101.3	87.9*	62.0*
	pyrimethanil	2.41	2.0 [/]	64.3*	0	0	n.d.

[§]Data from at least three replicates, unless otherwise stated (see Appendices B4-B7). Values show mean value of measurements taken from cultures grown on PDA (potato dextrose agar) and GGA (20 g.l⁻¹ agar containing 3.0 mM MgSO₄, 13 mM KH₂PO₄, 4 g.l⁻¹ D-glucose and 4 g.l⁻¹ gelatin) for 3 and 4 days respectively.

[†]Agar only.

[‡]Agar supplemented with solvent (100 µl.ml⁻¹ absolute ethanol or 10 µl.ml⁻¹ DMSO as appropriate).

Asterix denotes significance at *P* < 0.05 (Mann-Whitney U test). In statistical analysis, data from drug treatments and Control A were compared, except for 100 µg.ml⁻¹ BFA and 10 µg.ml⁻¹ pyrimethanil where the appropriate Control B was used.

[/]One value only.

n.d. = not determined.

4.2.7 Effects of BFA and pyrimethanil on the secretory pathway of young hyphae of *B. cinerea* prepared for TEM using conventional methods

Ultrastructural observations of ultra-thin sections of conventionally-fixed hyphae of *B. cinerea* grown on PDA supplemented with either 10 µg.ml⁻¹ brefeldin A or 0.1 µg.ml⁻¹ pyrimethanil, revealed little or no effect of these inhibitors on the structural components of the secretory pathway of the fungus compared to that of the controls (Plate 4.5, Figs 1-4). In both control and drug treatments, endoplasmic reticulum was abundant and the smooth membrane cisternae constituting the Golgi equivalents remained intact (Plate 4.5,

Figs. 1, 3 & 4). However, in some cases, in the presence of brefeldin A, areas largely devoid of ribosomes were apparent (Plate 4.5, Fig. 2). Such areas may correspond to possible sites of assembly/disassembly of Golgi equivalents. Growing apices of BFA-treated hyphae were rarely observed.

4.2.8 Effects of BFA and pyrimethanil on the secretory pathway of sporelings of *B. cinerea* prepared for TEM using conventional methods: Postembedment labelling using Concanavalin A (Con-A)

The ultrastructure of *B. cinerea* sporelings grown in suspension culture (Plate 4.6, Figs 1-4) and prepared for TEM using conventional preparative methods (i.e. glutaraldehyde/OsO₄ fixation and embedding in Spurr resin) compared reasonably well to that observed in young hyphae grown on agar (Plate 3.8, Figs 1 & 2, Plate 4.2, Fig. 1 & Plate 4.3, Figs 1 & 2). All components of the cytoplasm including mitochondria, multivesicular bodies, lipid droplets, endoplasmic reticulum and the Spitzenkörper were particularly well-preserved in these sporelings (Plate 4.6, Figs 1 & 2). In addition, abundant fibrillar matrix material was observed at their surface (Plate 4.6, Fig. 1). On closer examination, numerous large vacuoles were also apparent throughout the cytoplasm (Plate 4.6, Fig. 3). These vacuoles contained electron-opaque particulate deposits (Plate 4.6, Fig. 3) and were only occasionally observed in young hyphae grown on agar (not shown here). Golgi elements were also observed throughout the cytoplasm and associated with the endoplasmic reticulum (Plate 4.6, Fig. 4).

Postembedment labelling of control sporelings, using Concanavalin A (Con-A), revealed irregular labelling of the fungal cell wall and fibrillar matrix (Plate 4.7, Figs 1 & 2). Traces of Con-A labelling were also observed throughout the cytoplasm (Plate 4.7, Fig. 1). However, it was evident that Con-A bound more heavily to lipid droplets and multivesicular bodies (Plate 4.7, Figs 3 & 4). In vesiculated areas of the hyphal tip, Con-A labelling was often detected in both small (Plate 4.7, Fig. 5, small arrows) and large (Plate 4.7, Fig. 5) vesicles. In addition, Con-A binding-sites appeared to be located in the cytoplasm surrounding these vesicles (Plate 4.7, Fig. 5). The endoplasmic reticulum and Golgi equivalents were sparsely labelled (Plate 4.7, Fig. 4 & 6). In addition Con-A-binding sites were rarely associated with mitochondria or nuclei. With the omission of

Con-A (Plate 4.7, Fig. 7) or following pre-adsorption of Con-A binding sites with the hapten, α -methyl-D-mannoside (Plate 4.7, Fig. 8), no labelling of the fungal cell wall, fibrillar matrix or cytoplasmic contents was observed.

TEM observations of BFA-treated sporelings (Plate 4.8, Figs 1 & 2) revealed a significant difference in their ultrastructural morphology compared to that of the controls (Plate 4.6, Figs 1 & 3). Although the ultrastructure of the fungal cell wall and fibrillar matrix material appeared unaffected by the drug treatment, the cytoplasm appeared highly vacuolate (Plate 4.8, Figs 1 & 2), more so than the controls (Plate 4.6, Fig. 3). In BFA-treated sporelings, large vacuoles filled the cytoplasm to the exclusion of many organelles. In addition, they appeared similar to vacuoles observed in control sporelings (Plate 4.6, Fig. 3), since they also contained electron-opaque particulate deposits. Growing apices of BFA-treated sporelings were rarely observed.

Post-embedment Con-A-labelling of BFA-treated sporelings showed that Con-A-binding sites were abundant in regions of the cytoplasm largely devoid of ribosomes (Plate 4.8, Fig. 3). Such regions were absent from control treatments and appeared to represent areas of copious vesiculation (Plate 4.8, Fig. 4, arrows). Irregular labelling of the cell wall and fibrillar matrix was also observed (Plate 4.8, Fig. 3). Smooth membrane cisternae resembling Golgi equivalents were also present throughout the cytoplasm. Such smooth membrane cisternae were often surrounded by cytoplasmic areas heavily-labelled with Con-A (Plate 4.8, Fig. 5). In these areas, it could not be readily determined whether the labelling occurred free in the cytoplasm or if it was restricted to a set of intracellular structures. Extensive Con-A-binding sites were also associated with multivesicular bodies (Plate 4.8, Fig. 6) and located within the lumen of large vacuoles containing electron-opaque particulate deposits (not shown here).

4.2.9 Effects of BFA on the secretion of cell wall components and cell surface antigens in young hyphae of *B. cinerea*

IEM studies of BFA-treated hyphae of *B. cinerea*, prepared for immunocytochemistry using mild aldehyde fixation, PLT-dehydration and embedding in LR White resin and subsequently immunolabelled with the BC-KH4 MAb or a polyclonal antiserum directed against polymers containing $\beta(1\rightarrow3)$ glucose linkages, revealed a marked effect of the

drug on the ultrastructural location of the corresponding antigens [BC-KH4-labelling: Plate 4.9, $\beta(1\rightarrow3)$ glucan antibody-labelling: Plate 4.10]. TEM observations of BC-KH4 labelled and BFA-treated hyphae indicated an increase in labelling throughout the translucent fungal cell wall and the fibrillar matrix material surrounding the germ tube (Plate 4.9, Fig. 1) compared to the controls (Plate 4.9, Fig. 2). In addition, prominent cytoplasmic alterations were observed as a result of exposure of the sporelings to BFA (Plate 4.9, Fig. 1). Such alterations were apparent as translucent areas in the cytoplasm and they were often associated with the lateral walls of the hypha (Plate 4.9, Fig. 1). However, they were rarely observed in the cytoplasm of control treatments (Plate 4.9, Fig. 2). On closer examination, this translucent matrix appeared to be composed of numerous vesicles or small vacuoles (Plate 4.9, Figs 3 & 4). However, as a result of the low contrast and lack of membrane preservation of the LR White preparations, as mentioned previously, it was difficult to ascertain whether or not such cytoplasmic alterations corresponded to membrane-bounded compartments. Regardless of this fact, it was apparent that following BFA treatment of sporelings, BC-KH4 MAb-binding sites were located intracellularly and they appeared to be associated to some extent with these areas of cellular distortion (Plate 4.9, Fig. 3). It must also be noted that sparse labelling of such areas also occurred in control hyphae following omission of the BC-KH4 MAb (Plate 4.9, Fig. 4).

Similarly, TEM observations of ultra-thin sections of BFA-treated hyphae, immunolabelled with the antiserum to $\beta(1\rightarrow3)$ oligosaccharides, indicated the presence of further antibody-binding sites throughout the copious areas showing cytoplasmic alterations and associated with the lateral walls of hyphae (Plate 4.10, Figs 1 & 2). In control treatments, i.e. in untreated hyphae, $\beta(1\rightarrow3)$ glucan antibody-labelling was generally restricted to the translucent cell wall (Plate 4.10, Fig. 3) and septa (not shown here). Although occasionally, antibody-binding was also located in small vacuoles or large vesicles.

Furthermore, following immunolabelling of BFA-treated hyphae with the polyclonal antiserum directed against polymers containing $\beta(1\rightarrow4)$ glucose linkages, gold labelling was also detected in these translucent areas showing cellular distortion (Plate 4.10, Fig. 4). In contrast, very few antibody-binding sites were observed in such regions when either polyclonal antiserum was omitted (Plate 4.10, Fig. 5). It was interesting to note that such prominent regions of cytoplasmic alterations, which were

also characteristic of PLT-dehydrated and -embedded BFA-treated hyphae, were rendered electron-opaque following *en bloc*-staining with aqueous uranyl acetate, post-fixation in osmium tetroxide and post-staining with lead citrate (Plate 4.10, Fig. 6). However, these regions exhibited no discernible structure comparable to an individual or subset of membrane-bounded compartments.

4.2.10 Immunolocalisation of fungal cell wall components and extracellular surface molecules in young hyphae of *B. cinerea* prepared for IEM using cryo-fixation and molecular distillation drying

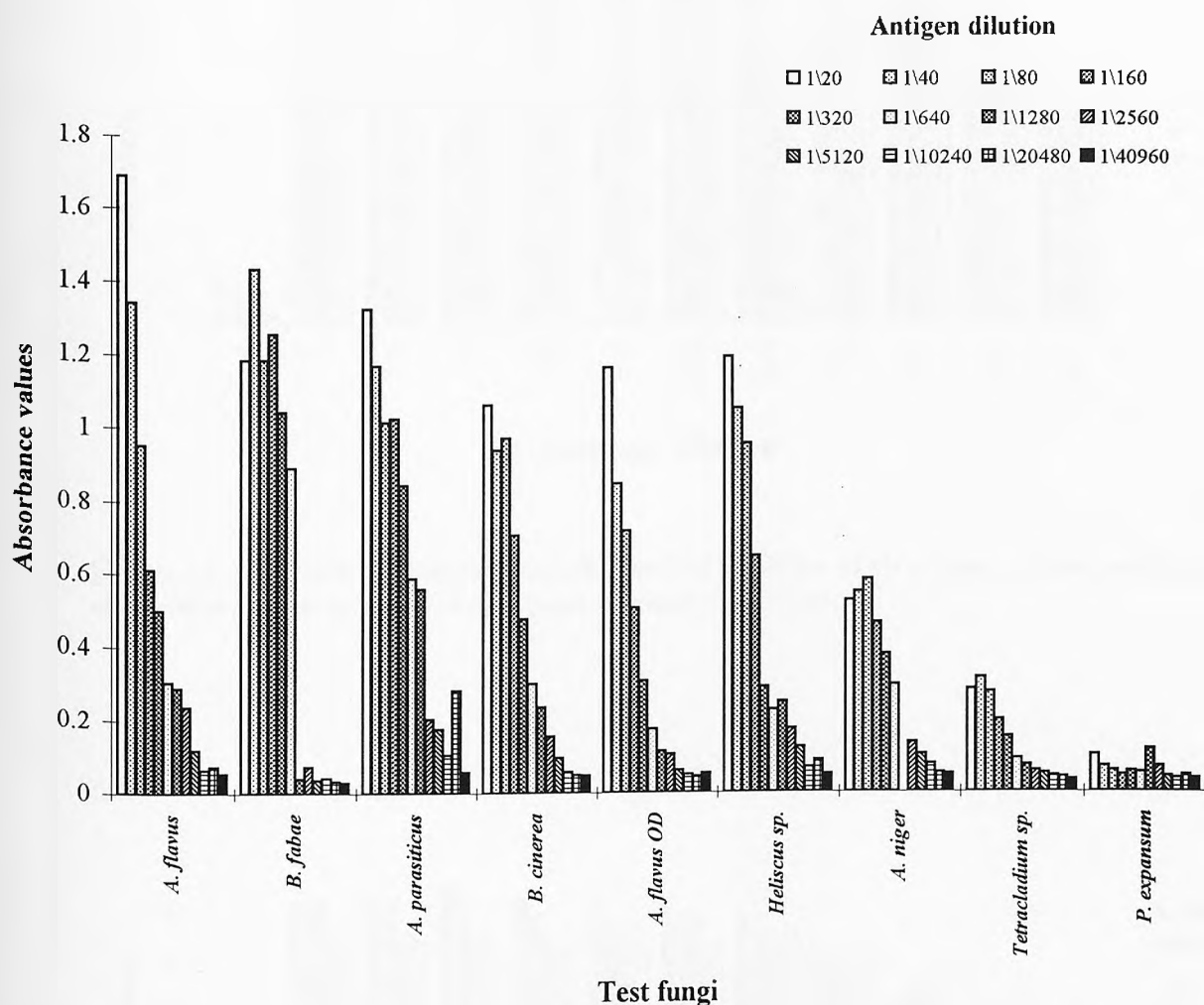
Immunoelectron microscopy of young hyphae of *B. cinerea* prepared by cryo-fixation and molecular distillation drying, using BC-AF7 or BC-KH4 MAbs, revealed a significant increase in the retention and preservation of antigenic sites (Plate 4.11, Figs 1-4) compared to that observed in PLT-dehydrated and embedded samples (Plate 3.9, Figs 1-4). Profuse labelling of the fibrillar matrices surrounding the hyphae was observed (Plate 4.11, Fig. 2-4). In addition, abundant MAb-binding sites were located free in the agar medium, and often at some distance from the fungal cell wall and fibrillar matrices (Plate 4.11, Fig. 1). Gold labelling was rarely observed inside the fungal cell. In control treatments, i.e. when the primary antibody was omitted, relatively few antibody-binding sites were observed at the fungal cell wall and fibrillar matrices (Plate 4.11, Figs 5 & 6). In many cases, the plasma membrane, fibrillar matrix material and certain membrane-bounded organelles e.g. small vacuoles and mitochondria were especially well-preserved (Plate 4.11, Figs 1-6).

IEM studies of cryo-fixed and low-temperature freeze-dried *B. cinerea* hyphae, using the polyclonal antiserum directed against $\beta(1\rightarrow3)$ glucan also showed profuse labelling of the fungal cell wall (Plate 4.12, Fig. 1). Using an antiserum directed against purified fimbriae of *Microbotryum violaceum* (Plate 4.12, Figs 2 & 3), no specific labelling of the fibrillar matrices was detected in such low-temperature freeze-dried preparations of *B. cinerea* compared to the controls (Plate 4.12, Fig. 5) and BC-KH4-immunolabelled material (Plate 4.12, Fig. 4).

4.2.11 Specificity of BC-KH4 MAb for *Botrytis* species and other fungi

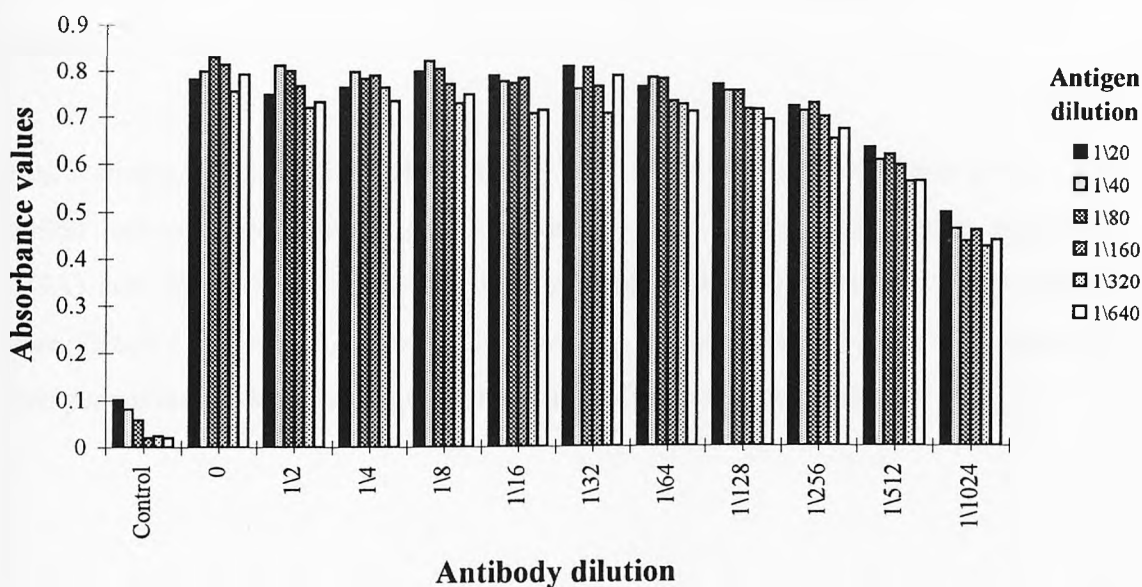
When the MAb BC-KH4 was applied at full-strength in a plate-trapped antigen enzyme-linked immunosorbent assay (ELISA), using a checkerboard titration system for both the antibody and antigen, the hybridoma supernatant recognised surface antigens from *Botrytis cinerea*, *B. fabae*, two isolates of *Aspergillus flavus*, *A. parasiticus*, and a species of *Heliscus*, but not *Tetracladium marchalianum* or *Penicillium expansum* (Graph 4.2.; see also Appendix C). Low absorbance values were observed with surface washings of *Aspergillus niger* (Graph 4.2; Appendix C). The undiluted BC-KH4 MAb gave high absorbance values in ELISA tests against surface antigens of *B. fabae* diluted approximately 600-fold. In contrast, when undiluted BC-KH4 MAb was tested against surface washings from a range of other fungi including *B. cinerea*, at this antigen dilution, only low absorbance values were recorded (Graph 4.2).

Further ELISAs, using checkerboard titration systems in order to determine the optimum reactant concentrations of the BC-KH4 MAb and the corresponding antigen, revealed that a 130-fold and 60-fold dilution of the BC-KH4 MAb gave absorbance values equal to or greater than 90% of the maximum values observed, when tested against surface washings of *B. cinerea* and *B. fabae* (diluted 1:20 in PBS) respectively (Graph 4.3. & 4.4; Appendix D). Moreover, at such antibody dilutions, the BC-KH4 MAb was sufficient to detect the corresponding antigen in surface washings of *B. cinerea* and *B. fabae* diluted up to 80- and 20-fold respectively.

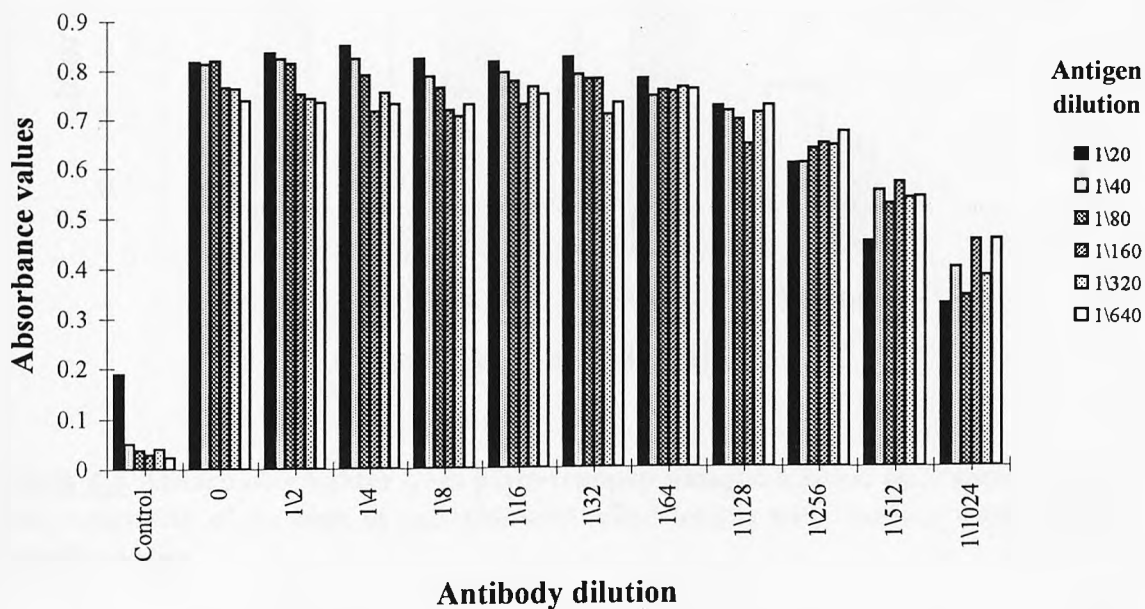


Graph 4.2 Absorbance values from plate-trapped antigen ELISA tests showing the cross-reactivity of the BC-KH4 MAb with surface antigens of *Botrytis* species and other fungi

The BC-KH4 MAb was employed at full-strength and a checkerboard titration system was employed for dilution of antigen (e.g. 1/20 equals a 1 in 20 dilution). Absorbance values equal to less than 0.5 corresponded to no specific recognition.



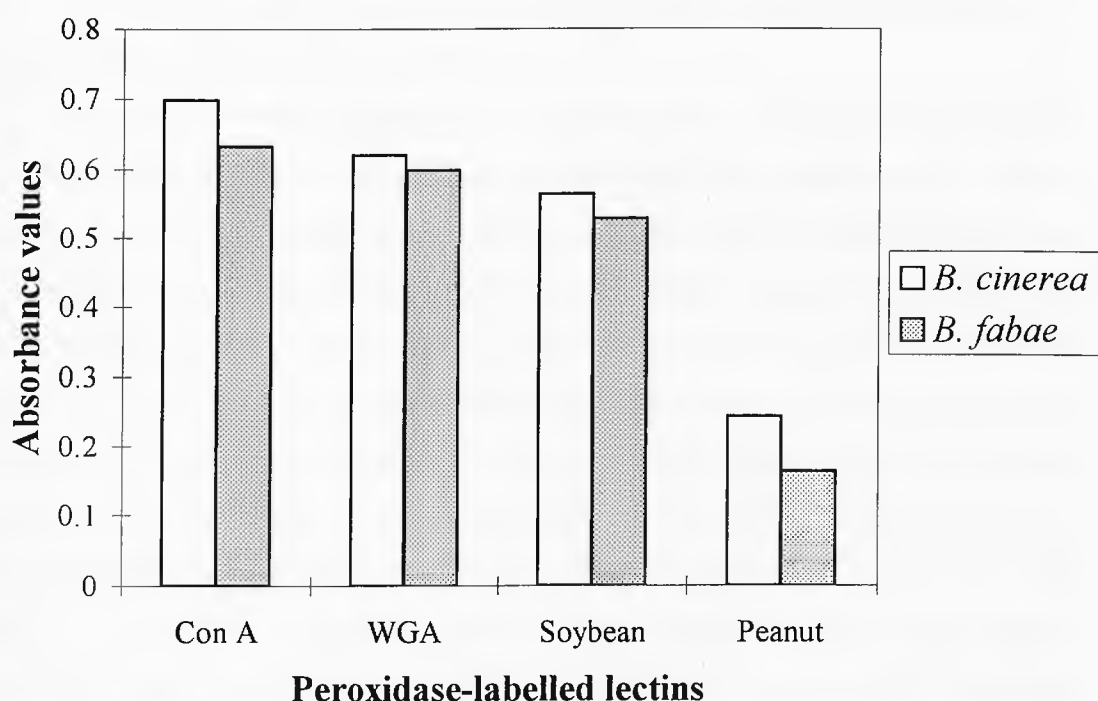
Graph 4.3 Absorbance values from ELISA tests of BC-KH4 MAb against surface washings of *B. cinerea*. Maximum absorbance value observed equals 0.83.



Graph 4.4 Absorbance values from ELISA tests of BC-KH4 MAb against surface washings of *B. fabae*. Maximum absorbance value observed equals 0.85.

4.2.12 Plate-trapped antigen ELISAs and the use of enzyme-labelled lectins for the characterisation of cell surface antigens of *Botrytis* species

Using a simple direct ELISA method and a range of commercially-available peroxidase-labelled lectins, it was found that Concanavalin A (Con-A), wheat germ agglutinin (WGA) and the soybean lectin recognised surface antigens of both *B. cinerea* and *B. fabae* (Graph 4.5; see also Appendix E). However, low absorbance values were observed when peanut lectin was tested against both fungi (Graph 4.5; Appendix E).



Graph 4.5 Absorbance values from plate-trapped antigen ELISA tests showing the cross-reactivity of a range of peroxidase-labelled lectins with surface washings of *Botrytis* species

Con-A: Concanavalin A. WGA: wheat germ agglutinin. Surface washings of fungus were diluted 1:20 in PBS. Peroxidase-labelled lectins were diluted to give a final concentration of 1.5 units/ml before use [One unit formed 1 mg of purpurogallin in 20 sec from pyrogallol at pH 6.0 at 20°C]. Absorbance values equal to less than 0.5 corresponded to no specific recognition.

4.3 Discussion

4.3.1 β -(1→3)-glucan is evenly distributed throughout the walls of *Botrytis* and is located intracellularly in small vesicles/vacuoles

The results of IEM studies presented here, investigating the wall composition of *Botrytis* hyphae, using polyclonal antisera to oligosaccharides containing β -(1→3)- and β -(1→4)-glucose, were consistent with data known on the chemical composition of the hyphal wall of ascomycetous fungi (Bartnicki-Garcia, 1968; Wessels, 1994). In brief, β -(1→3)-glucan was evenly distributed throughout the translucent walls of *Botrytis* hyphae, including septa. However, β -(1→4)-glucan was absent from the fungal cell walls. The presence of β -(1→3)/ β -(1→6)-glucan and the absence of cellulose [a polymer composed of β -(1→4)-glucose subunits] in the walls of ascomycetous hyphae are in fact both essential criteria for the taxonomy of fungi (Bartnicki-Garcia, 1968).

The observation that oligosaccharides containing β -(1→3)- and β -(1→4)-glucose were immunolocalised intracellularly in *Botrytis* hyphae and located predominantly in discrete compartments i.e. small vacuoles or large vesicles, suggests that these oligosaccharides may be synthesised intracellularly and transported to the cell surface via exocytotic vesicles. It is well known in plant cells, that the major polysaccharidic components of the extracellular matrix (i.e. the plant cell wall) are synthesised in the Golgi apparatus and transported to the cell surface via the exocytosis of vesicles (Driouich, Faye & Staehelin, 1993). In filamentous fungi, as mentioned previously, it has been suggested that the Golgi equivalents are the sites of polysaccharide synthesis (Grove & Bracker, 1970; Sewall, Roberson & Pommerville, 1989; see introduction to this chapter, Section 4.1.2). Vesicles carrying polysaccharidic material are thought to bud off from the Golgi complexes and are subsequently transported vectorially to the growing apex of the hypha where they fuse with the plasma membrane (Grove & Bracker, 1970; Sewall, Roberson & Pommerville, 1989; see also Diagram 4.1). In this present study, numerous vesicles appeared to be associated with the Golgi bodies of *Botrytis* hyphae and sporelings observed by TEM and prepared using conventional fixation and embedding protocols. Furthermore, similar vesicles were also located at the apical dome in hyphal tips.

Studies on glucan biosynthesis³ in yeast and other fungi have, however, shown little evidence for the synthesis of oligosaccharides of β -(1 $\rightarrow$ 3)-glucose inside the fungal cell. Moreover, autoradiographic studies on fungi, yeast and oomycetes have shown that such oligosaccharides (and chitin) are directly deposited outside the plasma membrane and predominantly at the growing apices (Bartnicki-Garcia & Lippman, 1969; Gooday, 1971; Shematek, Braatz & Cabib, 1980; Fèvre & Rougier, 1982; Cabib, Bowers & Roberts, 1983; Wessels, Sietsma & Sonnenberg, 1983). In a recent study, MAbs prepared against β -glucan synthases have also been observed to bind preferentially to the plasmalemma of protoplasts released from apical cells of *Saprolegnia* (Fèvre, Girard & Nodet, 1990). This result also suggests that these enzymes are localised in growing apices only. In contrast, there is some evidence that β -(1 $\rightarrow$ 6)-glucan chains are made within the cytoplasm (Boone *et al.*, 1990; Brown *et al.*, 1993). In view of the available data, it is speculated that the antisera to oligosaccharides of β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucose recognise appropriate domains on glycoproteins or proteoglycans manufactured inside the fungal cell. It is well-known that filamentous fungi produce large amounts of polysaccharides, glycoproteins and proteoglycans which perform a wide variety of biological functions.

4.3.2 Are the surface antigens recognised by the *Botrytis*-MAbs secreted at hyphal tips?

A summary of the distribution of the *Botrytis*-MAb (i.e. BC-KH4, BC-FD7-G9 and BC-AF7) binding sites in the cell wall of *Botrytis* hyphae is illustrated in Diagram 4.3. Previous IEM studies of *Botrytis* hyphae confirmed that the fibrillar matrix is highly reactive to MAbs BC-KH4, BC-FD7-G9 and BC-AF7, thus indicating the presence of a glycoprotein component

³ Previous research on the ascomycete yeast, *Saccharomyces cerevisiae* and the basidiomycete, *Schizophyllum commune* has shown that initially, β -(1 $\rightarrow$ 3)-glucan is synthesised as a water/alkali-soluble precursor (Wessels & Sietsma, 1979; Wessels, Sietsma, & Sonnenberg, 1983). Chains of β -(1 $\rightarrow$ 3)-glucan are synthesised at the plasma membrane by a UDP-glucose-dependent glucan synthetase (Shematek, Braatz & Cabib, 1980; Orlean, 1982) which extrudes its product out from the membrane by a vectorial process that allows only linear molecules to be formed (Cabib *et al.*, 1988). During hyphal wall growth a steady-state amount of plastic wall material is continuously produced at the apex, this process has been dubbed the “steady-state theory of apical growth” by Wessels (1986). Sub-apically, the nascent β -(1 $\rightarrow$ 3)-glucan chains become covalently linked to chitin (or more precisely glucosaminoglycan) to form an alkali-insoluble complex (Sietsma & Wessels, 1979, 1981; Surarit, Gopal & Shepherd, 1988). Little is known about the process by which β -(1 $\rightarrow$ 6)-glucan branches are attached to nascent β -(1 $\rightarrow$ 3)-glucan chains or the timing of this process. However, recently a glucosyltransferase has been discovered which may be responsible for the attachment of single β -(1 $\rightarrow$ 6)-glucose branches to linear β -(1 $\rightarrow$ 3)-glucan (Hartland, Emerson & Sullivan, 1991).

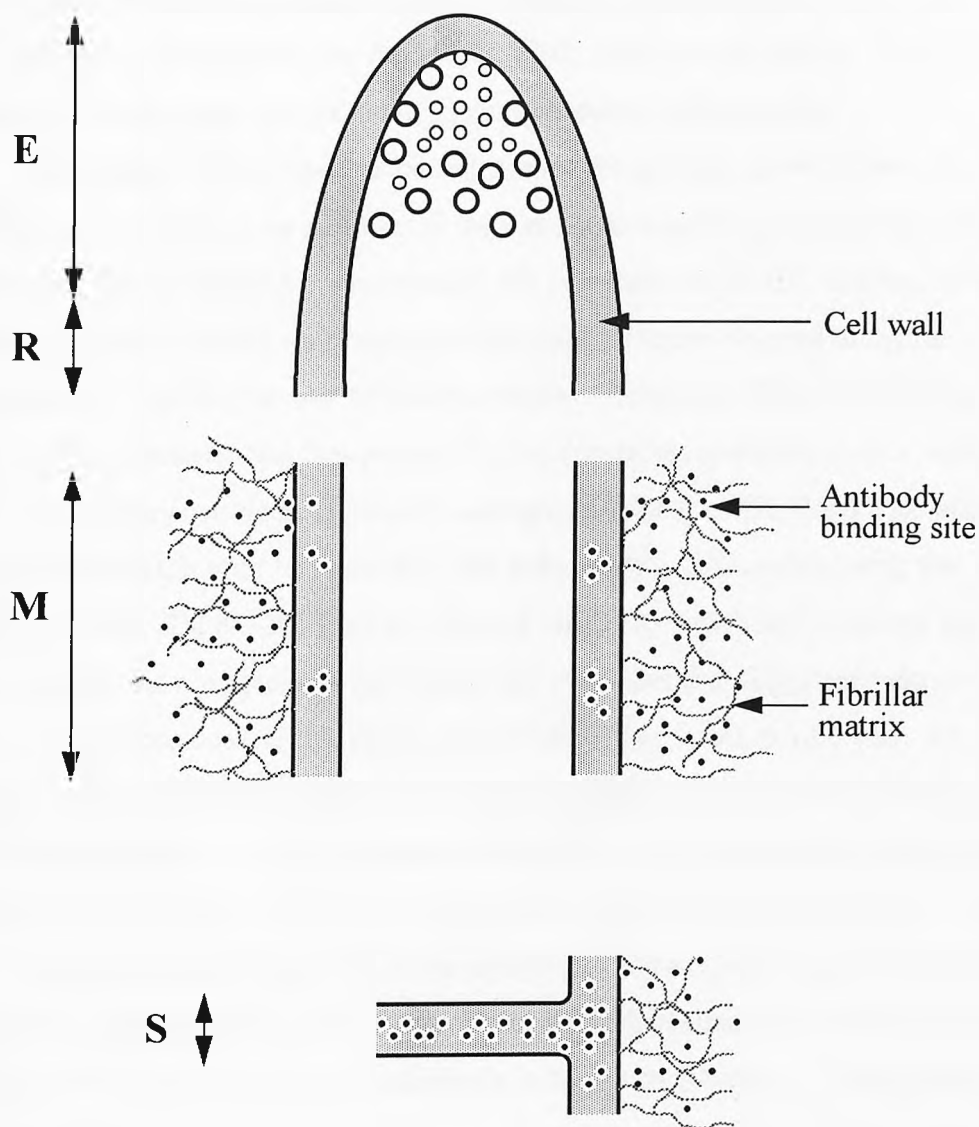


Diagram 4.3 Summary of *Botrytis*-MAb (BC-KH4, BC-FD7-G9 & BC-AF7) binding sites in young hyphae of *Botrytis* species

IEM studies showed that the surface antigens were not detected at hyphal tips. Instead, they were located subapically, in discrete regions of the hyphal walls and throughout the fibrillar matrix. **E:** Extension zone showing growing apex of hypha. Vesicles accumulate in the apical dome to the exclusion of all other organelles. **R:** Rigidification zone. **M:** Maturation zone. **S:** Cross-wall formation zone. Antibody-binding sites were observed throughout the septum.

(See Chapter Three, Section 3.3.2.4). Traces of oligosaccharides containing β -(1 $\rightarrow$ 4)-glucose were also detected throughout the fibrillar matrix. Since it is likely that the β -(1 $\rightarrow$ 4)-glucan antiserum recognised corresponding domains on glycoproteins located in the extracellular matrix, it is suggested that the extracellular matrix of *Botrytis* species, like that of many pathogenic fungi (Nicholson & Epstein, 1991; Mendgen & Deising, 1993), may be composed of a heterogeneous mixture of polysaccharides and glycoproteins.

The absence of *Botrytis*-MAb binding sites at the growing apices of *Botrytis* hyphae (see Diagram 4.3) seems to be paradoxical⁴ since in filamentous fungi, it is widely known that proteins (*viz.* glycoproteins) are synthesised in the cytoplasm on the ER, directed to the cell surface by secretory vesicles via Golgi equivalents and ultimately secreted at hyphal tips (see introduction to this Chapter and references therein). Moreover, TEM observations of *B. cinerea* hyphae confirmed that they possessed an accumulation of vesicles at their hyphal tips and all the necessary machinery required to undergo secretion (*i.e.* ER, Golgi equivalents and exocytotic vesicles). It must be stated here that unfortunately IEM studies, using the specific marker antibodies, JIM 84, 2E7 (anti-HDEL) and MAC 256 (anti-KDEL) showed that these plant antibodies did not recognise the appropriate organelles (*i.e.* Golgi equivalents or ER) under the conditions used in this study, and therefore they could not be used for further investigations of the secretory pathway in *Botrytis* hyphae. It is conceivable that the lack of BC-KH4 binding sites at the tips of growing hyphae of *B. cinerea* may simply reflect a loss of antigenic determinants as a result of the mode of preparation of the sample for TEM. Such a loss of antigen may occur as a result of the solubility of the antigen in aqueous fixatives, its sensitivity to chemical fixation and/or organic solvents. Furthermore, the finding that these surface molecules were only located subapically in this study (Diagram 4.3) may adequately be explained if the antigen was secreted in a water-soluble form at the hyphal tips. Subsequently, this water-soluble component may have become modified sub-apically to form an insoluble complex in the fungal cell wall and fibrillar matrix [*c.f.* the cross-linking of the alkali-soluble β -(1 $\rightarrow$ 3)-glucan to chitin to form an alkali-insoluble complex (Sietsma, Sonnenberg & Wessels, 1985)].

An alternative suggestion is that the cell surface antigens may be synthesised and/or modified *in muro*. In an early report, Hunsley & Kay (1976) intimated that during maturation of the hyphal wall, new material may be synthesised on the outside of the existing wall remote

⁴ In view of the glycoprotein content of the fibrillar matrices of *Botrytis* hyphae, as shown by BC-KH4, BC-FD7-G9 and BC-AF7 MAb recognition, it seems highly likely that these antigenic determinants are, like all proteins/glycoproteins, secreted at the hyphal tips.

from cytoplasmic contact. However, there has been no further evidence to support this idea. Methods (e.g. freeze-substitution, molecular distillation drying) are required which allow increased retention of soluble antigens in fungal material prepared for immunoelectron microscopy before a definitive answer can be given to the question of whether or not these specific surface antigens are secreted at hyphal tips.

4.3.3 Possible role(s) of the surface antigens in biological processes

The location of specific cell surface antigens in walls of *Botrytis* hyphae may provide clues to the possible role(s) of these molecules in biological processes. At hyphal tips, proteins/glycoproteins readily diffuse through the loosened wall region into the surrounding medium by bulk flow, without going through pores (Wessels, 1990). However, not all of the proteins/glycoproteins that are vectorially transported to the hyphal tip are immediately released into the surrounding medium (Wessels, 1994; see introduction to this Chapter, Section 4.1.1). Indeed, some may be simply caught in the wall fabric during secretion as a result of the presence of relatively impermeable outer wall components (Nobel & Barnett, 1991). Such proteins/glycoproteins may possess assimilatory functions or they may be involved in infection or recognition processes. Analogously, *Botrytis*-antigens may become trapped in the cell wall in a similar manner and possess similar functions.

On the other hand, it has been reported that proteins may be targeted to the fungal cell wall in order to form specific cross-linkages (Schreuder *et al.*, 1993) or insoluble complexes like hydrophobins (Wessels, 1994). There is as yet no evidence to suggest that the surface molecules of *Botrytis* hyphae and sporelings resemble hydrophobin-like molecules. Nevertheless, in view of their external location, it is probable that the surface molecules of *Botrytis* species contribute to specific biological functions, e.g. morphology, attachment and penetration of the host (Farkas, 1979). The possible role of the surface molecules of *Botrytis* hyphae and sporelings in adhesion processes has already been alluded to (see Chapter Three, Section 3.3.2.2). Furthermore, the likelihood that such molecules play adhesive and other roles in the development of the host-pathogen relationship is discussed in Chapters Five & Six.

4.3.4 Mode of action of brefeldin A and Pyrimethanil on conidial germination and radial growth of *B. cinerea*

Mode of action studies using the secretory inhibitors, brefeldin A (BFA) and pyrimethanil, revealed that of the treatments applied, only a high concentration of BFA ($100 \mu\text{g}.\text{ml}^{-1}$) affected *B. cinerea* conidial germination and germ tube growth. Although it has been reported previously that pyrimethanil, when applied at a concentration of ($1 \mu\text{g}.\text{ml}^{-1}$), has no effect on conidial germination of *B. cinerea* grown *in vivo* (Milling, Richardson & Daniels, 1993), the effects of BFA on conidial germination and germ tube growth of *Botrytis* species were hitherto, unknown. Preliminary experiments also revealed that BFA did not affect adhesion of *Botrytis* sporelings to glass surfaces. At this stage, the primary mode of action of BFA on conidial germination and germ tube growth cannot be fully elucidated.

As mentioned previously, BFA is a heterocyclic lactone. When BFA is added to the medium of cultured animal cells, it induces a reversible block in secretion, disrupts the Golgi apparatus and causes a redistribution of Golgi-resident proteins into the ER (Lippincott-Schwartz *et al.*, 1989; Klausner, Donaldson & Lippincott-Schwartz, 1992; Rothman & Orci, 1992). As a result of rapid advances in molecular genetics of the yeast *Saccharomyces cerevisiae*, much research into the mode of action, i.e. the molecular targets of BFA has been carried out on yeast (Shah & Klausner, 1993; Vogel *et al.*, 1993; Rambourg *et al.*, 1995). Current evidence suggests that the drug acts to prevent the association of coat proteins, which are required to facilitate vesicle budding from Golgi cisternae, with their respective membrane (Donaldson *et al.*, 1990; Orci *et al.*, 1991), thus causing a block in the secretory pathway. In contrast, the anilino-pyrimidine fungicide, pyrimethanil, has been described as a potential inhibitor of the secretion of proteins associated with pathogenesis in *B. cinerea* (Milling & Richardson, 1995). In this report, these authors suggested that pyrimethanil inhibits protein secretion at a post-translational stage in the secretory pathway of *B. cinerea*.

The results of plate assays showed that the radial growth of *B. cinerea* on PDA or GGA media was extremely sensitive to BFA and pyrimethanil at inhibitor concentrations as low as 10 and $0.1 \mu\text{g}.\text{ml}^{-1}$ respectively over the initial growth period⁵. At this stage, an acute comparison of *B. cinerea* radial growth on the two different media cannot be made since the growth period was different in each case. Nonetheless, it is interesting to discuss briefly such a potential difference in the sensitivity of *B. cinerea* radial growth which was observed when

⁵ *B. cinerea* was grown on PDA and GGA supplemented with the inhibitor for 72 and 96 h respectively.

the fungus was grown on two different agar media supplemented with pyrimethanil. Recently, it was reported that this fungicide was most active in media where the fungus had to utilise extracellular enzymes in order to mobilise the nutrients it required for growth (Milling & Richardson, 1995). Thus, when the fungus was grown on PDA, it required few extracellular enzymes since this medium contains many simple sugars and amino acids. In the current study, the fact that the reverse situation was apparent when the fungus was exposed to BFA on different agar media, i.e. growth inhibition was greater when the fungus was grown on PDA (compared to GGA) supplemented with BFA, may indicate that the modes of action of pyrimethanil and BFA are somewhat different. However, further research on the precise mode(s) of action of both pyrimethanil and BFA is required.

It must also be mentioned here that in a recent report, it was observed that the growth inhibition of the rice blast fungus, *Magnaporthe grisea* on PDA supplemented with BFA ($50 \mu\text{g.ml}^{-1}$), decreased after 48 h and during a subsequent 72 h growth period (Bourett & Howard, 1995). Concurrent to this, in the present study, ultrastructural observations indicated that elements of the secretory pathway (e.g. ER and Golgi equivalents) of *B. cinerea* appeared, in most cases, to be unaffected by the presence of BFA ($10 \mu\text{g.ml}^{-1}$) or pyrimethanil ($0.1 \mu\text{g.ml}^{-1}$) when the fungus was exposed to either compound for a prolonged period (72 h). It must be remembered here that the radial growth of *B. cinerea* was significantly arrested at these inhibitor concentrations. In view of these results and despite the fact that Golgi equivalents appeared on a few occasions to be disrupted, it is presumed that the fungus was able to grow normally in the presence of the inhibitor, but at a much reduced rate. In addition, the possibility that *B. cinerea* can metabolise BFA and/or recover from the effects of prolonged exposure to BFA treatment (or pyrimethanil) cannot be ignored. It must be stated that it is also feasible that additional target site(s) of BFA, for example the site of accumulation of vesicles at hyphal tips (Bourett & Howard, 1995) remained undetected in this study. A possible heterogeneity in the target sites of BFA in filamentous fungi compared to animal, plant and yeast cells has also already been described (Bourett & Howard, 1995; Satiat-Jeunemaitre *et al.*, 1996).

4.3.5 Comparison of the ultrastructure of *Botrytis* sporelings and hyphae grown *in vitro*

The overall ultrastructure of *Botrytis* sporelings, as revealed by TEM, was dependent upon the culture conditions, i.e. whether the fungus was grown in liquid culture or on solid agar. In both cases, Spitzenkörper were located at the growing apices and all elements of the secretory pathway (ER, Golgi equivalents, exocytotic vesicles) were readily identified throughout the cytoplasm. Yet, when sporelings were grown in liquid suspension, numerous intracellular vacuoles containing electron-opaque particulate deposits, were also observed. Only a few of these vesicles were apparent in sporelings grown on agar. Both solid and liquid media represent two very different environments which can support fungal growth. Thus, it is perhaps not surprising that the gross morphology of the cytoplasm of *Botrytis* hyphae varied according to the growth conditions applied. Fungal vacuoles perform a variety of tasks including osmotic and pH regulation, and the storage of certain ions, basic amino acids and insoluble polyphosphate granules (Klionsky, Herman & Emr, 1990). It is suggested that an increase in number of vacuolar compartments may simply reflect an adaptation of the fungal sporelings to growth in liquid culture.

4.3.6 Use of Concanavalin A as a marker for the endomembrane system and as an aid to understanding the effect(s) of BFA on the ultrastructure of *Botrytis* hyphae

In the present study, the application of a single-sided grid-labelling method in conjunction with concanavalin A (Con-A) to specifically label the endomembrane system of conventionally-fixed sporelings of *B. cinerea* revealed that numerous Con-A binding sites were located in multivesicular bodies and lipid droplets in *B. cinerea* sporelings indicating the occurrence of α -D-mannosyl and/or α -D-glucosyl residues in these areas. However, the lectin showed no specificity towards the endomembrane system as described previously (Bourett & Howard, 1994). This discrepancy of results may be explained by the fact that the previous researchers carried out a more sensitive double-sided Con-A labelling protocol and employed fungal material prepared by freeze substitution. The detection of only low levels of Con-A receptors in the walls and fibrillar matrices of *Botrytis* species suggests that the

polysaccharide moieties of the glycoproteins located in the walls and fibrillar matrices may consist of other sugars e.g. xylose, arabinose, rhamnose. Naturally, such a suggestion requires further investigation.

When BFA was added to *B. cinerea* sporelings in liquid suspension, a vesiculation of the cytoplasm was observed. Con-A binding sites accumulated in these areas and therefore indicated an abundance of α -D-mannosyl and/or α -D-glucosyl residues. Prominent vesiculation of the cytoplasm was observed previously following the BFA treatment of most plant root cells (Satiat-Jeunemaitre & Hawes, 1993; Satiat-Jeunemaitre *et al.*, 1996). Such vesicular areas were characteristic of BFA-treated root cells and termed “BFA compartments”. It was also suggested that the “BFA compartment” was derived from a trans-driven vesicular fragmentation of the Golgi cisternae (Satiat-Jeunemaitre & Hawes, 1992a,b). Similarly, in BFA-treated *B. cinerea* sporelings, the highly vesiculated areas are thought to correspond to the breakdown of a subcellular compartment. However, there was no specific relationship between these areas and the ER and/or Golgi equivalents. Even so, it is still possible that BFA blocks the secretory pathway in *B. cinerea* sporelings by causing a disruption of vesicle trafficking. Thus, in this case, it is conceivable that the transport of vesicles carrying polysaccharidic/glycoprotein material to the cell surface was prevented and as a consequence, led to the formation of these Con-A positive vesiculated areas. Evidently, the precise mode of action of BFA on the endomembrane compartments remains to be determined. In addition, since the cytoplasm of BFA-treated sporelings consisted of numerous vacuoles which contained electron-opaque deposits, it was considered that these sporelings may have been subjected to severe constraints as a result of the presence of the drug.

4.3.7 Immunocytochemical evidence to suggest that BFA blocks vesicular transport to the cell surface in sub-apical regions of *Botrytis* hyphae

The morphological effects of BFA on membrane-bounded compartments within the hyphal tips of two fungi, *Schizophyllum commune* (Rupeš *et al.*, 1995) and the rice blast fungus, *Magnaporthe grisea* (Bourett & Howard, 1995) have been characterised ultrastructurally in cells prepared by freeze substitution using osmium tetroxide. These authors reported that BFA had a profound effect on membrane trafficking. Freeze substitution using osmium

tetroxide is however, generally avoided if immunocytochemical studies are to be carried out, since the antigenicity of the samples may be severely compromised by aqueous fixatives, solvents and/or epoxy resins. In this study, the PLT method of preparation involving mild aldehyde fixation and embedding in LR White acrylic resin was employed for the preparation of *B. cinerea* hyphae for immunocytochemistry. As a consequence of the low contrast afforded by these specimens, the effects of BFA on the endomembrane compartments *per se* could not be ascertained. Nevertheless, it was clear that the drug had a deleterious effect on the morphology of the cytoplasm. TEM revealed that the inhibitory effects of BFA ($100\text{ }\mu\text{g.ml}^{-1}$) on radial growth of *B. cinerea* after 4 days was accompanied by cytoplasmic malformations throughout the hyphal cells. These cellular distortions were thought to correspond to a further elaboration and breakdown of the Con-A-positive vesiculated areas observed when sporelings were exposed to BFA at a lower concentration and for a shorter time interval. IEM studies revealed that oligosaccharides of β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucose were also located throughout these perturbed areas of cytoplasm.

It is suggested that the fungus was severely affected by both the presence of a high dose of BFA ($100\text{ }\mu\text{g.ml}^{-1}$) and a prolonged exposure to the drug. As a result, these BFA-treated cells showed signs of autolysis. In a previous study, using BFA-sensitive mutants of *Saccharomyces cerevisiae*, it was shown that after 30-40 min exposure to BFA at a concentration of $100\text{ }\mu\text{g.ml}^{-1}$, these cells displayed non-specific transport defects (Rambourg *et al.*, 1995). In contrast, after only a short period of BFA treatment (0.5-5 min), the drug has been shown to induce, in these mutants, a hypertrophy of the *cis*-Golgi networks resulting from a block of protein transport at the exit end of the *cis*-Golgi compartment (Rambourg *et al.*, 1995). Evidently, in order to fully characterise the effects of BFA on the secretory pathway of *B. cinerea* a range of concentrations of BFA must be tested, in combination with a variety of drug exposure times. In short, it is proposed that after prolonged exposure of *B. cinerea* hyphae to a high dose of BFA, the drug caused a severe block in the exocytosis of vesicles to the cell surface in sub-apical regions of hyphae. Further studies are required in order to observe the effects of BFA on the growing apices of *Botrytis* hyphae.

4.3.8 Does BFA block the secretion of *Botrytis*-antigens?

It was interesting to note that IEM studies revealed that BFA did not cause a build-up of BC-KH4 MAb-binding sites inside the fungal cell. Instead, an increase in BC-KH4-binding sites was observed throughout the cell wall and fibrillar matrix. In view of this result, it is predicted that BFA does not directly affect production of the corresponding antigens. This result provides further evidence for the idea that the surface antigens of *B. cinerea*, recognised by the specific *Botrytis*-MAbs, are synthesised and/or modified *in muro*.

4.3.9 Molecular distillation drying: a novel technique for the improved preservation of fungal extracellular matrices?

Experiments involving cryo-fixation and low-temperature freeze-drying methods for the preparation of samples of *B. cinerea* hyphae for immunocytochemistry using the *Botrytis*-MAbs, revealed a significant increase in the retention and hence, preservation of the corresponding antigens at hyphal walls and throughout the fibrillar matrices. More interestingly, an abundance of antigens was located some distance from the fungal wall and fibrillar matrix in the surrounding agar. This exuberant production of antigenic determinant was not detected previously in fungal hyphae prepared for IEM by the PLT-method of dehydration and embedding in acrylic resin and immunolabelled with the *Botrytis*-MAbs. It is suggested that these additional MAb-binding sites indicated the presence of a soluble antigen, possibly a glycoprotein, which was heretofore undetected. The possibility that such soluble antigens are secreted at the growing apices of *Botrytis* sporelings would be an interesting area for future investigations.

Preliminary studies using the molecular distillation drying apparatus revealed that certain membrane-bounded organelles e.g. mitochondria, Golgi equivalents and small vacuoles were especially well preserved. In addition, it was noted that the ultrastructure of fungal membranes and fibrillar matrices was considerably enhanced following the application of this technique. In short, this low-temperature freeze-drying method offers both a means of enhancing the retention of soluble surface antigens and improving the preservation of *Botrytis* fibrillar matrices.

IEM studies of cryo-fixed and low-temperature freeze-dried hyphae of *B. cinerea*, using an antiserum directed against fimbriae of *Microbotryum violaceum* (M. Celerin, unpublished data) revealed that the fimbrial epitope could not be detected in *B. cinerea* fibrillar matrices. Previous research has established that the major component of the fimbriae of *M. violaceum* is a 74 kDa glycoprotein subunit (Gardiner & Day, 1985). The carbohydrate moiety is composed of mannose and possibly some acetylated sugars. It comprises 35% of the fimbrial subunit; the aglycone has a molecular mass of 47 kDa (Celerin *et al.*, 1995). The Av-95 antibody employed in this study has been found to mainly recognise the fimbrial protein, but it may also detect the carbohydrate moiety (M. Celerin, personal communication). The absence of the Av-95 antigen therefore suggested that the fibrils observed at the surface of *B. cinerea* hyphae were dissimilar to *M. violaceum* fimbriae. This is somewhat surprising since previous studies have suggested that the protein moiety of the fimbrial subunit is highly conserved amongst fungal species including *B. cinerea* (Celerin *et al.*, 1995). Further characterisation of the glycoprotein component of *Botrytis* fibrillar matrices is required.

4.3.10 Specificity and characterisation of BC-KH4 MAb-binding sites by plate-trapped antigen ELISAs

The results of indirect plate-trapped antigen ELISAs, using surface washings of a range of fungi, including *Botrytis* species, as the antigen and BC-KH4 as the test antibody, revealed reactivity of the BC-KH4 MAb with both *B. cinerea* and *B. fabae* but also cross-reactivity of the BC-KH4 MAb with several other fungi, notably *Aspergillus* species. In view of these results, it is evident that the *Botrytis*-MAb, BC-KH4, is specific but not genus-specific as previously thought (Bossi & Dewey, 1992). Previous IEM studies have also shown that the BC-KH4 MAb recognises fibrillar matrices associated with *Aspergillus niger* grown *in vitro* (B. Martin, personal communication). However, whether this sensitivity of *A. niger* fibrillar matrices to the BC-KH4 MAb merely reflects the immunodominant nature of the molecules comprising such matrices or a similar glycoprotein matrix to that found in *Botrytis* species cannot as yet, be determined. Evidently, further characterisation of the soluble surface antigens of both *Botrytis* and *Aspergillus* species are warranted here.

ELISA results have also emphasised the sensitivity of the BC-KH4 MAb for surface washings of *B. cinerea* and *B. fabae*. High absorbance values were obtained even when the antibody was diluted several-fold. Furthermore, lectin-binding assays revealed that the surface washings of both *B. cinerea* and *B. fabae* consisted of a large amount of α -D-mannosyl and/or α -D-glucosyl, *N*-acetylglucosamine and *N*-acetylgalactosamine residues, but relatively small amounts of α -D-galactosyl and/or α -lactose subunits. Additional experiments e.g. hapten-inhibition studies and enzyme treatments of surface antigens, are now required to characterise the BC-KH4 MAb-binding site(s) further.

Chapter Five

Chocolate spot disease of broad bean (*Vicia faba* L.)

I. Pre-penetration & pre-infection processes of wet & dry conidia of *Botrytis* species and the role of fungal extracellular matrices in the *V. faba*-*Botrytis* interaction

5.1 Introduction

Botrytis cinerea Pers.:Fr. and *B. fabae* Sard. were first considered to be the causal agents of chocolate spot disease of broad bean over 65 years ago (Sardiña, 1929, 1930). At this time, *B. fabae* was regarded as the more serious pathogen. Subsequent reports have also confirmed the greater pathogenicity of *B. fabae* on broad and field beans compared to that of *B. cinerea* (Ogilvie & Munro, 1947; Leach 1955; Deverall, 1960; Wastie, 1962). In the field, *B. fabae* forms rapidly spreading or “aggressive” lesions on the leaf surface (Sardiña, 1929, 1930). In contrast, *B. cinerea*, often forms limited or “non-aggressive” lesions only (Wilson, 1937; Leach, 1955; Deverall & Wood, 1961b; Purkayastha & Deverall, 1965; Mansfield & Deverall, 1974). The ability of *B. fabae* to kill host epidermal cells soon after penetration is the major determinant of the specificity of the fungus to *V. faba* (Hutson & Mansfield, 1980). This difference in the pathogenicity of the two fungi on *V. faba* leaves may depend upon the sensitivity of the fungus to the phytoalexin, wyerone acid and the ability of the fungus to metabolise this compound (Deverall & Vessey, 1969; Mansfield & Widdowson, 1973; Mansfield & Deverall, 1974a).

In the laboratory, the infection of bean plants (*Vicia faba* L.) by *Botrytis* species can be studied both macro- and microscopically following inoculation of excised leaves with droplets of conidial suspensions and their subsequent incubation in a moist chamber in order to allow infection to occur (Deverall & Wood, 1961a). If this simple approach is to be adopted for studies of the *V. faba*-*Botrytis* interaction, however, some forethought

is required prior to inoculation since a number of factors e.g. concentration of the inoculum, age of leaf to name but a few, have been reported to influence the infectivity of *Botrytis* species on broad bean leaves (Harrison, 1988). At high spore concentrations, germination of *Botrytis* conidia may be reduced as a result of nutrient competition or by the presence of a self-inhibitor of germination. Such an inhibitor has been extracted from conidia of *B. cinerea*; it was heat-stable but its identity has still to be determined (Masuta, 1984). On the other hand, low levels of inoculum may be unable to kill the large number of plant host cells which are required to suppress active host resistance (Mansfield & Hutson, 1980). Young *V. faba* leaves have also been shown to be less susceptible to infection by sporelings of *B. cinerea* and *B. fabae* compared to older leaves (Purkayastha & Deverall, 1965).

Previous laboratory studies have shown that the pathogenicity of *B. cinerea* on bean leaves may be manipulated by the addition of nutrients to the inoculum e.g. simple sugars (e.g. glucose, mannose), yeast extract, boric acid or pollen grains, (Deverall, 1960; Purkayastha & Deverall, 1965; Chu Chou & Preece, 1968; Mansfield & Deverall, 1971; Warren, 1972, 1976; Rossall & Mansfield, 1980) or by using very young conidia (Harrison, 1983a). In addition, germination of *Botrytis* conidia on the leaf surface and hence their ability to infect the host plant may be influenced by the presence of epicuticular waxes, leaf exudates and micro-organisms (Blakeman, 1975; Rossall & Mansfield, 1980). The effect of wiping the leaf surfaces to remove surface waxes prior to inoculation has also been reported to increase the incidence of infection of plant host surfaces by *Botrytis* species (Sutton, Rowell & James, 1984). In the field, weathering and abrasion may cause a loss of wax from the leaf surface and thus, predispose the plant to infection by *Botrytis* species.

The humidity of the air surrounding infected leaves has also been shown to have a major effect on lesion expansion (Harrison, 1988). Indeed, there have been several reports that chocolate spot disease of broad bean is more severe in humid or wet weather (Sardiña 1930; Wilson, 1937). Furthermore, it has been shown that when broad bean leaves are kept continuously in air at or close to saturation after inoculation with *B. cinerea* conidia, aggressive lesions develop (Harper, Strange & Langcake, 1981; Brown & Swinburne, 1982; Harrison, 1983a). However, this is not always the case, since only limited i.e. non-expanding lesions appeared following inoculation of *V. faba* leaves with *B. cinerea* conidia and incubation under conditions of high humidities (Mansfield & Deverall, 1974). This chapter describes studies of the pre-penetration and pre-infection

processes of wet and dry conidia of *B. cinerea* and *B. fabae* on *V. faba* leaves using a simple sandwich box-technique (Deverall, 1961a) to provide a humid chamber for the initiation of the disease process. The design of experiments was carefully considered in view of the aforementioned factors which may alter the infectivity of *Botrytis* species on the host plant.

Both *B. fabae* and *B. cinerea* are regarded as facultative necrotrophs since they can invade plant tissue through wounds or via senescent or dead plant parts (Verhoeff, 1980). In an early study (Wilson, 1937), it was observed that both fungi flourished as saprophytes on fallen leaflets in bean fields. Conidia are a primary source of inoculum. Therefore, it is not surprising that the mechanisms by which conidia are dispersed and the processes by which conidial germ tubes invade undamaged plant tissues are of great interest to plant pathologists and plant growers alike.

Although convincing evidence has been produced which suggests that rain droplets are important vectors for the effective dispersal of *Botrytis* conidia in field bean crops (Fitt, Creighton, & Bainbridge, 1985), it is generally assumed that once conidia of *B. fabae* and *B. cinerea* are released from infected plant material they are disseminated principally as air-borne conidia (De Bary, 1884; Sardiña 1930). Furthermore, vast numbers of conidia may be blown by the wind for considerable distances (Wilson, 1937).

The cascade of histological events which constitute the pre-penetration (i.e. germination of conidia on the leaf surface, formation of appressoria, production of adhesive matrices), penetration (e.g. formation of infection peg) and post-penetration processes (e.g. development of infection hyphae and lesion formation) of plant-*Botrytis* interactions following wet and/or dry inoculation are well-documented and all stages have been determined by light and/or electron microscope methods (see Table 5.1 and references therein). However, relatively few research studies have been conducted on the infection of host plants by dry-inoculated conidia of *Botrytis* species under conditions of high humidities [e.g. bean leaves (Harrison, 1984b), plum, nectarine (Fourie & Holz, 1994) and tomato fruits (Rijkenberg, De Leeuw & Verhoeff, 1980) and gerbera (Salinas & Verhoeff, 1995) and rose petals (Pie & De Leeuw, 1991; Williamson *et al.*, 1995); see Table 5.1]. Further information on the development of the *V. faba*-*Botrytis* interaction by dry-inoculated conidia is required for the development of effective disease control.

Table 5.1 Overview of relevant data from previous microscope studies describing the infection of host plants by conidial germ tubes of *Botrytis cinerea* and *B. fabae*

<i>Botrytis</i> spp.	Plant host	Inoculation method	Microscope method	Observations/Comments ^f	References
<i>B. cinerea</i>	<i>Vicia faba</i> leaves	Wet:(glucose-peptone solution)	LM	Mechanical rupture of cuticle. Epidermal cell wall dissolved enzymatically.	Brown, 1915, 1916
		Wet:(turnip juice)	LM	Simple or no appressoria. Mucilaginous layer on germ tubes. Fine peg-like outgrowths often developed from appressoria. Fungus penetrated cuticle solely by mechanical pressure. After penetration, walls of epidermal cells became swollen as a result of the action of a cell-wall dissolving enzyme.	Blackman & Welsford, 1916
		Wet:(sterile H ₂ O)	LM	'Appressoria' formed. No mucilage apparent.	Wilson, 1937
		Wet:(turnip juice)	LM, TEM	No appressoria. Germ tube attached to cuticle by mucilage. Cutin-degrading enzymes implicated in penetration process.	McKeen, 1974
		Wet:(sterile H ₂ O)	TEM	Penetration of cuticle often occurred at cell junctions. On penetration, cuticle around infection peg was pushed down into epidermal cell wall. Enzyme activity involved in penetration process. Dense fibrillar material associated with walls of infection hyphae.	Mansfield & Richardson, 1981
	onion leaves	Wet (yeast extract)	LM	Mucilaginous sheath on appressoria. A very thin penetration peg grew from tip of appressorium and penetrated leaf directly via anticlinal cell wall junctions.	Clark & Lorbeer, 1976

continued overleaf

Table 5.1 continued

<i>B. cinerea</i>	bean (<i>Phaseolus vulgaris</i>) hypocotyls	Wet:(Czapek -broth)	SEM	Germ tubes/simple appressoria left imprints and penetration holes in cuticle. Superficial hyphae formed multicellular lobate appressoria and infection cushions. Mechanical and enzymatic factors involved in penetration.	Garcia-Arenal & Sagasta, 1980.
	immature tomato fruits	Dry	LM, SEM, TEM	Conidia germinated to produce short germ tubes which were attached to host by electron-opaque material. Fine penetration peg formed at tip of germ tube. Enzymatic dissolution of cuticle. Occasionally, papillae formed. After penetration, infection peg dilated in cell wall, discolouration of cell wall occurred and spread to nearby cells. Penetration tube dilations often devoid of cytoplasmic contents.	Rijkenberg, De Leeuw & Verhoeff, 1980
	nectarine flowers, nectarine & plum fruits	Wet:(sterile H ₂ O) & Dry	LM, FM, TEM, SEM	Protoappressoria, simple appressoria and multicellular lobate appressoria. Very thin infection peg from inner appressorium wall and penetrated cuticle directly. Restricted cutinase activity.	Fourie & Holz, 1994, 1995.
	rose flowers	Wet:(H ₂ O) & Dry	LM, SEM	Slight appressorium-like swelling at tip of germ tubes. Infection peg produced from lower side of swollen tip and penetrated epidermal cell wall.	Pie & De Leeuw, 1991
		Dry	LTSEM	No appressoria. No extracellular matrix material on germ tubes but apparent on infection hyphae. Intracellular hyphae may secrete cell wall-degrading enzymes.	Williamson <i>et al.</i> , 1995

continued overleaf

Table 5.1 continued

<i>B. cinerea</i>	gerbera petals	Dry	SEM	No appressoria. Cutinase produced by fungus which may be involved in penetration process.	Salinas & Verhoeff, 1995
<i>B. fabae</i>	<i>Vicia faba</i> leaves	Wet:(sterile H ₂ O)	TEM	Enzyme activity involved in penetration process. Although, some mechanical pressure may also be applied by fungus. Intercellular hyphae surrounded by fibrillar material which adhered to host walls.	Mansfield & Richardson, 1981.
		Wet:(H ₂ O)	FM, LTSEM	Swollen germ tube apices; characteristic of 'appressoria'. Cell wall-degrading enzymes secreted by infection hyphae.	Daniels & Lucas, 1995.

^f Where there is no mention of appressorial formation, presence or absence of extracellular matrix material or mode of entry into host plant, then no citation made in text.

In a recent study, Williamson and his co-workers (1995) emphasised the importance of low-temperature SEM (LTSEM) for studies on the effect of humidity on the infection of rose petals by dry-inoculated conidia of *B. cinerea*. They employed LTSEM in order to preserve any free surface water that may have formed on the petals under the range of humidities tested. LTSEM has also been used widely for investigations of a number of plant host-pathogen interactions, including the *V. faba*-*B. fabae* interaction (Daniels & Lucas, 1995), and for the observation of fungal extracellular matrices (see Chapter One, Section 1.3 and references therein). In Chapter Three, LTSEM was employed successfully to observe the copious fibrillar matrix material ensheathing the germ tubes of *B. cinerea* sporelings grown *in vitro* (see Section 3.2.3). In this present study, DIC light microscopy (samples fixed, cleared and stained with aniline blue in lactophenol) and LTSEM of infected *V. faba* L. leaves were carried out to provide further information on the pre- penetration and pre-infection processes of wet

and dry-inoculated conidia of *B. cinerea* and *B. fabae*. Particular attention was paid to the role of extracellular matrices produced by the fungal spore during the early stages of development of the disease process. Cross-fractures of both infected and non-infected bean leaves were also observed by LTSEM in order to study lesion formation, i.e. the spotting of bean leaves, in more detail.

It is clear from the data available on the infection mechanisms of *Botrytis* species on a variety of host plants (see Table 5.1 and references therein) that there is considerable variation in the phases involved in the pre-penetration and pre-infection processes. For example, the shape of the appressorium varied from simple swollen tips of germ tubes or hyphae to multi-lobed structures or infection cushions (see Table 5.1). In addition, it has been observed that the germ tubes of *B. cinerea* appeared to penetrate the leaf surface directly without the formation of an infection peg (McKeen, 1974). Although in most plant-*Botrytis* interactions, the formation of a fine infection peg or hypha was frequently observed on penetration of the host surface (see Table 5.1 and references therein). Fibrillar-like adhesive material was also observed at the surface of extracellular germ tubes, hyphae and appressoria of *B. cinerea* and *B. fabae* in some plant-*Botrytis* interactions (Blackman & Welsford, 1916; McKeen, 1974; Clark & Lorbeer, 1976), but not others (Williamson *et al.*, 1995) and not in all cases. Penetration of the plant cuticle by *Botrytis* species may involve mechanical and/or enzymatic processes (see Table 5.1). Such differences in the early events of the infection processes are likely to be the result of a number of factors including the choice of *Botrytis* species and the respective plant host, the inoculation conditions, and the microscope method used to observe infected plants. The possibility that *Botrytis* extracellular matrices may not have been readily observed in previous studies as a result of the loss of such matrices during light and electron microscope preparation protocols has already been alluded to (Chapter Four, Section 4.1.4).

Immunocytochemical studies using monoclonal antibodies directed against fungal pathogens have proved to be useful for investigating plant host-pathogen interactions (Benhamou *et al.*, 1985a,b; Mendgen *et al.*, 1988; Mackie *et al.*, 1993; Pain *et al.*, 1994; Green *et al.*, 1995; see also Table 1.1 for further references) including the *Vicia faba*-*Botrytis* relationship (Svircev *et al.*, 1986b) by light and electron microscopy. The results from Chapter Three have also confirmed the great value of the *Botrytis*-MAbs (BC-KH4, BC-AF7 & BC-FD7-G9: Bossi & Dewey, 1992) as immunocytochemical markers for the location of specific components of the cell wall and extracellular matrices of *Botrytis*

sporelings grown *in vitro* at both the light and/or electron microscope levels (see Sections 3.3.2.2. and 3.3.2.4).

This chapter presents similar immunocytochemical studies (indirect IF and IEM) as described in Chapter Three. However, here the *Botrytis*-MAbs, were employed to investigate the spatial distribution of corresponding antigens throughout the *Vicia faba*-*Botrytis* interface and the role(s) of such molecules in the early stages of development of chocolate spot disease. Laser scanning confocal microscopy (LSCM) in the epifluorescence mode was employed previously (see Chapter Three) to observe the location of specific surface molecules at the cell wall and throughout the extracellular matrices of *B. cinerea* sporelings grown *in vitro* after immunolabelling with the BC-KH4 MAb. In preliminary investigations, this microscope technique was employed to view small areas of *V. faba* leaves after inoculation with dry or wet conidia of *Botrytis* species and immunolabelling using the BC-KH4 MAb. Both TEM and IEM studies of the pre-penetration and pre-infection processes of *B. fabae* on *V. faba* leaves were also carried out.

5.2 Results

5.2.1 Symptoms of chocolate spot disease of broad bean: Formation of macroscopic lesions

Excised leaves of *Vicia faba* L. were observed 21 h after inoculation with unwashed dry and wet conidia (i.e. in the presence of aqueous glucose) of *B. fabae* or *B. cinerea* (Plate 5.1, Figs 1-6). In both fungi, in the presence of aqueous glucose, dark-brown to black-coloured “spots” were clearly visible beneath the infection drops on the adaxial surface of leaves (Plate 5.1, Figs 1 & 2). In addition, neighbouring “spots” or necrotic lesions often coalesced to form larger lesions (Plate 5.1, Figs 1 & 2). In contrast, however, following inoculation of leaves with unwashed dry conidia, small discrete dark-brown to black-coloured spots were observed on the leaf surface (Plate 5.1, Figs 3 & 4). Furthermore, as expected, a greater number of lesions were observed following

inoculation of dry conidia of *B. fabae* compared to *B. cinerea* (Plate 5.1, cf. Figs 3 & 4). As a result, *B. fabae*-infected leaf material was selected for further immunocytochemical studies involving laser scanning confocal microscopy (LSCM) and immunogold labelling at the electron microscope level. In control treatments, no necrosis or “spotting” of leaves was observed (Plate 5.1., Figs 5 & 6).

5.2.2 DIC microscopy of whole-mount preparations

DIC microscopy of whole-mount preparations of inoculated leaf discs (fixed, cleared and stained with aniline blue in lactophenol) revealed that conidia of both *Botrytis* species were unable to germinate on the leaf surface when they were inoculated in water only (Plate 5.2, Figs 1 & 2). However, following inoculation in the presence of aqueous glucose, conidia of both fungi germinated to produce one or more long germ tubes which often extended over the surface of the leaf (Plate 5.2, Figs 3 & 4). Prior to penetration, the long germ tubes often appeared swollen at their tip (not shown here), but not in all cases (Plate 5.2, Fig. 6). Penetration sites often occurred at anticlinal cell wall junctures (see Diagram 5.1). However, direct penetration of the plant cuticle and epidermal cell was also observed (Plate 5.2, Figs 5 & 6; see also Diagram 5.1). Occasionally, by DIC microscopy, the sites of penetration were visible as small circles beneath the tip of the germ tube (not shown here). However, in most cases, they were difficult to discern. In all cases, infection hyphae extended through the leaf tissue (Figs 5 & 6). In addition, the walls of both penetrated and neighbouring epidermal cells appeared swollen and dark-brown in colour (Plate 5.2, Figs 5 & 6, arrowheads). The cytoplasm and nucleus of these wounded cells also showed visible signs of decay, i.e. they often appeared granular and dark-brown in colour (Plate 5.2, Figs 5 & 6).

When excised bean leaves were inoculated with unwashed dry conidia of *B. cinerea* (not shown here) and *B. fabae* (Plate 5.3, Figs 1-3) conidia germinated to produce short germ tubes only which in turn, penetrated the leaf surface directly (Plate 5.3, Fig. 1, large arrow). Infection hyphae were observed to extend from the sites of penetration through the host tissue (Plate 5.3, Figs 2 & 3). Furthermore, both penetrated and neighbouring epidermal cells showed signs of necrosis. The precise timing of penetration of germ tubes produced by wet or dry conidia was not determined.

5.2.3 Observation of *B. cinerea*-infected *V. faba* leaves by LTSEM

LTSEM revealed that in the presence of aqueous glucose, *B. cinerea* conidia germinated on the surface of *V. faba* leaves to produce one or more long germ tubes (Plate 5.4, Figs 1 & 2). Furthermore, these germ tubes were surrounded by an extensive veil-like covering of fibrillar material which appeared to provide close adherence of the sporelings to the leaf surface (Plate 5.4, Figs 1 & 2). In contrast, no or relatively few, fine strands of matrix material were observed to be associated with the short germ tubes produced by dry-inoculated conidia (Plate 5.4, Fig. 3, 5 & 6). The short germ tubes produced by dry-inoculated conidia also appeared to penetrate the leaf surface directly to form clearly defined necrotic lesions (Plate 5.4, Fig. 4). In some cases, the short germ tubes also served as the only point of attachment of the sporelings to the leaf surface (Plate 5.4, Figs 5 & 6). In addition, subsequent infection often led to the collapse of the conidium on the leaf surface, possibly as a result of the passage of conidium contents into the penetration/infection hypha beneath the leaf surface (Plate 5.4, Fig. 6).

LTSEM of advanced lesions formed by wet or dry-inoculated conidia showed that the necrotic lesions or “spotting” on the leaf surface observed previously with the naked eye (Plate 5.1, Figs 1-4) corresponded to sunken areas on the leaf surface (Plate 5.5, Figs 1 & 2). Observation of cross fractures of infected and healthy leaf tissue revealed that these sunken areas were caused by the collapse of infected and neighbouring epidermal cells (Plate 5.5, Fig. 3). Since, in healthy tissue, the epidermal cells remained fully turgid and showed no visible signs of collapse (Plate 5.5, Fig. 4).

5.2.4 Indirect IF microscopy of infected bean leaves using the *Botrytis*-MAbs

Indirect IF microscopy of *V. faba* leaves previously inoculated with *Botrytis* conidia in the presence of aqueous glucose and immunostained with the *Botrytis*-MAb BC-KH4, BC-FD7-G9, or BC-AF7, revealed that in all cases fluorescent labelling was apparent at the surface of conidia and germ tubes. (Plate 5.6, Figs 1-7). In addition, labelling was detected throughout the fibrillar matrix which often extended from the periphery of the conidia and germ tubes to the leaf substratum (Plate 5.6, Figs 1, 3 & 4). MAb-binding

was also prominent at regions where septa were formed (Plate 5.6, Figs 2 & 4, large arrow) and occasionally at hyphal tips (Plate 5.6, Figs 2 & 7, arrowheads). In some cases, a distinct fluorescent ring which was thought to correspond to the penetration/infection peg could be detected at the base of a swollen germ tube tip (Plate 5.6, Fig. 6; see also frontispiece, Figs 1 & 2). The diameter of this ring often appeared to be much smaller than that of the germ tube on the leaf surface (Plate 5.6, Fig. 6; see also frontispiece, Figs 1 & 2). In heavily-infected leaf material (i.e. samples immunolabelled 48 h post-inoculation) extensive MAb-binding sites were located at the external surface of the infection hyphae (see frontispiece, Fig. 4). These infection hyphae ramified throughout the plant host tissue.

Similarly, when leaves inoculated with dry conidia of *Botrytis* species were immunostained with either of the *Botrytis*-MAbs as described above, and observed by IF microscopy, fluorescent labelling was located at the surface of conidia (Plate 5.7, Figs 2, 3, 5, 6, 8 & 9). In contrast however, and in most cases, no labelling was apparent at the surface of the short germ tubes (Plate 5.7, Figs 2, 3, 5 & 6). Instead, MAb-binding sites were located at sites of penetration of the host leaf tissue (Plate 5.7, Figs 3, 8 & 9) and at the surface of infection hyphae (Plate 5.7, Figs. 5 & 6). In control treatments, i.e. in the absence of the primary antibody, no fluorescence was detected at the surface of sporelings (Wet: Plate 5.6, Fig. 8. Dry: Plate 5.7, Fig. 11). Furthermore, in both immunolabelled and control treatments, only low levels of autofluorescence of the leaf tissue were observed.

Laser scanning confocal microscopy (LSCM), in the epifluorescence mode, of BC-FD7-G9-immunolabelled and *B. fabae*-infected leaf material confirmed that numerous MAb-binding sites were located at the surface of conidia and germ tubes on the leaf substratum (Wet: Plate 5.8, Figs 1-6). In addition, this technique emphasised the association of the MAb-binding sites with the surface of the penetration and infection hyphae (Wet: Plate 5.8, Figs 1-6).

5.2.5 Light microscopy of semi-thin sections of infected *V. faba* leaves

Toluidine blue-stained semi-thin sections of LR White-resin embedded and *B. fabae*-infected *V. faba* leaves were observed by light microscopy. Cross-sections of the plant host-pathogen interface illustrated the dramatic killing action of the fungus (Plate 5.9, Fig. 1). Following penetration of the plant cuticle and epidermis, the infection hyphae invaded the leaf tissue causing the death of neighbouring epidermal and palisade mesophyll cells (Plate 5.9, Fig. 1). Dead cells were recognised by a loss of form i.e. turgidity, and their cytoplasmic contents appeared disrupted and granular (Plate 5.9, Fig. 1). In control treatments, i.e. non-inoculated leaves, no death or collapse of leaf tissue was observed (not shown here).

5.2.6 TEM studies of the *V. faba*-*B. fabae* interaction

5.2.6.1 Pre-penetration and pre-infection processes of wet conidia

TEM observations of ultra-thin sections of *B. fabae*-infected bean leaves, prepared using mild aldehyde fixation followed by PLT-dehydration and embedding in LR-White resin, revealed that the ultrastructure of both the fungus and plant host-pathogen interface compared favourably to that observed in sections of glutaraldehyde-OsO₄-fixed and Spurr resin-embedded samples (e.g. Plate 5.10, cf. Figs 1 & 3). In the former case, and where conidia were inoculated in the presence of aqueous glucose, abundant fibrillar matrix material was observed to ensheath the germ tube on the leaf surface (Plate 5.10, Fig. 1 and Plate 5.12, Figs 1 & 3). This fibrillar sheath extended across and remained firmly attached to the leaf surface even after the rigorous procedures encountered during preparation for TEM.

At sites of penetration, the base of the germ tube was also closely adhered to the leaf surface. Furthermore, a thin layer of amorphous material was often seen between the base of the germ tube and the leaf surface (Plate 5.10, Fig. 2). In addition, narrow penetration pegs or hyphae often developed from the base of these germ tubes and appeared to penetrate the plant cuticle directly. Although, the plant cuticle showed signs of curling (Plate 5.10, Figs 1 & 2), the total length of these curled edges was always less

than the diameter of the penetration peg or hypha (Plate 5.10, Figs 1 & 2). Dissolution or breakdown of the plant cuticle, other than at sites of penetration, was observed only occasionally (Plate 5.12, Fig. 1). In some cases, the penetration peg or hypha appeared devoid of cytoplasmic contents, particularly in their early stages of development (Plate 5.10, Fig. 2).

In many instances, infection hyphae penetrated the epidermal cell layer via anticlinal cell wall junctures (Plate 5.10, Fig. 1; see also Diagram 5.1), although direct penetration of the epidermis at the centre of epidermal cells was observed (Diagram 5.1). In some cases following penetration, the intruding penetration peg or hypha grew intramurally via the outer periclinal epidermal cell wall prior to penetration of the anticlinal wall juncture or lumen of the epidermal cell (Diagram 5.1). Once inside the host, the penetration hypha formed a swollen infection hypha which became delimited from the penetration hypha by a septum (Plate 5.10, Fig. 1). Both infected and neighbouring epidermal cells showed signs of necrosis, i.e. the cytoplasmic contents became highly disorganised (Plate 5.10, Fig. 1 and Plate 5.14, Fig. 1). As infection hyphae penetrated the layers of the outermost epidermal cell wall, the host cell wall showed signs of swelling or loosening (Plate 5.10, Fig. 1). No swelling or loosening of epidermal cell walls was observed in non-inoculated leaves (Plate 5.12, Fig. 6).

5.2.6.2 Pre-penetration and pre-infection processes of dry conidia.

Excised *V. faba* leaves inoculated with dry conidia of *B. fabae* and subsequently (18 h post-inoculation) prepared by mild aldehyde fixation and embedding in LR White resin, were examined by TEM. TEM observations revealed that in all cases, dry-inoculated conidia germinated on the leaf surface to produce one or more germ short tubes (Plate 5.11, Fig. 1). Occasionally, the tip of the short germ tube appeared swollen and thus, similar to an appressorium (Plate 5.11, Fig. 1). In many cases, the germ tube was surrounded by a pad of amorphous-material which was closely adhered to the leaf surface (Plate 5.11, Fig. 1). In previous experiments using DIC and indirect IF microscopy and LTSEM, such matrix material was not readily detectable at infection sites of dry-inoculated conidia. Observation of numerous infection sites suggested that in such cases where little or no matrix material was produced by the dry-inoculated spore,

the sporeling was probably more easily dislodged from the leaf surface during the severe and extensive washing steps involved in TEM preparation protocols.

At the base of the short germ tube, a penetration hypha was formed which often entered the lumen of the epidermal cell directly (Plate 5.11, Fig. 1). It was interesting to note that, in some cases, this hypha exhibited an irregular variation in diameter (Plate 5.11, Fig. 1). It often lacked cytoplasm and was delimited from the infection hypha by a septum (Plate 5.11, Fig. 1). At the site of penetration, as observed previously in infections formed by wet conidia (see Plate 5.10, Fig. 2), the leaf cuticle showed signs of curling (Plate 5.11, Fig. 2). The total length of the curled edges of the leaf was less than the diameter of the penetration hypha (Plate 5.11, Fig. 1). The plant cuticle remained intact other than at the sites of penetration (Plate 5.11, Fig. 1). In addition, the cytoplasmic contents of both infected and neighbouring cells were highly disrupted (Plate 5.11, Fig. 1).

5.2.7 IEM studies of the *V. faba*-*B. fabae*-interaction, using the *Botrytis*-MAbs

IEM studies of *B. fabae*-infected *V. faba* leaves prepared by mild aldehyde fixation, PLT dehydration and embedding in LR White resin, revealed that all three *Botrytis*-MAbs, BC-KH4, BC-AF7 and BC-FD7-G9, exhibited a high degree of specificity for the fungus and its site(s) of infection (Wet-infections: Plate 5.12, Figs 2-4 and Plate 5.13, Fig. 2; Dry-infections: Plate 5.15, Figs 1-3). In all cases, little or no labelling of the plant host tissue was observed. Such specificity was particularly prominent when a silver enhancement step was applied to the immunostaining procedure, after indirect immunogold-labelling and prior to observation of sections by TEM (Wet-infections: Plate 5.14, Figs 1 & 2; Dry-infections: Plate 5.16, Figs 1-5).

When *B. fabae* conidia were inoculated onto the leaf surface in the presence of aqueous glucose, as expected, they germinated to produce one or more long germ tubes which were enclosed by a sheath of fibrillar matrix material (Plate 5.12, Fig. 1). Such matrix material also extended over the surface of the conidium (Plate 5.12, Fig. 2). In all cases, this fibrillar matrix material was readily immunolabelled with the *Botrytis*-MAbs (Plate 5.12, Fig. 2-4 and Plate 5.14, Figs 1 & 2). Interestingly, the pad of amorphous

material surrounding the short germ tubes of dry-inoculated conidia at sites of penetration of the leaf surface, frequently observed in TEM preparations (see Plate 5.11, Fig. 1), was also immunolabelled using the *Botrytis*-MAbs (Plate 5.15, Fig. 2 and Plate 5.16, Fig. 2). In both wet and dry-infections, abundant MAb-binding sites were also located throughout the translucent conidial and germ tube walls of external sporelings (Wet: Plate 5.12, Fig. 2 and Plate 5.14, Figs 1 & 2; Dry: Plate 5.15, Figs 1 & 2 and Plate 5.16, Figs 1 & 2).

Further observations of both wet and dry-infections revealed that the translucent walls of the penetration and infection hyphae were also immunolabelled with the *Botrytis*-MAbs (Wet: Plate 5.12, Fig. 5, Plate 5.13, Fig. 2 and Plate 5.14, Fig. 1. Dry: Plate 5.15, Fig. 3 and Plate 5.16, Figs 3-6). At sites of penetration, and in particular where penetration hyphae entered the outer host epidermal cell wall, it was intriguing to observe that extensive labelling was also located throughout the host wall and at some distance from the penetration hyphae (Plate 5.12, Fig. 5). No such labelling was observed in the outer epidermal cell wall of non-inoculated *V. faba* leaves (Plate 5.12, Fig. 6). Numerous MAb-binding sites were located throughout the fibrillar matrix material associated with the walls of the infection hyphae (Wet: Plate 5.13, Fig. 2 and Plate 5.14, Fig. 1. Dry: Plate 5.15, Fig. 3 and Plate 5.16, Fig. 3). Similar immunolabelled matrix material was also located in infected host walls (Wet: Plate 5.13, Fig. 4) and often some distance from infection hyphae (Dry: Plate 5.16, Fig. 4) or apparently separated from the infection hyphae by physical barriers e.g. host cell wall or degenerate cytoplasm (Wet: Plate 5.13, Fig. 5. Dry: Plate 5.16, Fig. 5). In all control treatments, i.e. in the absence of the primary antibody, no labelling of fungal cell walls or fibrillar matrix material was detected in infected leaf material (Wet: Plate 5.13, Fig. 6; Dry: Plate 5.15, Fig. 4 and Plate 5.16, Fig. 6).

In brief, the results of the aforementioned microscope studies investigating the infection processes of wet and dry conidia of *Botrytis* conidia on broad bean leaves are summarised in Table 5.2. In addition, the possible pathways followed by *Botrytis* germ tubes on infection of broad bean leaves are outlined in Diagram 5.1.

Table 5.2 Summary of results from microscope studies describing the infection of *Vicia faba* L. plants by conidial germ tubes of *Botrytis cinerea* and *B. fabae*

<i>Botrytis</i> spp.	Plant host	Inoculation method	Microscope method	Observations/Comments ^f
<i>B. cinerea</i>	<i>Vicia faba</i> leaves	Wet:(0.5 % glucose)	LM, IF, LTSEM	Conidia germinated to produce long germ tubes. Simple or no appressoria. Fibrillar material located at surface of conidia, germ tubes and appressoria on leaf surface. Fibrillar material provided firm attachment of sporeling to leaf substratum. Germ tube/appressorial penetration sites occurred mainly at anticlinal wall junctures. In some cases, fungus penetrated leaf surface at the centre of epidermal cells. Penetration led to the death and collapse of host epidermal cells.
		Dry	LM, IF, LTSEM	Conidia germinated to produce short germ tubes which penetrated the leaf surface directly. Occasionally proto-appressoria were formed prior to penetration. Only traces of matrix material observed at surface of germ tubes Penetration led to the death and collapse of host epidermal cells.
<i>B. fabae</i>	<i>Vicia faba</i> leaves	Wet:(0.5 % glucose)	LM, IF, LSCM, TEM, IG	Conidia germinated to produce long germ tubes. Simple or no appressoria. Fibrillar material located at surface of conidia, germ tubes and appressoria on the leaf surface. Matrix material provided firm attachment of sporeling to leaf substratum. Thin penetration peg grew from base of swollen germ tube tip and penetrated host cuticle and epidermal cell wall directly or via anticlinal cell wall junctures. Hyphae appeared to penetrate host cuticle by mechanical pressure. Restricted enzyme activity may also be involved in the penetration process. After penetration, walls of epidermal cells became swollen, probably as a result of the action of cell-wall degrading enzyme(s). Both penetration and infection hyphae were surrounded by a sheath of matrix material. This fibrillar sheath often extended some distance from infection hyphae and passed through swollen walls of infected cells.

continued overleaf

Table 5.2 continued

<i>B. fabae</i>	<i>Vicia faba</i> leaves	Dry	LM, IF TEM, IG	Conidia germinated to produce short germ tubes which penetrated plant cuticle and epidermal cell wall directly. Occasionally, simple appressoria formed prior to penetration. No fibrillar material observed at surface of conidia, germ tubes and appressoria on leaf surface. By TEM, an amorphous pad of matrix material was observed to surround short germ tubes at site(s) of attachment to leaf substratum. This matrix material provided tight adherence of the sporeling to the leaf surface during penetration. On penetration, a peg grew from base of swollen germ tube tip and penetrated host cuticle and epidermal cell wall directly. Hyphae appeared to penetrate host cuticle by mechanical pressure. Restricted enzyme activity may also be involved in the penetration process. After penetration, walls of epidermal cells became swollen, probably as a result of the action of cell-wall degrading enzyme(s). Both penetration and infection hyphae were surrounded by a sheath of matrix material. This fibrillar sheath often extended some distance from infection hyphae and passed through the swollen walls of infected cells.
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5.3 Discussion

5.3.1 Pre-penetration processes of wet and dry conidia of *Botrytis* species on *V. faba* leaves

Light and electron microscopy of the infection mechanisms of *B. cinerea* and *B. fabae* on *V. faba* leaves revealed that the growth and behaviour of the fungus on the leaf surface varied according to the mode of inoculation applied. Conidial germination was prevented when conidia were inoculated onto leaves in the presence of sterile water only. It is unlikely that inhibitory factors (e.g. leaf exudates, epicuticular waxes and other micro-

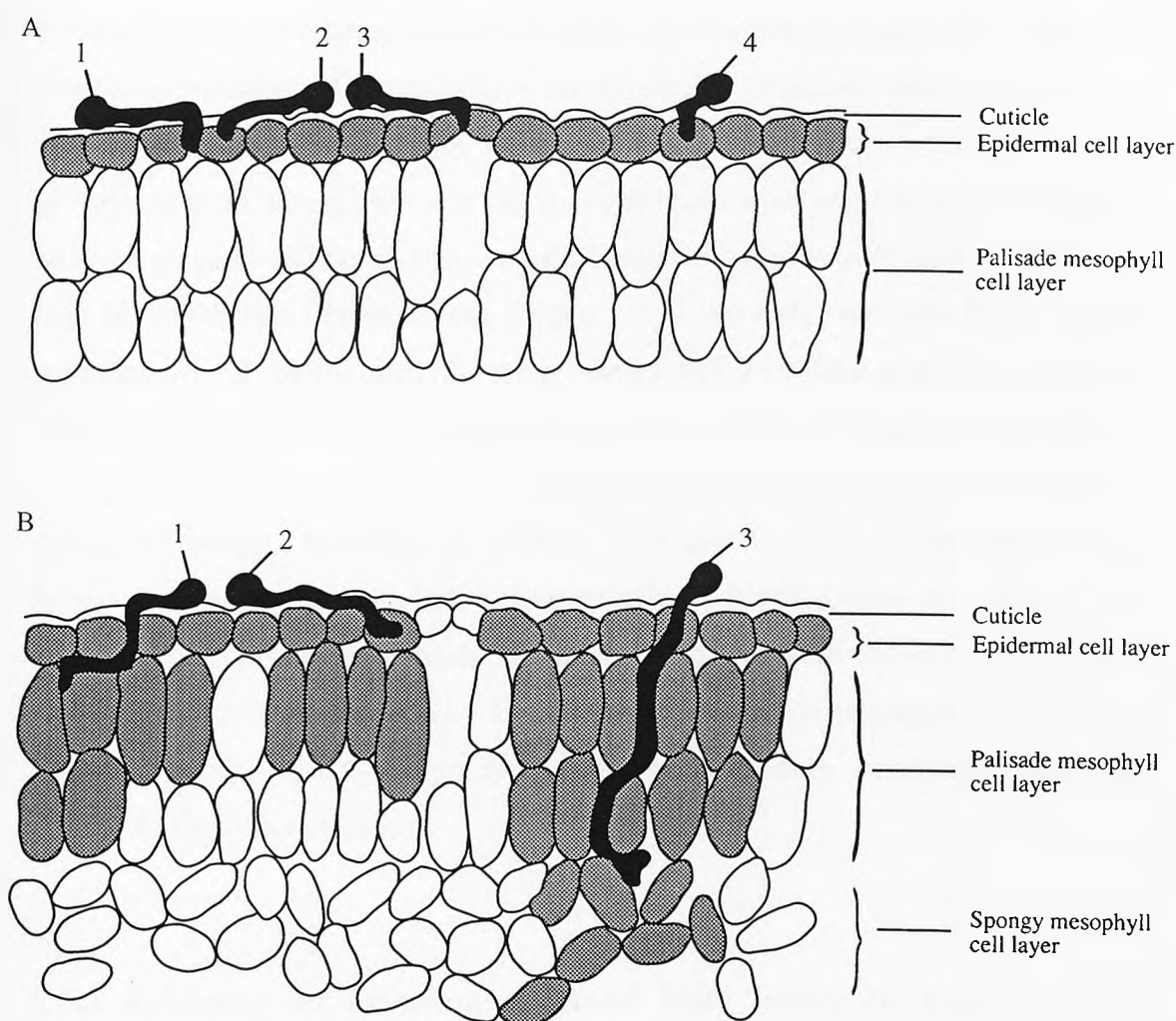


Diagram 5.1 Diagrammatic representation of the pathways observed following infection of broad bean by *Botrytis* germ tubes and infection hyphae

Shaded area represents extent of necrosis of leaf tissue. [A] Early stages of infection process. In the presence of aqueous glucose, sporelings (1-3) formed long germ tubes which penetrated the epidermal cell layer via antichinal wall junctions (1), the centre of the epidermal cell (2) or via a stoma (3). In contrast, dry-inoculated conidia (4) produced short germ tubes which penetrated the leaf surface directly. In many cases, on early penetration of the epidermal cell layer, only infected and neighbouring epidermal cells were affected. [B] Later stages of infection process. Wet-infections (1 & 2): Infection hyphae often penetrated the palisade mesophyll cell layer via an intra-mural route (1). Infection hyphae also grew through the outermost periclinal wall prior to entry into host cells (2). Dry-infection (3): Infection hyphae extend through the leaf tissue to the spongy mesophyll cell layer via both intra- and intercellular routes. Extensive necrosis of leaf tissue was observed.

organisms) exerted by the plant host surface affected conidial germination since dry-inoculated conidia germinated readily and penetrated the leaf surface directly. The inability of conidia to germinate in the presence of sterile water only may therefore be explained by a loss of nutrients from the fungal spore to the aqueous environment. It is suggested that such a nutrient loss occurred during isolation of conidia and resuspension procedures. The fact that conidial germination and the growth of the developing germ tube was promoted by the addition of glucose to the spore suspension supports this theory. A similar effect of glucose on germ tube growth was reported previously when wet (i.e. in the presence of aqueous glucose) and dry *Botrytis* conidia were germinated *in vitro* under conditions of high humidities (see Chapter Three, Section 3.3.1). An early report has shown that the germination of *B. cinerea* conidia was prevented during leaching with water or mineral solutions, but unaffected by leaching with glucose solution (Sztejnberg & Blakeman, 1973). On *V. faba* leaves, it is suggested that aqueous glucose enhanced the superficial growth of *Botrytis* sporelings. In addition, it is possible that glucose prevented the fungus from penetrating the leaf surface directly. It is well-known that glucose is an efficient repressor of cutinase production in mycelia of *Fusarium solani* f. sp. *pisi* (Lin & Kolattukudy, 1978; Köller, 1991). The effects of glucose on pathogenicity of *Botrytis* species in relation to cutinase production by the fungal spore would be an interesting project for future investigations.

5.3.2 Evidence for infection of broad bean leaves by unwashed dry conidia of *Botrytis* species

The pathogenesis of *B. fabae* and *B. cinerea* on *V. faba* leaves is well documented (Harrison, 1988; see also Table 5.1 and references therein). However, the importance of a knowledge of the disease process by dry-inoculated conidia has not been emphasised previously. It has been suggested that early studies on the pathogenesis of dry-inoculated conidia of *Botrytis* species may have been avoided or hindered as a result of the difficulties in controlling and obtaining precise measurements of humidity (Williamson *et al.*, 1995). Previous researchers have revealed that condensation droplets¹ may be formed at high humidities on plant surfaces previously inoculated with dry conidia of *B. cinerea* (Pie

¹ Here, the quantities of surface water present on the leaf surface were probably not sufficient to remove nutrients from the fungal spore.

& De Leeuw, 1991; Williamson *et al.*, 1995). It must be stated that in the present study, surface water was rarely observed on the surface of *V. faba* leaves previously inoculated with dry conidia of *Botrytis* species, yet infection still occurred. Other research has indicated that *B. fabae* conidia can infect leaves without surface water (Harrison, 1984b). In short, the results of the present study provide evidence for the infection of *V. faba* leaves by unwashed dry conidia of *Botrytis* species under the conditions employed. The infection of broad bean leaves by unwashed dry conidia of *B. cinerea* and *B. fabae* has been hitherto unrecognised.

5.3.3 Is appressorial formation a prerequisite for penetration of the leaf surface?

When conidia were inoculated onto broad bean leaves in the presence of aqueous glucose, simple appressoria or proto-appressoria (Emmett & Parbery, 1975) were occasionally observed at the tips of germ tubes of *Botrytis* sporelings prior to penetration of broad bean leaves. However, in many cases, *Botrytis* germ tubes exhibited no such apical swelling prior to penetration of the leaf surface. Furthermore, in dry-infections, appressoria were rarely formed. Instead, the short germ tubes penetrated the leaf surface directly (see also Williamson *et al.*, 1995). Previous studies of plant-*Botrytis* interactions have shown that appressoria were formed by *Botrytis* germ tubes prior to infection of a variety of plant host surfaces (see Table 5.1). There is considerable evidence that, in a large number of plant pathogenic fungi, appressorial formation occurs in response to contact stimuli and prior to penetration of the leaf surface (Emmett & Parbery, 1975; Wynn, 1981; Staples *et al.*, 1983; Epstein *et al.*, 1987; Hoch & Staples, 1987; Hoch *et al.*, 1987). However, in the *Vicia faba*-*Botrytis* interaction, it seems unlikely that this general rule applies.

It was also interesting to note that in the present study, when *B. cinerea* or *B. fabae* conidia were inoculated on to broad bean leaves in the presence of aqueous glucose, penetration sites were frequently observed at anticlinal wall junctures. Future investigations of the pre-penetration processes of *Botrytis* species grown on both natural and synthetic substrata may aid in the elucidation of the conditions required for appressorial formation and penetration.

5.3.4 Role(s) of *Botrytis* extracellular matrices in the pre-penetration processes of *Botrytis* species

The suggestion that mucilaginous-like extracellular matrices produced by *Botrytis* species provide firm attachment of sporelings to the host surface was first proposed by Ward in 1888. Since then, several researchers have confirmed that such a phenomenon exists in plant-*Botrytis* relationships and that it is a prerequisite to infection (Blackman & Welsford, 1916; McKeen, 1974). In the present study, extracellular matrices were not apparent on the surface of *Botrytis* sporelings grown *in vivo* by DIC microscopy of whole mount preparations. This was probably a result of the extraction of such material during the fixation and clearing protocols. Nevertheless, matrix material was detected by at the surface of glucose-supplemented sporelings on *Vicia faba* leaves by indirect IF microscopy using the *Botrytis*-MAbs and by LTSEM and TEM. It is likely that such matrix material ensured tight adhesion of the sporelings to the leaf substratum, particularly where matrix material was abundant, since the sporelings and matrix material were rarely dislodged by the washing steps involved in the light and electron microscope preparation protocols.

LTSEM revealed that in the case of dry-inoculated conidia, in some instances, the short germ tube appeared to be the only point of attachment of the sporeling to the leaf surface at the site of penetration. In a recent report, Williamson and co-workers (1995) attributed the loss of contact of *B. cinerea* sporelings to the surface of rose petals except at the point of attachment to (i) a disturbance of the specimen during preparation for LTSEM or (ii) stresses exerted during penetration. In the present study, the former suggestion seems highly unlikely since the LTSEM preparation protocol applied here ensured minimal handling and disturbance of specimens prior to freezing and observation. Furthermore, TEM observations of the *V. faba*-*B. fabae* interaction revealed that an amorphous pad of matrix material was associated with the short germ tubes of dry-inoculated conidia on the leaf surface. This amorphous pad provided firm attachment of the sporeling to the leaf surface at the site of penetration. Thus, it is conceivable that as the *Botrytis* germ tube exerted the necessary forces for penetration of the host surface, the sporeling was lifted off the leaf surface. As a result, the sporeling remained attached to the leaf surface only at its site of penetration. In view of these

results, the latter explanation by Williamson and co-workers (1995) for the loss of contact of *Botrytis* sporelings to the plant surface is considered to be the most plausible.

5.3.5 Penetration processes of wet and dry conidia of *Botrytis* species: Mechanical or enzymatic?

In order for any fungus to enter the leaf surface directly, it must possess a means of breaching the cuticular barrier by mechanical and/or chemical (i.e. enzymatic) action (See Chapter One). The nature of the plant cuticle has already been described in detail in Chapter One. In simple terms, the plant cuticle consists of wax on the outside and a layer of cutin close to the epidermal cells. In view of the pathogenesis of *Botrytis* species on a variety of intact, living plant host tissues (see above results and Table 5.1), it is clear that the plant cuticle does not form an impenetrable barrier to infection by *Botrytis* species.

In this study, LTSEM observations of the ingress of short germ tubes of dry-inoculated conidia of *Botrytis* species on *V. faba* leaves suggested that physical pressures may be exerted during penetration of the leaf surface (see Section 5.3.4). In addition, TEM observations revealed signs of inward curling of the plant cuticle at sites of penetration. Although, no indentation of plant cuticle was observed at penetration sites, this inward curling of the cuticle suggested that mechanical pressure was involved in the penetration process. Early scientists observed the infection of broad bean leaves by *B. cinerea* microscopically and concluded that the cuticle was penetrated solely by mechanical pressure yielded by the fungus (Brown, 1915, 1916; Blackman & Welsford, 1916). However, rigorous proof of purely mechanical action of any fungal parasite on penetration may only be achieved by observing fungal penetration of artificial membranes resistant to chemical secretions. Research on the rice blast fungus, *Magnaporthe grisea* has shown that penetration pegs, formed at the base of swollen appressoria, indented hard substrata e.g. plastic coverslips composed of poly(vinyl chloride) and poly(vinyl acetate) and non-biodegradable Mylar membranes exhibiting a range of surface hardness (Howard, Bourett & Ferrari, 1991; Howard *et al.*, 1991). Indirect evidence indicated that the appressoria generated turgor pressures in excess of 8.0 MPa or 80 bars (Howard *et al.*, 1991). It was therefore concluded that penetration of synthetic membranes and host epidermal cells was accomplished by the application of the physical force derived from appressorial turgor (Howard *et al.*, 1991).

Miyoshi (1895) reported that *B. cinerea* was able to penetrate a gold film via the formation of a small infection peg, however this work is often overlooked. In the present study, it seems likely that mechanical pressures are involved in the penetration of broad bean leaves by *Botrytis* species. Nevertheless, the involvement of chemical i.e. enzymatic activity cannot be ruled out completely.

In early TEM studies of the infection of *V. faba* leaves by conidial germ tubes of *B. cinerea*, McKeen (1974) always observed a clean penetration pore i.e. with no curled edges and no indentation of the cuticle during penetration. McKeen (1974) also detected esterase activity at the tips of germ tubes 7 h after inoculation. Thus, it was concluded that the plant cuticle was enzymatically dissolved rather than mechanically broken. Previous researchers isolated a cutinase-like enzyme from germinating conidia of *B. cinerea* (Salinas, 1992; Salinas & Schots, 1992; Salinas & Schots, 1994). In addition, it was reported that lesion formation by *B. cinerea* was prevented by spraying the florets with an antiserum against cutinase (Salinas, 1992). Interestingly, these researchers observed no signs of cuticle degradation around the penetration germ tubes of *B. cinerea* sporelings on infection of gerbera ray florets by SEM (Salinas & Verhoeff, 1995). It was therefore assumed that cutinase was only secreted at the tip of germ tubes. Other researchers have also reported that *Botrytis* species exhibited cutinase activity (Baker & Bateman, 1978; Shishiyama, Araki & Akai, 1970; Verhoeff, Warnaar & Schijven, 1979; Rijkenberg, De Leeuw & Verhoeff, 1980; Coleman, Hiscock, & Dewey, 1993). The results of the present study revealed that cuticular material was absent from the inoculated leaf surface at or near sites of attachment of *B. fabae* sporelings in only a few cases. Although it is feasible such a loss of host cuticular material may have been the result of the action of cutin-degrading enzymes produced by the fungus, this seems unlikely since there was no loss of cuticular material around the penetrating hyphae. Alternatively, it is suggested that the cuticle was damaged during the TEM preparation protocol².

While in this study there is no direct proof that cutinolytic enzymes were produced during the *V. faba*-*B. fabae* interaction, it is conceivable that such enzymes may have been confined to the extreme tip of the germ tube, as proposed by previous workers (McKeen, 1974, Mansfield & Richardson, 1981). Mansfield & Richardson (1981) observed the ultrastructure of interactions between broad bean leaves and *Botrytis* species. They suggested that enzymatic dissolution of a small area of cuticle

² It must be stated here that it is unlikely that gentle washing and wiping of the leaf surfaces prior to inoculation altered the plant cuticle (Sutton, Rowell & James, 1984).

beneath the swollen germ tube tip of the fungus would create a zone of weakness susceptible to further physical breach by pressures exerted by the expanding hypha. Similarly, in the present study, it is suggested that the observed passage of the penetration peg through the cuticle may have not been accomplished by mechanical means alone but by the pathogen pressing against the host cuticle in a relatively small area and secreting a limited amount of cutinolytic enzyme. Immunocytochemical studies using antibodies to cutinase may shed more light on the role of this enzyme in the penetration of broad bean leaves by *Botrytis* species.

5.3.6 Early events in the post-penetration processes of *Botrytis* species

TEM observations of the *V. faba*-*B. fabae* interaction revealed that, as reported previously in other plant-*Botrytis* interactions (Blackman & Welsford, 1916; McKeen, 1974; Rijkenberg, De Leeuw & Verhoeff, 1980; Pie & De Leeuw, 1991), after the penetration peg passed through the cuticle the penetration enlarged to about normal mycelial diameter or slightly larger. In addition, the infection hole increased in diameter. Such penetration tube dilations were often devoid of cytoplasm³. Furthermore, the penetration hypha resembled an “inverted funnel”; a phenomenon described by Dickinson in 1960. Irregular variation in the diameter of the penetration hyphae of the short germ tubes from dry-inoculated conidia was also observed. Such irregular variation in diameter of penetration hyphae has also been recorded previously during the infection processes of the necrotroph *Venturia inaequalis*, the apple scab fungus (Wiltshire, 1915). Dickinson (1960) stated that the variations in original diameter of the penetration tube in the cuticle, as well as the increase in diameter in the pectin-cellulose layers of the wall depended upon (a) the ratio of pectin-cellulose in the epidermal cell wall, (b) the potentialities of the enzyme system of the pathogen and (c) the potential force that the pathogen was able to exert. It was considered that the “inverted funnel” appearance of a penetration hypha corresponded to evidence of enzyme activity during penetration. Such enzyme activity resulted in widespread softening and swelling of the epidermal cell wall layers (Dickinson, 1960). It is well-known that cell wall degrading enzymes are produced by *B. cinerea* on infection of *V. faba* leaves (see Table 1.1 and references therein). The role(s) of

³ It has been suggested previously that such penetration hyphae may be formed as a result of attack by the plant host or unfavourable environmental conditions (Rijkenberg, De Leeuw & Verhoeff, 1980).

cell wall-degrading enzymes in the *V. faba*-*B. fabae* interaction is discussed further in Section 5.3.7 (see also Chapter Six).

LTSEM of dry-infections revealed that the superficial conidia and germ tubes of *B. cinerea* frequently collapsed following penetration of *V. faba* leaves, possibly as a result of the passage of cytoplasm into the penetration and infection hyphae inside the host tissue. Such a phenomenon has been observed in other plant-*Botrytis* interactions and may function as a measure of conservation on the part of the pathogen (Ward, 1888; Clark & Lorbeer, 1976).

B. fabae infection hyphae often followed an intra-mural route following penetration of anticlinal wall junctures or the outermost layer of the epidermal cell wall. In the latter case, infection hyphae ramified within the outer periclinal walls of the epidermal cells prior to invasion of the epidermal cell layer via anticlinal wall junctures or entry into cavities of epidermal cells. Since, in many cases, infection hyphae entered the lumen of epidermal and mesophyll cells, it is concluded that *B. fabae* infection hyphae do not follow exclusively an intra-mural route on infection of *V. faba* leaves (see also Harrison, 1988). Ramification of *B. cinerea* infection hyphae within the outer periclinal wall of the onion leaf epidermis was reported previously (Clark & Lorbeer, 1976). These authors suggested that orientation of the cellulose microfibrils which comprise the epidermal cell walls may determine the course of growth of the infection hyphae (Clark & Lorbeer, 1976). It is also likely that the growth of *B. fabae* infection hyphae through plant cell walls follows a path of least resistance.

5.3.7 *Botrytis*-infection processes cause extensive swelling of host cell walls and widespread necrosis of host tissue

DIC microscopy and TEM of infected leaves showed that following penetration of the epidermal cell wall, swelling of the epidermal and contiguous cell walls occurred, as observed previously in plant-*Botrytis* interactions (Ward, 1888; Brown 1916; Blackman & Welsford, 1916; Wilson, 1937; McKeen 1974; Mansfield & Richardson, 1981). It has been reported that these host cell responses only occur after the penetrating hyphae have reached the epidermal cell walls, i.e. only after penetration of the cuticle (Brown, 1916; Blackman & Welsford, 1916; Verhoeff, 1980).

Swelling or loosening of plant host cell walls is a characteristic symptom of attack by pectic enzymes (Cooper, 1989). Swelling of isolated walls of soybean or rice, or the walls of apple suspension culture cells was reported following their treatment with highly purified endo-pectate and endo-pectin lyases from *Erwinia chrysanthemi* (Baker, Aist & Bateman, 1980) and *Monilinia fructigena* (Hislop, Keon & Fielding, 1979) respectively. Pectin-degrading enzymes or polygalacturonases are produced by conidia of *B. cinerea* grown *in vitro* and *in vivo* (Hancock, Millar & Lorbeer, 1964; Verhoeff & Liem, 1978; Movahedi & Heale, 1990; Johnston & Williamson, 1992). It has been reported that *B. fabae* produces twenty times more endo-polygalacturonase compared to *B. cinerea* (Cooper, 1984). Early microscope studies investigating *V. faba*-*Botrytis* interactions reported that the swelling of epidermal cell walls occurred as a result of the production of “wall-dissolving” enzymes by infection hyphae on penetration of the leaf tissue (Blackman & Welsford, 1916; Brown, 1916; Mansfield & Richardson, 1981). The role(s) of cell wall-degrading enzymes in the deconstruction of plant cell walls is discussed further in Chapter Six.

Microbial pectic enzymes not only macerate plant tissue but they also kill plant cells and elicit synthesis and accumulation of phytoalexin (Brown, 1915; Tribe, 1955; Mount, Bateman & Basham, 1970; Hahn, Darvill & Albersheim, 1981; Wijesundera *et al.* 1989; Alghisi & Favaron, 1995). The cell-killing action of pectic enzymes has often been questioned (Hall & Wood, 1973). In addition, it has been reported that phytotoxins produced by plant pathogenic fungi are also capable of killing host protoplasts (Wood, 1967). Harrison (1980) showed that extracts from liquid cultures of *B. fabae* and from aggressive chocolate spot lesions contained heat-stable phytotoxins which controlled lesion expansion depending on the humidity of the air surrounding a leaf. Harrison (1983b) partially-purified the heat-stable phytotoxin produced by *B. cinerea* and showed that their molecular weight were within the range 10 000 to 30 000 daltons and that they were unlikely to have a protein content. It was also observed that polygalacturonase produced by *B. cinerea* also killed leaf tissues (Harrison, 1983b). It was proposed that toxins and cell wall-degrading enzymes may act together to control lesion development in chocolate spot disease of broad bean. In an early report, Nordhausen (1899) also considered that *B. cinerea* killed leaf tissues of *V. faba* by secreting both cell wall-dissolving enzymes and toxins. Further studies are necessary for the elucidation of the role of pectin-degrading enzymes and toxins in the *V. faba*-*B. fabae* interaction.

5.3.8 Lesion formation

The macroscopic symptoms of chocolate spot disease caused by *B. fabae* or *B. cinerea* are well documented (Wilson, 1937; Harrison, 1988). In the present study, LTSEM observations of the surface of developing lesions caused by *B. cinerea*-infection of *V. faba* leaves revealed that the macroscopic “chocolate spots” or necrotic lesions corresponded to discrete areas of collapsed epidermal cells. Localised collapse of both epidermal and mesophyll cells has been described previously during the early stages of *B. cinerea*-infection of rose petals (Pie & De Leeuw, 1991), onion leaves (Clark & Lorbeer, 1976), immature tomato fruits (Rijkenberg, De Leeuw, & Verhoeff, 1980) and cucumber flowers (Elad, 1988). Recently, by LTSEM, Daniels & Lucas (1995) observed the collapse of epidermal cells of *V. faba* leaves following infection by *B. fabae*. However, hitherto, no such collapse of epidermal cells has been reported to occur during the early events in the establishment of the *V. faba*-*B. cinerea* relationship.

5.3.9 Immunocytochemical studies of the *Vicia faba*-*Botrytis* interaction using the *Botrytis*-MAbs: Do they tell us something new?

Immunocytochemical studies of the *Vicia faba*-*Botrytis* interaction using the *Botrytis*-MAbs, BC-KH4, BC-AF7 and BC-FD7-G9, revealed the location of the *Botrytis*-antigens throughout the fungal structures involved in the pre-penetration and pre-infection processes. Preliminary studies using LSCM in the epifluorescence mode indicated that this technique was particularly useful for the observation of the spatial distribution of the surface antigens recognised by the *Botrytis*-MAbs throughout the infection site. The results of these immunocytochemical studies are summarised in Diagrams 5.2 and 5.3 for wet- and dry-infections respectively. No difference in labelling was observed between the MAbs tested. Since the BC-KH4 MAb was used most extensively, the aforementioned diagrams indicate the distribution of BC-KH4 MAb-binding sites.

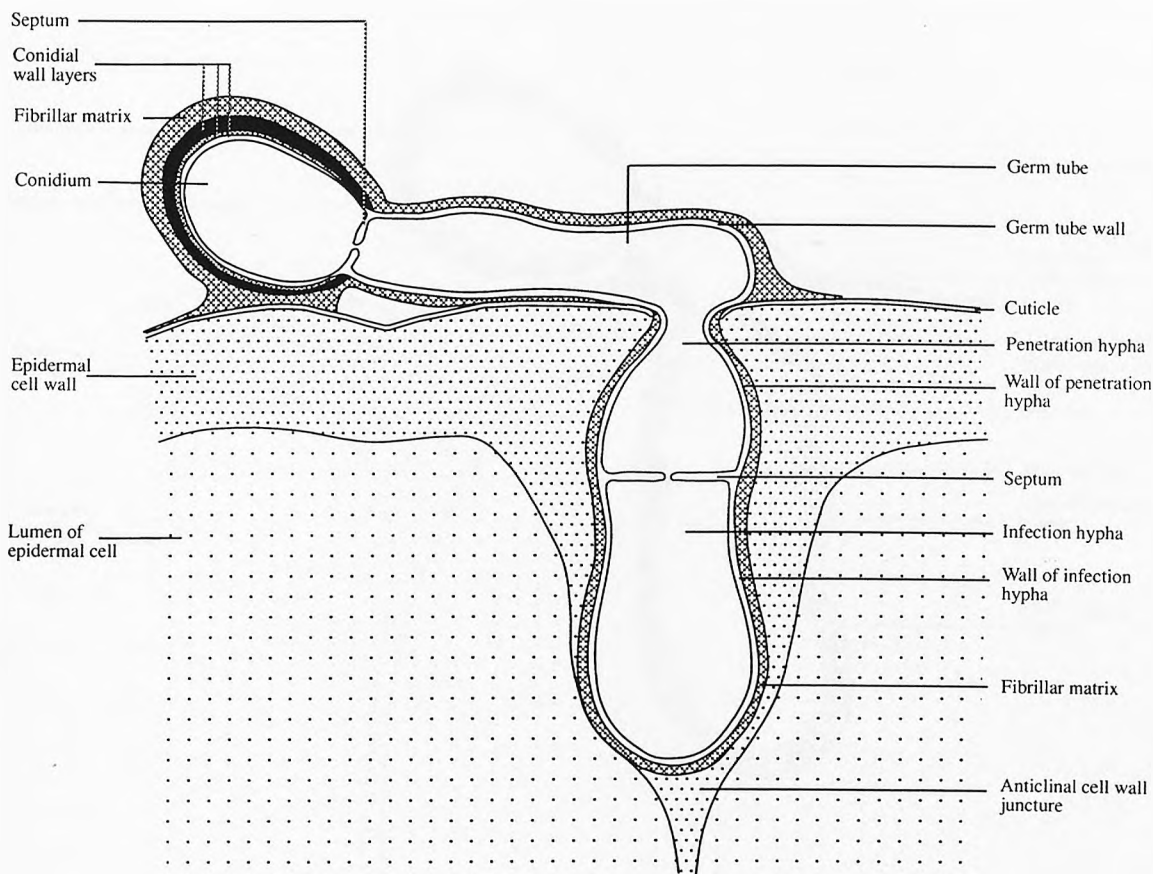


Diagram 5.2 Location of extracellular matrix material and BC-KH4 MAb-binding sites on external and internal fungal structures formed following inoculation of wet conidia of *B. fabae* on *V. faba* leaves

BC-KH4 MAb-binding sites were located throughout the fibrillar matrix material surrounding the sporeling on the leaf surface. This immunolabelled fibrillar matrix formed a veil-like covering over the germinating conidium and the developing germ tube. At the site of penetration a narrow penetration hypha was often formed. This penetration hypha formed a swollen infection hypha and was delimited from it by a septum. Both the penetration and infection hyphae were surrounded by a fibrillar sheath which was also immunolabelled with the BC-KH4 MAb.

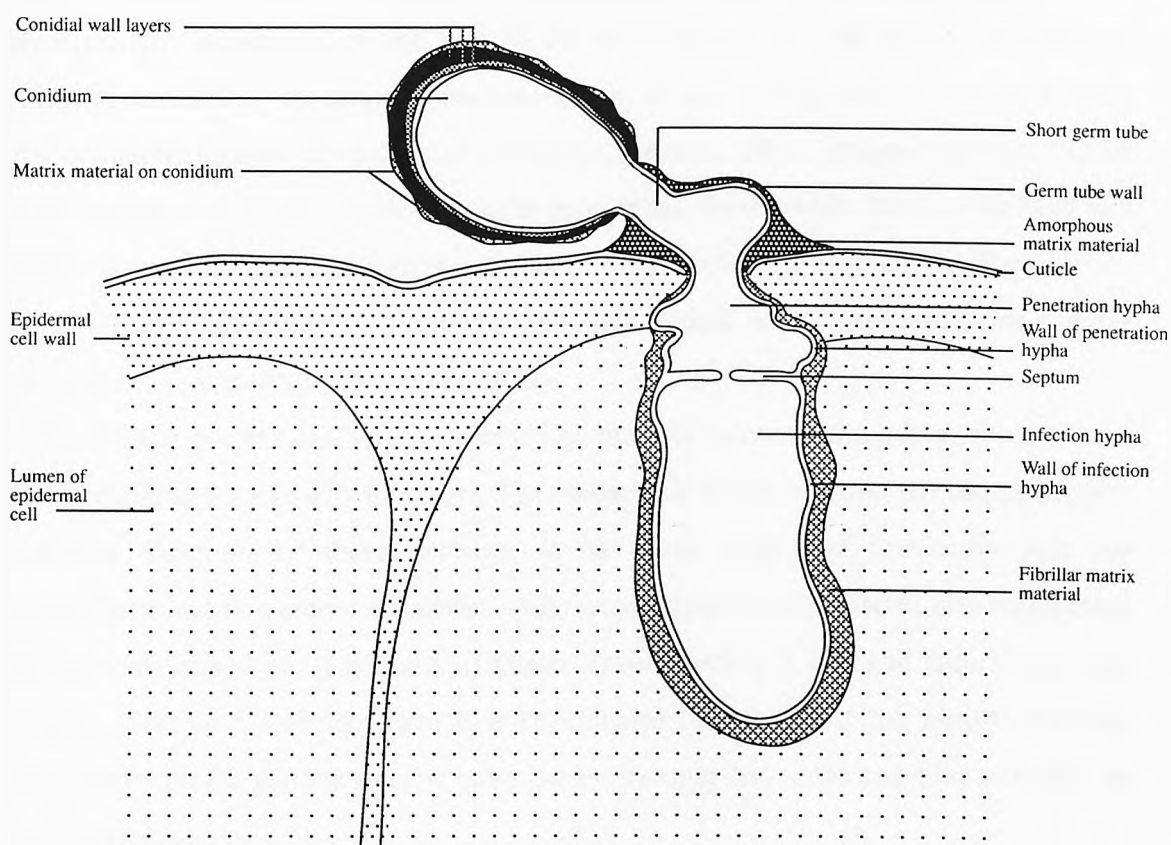


Diagram 5.3 Location of extracellular matrix material and BC-KH4 MAb-binding sites on external and internal fungal structures formed following inoculation of dry conidia of *B. fabae* on *V. faba* leaves

BC-KH4 MAb-binding sites were located at patches of matrix material on the surface of the conidium. A distinct pad of amorphous matrix material surrounded the short germ tube at its site of penetration of the leaf surface. This amorphous pad was also immunolabelled. On ingress into the cuticle and epidermal cell wall, the penetration hyphae formed a swollen infection hypha and was delimited from it by a septum. Both the penetration and infection hyphae were surrounded by a fibrillar sheath which was also immunolabelled with the BC-KH4 MAb.

In all cases, and in the presence of aqueous glucose, immunolabelled matrix material was apparent at the surface of conidia, germ tubes and infection hyphae. Similar matrix material was observed previously at the surface conidia and germ tubes when *Botrytis* sporelings were grown *in vitro* (see Chapter Three, Sections 3.3.2.2 & 3.3.2.4). Previous immunocytochemical studies, using specific MABs (UB20 and UB22) raised against homogenates of *Colletotrichum lindemuthianum* sporelings, have shown that the glycoproteins recognised by the two MABs were present on the surface of different infection structures in varying amounts (Pain *et al.*, 1992). UB20 antigens were predominantly located on conidia of sporelings, whereas UB22 antigens appeared to be most abundant in fibrillar material around germ tubes. Both MABs labelled the wall and matrix around intracellular infection hyphae in infected bean hypocotyls (Pain *et al.*, 1992). It was concluded that conidia and germ tubes of *C. lindemuthianum* differ markedly in composition (Pain *et al.*, 1992).

In the present study, the observed similarities between the composition of the matrix material associated with external and internal *Botrytis* infection structures suggest a similar function for these matrices. It has been suggested previously that the extracellular matrix material associated with *Botrytis* sporelings provided firm attachment for the sporelings to both synthetic (Chapter Three, Section 3.3.2) and natural (see this Chapter, Section 5.3.4) substrata. It is conceivable therefore that the fibrillar material associated with the penetration and infection hyphae may have also provided adhesion to the infection site.

It was interesting to note that the results of IF studies of the infection processes of dry-inoculated conidia of *Botrytis* species grown *in vitro* (see Chapter Three, Section 3.3.2.2) and *in vivo* (see this Chapter, Section 5.2.4) revealed that *Botrytis*-MAB-binding sites were located at the surface of conidia. However, the short germ tubes were rarely immunolabelled. Ultrastructural observations revealed the presence of small amounts of immunolabelled matrix material at the surface of the germ tubes and at points of contact of the short germ tubes to the leaf surface. Thus, it is likely that such material was also present in IF preparations but that the fluorescence signal was below the limit of resolution of this microscope technique.

IEM studies of the *V. faba*-*B. fabae* interaction showed that both the fibrillar and amorphous matrices produced during the pre-penetration processes of dry and wet conidia respectively were successfully immunolabelled with the *Botrytis*-MAB BC-KH4 (see Diagram 5.2 and 5.3). Since it has been reported previously that all three MABs

recognise a carbohydrate epitope on a glycoprotein (Bossi & Dewey, 1992), it therefore follows that both types of matrices contain a glycoprotein component. It is suggested that the amorphous material associated with the short germ tubes of dry-inoculated conidia consisted of the fibrillar material produced by conidia grown in the presence of aqueous glucose, but in a more condensed form as a result of the absence of free water.

Beckett and colleagues (1990) observed that dry-inoculated urediniospores of *Uromyces viciae-fabae* germinated on hydrophobic surfaces under conditions of high humidities to form a thick, viscous mucilaginous deposit between the germ tube and the substratum (Beckett, Tatnell & Taylor, 1990). They concluded that the amount and form of extracellular matrix material produced by the fungus varied according to the availability of free water and the nature of the substratum surface. Previous studies of the production of extracellular matrices by *Botrytis* sporelings indicated that dry-inoculated conidia released a thin pool of amorphous material following germination on glass surfaces (see Chapter Three 3.3.2). However, no such material was observed at the surface of *V. faba* leaves following the germination of dry-inoculated conidia of *B. cinerea* or *B. fabae* and immunolabelling using the *Botrytis*-MAbs. It is suggested that the amount and form of extracellular matrix material produced by conidia of *Botrytis* species may also vary according the nature of the substratum surface. Such an hypothesis is further corroborated by immunocytochemical studies of both wet and dry-infections, since in every case, fibrillar matrix material was associated with the penetration and infection hyphae. This fibrillar material was immunolabelled using the *Botrytis*-MAbs (Diagrams 5.2 and 5.3). Further analyses of the composition of the extracellular matrix material produced by wet and dry-inoculated conidia of *B. cinerea* and *B. fabae* on both artificial and natural substrata are required.

Close examination of the *V. faba*-*B. fabae* interface inside the plant host tissue revealed that the fibrillar material extended some distance from the surface of the infection hyphae and in some cases, through the swollen epidermal and mesophyll cell walls. In a early IEM study of infection of *V. faba* leaves by *B. cinerea*, two antisera were employed, one prepared against surface components of *B. cinerea* and the other against fimbriae of the smut fungus *Ustilago violacea* (Svircev *et al.*, 1986b). It was observed that both antisera labelled host cells not penetrated by the fungus and were some distance away from the nearest hypha. They suggested two alternative hypotheses for their results; (i) fimbrial protein, either as intact fibrils or as dissociated subunits, entered host cells well ahead of the hyphae or (ii) infection by the fungus stimulated a

host response that included the synthesis of host proteins with antigenic regions similar to those of fimbriae of *U. violacea* and the surface proteins of *B. cinerea*. In the present study, it seems highly unlikely that the plant host synthesised proteins with similar antigenic determinants to those of the fibrillar matrices associated with the infection hyphae as a response to infection since rapid necrosis of host cells was observed immediately upon *B. fabae*-infection. Furthermore, no such molecules were located inside unaffected i.e. healthy host cells at the edge of the developing lesion. It must also be mentioned that no such antigens were located in non-inoculated leaves following immunolabelling using the *Botrytis*-MAbs. In all cases, although immunolabelled fibrillar material was located some distance from the infection hyphae, TEM observations showed that this fibrillar material was always associated with the infection hyphae and not distributed free throughout the infection site. Thus, it was considered that these antigens were of fungal origin. The penetration of the fibrillar material associated with the infection hyphae through the plant cell wall is discussed further in Chapter Six, see Section 6.3.5).

The location of *Botrytis*-MAb binding sites throughout the walls of the infection hyphae, including septa, and their absence from inside fungal cells suggested that these antigens were synthesised *in muro*. However, the possibility that such surface molecules were secreted at the hyphal tips cannot be ruled out (see Chapter Four, Section 4.3.2) and further studies are required.

Chapter Six

Chocolate spot disease of broad bean (*Vicia faba* L.)

II. Further investigations of the post-penetration processes involved in the *V. faba*-*Botrytis fabae* interaction

6.1 Introduction

During penetration and colonisation of plant tissues, pathogenic fungi come into contact with the host cell walls. In many cases, breakdown of the plant cell wall is an essential process, not only to ensure spread of the fungus throughout the host tissue, but also for the release of assimilable carbohydrates or accessibility to the host cell protoplast and cell killing. In the previous Chapter, some attention was paid to the host cell wall dissolution following penetration of the epidermal cell wall of *Vicia faba* leaves by *B. fabae* (see Chapter Five, Section 5.3.7). This Chapter examines further the mechanisms by which cell wall deconstruction occurs during the development of the *V. faba*-*B. fabae* interaction.

6.1.1 The plant cell wall

In order to understand the possible mechanisms by which cell wall breakdown occurs, it is essential to have some knowledge of the macromolecular composition of plant cell walls, the nature and structural significance of these molecules, their spatial arrangement and how these molecules interact with each other. The chemical composition and molecular architecture of plant cell walls has been reviewed extensively (Northcote, 1972; Preston, 1974; McNeil *et al.*, 1984; Bacic, Harris & Stone, 1988; Fry, 1988; Roberts, 1989; Varner & Lin, 1989).

Plant cell walls can be divided into two types: primary and secondary. The primary cell walls of plants are thin and accommodate cell expansion. Secondary walls, on the other hand, are deposited on the inside of the primary wall of cells when they have stopped expanding. In mature fully differentiated cells e.g. collenchyma cells, tracheids and phloem fibres, the secondary wall forms the bulk of the whole cell and has a laminated and complex structure. However, in some cells, e.g. isodiametric parenchymatous cells, the secondary walls are poorly developed. Throughout the plant tissue, the primary walls of contiguous cells are separated by a common layer termed the middle lamella, which cements the cells together. Although the primary wall forms a thin envelope around the growing cell, this delicate layer remains present in a mature, fully differentiated cell as the outer limiting layer of the wall. The primary wall is therefore the first barrier that a plant pathogenic fungus encounters following its passage through the plant cuticle.

Some advances in the knowledge of the macromolecular composition and the molecular architecture of plant cell walls have come from studies on primary cell walls using cultured sycamore cells (Talmadge *et al.*, 1973; Bauer *et al.*, 1973; Keegstra *et al.*, 1973). It can be assumed that the secondary wall is essentially composed of macromolecules laid down in the primary wall, with the exception of lignin which is usually confined to secondary walls (Preston, 1974). The relative proportion of these substances, their relative disposition and degree and type of orientation is also likely to vary between primary and secondary walls (Preston, 1974).

6.1.2 Macromolecular composition of plant cell walls

The primary cell walls of all higher plants are composed largely of polysaccharidic material (Fry, 1988; Roberts, 1989). The main three types of polysaccharides are cellulose, hemicelluloses and pectins (Northcote, 1972; Fry, 1988):

6.1.2.1 Cellulose

Cellulose is an unbranched polymer of D-glucopyranose residues joined by β -(1 $\rightarrow$ 4) linkages (see Appendix A). Intermolecular hydrogen bonding between adjacent cellulose polymers causes the polymers to strongly adhere to each other in overlapping parallel arrays forming bundles of 60-70 cellulose chains, all of which have the same polarity. These bundles form the *cellulose microfibrils*; they are highly-ordered crystalline aggregates and may be many micrometres long. Cellulose is particularly abundant in secondary walls and constitutes usually 20-30 % of the dry weight of most primary walls (Preston, 1974; McNeil *et al.*, 1984; Fry, 1988). The biological function of cellulose is presumed to be skeletal, since it provides both shape and strength to the wall (Fry, 1988).

6.1.2.2 Hemicelluloses

Hemicelluloses are functionally defined as those polysaccharides that form strong hydrogen-bonded complexes with cellulose fibres (McNeil *et al.*, 1984; Fry, 1988). The hemicelluloses of the primary walls of higher plants are xyloglucan (XG) and arabinoxylan (AX). XGs are composed of a branched 4-linked β -D-glucan backbone. In many plants, approximately 75% (fewer in grasses) of the backbone glucosyl residues are substituted at O-6 with an α -D-xylosyl residue (Bauer *et al.*, 1973; McNeil *et al.*, 1984; Fry, 1988). XGs represent 20% of the primary walls of dicots and their function is presumed to be structural (McNeil *et al.*, 1984; Fry, 1988). AXs on the other hand are composed of a backbone of β -(1 $\rightarrow$ 4)-linked D-xylopyranose residues: they form only a small percentage (5%) of the primary walls of dicots (Darvill *et al.*, 1980; Fry, 1988).

6.1.2.3 Pectins

Pectins are a major matrix component in the primary walls of dicotyledonous plants. Three different classes of pectic polysaccharides have been isolated from the primary cell walls of dicots: homogalacturonan also known as pectate, polygalacturonate or

polygalacturonic acid [PGA] and two types of rhamogalacturonans [RG-I and RG-II] (Varner & Lin, 1989; Roberts, 1990). Homogalacturonan is a chain of partially methyl-esterified 1,4-linked α -D-galacturonic acid residues (Jarvis, 1984; McNeil *et al.*, 1984; see Appendix A). Non-esterified homogalacturonan sequences of pectins can self-associate to form dimers and aggregates through calcium binding to form the junction zones that hold a gel together (Jarvis, 1984). This propensity of homogalacturonan to form gels may reflect its function in wall structure. Pectins are considered to play an important role in cell wall hydration, adhesion of contiguous cells and wall plasticity during growth (Jarvis, 1984). They may also act as a source of biologically active molecules (Nothnagel *et al.*, 1983; McNeil *et al.*, 1984; Fry, 1988). However, the contention that pectins are simply matrix polymers i.e. with no skeletal function is now largely contested (Roberts, 1990) in view of recent studies: Research by Shedletzky and colleagues (1990) has shown that cultured tomato cells adapted to the presence of 2,6-dichlorobenzonitrile, a potent inhibitor of cellulose synthesis. These adapted cells formed coherent walls with virtually no cellulose or hemicellulose and a much reduced protein content. It was shown that the walls of these adapted cells consisted solely of a cross-linked meshwork of the three classes of pectin (Shedletsky *et al.*, 1990).

Pectin molecules may be glycosidically linked to XG chains (Keegstra *et al.*, 1973). Since, XG chains can bind non-covalently to cellulose, pectin molecules may therefore become linked to cellulose indirectly (Keegstra *et al.*, 1973). Recent research on dicot walls has also revealed surprising results concerning interactions between pectin and cellulose molecules (Seymour *et al.*, 1990): By a series of mild chemical extractions, it was found that a highly branched neutral polysaccharide remains very tightly complexed to the cellulose residue.

6.1.2.4 Other polysaccharides

Other polysaccharides which may be located in the cell walls of plants include callose, lignin and small amounts of arabinans, galactans and arabinogalactans. However, only callose will be discussed further here. Callose is an unbranched polymer of D-glucopyranose residues joined by β -(1 $\rightarrow$ 3) linkages and is normally only present in healthy cell walls in small amounts where it occurs in specialised walls (e.g. sieve plates) or parts of cell walls (e.g. plasmodesmata).

6.1.2.5 Glycoproteins

In addition to polysaccharidic material, glycoproteins e.g. extensins, arabinogalactan proteins [AGPs], lectins and certain enzymes (e.g. β -1,3-glucanases, phosphatases, peroxidases), are present in specific locations throughout the primary walls and middle lamellae of plants (McNeil *et al.*, 1984; Fry, 1988). Extensins are a family of hydroxyproline-rich glycoproteins [HRGP] (Cooper *et al.*, 1987; Tierney & Varner, 1987; Cassab & Varner, 1988; Varner & Lin, 1989). They occur in the primary walls of dicots in widely varying amounts (1-10 %) and are particularly abundant in epidermal cell walls (McNeil *et al.*, 1984; Fry, 1988). Extensins may form a protein network in the primary wall, possibly as a result of inter-polypeptide cross-linking via iso-dityrosine bridges (McNeil *et al.*, 1984; Fry, 1988; Varner & Lin, 1989). Analyses of fragments released by proteolytic action from endopolygalacturonase-endoglucanase-treated cell walls of suspension-cultured sycamore cells have also provided evidence for a covalent-connection between the pectic polysaccharides and structural protein (Keegstra *et al.*, 1973). Furthermore, since extensins are positively-charged molecules, it has been suggested that they may be ionically bound to negatively-charged polysaccharides in the wall (Varner & Lin, 1989). A number of roles have been suggested for extensins in the primary cell wall: they may provide structural support, restriction of growth, exclusion of invading pathogens and/or they may be involved in the organisation of the cellulose microfibrils (Fry, 1988).

A simplified diagram showing the distribution of the different polymers throughout the walls (primary and secondary) and middle lamella of two adjacent cells of a dicotyledonous plant is given below (Diagram 6.1).

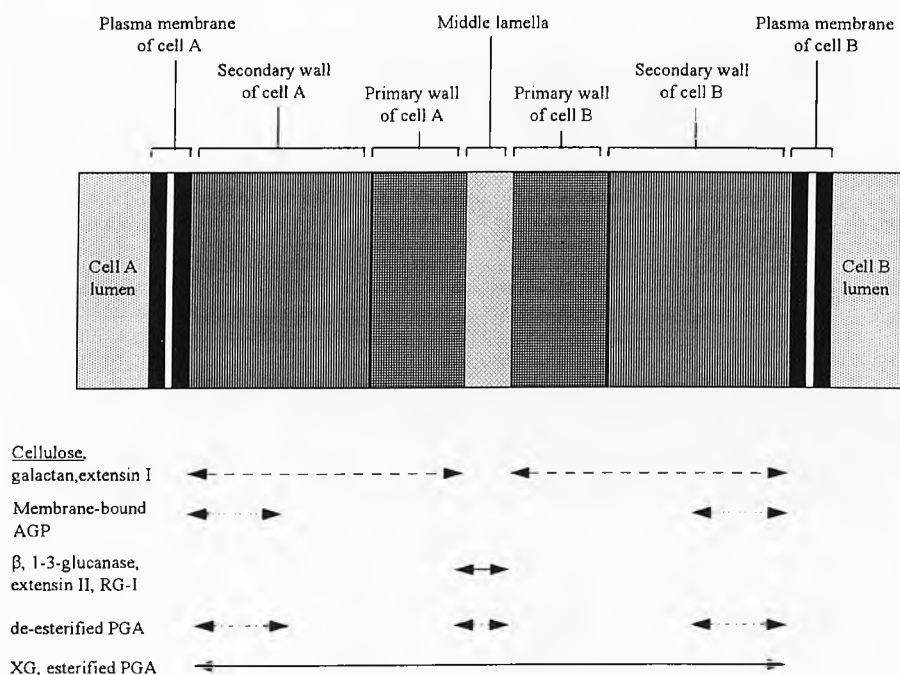


Diagram 6.1 A proposed model of the walls of two adjacent cells, A and B, of a dicotyledonous plant, showing the localisation of the different polymers

The localisations shown are based on immunogold labelling data from various groups (adapted from Roberts, 1989). In some cells e.g. parenchyma cells, the secondary wall may be poorly developed. However, in highly differentiated cells e.g. xylem elements, the secondary wall thickenings are formed. These wall thickenings often contain lignin. AGP, arabinogalactan protein; RG-I, rhamnogalacturonan I; PGA, polygalacturonic acid; XG, xyloglucan. The two polymers of interest in this study, cellulose and esterified PGA are underlined.

6.1.3 Molecular architecture of plant cell walls

In simple terms, the molecular architecture of the plant cell wall consists of a framework of cellulose microfibrils held together by hemicellulose which is co-extensive with a network of pectic polysaccharides (Roberts, 1989). If however, the protein network formed by extensin molecules is included in this model of the architecture of the primary cell wall, as considered by Varner & Lin (1989), then it is clear that the primary wall is best regarded as a *multi-network gel* (Roberts, 1989; Varner & Lin, 1989; see Diagram 6.2).

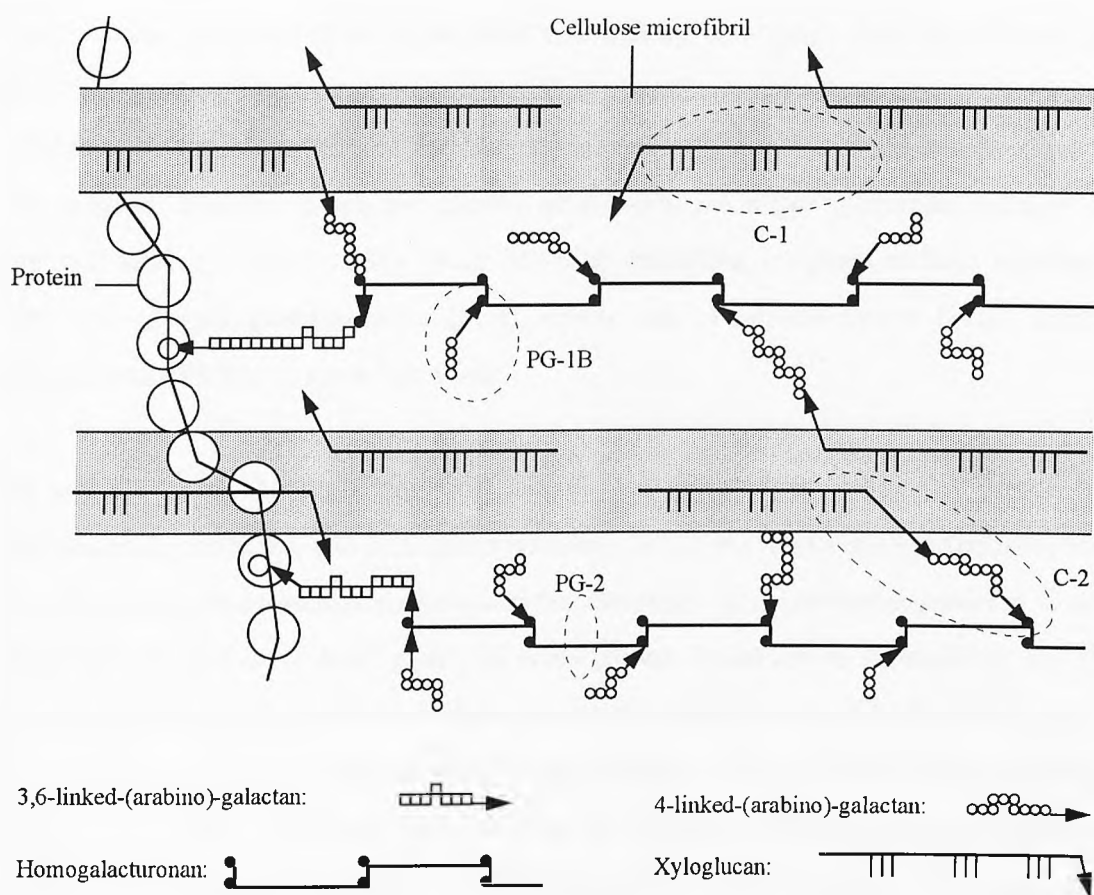


Diagram 6.2 Proposed model of a primary cell wall showing the two major polysaccharide networks, interaction of the protein network and sites of action of cell wall-degrading enzymes

Sets of microfibrils are arranged in layers or "lamellae" and each *cellulose microfibril* is attached to each other by cross-linking *via* hydrogen bonds with the long hemicellulosic (*xyloglucan*) molecules. The layers of cellulose microfibrils are orthogonally arranged. This cellulosic and hemicellulosic network is co-extensive with a network of pectic polysaccharides (*homogalacturonan*) and a network of hydroxyproline-rich *protein* molecules. The xyloglucan and pectic polysaccharides are often inter-linked *via* 4-(arabino)galactan bridges. The galactan side chains of protein molecules may interconnect with the terminal end of pectic polysaccharides to form 3,6-(arabino)galactan cross-linkages. Pectic polysaccharides and cellulose microfibrils may also interact with each other, possibly *via* 4-(arabino)galactan bridges. The cellulose and hemicellulose network provides tensile strength while the pectin network resists compression. The protein network may also play a structural role in cell wall architecture. The circled areas show wall fractions (PG-1B and PG-2) and (C-1 and C-2) released by the action of degradative enzymes: endo-polygalacturonase (endo-PG) and endo-glucanase respectively. The endo-PG attacks the galacturonosyl linkages of the main pectin chain releasing tri-, di- and mono-galacturonic acid (fraction PG-2) as well as the arabinan and galactan chains attached to the acidic fragments of the main chain (fraction PG-1B). Following endo-PG activity, endo-glucanases can more readily degrade xyloglucan releasing neutral oligosaccharides (fraction C-1) and pectic fragments that may be held insoluble by their connection to xyloglucan (fraction C-2). (Redrawn from Keegstra *et al.*, 1973).

6.1.4 Cell wall degradation by phytopathogenic fungi

In many cases, deconstruction of the plant cell wall by pathogenic fungi is achieved by cell wall degrading enzymes (CWDEs) which are produced constitutively by the fungus or induced by the substrate¹. Many, but not all, phytopathogenic fungi can produce a wide range of CWDEs which are capable of cleaving the major glycosidic linkages in plant cell walls (Cooper, 1984). Such cell-wall degrading enzymes include cellulase, endo- and exo-polygalacturonases (PGs), endo- and exo-pectin lyases (PLs), pectin methylesterase(PME), to name but a few.

It is generally assumed that the plant wall structure is loosened by the activity of PGs and PLs and the remaining components are exposed to sequential attack by hemicellulases, proteases, and cellulases (Wheeler, 1975). PG has been reported to be the first polysaccharide-degrading enzyme which is secreted by *Fusarium oxysporum* f. sp. *lycopersici* cultured on isolated plant cell walls (Jones, Anderson & Albersheim, 1972). Evidence has also been obtained which indicates that in some cases, PG acts before other CWDEs can attack their substrates (Karr & Albersheim, 1970). Furthermore, it has been suggested that the production of CWDEs by several pathogenic fungi including *Colletotrichum lindemuthianum* and *Verticillium albo-atrum* occurs in the sequence: pectic enzymes, hemicellulases and finally cellulases (English, Jurale & Albersheim, 1971; Cooper & Wood, 1975). It is clear that deconstruction of plant cell walls by pathogenic fungi is a complex process which may involve a sequence of enzymatic reactions. It is noteworthy here that CWDEs, in particular endo-PGs and endoglucanases have proved to be invaluable tools for the molecular dissection of walls of suspension-cultured sycamore cells (Talmadge *et al.*, 1973; Bauer *et al.*, 1973; Keegstra *et al.*, 1973). As a consequence, the action of CWDEs have contributed significantly to the knowledge of the composition and architecture of the primary wall of plants. The sites of action of an endo-PG and endo-glucanase isolated from *Colletotrichum lindemuthianum* and *Trichoderma viride* respectively are shown in Diagram 6.2.

CWDEs are considered to be of particular importance to parasitic fungi that do not produce appressoria (Mendgen & Deising, 1993). Appressoria provide support for

¹ Regulation of the production of pectic enzymes by fungal pathogens has been reviewed extensively (Cooper, 1983; Collmer & Keen, 1986). In brief, basal or constitutive pectinolytic activity results in the detachment of the monomer D-galacturonate from the pectic polymer. This monomer, in turn induces the synthesis of PG and PL, which can demolish the pectin polymer further. Nutritional, environmental and plant factors may also interfere with this regulatory mechanism and influence the amount of production of, as well as the sequence of synthesis of, the enzymes and iso-enzymatic forms.

the pressure applied by the infection peg during penetration (Howard *et al.*, 1991; Mendgen & Deising, 1993). Nonetheless, it has been suggested that localised secretion of CWDEs may be necessary for penetration of plant host cell walls by some parasitic fungi which form distinct appressoria e.g. *Erysiphe* species (Staub, Dahmen & Schwinn, 1974). Indeed, most plant pathogenic fungi which penetrate the plant cell wall by means other than purely mechanical, possess one or more pectic enzymes which can attack the plant cell wall.

6.1.5 Cell wall degrading enzymes produced by *B. fabae*

It is widely accepted that the plant pathogenic fungus *B. fabae* produces large amounts of pectin-degrading enzymes during the development of chocolate spot disease of broad bean (Deverall & Wood, 1961b; Balasubramani, Deverall & Murphy, 1971; Harrison, 1988). These enzymes are known to cause cell separation and ultimately cell death (see Chapter Five, Section 5.3.7). In contrast, there is as yet no evidence that other CWDEs e.g. hemicellulases and/or cellulases are produced by this fungus.

6.1.6 Ultrastructural evidence for the action of CWDEs

The results of early ultrastructural studies have indicated that swelling of plant cell walls is a symptom of attack by pectic enzymes (Hislop, Keon & Fielding, 1979; Baker, Aist & Bateman, 1980). Similarly, researchers investigating plant host-pathogen interactions by electron microscopy have attributed the swelling of host walls to the degradative action of pectin-degrading enzymes produced by the pathogen (Mansfield & Richardson, 1981; O'Connell, Bailey & Richmond, 1985). Research using the *Aplysia depilans* gonad lectin-gold complex has provided convincing evidence for extensive pectin breakdown of tobacco root tissues following colonisation by *Phytophthora parasitica* var. *nicotianae* (Benhamou & Côté, 1992). Furthermore, using the PG-binding protein (PGIP) as a probe, a PG enzyme has been located in bean leaf cells where extensive breakdown or cell separation occurred following the necrotrophic phase of growth of *Colletotrichum lindemuthianum* (Benhamou *et al.*, 1991). More recently, endo-PG activity has been

detected in soybean infected with *Sclerotinia sclerotiorum* using an anti-PG antibody (Favaron *et al.*, 1993). Other possible markers for pectic molecules that could be applied to studies of the role of pectin-degrading enzymes in the development of plant-host pathogen relationships include the pectinase-gold complex and antibodies to pectin. The MAbs JIM 5 and JIM 7 which recognise non-esterified and methyl-esterified pectins respectively have been employed previously to locate pectic polysaccharides in plant cell walls in a number of different plant species and in specific cell types (VandenBosch *et al.*, 1989; Bonfante-Fasolo *et al.*, 1990; Knox *et al.*, 1990; Vian & Roland, 1991).

Evidence for cellulose degradation by plant pathogens has come from ultrastructural studies using an exo-glucanase, cellobiohydrolase, isolated from *Trichoderma* and conjugated directly to colloidal gold for the detection of cellulose (Benhamou & Ouellette, 1987b; Benhamou *et al.*, 1987; Benhamou & Côté, 1992). At present, the cellobiohydrolase-gold (CBH1-gold) conjugate technique appears to be the best method for visualising the abundant cellulose in plant cell walls at the ultrastructural level (Chanzy *et al.*, 1983; Chanzy, Henrissat & Young, 1984; Berg *et al.*, 1988; Berg, 1990; Vian & Roland, 1991). A polyclonal antiserum raised against oligosaccharides containing β -(1 $\rightarrow$ 4) glucose has been produced by Northcote and his co-workers (1989) but unfortunately, this antiserum did not recognise native cellulose when tested by immunogold labelling and TEM (Northcote, Davey & Lay, 1989).

In this present study, the *in situ* modification of the pectic and cellulosic components of the epidermal and mesophyll cell walls of *V. faba* leaves by *B. fabae* were investigated at the ultrastructural level using the anti-pectin antibody, JIM 7 and a cellobiohydrolase-gold complex directed against cellulose. Double-labelling studies using varying combinations of molecular markers which recognise components of (i) the fungal cell wall [e.g. anti- β -(1 $\rightarrow$ 3)-glucan antibody (Northcote, Davey & Lay, 1989; see also Chapter Four], (ii) *Botrytis* extracellular matrices [BC-KH4 MAb (Bossi & Dewey, 1992; Bossi *et al.*, 1994; see also previous Chapters)] and (iii) the plant cell wall (e.g. JIM 7 and cellobiohydrolase-gold complex) were employed in order to investigate the post-penetration processes further. Attention was also paid to the role of the fungal extracellular matrices in the *V. faba*-*B. fabae* interaction.

6.1.7 Host defence responses to infection by plant pathogenic fungi

Just as parasitic fungi have evolved the ability to produce a wide variety of cell wall-degrading enzymes necessary to breach the barrier afforded by the plant host wall, plants have evolved means of defending themselves from attack by fungal pathogens and in response to wounding. A plant's response to penetration by a fungal pathogen or wounding may involve changes in the plant cell wall, ranging from the deposition of callose, HRGP and lignin, for example, to more subtle alterations resulting from wall-degrading enzymes (Bowles, 1990). The large family of pathogenesis-related proteins made by infected plants are now known to consist of several wall-degrading enzymes targeted to the fungal cell wall e.g. β -1,3-glucanases and chitinases (Legrand *et al.*, 1987). In this study, an antiserum directed against oligosaccharides containing β -(1 $\rightarrow$ 3) glucose (Northcote, Davey & Lay, 1989) was employed in order to locate the distribution of callose throughout the walls of healthy and *B. fabae*-infected *V. faba* leaf cells.

6.2 Results

6.2.1 Ultrastructure of *Vicia faba* L. leaves showing specific cell types

TEM observations of ultra-thin sections of PLT-dehydrated and LR White-embedded non-inoculated *V. faba* leaves and "green" or non-necrotic leaf tissue revealed a typical leaf anatomy (not shown here) including specific cell types (Plate 6.1, Figs 1-4). The adaxial and abaxial epidermis were composed of a single layer of tightly-packed non-isodiametric epidermal cells which were occasionally interspersed by stomata and gland cells (not shown here). The outermost wall of each epidermal cell was thickened, especially at anticlinal wall junctions, and continuous with contiguous cells (Plate 6.1, Fig. 1). The outermost wall of the epidermal cell was covered by a cuticular layer (Plate 6.1, Fig. 1). The remaining walls of the epidermal cell were thin, like those of both the palisade and spongy mesophyll cells (not shown here).

Other specialised cells e.g. transfer cells (Plate 6.1, Fig. 2) and xylem elements or vessels (Plate 6.1, Fig. 3) possessed wall thickenings. In addition, the phloem cells, composed of sieve tube elements, companion and transfer cells, were readily distinguishable from other cell types. Each sieve tube element was separated from a companion or transfer cell by specialised wall regions termed “sieve areas” or “sieve plates” (Plate 6.1, Fig. 4).

6.2.2 Identification of β -(1 $\rightarrow$ 3)-glucan in *B. fabae*-infected *V. faba* leaves by IEM using a polyclonal antiserum against oligosaccharides containing β -(1 $\rightarrow$ 3)-glucose

IEM studies of *B. fabae*-infected *V. faba* leaves using anti- β -(1 $\rightarrow$ 3)-glucan antibody revealed that in non-necrotic cells, i.e. “green” regions of inoculated leaf tissue, antibody-binding sites were located at (i) plasmodesmata interconnecting adjacent mesophyll cells or sieve tubes and transfer cells (Plate 6.2, Fig. 1), (ii) at the plasmodesmata and other electron-translucent wall areas located within primary pit-fields (Plate 6.2, Fig. 2) and (iii) at sieve pores located in sieve plates (Plate 6.2, Figs 3 & 4). In control treatments, where sections of necrotic and non-necrotic leaf tissue were incubated with PBST in lieu of the primary antibody, no gold labelling was observed throughout the fungal or plant host tissue (Plate 6.2, Figs 5 & 6). Similar labelling patterns were observed when ultra-thin sections of non-infected leaf tissue were immunostained using the anti- β -(1 $\rightarrow$ 3)-glucan antibody (data not shown).

Close inspection of the *V. faba*-*B. fabae* interface revealed that antibody-binding sites were located throughout the translucent fungal cell wall (Plate 6.3, Figs 1 & 2) and in distinct regions of the highly modified walls of infected mesophyll cells (Plate 6.3, Fig. 2). In many cases, these distinct regions appeared to correspond to sites of plasmodesmata (Plate 6.3, Fig. 3). At the limiting edge of necrotic lesions, a sharp boundary existed between the necrotic and intact mesophyll cells (Plate 6.3, Fig. 4). Plasmodesmatal canals interconnecting necrotic and intact cells were also immunolabelled by the anti- β -(1 $\rightarrow$ 3)-glucan antibody (Plate 6.3, Fig. 4). It was interesting to note that antibody-binding sites were particularly abundant at canal

openings in the necrotic cell (Plate 6.3, Fig. 4). In all cases, no labelling was observed throughout the fibrillar matrix associated with the infection hyphae (Plate 6.3, Figs 1 & 2).

6.2.3 Double labelling of components of the *Botrytis* cell wall and extracellular matrices using anti- β -(1 $\rightarrow$ 3)-glucan antiserum and BC-KH4

In broad terms, when both the *Botrytis*-MAb, BC-KH4 and the anti- β -(1 $\rightarrow$ 3)-glucan antibody were applied directly and simultaneously to ultra-thin sections of the *V. faba*-*B. fabae* interface, BC-KH4 and the anti- β -(1 $\rightarrow$ 3)-glucan antibody labelled the fibrillar matrix material associated with the infection hyphae and the fungal cell walls respectively (Plate 6.4, Figs 2-4). In some cases, BC-KH4 MAb also labelled areas of the translucent fungal cell wall (Plate 6.4, Figs 2-4). Although BC-KH4 MAb-binding sites were rarely observed at any other sites throughout the necrotic or non-necrotic leaf tissue (not shown here), BC-KH4-immunolabelled matrix material often extended through the degenerate host cytoplasm (Plate 6.4, Fig. 3, arrows) and loosened host cell walls (Plate 6.4, Fig. 4). As observed previously (Plate 6.3, Figs 2 & 3), β -(1 $\rightarrow$ 3)-glucan-labelling was located at remnants of plasmodesmata (not shown here). In all cases, only traces of β -(1 $\rightarrow$ 3)-glucan-labelling were observed throughout the fungal cytoplasm, the fibrillar matrix material associated with the infection hyphae and the necrotic leaf tissue (Plate 6.4, Figs 2-4).

Similar labelling patterns were observed when the BC-KH4 MAb and the β -(1 $\rightarrow$ 3)-glucan antiserum were applied to sections separately (e.g. for β -(1 $\rightarrow$ 3)-glucan-labelling pattern: see Plate 6.3, Fig. 1). Furthermore, no gold labelling was located throughout the fungal or plant host tissue in control sections, i.e. those incubated with PBST or RPMI in lieu of the primary antibody (not shown here).

6.2.4 Comparison of the distribution of pectin in the walls of control and *B. fabae*-infected *V. faba* leaves by IEM using the anti-pectin MAb JIM 7

When ultra-thin sections of PLT-dehydrated and LR White-embedded non-inoculated (i.e. control) *V. faba* leaves were immunolabelled with the anti-pectin MAb JIM 7, abundant gold labelling was observed throughout the epidermal (Plate 6.5, Figs 1 & 2) and mesophyll cell walls (Plate 6.5, Fig. 3) and the middle lamella (Plate 6.5, Fig. 3). In addition, antibody-binding sites were evenly distributed throughout the primary walls including the elaborate wall ingrowths of transfer cells located in vascular tissue (Plate 6.5, Fig. 4). No labelling was observed at the walls of epidermal, mesophyll and transfer cells when JIM 7 was replaced with PBST only (Plate 6.5, Figs 5 & 6). Similar JIM 7-labelling patterns were observed throughout the “green” areas of infected leaf tissue (Plate 6.6, Figs 1 & 2). Regular JIM 7-labelling was observed throughout the walls and middle lamella of adjacent mesophyll cells (Plate 6.6, Fig. 1). The elaborate ingrowths of the primary wall of phloem and xylem transfer cells were also evenly labelled (e.g. Plate 6.6, Fig. 2). In addition, extensive MAb-binding sites were located throughout the walls supporting primary pit-fields (Plate 6.6, Fig. 3) and throughout sieve plates (Plate 6.6, Fig. 4).

In contrast, observations of infected leaf tissue revealed that JIM 7-binding sites were sparsely distributed throughout the swollen and loosened texture of the epidermal (Plate 6.7, Fig. 2). Occasionally abundant gold labelling was observed at the anticlinal wall junction between two epidermal cells (Plate 6.7, Fig. 3). Following fungal invasion of the palisade and spongy mesophyll cell layers, slight swelling and loosening of the walls of infected and contiguous mesophyll cells was observed (Plate 6.8, Figs 1, 2 & 4). Extensive collapse of mesophyll cells was frequently observed (Plate 6.8, Figs 3 & 4). In the collapsed mesophyll cells, JIM 7-labelling was restricted to the inner and outer layers of the swollen mesophyll walls and was absent throughout the open texture of the walls (Plate 6.8, Fig. 4). At the edge of lesions, where a clear distinction was observed between the swollen walls of the necrotic mesophyll cells and the intact walls of the “green” i.e. non-necrotic host tissue (Plate 6.8, Fig. 5), the pattern of JIM 7-labelling contrasted sharply: Although the JIM 7 MAb regularly labelled the walls of intact cells, the swollen walls of necrotic cell appeared to be sparsely labelled. In all cases, JIM 7-

labelling was restricted to the plant cell wall and was absent from the fungal cell wall (Plate 6.8, Figs 2 & 3). In control sections, where PBST or RPMI plus 5% FBS was employed in lieu of the primary antibody, no labelling was observed throughout the fungal or plant host tissue (Plate 6.8, Fig. 6).

The spatial distribution of JIM 7-labelling throughout infected and control leaf tissue was visualised more clearly using a secondary antibody 5 nm-gold conjugate followed by silver enhancement (Plate 6.9, Fig. 1-5). As expected, relatively sparse labelling was observed at the walls of the infected epidermal cell and contiguous epidermal and palisade mesophyll cells (Plate 6.9, Figs 1-3). In contrast, the walls of mesophyll cells located far from the infection site appeared intact and heavily-labelled (Plate 6.9, Figs 1 & 3). Observations of JIM 7-labelled control leaf tissue revealed that the walls of the epidermal and mesophyll cell walls were heavily-stained (Plate 6.9, Figs 4 & 5).

6.2.5 Comparison of the distribution of cellulose in the walls of non-infected and *B. fabae*-infected *V. faba* leaves by TEM using CBH1-colloidal gold

Colloidal gold particles (average particle size equal to 10-15 nm: Plate 6.10, Fig. 1) were prepared and successfully conjugated to the enzyme cellobiohydrolase (CBH1) to form the enzyme-gold complex (CBH1-colloidal gold). TEM observations of ultra-thin sections of non-inoculated *V. faba* leaves following staining with the CBH1 complex revealed that only traces of gold label were visible throughout the multi-layered outermost wall of the epidermal cell (Plate 6.10, Fig. 2). The inner walls of the epidermal cell which were thin and characteristically composed of only a few layers of cellulose microfibrils, were also only slightly stained with the CBH1 complex (Plate 6.10, Figs 3 & 4). No labelling was observed when sections were incubated with the CBH1-colloidal gold complex which had been previously adsorbed by carboxymethylcellulose (Plate 6.10, Fig. 5).

In contrast, when ultra-thin sections of *B. fabae*-infected *V. faba* leaves were similarly stained using the CBH1-colloidal gold complex, extensive gold labelling was detected throughout all walls of the infected and contiguous epidermal (Plate 6.10, Fig.

6; Plate 6.11, Figs 2-5 and Plate 6.12, Fig. 1) and mesophyll cells (Plate 6.12, Fig. 2). On fungal invasion of the epidermal cell wall layer, extensive modification of the host cell wall occurred (Plate 6.11, Fig. 1). The multi-layered structure of the thickened outer epidermal cell wall was readily apparent in infected (Plate 6.10, Fig. 6 and Plate 6.11, Fig. 2 & 5) compared to non-inoculated tissue (Plate 6.10, Figs 2 & 5). In all CBH1-colloidal gold-labelled sections, no label was located throughout the fungal fibrillar matrix material (Plate 6.12, Figs 1 & 2). However, in some cases, traces of label were detected at the fungal cell wall (Plate 6.12, Fig. 1).

Sections of PLT-dehydrated and LR-White embedded *B. fabae* sporelings (grown on agar) were stained with the CBH1 complex and observed by TEM in order to investigate the possibility that the CBH1 complex cross-reacted with components of the fungal cell wall. In these cases, no CBH1-gold labelling was observed throughout the fungal cell wall, the cell cytoplasm or extracellular matrices (not shown here).

In "green" areas of infected tissue, the walls of parenchyma cells and triple cell-junctions were labelled with the CBH1 complex (Plate 6.12, Fig. 3). In vascular tissue, secondary thickenings of xylem elements (Plate 6.12, Fig. 4) and the remains of the primary wall interconnecting these elements (Plate 6.12, Fig. 5) were also stained with CBH1-colloidal gold. However, no labelling of the elaborate wall ingrowths of phloem or xylem transfer cells was observed (not shown here). In control sections, no gold staining was observed when the CBH1 complex was pre-adsorbed with carboxymethylcellulose prior to labelling (Plate 6.11, Fig. 6 and Plate 6.12, Fig. 6).

6.2.6. Double labelling of the *V. faba*-*B. fabae* interface using BC-KH4 and CBH1-colloidal gold

Double labelling studies using both BC-KH4 and CBH1-colloidal gold showed that BC-KH4-immunolabelled matrix material surrounded the infection hyphae (Plate 6.13, Fig. 2) and was closely associated with the modified host cell wall (Plate 6.13, Figs 2-4). BC-KH4-immunolabelled material was often observed to extend through the swollen or loosened host cell walls (Plate 6.13, Figs 5 & 6). However, BC-KH4 MAb-binding sites were not observed at any other sites throughout the plant host tissue. In all cases, remnants of degraded host cell walls were clearly labelled by the CBH1-colloidal gold

complex (Plate 6.13, Figs 3-6). In some instances, traces of CBH1-colloidal gold labelling were also observed at the fungal cell wall (Plate 6.13, Figs 2-4). In control sections, where the BC-KH4 MAb was replaced with hybridoma culture medium and/or the CBH1 complex was pre-adsorbed with carboxymethylcellulose, little or no gold labelling was detected throughout the fungal or plant host tissue (not shown here).

6.2.7 Double labelling of the *V. faba*-*B. fabae* interface using the anti-pectin MAb JIM 7 and CBH1-colloidal gold

Double labelling studies of the *V. faba*-*B. fabae* interface, using the anti-pectin MAb JIM 7 and CBH1-colloidal gold, revealed that both JIM 7 and CBH1-gold labelling were observed throughout the swollen outer (Plate 6.14, Figs 1 & 2) and inner epidermal cell walls (not shown here). The modified walls of mesophyll cells were also stained with both JIM 7 and the CBH1-gold complex (Plate 6.14, Figs 3 & 4). JIM 7 MAb-binding sites appeared to be restricted to the plant host walls and middle lamella (Plate 6.15, Fig. 1 & 2). In addition, the level of JIM 7-labelling appeared to be less in the walls of infected cells compared to that of contiguous cells, especially in regions where the fungal hyphae were in close contact with the host cell walls (not shown here). In most cases, CBH1-colloidal gold labelling appeared to be evenly distributed throughout the walls of the necrotic cells (Plate 6.14, Figs 1-4). In a few cases, where host cell walls were highly modified as a result of fungal invasion, sparse labelling of the CBH1-gold complex was observed at the host walls (see Plate 6.13, Fig. 4). Traces of CBH1-colloidal gold labelling were also detected at the fungal cell wall (Plate 6.14, Fig. 3).

At the edge of a lesion: In necrotic tissue, the open texture of the modified walls of mesophyll cells was heavily-stained with both JIM 7 and the CBH1 complex (Plate 6.15, Figs 1-4). In contrast, the walls of "green" or non-necrotic mesophyll cells were labelled with JIM 7 only (Plate 6.15, Figs 3 & 4). Where slight loosening of mesophyll cell walls was observed, traces of CBH1-gold label were detected at the modified host wall (Plate 6.15, Fig. 4). In control sections, where the JIM 7 MAb was replaced with PBST only and/or the CBH1 complex was pre-adsorbed with carboxymethylcellulose, little or no gold labelling was detected throughout the fungal or plant host tissue (not shown here).

6.3 Discussion

6.3.1 Distribution of β -(1 $\rightarrow$ 3)-glucan-binding sites

6.3.1.1 Location of callose

Callose, a polymer of β -(1 $\rightarrow$ 3)-glucan, commonly occurs in higher plants as a component of specialised walls; it is found in the pores of sieve plates and at plasmodesmata located in pit-fields and in walls adjoining cells (Northcote & Wooding, 1966; Gunning & Steer 1975). In a previous report, immunogold labelling experiments using the β -(1 $\rightarrow$ 3)-glucan antiserum suggested that this antiserum specifically located callose in the young growing wall and in the developing cell plate of meristematic cells of bean (*Phaseolus vulgaris* L.) root (Northcote, Davey & Lay, 1989). In primary walls, callose was also detected at plasmodesmata (Northcote, Davey & Lay, 1989). The results of this present study provide further evidence to suggest that the β -(1 $\rightarrow$ 3)-glucan antiserum recognises callose at plasmodesmata in walls adjoining contiguous cells. In addition, it is shown here that β -(1 $\rightarrow$ 3)-glucan (i.e. callose) is located at plasmodesmata in pit-fields and at pores in sieves plates.

6.3.1.2. Is callose produced by the plant in response to fungal attack?

Deposition of callose and other components, namely suberin, lignin, and silica, have been reported to occur in a variety of host plants following mechanical injury and microbial infection (Faulkner & Kimmins, 1975; Aist, 1976; Heath, 1980; Bostock & Stermer, 1989). Mechanical injury may trigger the deposition of plugs of callose in the pores of sieve plates. This host defence response is often extremely rapid and it has been suggested that it may occur as a result of the manipulations involved in fixation of plant material for electron microscopy (Gunning & Steer, 1975). Thus, it is not surprising that it is difficult for electron microscopists to judge what sieve plates really look like (Gunning & Steer, 1975). It is suggested that the callose observed at pores in sieve plates and plasmodesmata located in pit-fields in the walls adjoining mesophyll cells of

non-inoculated *V. faba* leaves may have been deposited as a wound response to excision of leaves from the intact plant and/or as a response to the fixation protocol for TEM.

As mentioned above, callose deposition is also considered to be a common plant defence response to microbial infections. Usually, deposition of callose occurs at host walls in contact with fungal hyphae. Papillae² are often formed and in some cases, this plug-like material is thought to limit the interchange of substances between the pathogen and the host (Skipp & Deverall, 1972; Heath, 1980; Heath, 1984). In this present study, no callose deposition was observed at the *V. faba*-*B. fabae* interface. Instead, an accumulation of callose was observed at plasmodesmata adjoining injured and apparently healthy mesophyll cells. These observations may indicate that such callose deposition may play a role in plant host resistance in the *V. faba*-*B. fabae* interaction. However, this seems highly unlikely in view of the rapidly-spreading lesions formed by *B. fabae* following penetration of the leaf surface (see Chapter Five, Section 5.3.7). In addition, it has been widely reported that the *V. faba* plant shows little resistance to infection by *B. fabae* as a direct result of the relative insensitivity of *B. fabae* to the phytoalexin, wyerone acid and the ability of *B. fabae* to metabolise this phytoalexin to less toxic products (Mansfield & Widdowson, 1973; Mansfield & Deverall, 1974). Furthermore, papillae are rarely formed in *V. faba*-*B. fabae* interactions. In contrast, however, in some host and non-host species, it has been reported that papillae are formed in cell walls at the site of penetration of *B. cinerea* as a means of restricting growth and further penetration of *B. cinerea* hyphae (Mansfield & Hutson, 1980; Rijkenberg, Leeuw & Verhoeff, 1980).

In a previous histopathological study (Garcia-Arenal & Sagasta, 1977), callose deposition was observed in *B. cinerea*-infected *Phaseolus vulgaris* hypocotyls using the histochemical stain aniline blue, and fluorescence microscopy. Here, deposition of callose was observed in discrete areas of walls adjoining healthy cortical cells, but no callose was detected in unchallenged hypocotyls except in sieve tubes (Garcia-Arenal & Sagasta, 1977). It is conceivable that the callose spots observed in the *B. cinerea*-inoculated plant tissue co-localised with sites of plasmodesmata, but no suggestion of this was given by the authors (Garcia-Arenal & Sagasta, 1977). These researchers observed the same pattern of callose deposition in both resistant and healthy cultivars and therefore concluded that callose deposition could not be related to a differential host response to

² host cell thickenings which may include callose, lignin and tannins

infection. In the present study, extensive breakdown of host cell walls was observed. Furthermore, the plasmodesmata located at these damaged walls were highly disrupted. It is therefore suggested that the accumulation of callose at plasmodesmata in the walls adjoining necrotic and non-necrotic cells may have occurred as a result of the action of cell wall degrading enzymes. Such enzymes loosen host cell walls and inevitably release fragments of wall material including callose from the host wall.

6.3.2 Double-labelling studies using the β -(1 $\rightarrow$ 3)-glucan antiserum and BC-KH4: A simple technique for locating *Botrytis* infection hyphae?

Double immunolabelling studies of the *V. faba*-*B. fabae* interaction confirmed that the fungal cell wall and extracellular matrix components were both conveniently labelled and broadly distinguished using the polyclonal antiserum to β -(1 $\rightarrow$ 3)-glucan and the *Botrytis*-MAb, BC-KH4 respectively. Thus, this double-labelling technique represents a simple method for the dual location of fungal hyphae and associated matrix material inside the host leaf tissue. Interactions of fungal matrix components with plant host walls and/or cytoplasm were clearly visualised.

6.3.3 Distribution of JIM 7-MAb binding sites

6.3.3.1. Location of methyl-esterified pectin

IEM studies using the JIM 7 MAb allowed consistent mapping of the distribution of methyl-esterified pectin throughout healthy and *B. fabae*-infected bean leaf tissue. Previous IEM studies of the carrot (*Daucus carota* L.) root apex, using the JIM 7 MAb, showed that the JIM 7 epitope was located evenly throughout the cell walls including the middle lamella (Knox *et al.*, 1990; see also Diagram 6.1). A similar labelling pattern was observed at the walls of *V. faba* leaf tissue when the JIM 7 MAb was applied to sections of uninfected *V. faba* leaves in this study. The location of methyl-esterified pectin at the walls comprising pit-fields and sieve areas contrasted markedly with that of callose,

observed previously and detected using the β -(1 $\rightarrow$ 3)-glucan antiserum (see Section 6.3.1.1). The distribution of methyl-esterified pectin throughout the walls forming pit-fields, sieve areas and the elaborate primary wall thickenings of transfer cells has not been reported previously at the ultrastructural level.

6.3.3.2. Evidence for the action of pectin-degrading enzymes?

In an earlier report, the ultrastructure of the *V. fabae*-*B. fabae* interaction was described in detail (Mansfield & Richardson, 1981). Degradation and swelling of epidermal cell walls was observed. It was proposed that pectic enzymes were the principal cause of the swelling observed in the broad bean cell walls. Indeed, it is well-known that *Botrytis* species produce pectic enzymes (Hancock, Millar & Lorbeer, 1964; Balasubramani, Deverall & Murphy, 1971; Verhoeff & Liem, 1978). In the present study, close examination of both non-infected and *B. fabae*-infected *V. faba* leaves suggested a loss of methyl-esterified pectin from the swollen outermost walls of infected and contiguous epidermal cells. The levels of methyl-esterified pectin throughout the modified mesophyll cell walls also appeared to be less compared to that of healthy cells. Thus, the fact that swelling of host cell walls corresponded to a loss of pectin from these walls provides further confirmation for the role of pectin-degrading enzymes in the development of the *V. faba*-*B. fabae* interaction. Remote from the infection site, the levels of methyl-esterified pectin detected in the mesophyll cell walls appeared to increase to levels similar to that observed in the mesophyll cell walls of non-infected tissue. Thus, it was assumed that pectic enzyme activity was greater in regions close to the site of infection compared to that observed at the limiting edge of the lesion.

In order to understand fully the role of pectin-degrading enzymes in the processes of swelling or loosening of broad bean cell walls, the precise action of these enzymes must be considered. In simple terms, pectic polysaccharides of plant cell walls are degraded by hydrolytic polygalacturonases (PGs) or by lyases (Diagram 6.3). Lyases form an unsaturated bond between the C₄ and C₅ of the galacturonic acid at the non-reducing ends of the cleaved chains. PGs and lyases may be sub-divided into forms that attack internal regions of chains at random (endo-) or terminally (exo-) and on the basis of their specificity for polymers with esterified (methoxy) or non-esterified carboxyl groups; known as pectin and polygalacturonic acid respectively (Cooper, 1983). In short,

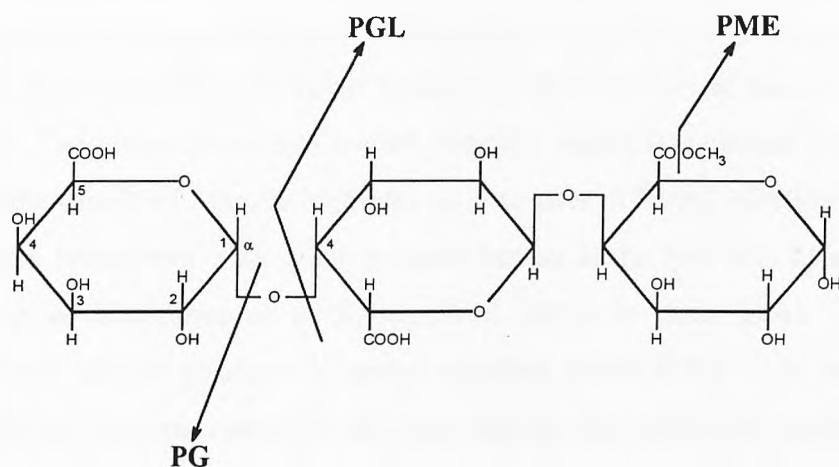


Diagram 6.3 Mode of action of pectinolytic enzymes

Polygalacturonase (PG) and polymethyl galacturonase (PMG) hydrolyse the α -(1 $\rightarrow$ 4)-bonds linking galacturonosyl moieties in unesterified and methyl-esterified pectin respectively. In contrast, lytic degradation of this glycosidic linkage is carried out by polygalacturonic acid *trans*-eliminases and pectin-methyl *trans*-eliminases which are also referred to as lyases [i.e. pectate lyase (PGL) and pectin lyase (PML) respectively]. PME hydrolyses the methyl esters of α -(1 $\rightarrow$ 4)-linked galacturonosyl residues at the carboxyl of the uronide moiety to yield a uronic acid carboxyl and methanol. (Redrawn from Cooper 1983, 1984).

PG and polygalacturonide or pectate lyase (PGL) attack polygalacturonic acid whereas polymethylgalacturonase (PMG) and pectin lyase (PL) attack pectin. Endo-PGs do not only solubilise galacturonides but they also result in a marked depletion of the covalently linked sugars e.g. arabinose, galactose and rhamnose (Talmadge *et al.*, 1973; see Diagram 6.2, fraction PG-1B). Interestingly, biochemical studies on the action of purified endo-PG³ on walls of suspension-cultured sycamore cells have indicated that endo-PG may also release small amounts of XG (Keegstra *et al.*, 1973). The methoxy groups of pectin are removed by the enzyme pectin methylesterase (PME; see Diagram 6.3) which can also act in concert with PG and/or PL.

In view of the results of IEM studies presented here, the mechanisms of action of the various pectinolytic enzymes detailed above and the fact that endo-PGs are produced by *Botrytis* species grown *in vivo* (see introduction to this chapter, Section 6.1.5), it is suggested that PME and/or PMG activity occurred in regions of infected tissue close to

³ isolated from *Colletotrichum lindemuthianum* culture medium (English *et al.*, 1972)

sites of fungal infection. It must also be mentioned that although the walls of mesophyll cells observed at the outer edges of the lesion were highly modified, the levels of methyl-esterified pectin in these walls appeared to be similar to that of unaffected cells. Thus, it is proposed that PG activity may occur throughout the necrotic leaf tissue and prior to PME action. The preponderance of methyl-esterified pectin at anticlinal wall junctions and the middle lamella of necrotic host cells close to sites of fungal infection may reflect such a gradual breakdown in the pectic polysaccharides in the host cell. Alternatively, it may indicate an abundance of methyl-esterified pectin in these areas. It must be mentioned here that the presence of methyl-esterified pectin at the outer edges of the swollen walls of collapsed mesophyll cells may indicate that such pectic molecules were cross-linked to other polysaccharides or proteins in the host cell wall. It would be interesting to determine whether or not such pectic molecules are lost from the host cell walls during later stages of the necrotrophic interaction by the action of other cell wall-degrading enzymes.

6.3.4 Distribution of CBH1-gold binding sites

6.3.4.1 Location of cellulose

Cellobiohydrolase (CBH1)-colloidal gold labelling is a non-destructive and direct labelling technique for the identification of cellulose exposed on the surface of thin sections (Berg *et al.*, 1988). It has been suggested previously that CBH1 binds and hydrolyses the β -(1 $\rightarrow$ 4) linkage of the cellulose polymer and subsequently disrupts hydrogen bonding between polymers (Schulein, 1988). Water can therefore enter the cellulose crystalline matrix and endo-glucanases can attack the polymers further to release cellobiose and glucose (Chanzy *et al.*, 1983; Schulein, 1988; see Diagram 6.4). In the present study, the specificity of CBH1-gold complex towards cellulose was corroborated by positive staining of secondary wall thickenings and the cellulosic remnants of the primary walls of xylem elements. Although CBH1 normally has a high affinity for insoluble cellulose (Chanzy *et al.*, 1983; Schulein, 1988), it is generally accepted that CBH1-binding sites may be masked by other polysaccharides in the native plant cell wall (Berg *et al.*, 1988). The results of this present study confirm that the

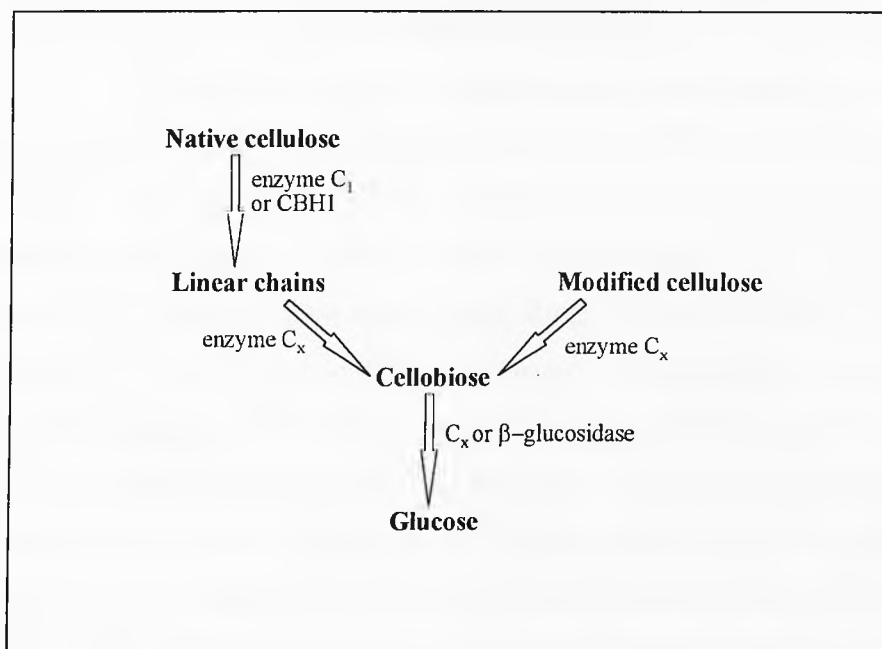


Diagram 6.4 The suggested mode of action of the enzyme cellobiohydrolase (CBH1) and the mechanism of cellulose degradation

Cellobiohydrolase (CBH1) behaves in a similar manner to enzyme C_1 which acts upon native cellulose and breaks down its crystalline structure to release cellulose fibrils. Enzyme C_x or β -(1 $\rightarrow$ 4)-glucanases degrade cellulose further to cellobiose or glucose. Cellobiose may be converted to glucose by β -glucosidases e.g. cellobiase. Modified celluloses are generally more susceptible to enzymatic degradation than are native celluloses. (Redrawn from Wood, 1967).

cellulose microfibrils constituting the epidermal and mesophyll cell walls of non-infected *V. faba* leaf tissue were heavily masked by polysaccharides (probably pectin) in the wall. It is feasible that such fibrils may have only been labelled by the CBH1-colloidal gold complex following exposure of the fibrils during the physical process of sectioning. This was a proposed explanation of the chitinase-gold labelling pattern in the wall of a fungal spore (Bonfante-Fasolo, Vian & Testa, 1986).

6.3.4.2 Further evidence for the wall-modifying activity of pectic enzymes

The “wall-modifying activity” of pectinolytic enzymes is well-known (Karr & Albersheim, 1970; Bateman & Basham, 1976). These enzymes are considered to be able to attack unaltered plant cell walls, loosen their structure and render other wall polymers

susceptible to enzymatic hydrolysis. Treatment of sycamore walls with an endo-PG isolated from *Colletotrichum lindemuthianum* has been shown to enhance ten-fold the subsequent degradation of the walls by endo-glucanase and protease and to enable several glycosidases, an exo-PG and cellulase to perform as CWDEs (Bauer *et al.*, 1973; Keegstra *et al.*, 1973). It has also been suggested previously that PG activity may facilitate the entry and action of other CWDEs by increasing wall porosity (Cooper, 1983). Endo-PG enzymes of most necrotrophic fungi are small (approx. 30 000 Da; Knee, 1978) and therefore clearly capable of penetrating the wall matrix. In a previous study, endo-PG digestion of the walls of mung bean hypocotyls exposed the individual microfibrils of the cellulose framework (Vian & Roland, 1991). In view of the results of JIM 7-, CBH1-gold and double labelling studies presented here, it is likely that during the early stages of development of chocolate spot disease of broad bean by *B. fabae*, the cellulose microfibrils of the epidermal and mesophyll cell walls were rendered more amenable to attack by cellulose degrading-enzymes e.g. cellobiohydrolase as a result of the wall-modifying action of pectin-degrading enzymes produced by the fungus. The parallel lamellae of cellulose microfibrils laid down in the outermost walls of the epidermal cells were clearly visible following ingress of broad bean leaves by *B. fabae*. That no such lamellae were observed in mesophyll cell walls may be explained by the fact that cellulose fibrils are randomly oriented in these walls.

6.3.4.3 Evidence for cellulase activity?

CBH1-labelling studies of the *V. faba*-*B. fabae* interface revealed limited cellulose degradation of host cell walls in wall regions in close contact with infection hyphae. Such localised wall breakdown is perhaps not surprising since cellulase activity is often only apparent in the later stages of the infection process, e.g. digestion of moribund tissue, in many soft-rot diseases and diseases of other related organisms (Cooper, 1983). It has been suggested previously that cellulase enzymes are probably restricted in their degradation of secondary walls as a result of their particle size (mean diam. equal to 6 nm) which is generally larger than the spaces⁴ offered by wall capillaries (diam. 3.5-5.2 nm) (Reese, 1977; Carpita *et al.*, 1979). Further evidence that wall porosity may be a

⁴ More recently, it has been reported that wall spaces may be as large as 7 nm and therefore large enough to allow the passage of cellulase enzymes (Tepfer & Taylor, 1981).

major factor in limiting enzymic activity has come from studies of infection of apple walls by *Phytophthora infestans* where minimal degradation of the host cell wall occurred (Knee *et al.*, 1975). This localised wall degradation was considered to be a result of the action of a PG which possessed a large particle size (>200 000 Da; Knee *et al.*, 1975). It is unlikely that wall porosity limited cellulase activity in the *V. faba*-*B. fabae* interaction as a result of the wall-modifying action of the pectin-degrading enzymes.

In a few cases, it has been reported that fungal cellulases are active during the early critical stages of the infection process e.g. lesions formed by *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and in tobacco stems invaded by *Pseudomonas solanacearum* (Kelman & Cowling, 1965; Cooper, 1983). It would be interesting to investigate whether or not cellulase activity increases during the later stages of infection of the *V. faba*-*B. fabae* interaction. It must be stated here that the observed CBH1-gold labelling of the walls of infection hyphae inside the host tissue indicated, most probably, a trapping of fragments of cellulose at the fungal cell wall. Furthermore, the possibility that other CWDEs e.g. hemicellulases were active during the early stages of development of the *V. faba*-*B. fabae* relationship cannot be ruled out.

6.3.5 Evidence for passage of *B. fabae* fibrillar matrices through the host cell wall

Double-labelling studies of the *V. faba*-*B. fabae* interaction by TEM, using both the CBH1-colloidal gold complex and the BC-KH4 MAb, confirmed the extension of *Botrytis* fibrillar matrix material throughout the swollen mesophyll cell walls (see also Chapter Five). In view of the wall-modifying action of the pectic enzymes produced by *B. fabae* on the walls of broad bean leaves, it is suggested that such activity altered the host wall barrier to fungal infection to such an extent to allow the passage of the fibrillar matrix material associated with the infection hyphae. In *B. fabae*, as indicated in other fungi, extracellular matrices may function as a "reservoir" for a number of compounds including enzymes involved in host wall breakdown (Ruel & Joseleau, 1991; Nicole *et al.*, 1993; Viret & Petrini, 1994). Thus, it is possible that the pervasion of host cell walls by *Botrytis* fibrillar matrices may have also occurred as a result of the localised action of other CWDEs e.g. hemicellulase, cellulases.

Concluding remarks

There is no doubt that the *Botrytis*-MAbs presented here are sensitive and specific tools for the location of surface molecules of *B. cinerea* and *B. fabae* in light (immunofluorescence) and electron microscope (immunogold labelling) studies of the *Vicia faba*-*Botrytis* interaction. Similarly, these MAbs may be applied to investigations of the spatial distribution of these surface antigens, and their role(s), in other plant-*Botrytis* relationships.

As MAbs to surface glycoproteins, the *Botrytis*-MAbs have allowed the observation of extracellular matrices associated with different fungal propagules (i.e. conidia and immature ascospores) and with both external and internal infection structures. In parallel with other microscope methods e.g. LTSEM or using specific probes for plant cell wall components, these studies have allowed the theories posed by early scientists regarding the role(s) of *Botrytis* surface molecules (i.e. as a fungal glue) and secretions (i.e. as wall-dissolving enzymes) to be re-addressed and new data to be obtained. For example, these studies have provided further evidence that *Botrytis* extracellular matrices ensure tight adhesion of sporelings to the substratum surface and that pectic enzymes possess wall-modifying properties. Further to this, they have shown that the fibrillar matrices around infection hyphae are able to pervade host walls. In addition, a knowledge of the adhesive properties of extracellular matrices has suggested a similar role for the matrices associated with conidia, ascospores and both internal and external infection structures. The application of the *Botrytis*-MAbs to immunofluorescence studies using the confocal microscope has permitted better visualisation of the extracellular matrices around sporelings. Confocal microscope studies are clearly beneficial for observing the spatial distribution of *Botrytis* surface molecules produced *in vivo* and future studies using this technique may be rewarding.

All of the aforementioned studies have emphasised the fibrillar nature of matrices associated with the conidia, germ tubes and infection hyphae of *Botrytis* species. In addition, they have allowed observation of changes in the form and content of matrices which may be affected by the supply of nutrients to the fungal spore and the contact substratum. Brefeldin A has proved to be a useful tool in studies of the mode of secretion of the *Botrytis* cell surface molecules. Other secretory inhibitors, e.g. monensin, may also

aid in the understanding of the secretory pathway of these surface molecules. Likewise, these inhibitors may be applied to studies of the mode of secretion of surface glycoproteins of other filamentous fungi.

The potential of low-temperature controlled freeze-drying as a method for improving the preservation of soluble fungal molecules and increasing retention of antigenicity of *Botrytis* fibrillar matrices for immunocytochemical studies has been demonstrated. Further immunostudies using this method and/or freeze substitution followed by careful orientated sectioning may allow the question of whether or not the surface molecules recognised by the *Botrytis*-MAbs are secreted at hyphal tips to be answered more fully.

In the near future, the *Botrytis*-MAbs may be applied to biochemical procedures e.g. Western blotting, immunoprecipitation and antigen modification, to identify the glycoprotein component of the *Botrytis* extracellular matrices observed in this study. These MAbs may be used to purify surface molecules of *Botrytis* species by immunoaffinity chromatography for carbohydrate analysis and protein sequencing. Mutagenesis may lead to the development of strains of *Botrytis* which are defective in the production of specific cell surface molecules and/or adhesion. Thus permitting another route for analyses of the role(s) of these surface molecules in plant pathogenesis.

Finally, it remains to be seen whether or not the *Botrytis*-MAbs may be employed as effective agents of disease control. If the former proves to be the case then, using molecular biology techniques, the genes encoding the protein sequence of the antigens of interest may be incorporated into the genome of crop plants, perhaps even the broad bean plant *Vicia faba* L., thereby conferring a genetically-engineered immune system to the plant.

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